The effect of a very low-calorie diet (VLCD) with and without concomitant resistance exercise training (RET) or high-intensity interval training (HIIT) on body composition, muscle function and metabolism

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Thesis Abstract

Background: Weight loss is beneficial for health in many aspects, including reducing the risk of various diseases such as type 2 diabetes mellitus, cardiovascular disease, respiratory problems and many more. A very low-calorie diet is one of the methods that can provide rapid weight loss. Unfortunately, there are concerns regards to muscle and lean mass loss with the weight loss process. Exercises particularly resistant exercise training (RET) is known to stimulate muscle growth; however, research combining RET with VLCD is scarce. High-intensity interval training (HIIT) has recently emerged as an 'efficient' exercise that can fit many busy lifestyles. However, no study has been done to combine HIIT with VLCD to compare its benefit with diet only or with RET.

Objectives: To assess for any significant differences in the outcomes from VLCD only, VLCD with RET and VLCD with HIIT interventions, focusing on changes in lean and muscle mass, muscle protein synthesis rate, muscle function in the form of muscle strength, metabolic markers including lipid profiles and glucose homeostasis, cardiovascular function, and cardiorespiratory fitness.

Design: Overweight/obese (BMI≥27) middle age (30 to 60-year old) males were recruited, randomly assigned to three groups either VLCD only (VLCD+O), VLCD with RET (VLCD+R) or VLCD+HIIT (VLCD+H). Interventions were for six weeks. Investigations and procedures include DXA scan, ultrasound scan of muscle structure and blood flow, echocardiogram of the heart, oral glucose tolerance test for insulin and glucose level, blood sampling, saliva collection and muscle biopsy involving stable isotope deuterium oxide to measure muscle protein synthesis rate.

Conclusion: All groups showed significant total weight, fat mass, and lean mass loss. Despite the lean mass loss, all groups had improvement in metabolic markers, including glucose homeostasis and lipid profiles and had no reduction in muscle strength. Incorporating HIIT with VLCD showed a statistically significant higher muscle protein synthesis rate compared to VLCD alone while incorporating RET showed a significant increase in strength compared to the other groups.

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Abbreviations

1-RM	One-repetitive maximum
4EBP1	4E-binding protein 1
AA	Amino acids
AFM	Android fat mass
AKT	Protein kinase B
AMPK	AMP-activated protein kinase
AT	Anaerobic threshold
ATG13	Autophagy-related protein 13
CEUS	Contrast-enhances ultrasound scan
cGMP	Cyclic guanosine-3’,-5’-monophasphate
CO	Cardiac output
CPET	Cardiopulmonary exercise test
CR	Caloric restriction
CT	Computer tomography
CT	Computer tomography
CVD	Cardiovascular disease
D_2O	Deuterium oxide
DXA	Dual-energy X-ray absorptiometry (DXA)
EAA	Essential amino acid
ECHO	Echocardiography
EDHF	Endothelial-derived hyperpolarizing factor

EF	Ejection fraction
eNOS	Endothelial nitric oxide synthase
ET	Endurance training
FA	Fatty acid
FAC	Fractional area change
FFA	Free-fatty acids
FFM	Fat-free mass
FM	Fat mass
FMD	Flow-mediated dilatation
FoxO	Forkhead box class O
FS	Fractional shortening
GLUT4	Glucose transporter type 4
HDL	High-density lipoprotein cholesterol
HIIT	High-intensity interval training
HOMA-IR	Homeostasis model assessment for insulin resistance
IGF-1	Insulin-like growth factor 1
IGF-1	Insulin-like growth factor 1
IR	Insulin resistance
LDL	Low-density lipoprotein cholesterol
LM	Lean mass
L-NMMA	Mono-methylarginine
LV	Left ventricular
MAFbx	Muscle atrophy box
MBF	Microvascular blood flow
MBV	Microvascular blood volume

MFV	Microvascular flow velocity
MI	Mechanical index
MICT	Moderate-intensity continuous training
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
MuRF-1	Muscle RING finger-1
MVC	Maximum voluntary contraction
NO	Nitric oxide
OGTT	Oral glucose tolerance test
P70S6K	Ribosomal protein S6 Kinase
PI3K	Phosphatidylinositol 3-kinase
PIL	Participant information leaflet
QUICKI	Quantitative insulin sensitivity check index
RET	Resistance exercise training
RMR	Resting metabolic rate
ROS	Reactive oxygen species
rpS6	Ribosomal protein S6
SMC	Smooth muscle cells
SV	Stroke volume
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TEE	Transoesophageal echocardiography
TG	Triglycerides

TSC	Tuberous sclerosis complexes
TTE	Transthoracic echocardiography
TW	Total weight
ULK1	Unc-51 like kinase-1
USS	Ultrasound scan
VCO_2	Respiratory carbon dioxide production
VE	Expired ventilation
VL	Vastus lateralis
VLCD	Very low-calorie diet
VO_2	Respiratory oxygen uptake
VO_2max	Maximum rate of oxygen consumption

Operational definitions

Several important and frequent terms that are used in this thesis:

Terms	Scope of definition in this thesis
Cardiometabolic health	A combination of cardiovascular and metabolic state of function.
Cardiorespiratory fitness	The capacity of the cardiovascular and respiratory systems to supply oxygen to vital organs to enable physical activity.
Cardiovascular disease	Abnormal cardiac and vascular function, including cardiac ischaemia, endothelial dysfunction, poor peripheral circulation, poor brain perfusion.
Dyslipidaemia	Abnormal lipid profiles involving any abnormality with high triglyceride, high LDL, or low HDL.
Fat-free mass	Total body mass minus fat mass on DXA result.
Glucose intolerance	A condition where the blood glucose is raised beyond normal levels, but not high enough to warrant a diabetes diagnosis.
Insulin resistance	The degree of cells within muscle, fat, or and liver not responding well to insulin causing impaired glucose uptake from the blood circulation.

Insulin sensitivity	The degree of cells within muscle, fat, or and liver responsiveness to circulating insulin for glucose uptake from the blood circulation.
Lean mass	Fat-free mass minus bone mass on DXA result.
Metabolic markers Metabolic effect Metabloic function	Used mainly in chapter 4 in relation to degree of glucose homeostasis and lipid metabolism.
Metabolic syndrome	Combination of several known cardiovascular risk factors, including insulin resistance, obesity, dyslipidaemia and hypertension.
VO_2max	The maximum rate of oxygen consumption measured using CPET.
$Watt_{max}$	Maximum wattage tolerated during gradual wattage increment using CPET.

Chapter 1: Introduction

1.1 Chapter synopsis

This chapter discusses the detrimental effect of obesity on various aspects of life, hence the importance of weight loss to eliminate the problem. A very low-calorie diet (VLCD), the primary focus of this thesis, is being introduced in this chapter as one of the known methods for rapid weight loss. Unfortunately, while weight loss comes mainly from fat mass, a quarter of the total loss is from lean mass, which includes muscle mass. Loss of muscle mass is very concerning, considering the importance of muscle functions physically and physiologically. With that interest in mind, this chapter also discusses muscle metabolism at the molecular level, focusing on nutrition and exercise stimulants that could affect the molecular pathway of muscle metabolism. With the understanding of the basic molecular mechanism of muscle synthesis and breakdown, this chapter then looks into available literature involving exercises as a way to mitigate muscle mass loss during VLCD intervention.

1.2 Obesity and health problems

Obesity is a new pandemic, causing massive health complications to the public. Data from the Health Survey for England (HSE) in 2015 showed that 62.9% of adults were overweight or obese (PHE 2016). Whilst the prevalence of overweight relatively plateau at 36–39% between 1993 and 2015, the prevalence of obesity shows a more worrying sign with the number rose from 14.9% to 26.9%. Obesity is predicted to affect 60% of adult men, 50% of adult women and 25% of children by the year 2050 (Butland et al. 2007).

Obesity has been linked to a multitude health issues including type 2 diabetes mellitus (T2DM) (Colditz et al. 1995; Nguyen et al. 2011), hypertension (do Carmo et al. 2016), dyslipidaemia (Grundy and Barnett 1990), coronary heart disease (Nordestgaard et al. 2012; McPherson 2015), heart failure (Kenchaiah et al. 2002; Aune et al. 2016), stroke (Kurth et al. 2002), dementia (Kloppenborg et al. 2008), various cancer types (Vainio et al. 2002; Wolin et al. 2010; Vucenik and Stains 2012), obstructive sleep apnoea (Li et al. 2010), asthma (Sideleva et al. 2013; Dias-Júnior et al. 2014), immune disorder (Huttunen and Syrjänen 2013; Versini et al. 2014), fatty liver disease (Fabbrini et al. 2010), gallstones (Bonfrate et al. 2014; Aune et al. 2015), gastroesophageal reflux disease (Lee and McColl 2015),

pancreatitis (Bonfrate et al. 2014), kidney stone (Semins et al. 2010), end stage renal disease (Hsu et al. 2006), reduced fertility (Semins et al. 2010; Bonfrate et al. 2014), osteoarthritis (Berenbaum et al. 2013), lower back pain (Shiri et al. 2013), rheumatoid arthritis (Lu et al. 2014), gout (Choi et al. 2005) and depression (Dixon et al. 2003).

Of all the known complications from obesity, T2DM itself is particularly important due to various health outcomes from the disease itself, particularly macrovascular and microvascular complications, which include coronary artery disease, peripheral vascular disease, retinopathy, and nephropathy, to name a few (Lotfy et al. 2016). T2DM implicates a huge financial burden to the nation with the cost of direct patient care amounted up to £8.8 billion, and the indirect costs were approximately £13 billion according to the data from 2010-2011, with the prescription related to diabetes mellitus medication covered 9.3% of the total prescription cost in England for the year 2012-13 (Mary et al. 2014). Obese adults are five times more likely to be diagnosed with T2DM compared to adults of a healthy weight; statistically, 90% of adults with T2DM are overweight or obese (Mary et al. 2014).

1.3 VLCD as one of the many tools to counter obesity

Extensive measures have been devised to stop the rising obesity number, ranging from lifestyle modification to surgical intervention. Lifestyle modification, also known as behavioural weight control, consists of diet, exercise, and behaviour therapy, while surgical intervention (commonly known as bariatric surgery) include procedures are gastric bypass, sleeve gastrectomy, and gastric band, to name a few.

Lifestyle modification has been shown to effectively reduce weight and risk of developing T2DM by 58% when compared to placebo, and in some cases, this to be more effective than pharmacological therapy in preventing T2DM (Tuomilehto et al. 2001; Knowler et al. 2002; Lean et al. 2017). Practice guidelines formulated by the US National Heart, Lung and Blood Institute and the North American Association for the Study of Obesity recommends that the association of diet, physical activity, and behavioural therapy should be considered as the primary option for treating obese and overweight people with two or more weight-related comorbidities (NIH 2000), which is also in line with the recommendation by England's National Institute for Health and Care Excellence (NICE

Guideline 2014). The Department of Health England recommends at least 150 minutes of moderate-intensity aerobic activity such as cycling or fast walking every week for adults. Physical activity that can be incorporated into everyday life, for example, brisk walking and cycling have been shown to be as effective as supervised exercise programmes in promoting weight loss (PHE 2017).

Dietary interventions include moderate calorie restriction and more intensive restriction such as a very low-calorie diet (VLCD), as well as an alteration in diet composition, for example, consuming low-fat instead of high-fat foods. The cornerstone of diet therapy is caloric intake below the amount required to maintain weight. Non-medical support groups, such as Weight Watchers, Lighter Life, or Cambridge Weight Plan, may provide support and encourage people to adhere to diets. VLCD limits daily calorie intake 800 or less - and can go as low as 400, which is inadequate to sustain a healthy body over the long period, itself not without controversy during the initial introduction a few decades ago, due to sudden death incidence attributable to cardiac arrhythmia. With improving safety profile, VLCD now has been widely accepted and has been used as a rapid weight loss program, for example, as a pre-surgical preparation to help with liver shrinkage (Colles et al. 2006).

1.3.1 The rise of VLCD

VLCD is defined as diets providing 800 kcal/day or less (SCOOP 2002). Because of variation in body mass hence total daily calories requirement would differ from person to person. This amount might seem insignificant for those with a low body weight compared to those with more weight. Therefore, an alternative definition of VLCD has also been proposed, defined as a diet that provides <50% of an individual's predicted resting metabolic rate (RMR) (Atkinson 1989). VLCD reached its popularity among the public in 1988 when famous talk show celebrity Oprah Winfrey announced to the world that she lost about 30kg in her weight following 'a liquid diet'. The interest among researchers goes back as far as 80 years ago by the work of the Pittsburgh group in the 1920-1930s. The first research published on VLCD appeared in 1929 as a new method for treating obesity, where a diet of 6 to 8 calories per kilogram of body weight, instead of the usual 14 to 15 calories per kilogram, was used which created rapid weight loss (Saris 2001). While VLCD is recognised as an effective way of losing weight and promoting health benefits from weight loss, people were initially worried about a huge incidence of sudden death related to VLCD attributable to cardiac arrhythmias (Sours et al. 1981; Saris 2001). Evolving studies on VLCD have contributed to the changes in using conventional food to a high-

quality protein containing all essential nutrients, making VLCD safe from previously associated poor complications.

1.3.2 VLCD improves glucose metabolism

In general, VLCD promotes weight loss between 1.4kg to 2.5kg per week (Saris 2001), in the form of fat and lean mass (LM) loss. Loss of LM is an important subject worthy of attention as LM also includes muscle mass, an important organ for overall body metabolic function and glucose homeostasis. Despite a reduction in muscle mass, there is an abundance of evidence that showed improvement in blood glucose control following weight loss. Lim et al. found that fasting plasma glucose and HbA1c normalised in the diabetic subjects following VLCD, and their plasma triacylglycerol levels halved, including reversal of fatty liver disease (Lim et al. 2011). In fact, normalisation of fasting plasma glucose occurred as early as one week following the VLCD. Large multicentre primary care study showing patients losing weight on an intense low-calorie diet (around 850 kcal/day, which is closed to VLCD definition of <800kcal/day) for 3-5 months had a better outcome in terms of remission in T2DM compared to patients on medications, with an odds ratio of 19.7 (Lean et al. 2017). Improvement in metabolic profile following weight loss can be explained via an increased in adipose tissue capacity for glucose uptake and fatty acid (FA) oxidation

(Bouwman et al. 2014). Under a normal energy-balanced state, glucose is the primary energy source of adipose tissue (Frayn et al. 1995); however, under conditions of calorie restriction such as VLCD, they turn to oxidation of FA as the source of energy (Magnusson et al. 2008; Franck et al. 2011). Using FA as an energy source reduces the FA load of the body, which contribute to improvement in insulin sensitivity of peripheral tissues (Maassen et al. 2007). After 3-week post VLCD on a low-fat weight maintenance diet, adipocytes remain to favour energy from mitochondrial β-oxidation of FA and not do not return to glucose as their major source of energy despite increasing glucose uptake (Bouwman et al. 2014).

Sasaki et al. found significant improvement in fasting plasma insulin from 85.8 to 64.8 pmol/L following 2-week VLCD on 11 obese patients (Sasaki et al. 2002). Improvement in insulin sensitivity also was seen during 2-week VLCD before bariatric surgery, where the whole-body insulin sensitivity was found to improve from 2.9 to 4.2 mg/kg/min using hyperinsulinemic-euglycemic clamp protocol (Pournaras et al. 2016). 6-week VLCD has been shown to help induced weight loss and contributed towards 0.47mmol/L decrease in fasting plasma glucose concentration (Raitakari et al. 2004), while 8-week VLCD followed by 9-week build up low calorie diet showed 0.22mmol/L reduction in fasting plasma glucose (Pekkarinen et al. 1998). Uusitupa et al. study on a

hypocaloric diet (500-800kcal/d) for three months helped with an average 14kg weight loss (~1kg/week) and declined in fasting plasma glucose from 12.3 to 10.5 in ten obese diabetic men and women (Uusitupa 1990).

Interestingly, higher fat distribution around a specific region, particularly the android region, or in a simple term, android fat mass (AFM), has been strongly linked with metabolic syndrome in the form of elevated plasma triglyceride (TG) level, elevated total cholesterol (TC) and impaired insulin sensitivity (Min and Min 2015; Sari et al. 2019).

1.3.3 VLCD and improvement in cardiovascular risk factor and cardiac profile

Obesity is a major risk factor for cardiovascular diseases (Lavie et al. 2009), increasing morbidity and mortality from underlying endothelial dysfunction and atherosclerotic cardiovascular disease (Hubert et al. 1983; Brook et al. 2001). It is well recognised that a critical early event in the pathogenesis of atherosclerosis is due to endothelial dysfunction, mainly via reduced bioavailability of the signalling molecule nitric oxide (NO), which is common in obesity (Williams et al. 2002). Dyslipidaemia, insulin resistance, hypertension, increased inflammatory state and increased oxidative stress are often present in obesity, and all of these

contributed to endothelial dysfunction (Raitakari et al. 2004). In general, weight loss has been shown to improve profiles that contribute to endothelial dysfunction, particularly blood pressure (Sasaki et al. 2002; Velazquez-Lopez et al. 2013; Luotolahti et al. 2016), glucose/insulin resistance (Velazquez-Lopez et al. 2013; Luotolahti et al. 2016; Pournaras et al. 2016), serum lipids (Dattilo 1992; Velazquez-Lopez et al. 2013), oxidized LDL (Vasankari et al. 2001), lipid mobilisation and oxidation (Bouwman et al. 2014) and C-reactive protein (Heilbronn et al. 2001; Tchernof et al. 2002).

A study by Sasaki et al. involving 11 obese hypertensive patients having 2-week VLCD intervention showed significant improvement in blood pressure with mean blood pressure from 118.4mmHg pre-intervention to 105.7mmHg post-intervention (Sasaki et al. 2002). Their work suggested that that caloric restriction improves endothelial-dependent vasodilation through an increased release of NO in obese hypertensive patients. Slightly contrary to the above findings, Pekkarinen et al. found no significant improvement in the blood pressure at the end of 8-week VLCD and 9-week of gradual build-up of a low-calorie diet, only at their one year follow up, the decrease in systolic blood pressure and diastolic blood pressure were significant at 4.1mmHg and 3.0mmHg respectively (Pekkarinen et al. 1998).

Fatty heart "cor adiposum" has been linked to obesity (Szczepaniak et al. 2007). Obesity itself is also known to be an independent risk factor for heart failure (Kenchaiah et al. 2002), while high triglyceride (TG) is predisposed to increase adipose tissue mass at the expense of left ventricular remodelling, which also can lead to cardiac failure (Szczepaniak et al. 2007). Increasing BMI is also associated with increasing severity of right ventricular dysfunction in overweight and obese subjects, independent of sleep apnoea (Wong et al. 2006). 6-week caloric restriction with VLCD was shown to decrease myocardial TG content by 31% (Viljanen et al. 2009). Luotolahti et al. demonstrated improvement in the cardiac systolic and diastolic function following an 8-week VLCD. These changes occurred at a more rapid rate than one could expect based on the traditional concept of the functions of the heart. The relative improvement in heart function and the relative decrease in epicardial fat were bigger than the relative decrease of BMI, suggesting that metabolic changes have some degree of contribution in addition to weight loss itself (Luotolahti et al. 2016).

1.3.4 VLCD and other health advantages

Interestingly caloric restriction (CR) in general has been reported to increase lifespan, maintain metabolism at a more youthful-like state and prevent chronic diseases (Fontana and Partridge 2015). Studies

involving monkeys showed a 2.6 to 2.9-fold increased risk of death in control animals compared to the CR group (Bodkin et al. 2003; Colman et al. 2014). CR also has been shown to improve lifespan in rodents (Weindruch et al. 1986). A study on rat muscles on lifelong CR demonstrated that CR increases the expression of some autophagic proteins attenuating the age-dependent decline in autophagy, decreasing oxidative damage as well as apoptotic DNA fragmentation, and is sufficient to reduce age-related myocyte degeneration, maintaining skeletal muscle homeostasis (Wohlgemuth et al. 2010). VLCD-induced weight loss in obese individuals has been shown to help to reduce colorectal inflammation and colorectal cancer risk via modulation of inflammatory and cancer-related gene pathways (Pendyala et al. 2011). A trial by Stenius-Aarniala et al. found beneficial effects on the control of asthma among obese subjects following VLCD (Stenius-Aarniala et al. 2000).

1.3.5 Problems and Pitfalls with VLCD

Early VLCD regime has been associated with sudden death. In the 1970s, VLCD products used 'low-quality protein' hydrolysed collagen as the only protein source and did not include an adequate amount of nutrients resulting in several sudden deaths (Sours et al. 1981). Of 60 persons who died in the United States, most developed cardiac

complications after a loss of about 30% of their initial weight (Gilden Tsai and Wadden 2006).

Fortunately, further evaluation of people on a modern VLCD of high-quality protein content for 16 weeks or less has not shown an increased incidence of cardiac ventricular dysrhythmias (NIH 1993). A study on 24 obese women on a 6-week VLCD has also demonstrated the safety of the diet when used with and without exercise (Moyer et al. 1989). VLCD now has been safely used as a rapid weight loss program, for example, as a pre-surgical preparation to help with liver shrinkage (Colles et al. 2006; González-Pérez et al. 2013; Tan et al. 2020).

Another recognised risk of VLCD is the formation of gallstones. The incidence varies from 11% to as high as 25%, with 25-50% of those who developed gallstones required cholecystectomy (Liddle et al. 1989; Kamrath et al. 1992). A large multicentre cohort on 3-month VLCD found a 1.5% incidence of gallstones requiring hospitalisation, and 60% of those gallstones incidents required cholecystectomy (Johansson et al. 2014). To reduce the risk of the gallstones formation, some studies suggested inclusion of ursodeoxycholic acid during the weight-loss period (Broomfield et al. 1988; Shiffman et al. 1995), as well as by making sure to include a moderate amount of fat in the diet (Hoy et al.

1994; Festi et al. 1998) and limiting weight loss to 1.5kg per week (Weinsier et al. 1995).

Whilst VLCD has been proven to be very effective for weight loss the in the short term, the long-term effect on net weight loss has been shown to be somewhat 'diluted'. People tend to regain 40% to 50% of the total weight loss 1 to 2 years after treatment in the absence of follow-up care (Anderson et al. 1994; Wadden and Frey 1997). Weight loss by VLCD produces better long-term results if the followed-up period incorporates nutritional education, behavioural therapy, and increase physical activity (Walsh and Flynn 1995). People who exercise maintained more than twice as much weight loss compared to non-exerciser after 30 months of VLCD (Flynn and Walsh 1993). The actual factor affecting the amount of weight regain remains a puzzle, although initially it was thought that the rate of weight loss would affect the total weight regains, this was later proven wrong (Purcell et al. 2014; Vink et al. 2016). The degree of metabolic adaptation at the end of the weight loss program also was not associated with weight regain (Fothergill et al. 2016). It is believed that the magnitude of early weight loss is the best predictor of long-term weight loss (Astrup and Rössner 2000; Nackers et al. 2010).

One main factor that contributes to weight regain is the reduction of resting metabolic rate (RMR) following weight reduction (Fothergill et al. 2016). Reduced RMR is often greater than the changes in body composition, a phenomenon is called "metabolic adaptation" or "adaptive thermogenesis," acting as a counter mechanism to the weight loss process (Rosenbaum and Leibel 2010; Müller and Bosy-Westphal 2013). RMR is depressed among sedentary obese individuals while on a VLCD; however, it is found to be reversible to the pre-dieting level when dieting includes exercises of sufficient frequency, intensity and duration (Molé 1990). Bosy-Westphal et al. reported a sustained RMR reduction after six months weight loss was observed among subjects who regained weight, while those who maintain weight recovered their expected RMR (Bosy-Westphal et al. 2013).

With all the above factors taken into consideration, a few key strategies for long-term success at weight loss were outlined, which include engaging in high levels of physical activity, eating a diet with calories and fat, eating breakfast, consistent eating pattern, regular weight monitoring, and catching "slips" in weight gain before they turn into larger regains (Wing and Phelan 2005). Maintaining weight loss would surely offer a massive health benefits.

1.3.6 Summary for pros and cons from VLCD

Table 1.1 – Pros and cons of VLCD.

Pro	Cons
• Rapid weight loss. • Improvement in glucose control and reversal of T2DM. • Reduce CVD risks, including lipid profiles and blood pressure. • Reduce cancer risk. • Improve in asthma control.	• Loss of lean mass including muscle mass. • Risk of gallstones formation. • Was associated with sudden death in the past. • Weight regain in the long term.

1.3.7 VLCD and lean mass loss

Whilst VLCD gives a multitude of benefits towards health, like any other caloric restriction, VLCD also causes loss of LM, which includes muscle tissue, as a large proportion of the total weight loss. Larger caloric deficits are also associated with larger LM loss (Chaston et al. 2007; Garthe et al. 2011). Interestingly, despite associated LM loss, Donnelly

et al. demonstrated 10%-17% improvement in muscle strength among subjects that underwent resistance exercise while on 90-day VLCD (Donnelly et al. 1991b, 1993). Krotkiewski et al. showed that VLCD was associated with a significant increase in isokinetic muscle endurance, despite the fact that LM contributed 30% from overall weight loss in their study (Krotkiewski et al. 1990).

Findings on endurance training affecting LM changes are rather contentious. The effects vary from augmentation of LM loss with VLCD (Hammer et al. 1988; Heymsfield et al. 1989), produced no effect (Henson et al. 1987; Van Dale et al. 1987; Phinney et al. 1988; Donnelly et al. 1991b; Jo et al. 2019), and attenuation of LM loss (Pavlou et al. 1985; Hill et al. 1987). Perhaps continuing research in this subject would give clearer guidance regarding the outcomes.

1.4 Skeletal muscle functions, sarcopenia and molecular metabolism

Skeletal muscle, being an important component of LM, is the largest organ in the human body, comprising about 30-40% of the total body weight (Eston et al. 1992; Janssen et al. 2000). Good overall muscle

mass is often associated with masculinity, strength and aesthetics. Beyond its physical appearance, skeletal muscle plays far more important roles in overall health and physiology, including in the regulation of electrolytes such as potassium (Clausen 1986) and calcium (Levy 1999), as well as glucose (Sinacore and Gulve 1993). Skeletal muscle also serves as the largest reserve of protein in the body (Rennie et al. 2004). Protein store, as a form of amino acids storage, can be broken down in times of fasting, infection and acute disease to provide energy to maintain other critical organs to maintain survival. Skeletal muscle accounts for up to 85% of glucose uptake under the influence of insulin and acts as a primary site of insulin resistance in the context of metabolic syndrome, in particular, T2DM and obesity (DeFronzo et al. 1981; Rennie et al. 2004; Bouzakri et al. 2005).

Despite all these important roles it meant to serve, unfortunately, muscle atrophy is an inevitable but somewhat modifiable process that occurs with ageing, referred to as primary sarcopenia (Sayer et al. 2008), or secondary sarcopenia, which resulted from reduced physical activity, immobility, or due to pathological causes, such as organ failure, inflammatory disease, severe infection, malignancy, endocrine problem, as well as malnutrition (Cruz-Jentoft et al. 2010; Rudrappa et al. 2016). Relative muscle mass starts to decrease in the third decade and is more

noticeable at the end of the fifth decade, with an approximate reduction of about 1.9 and 1.1 kg every decade in the men and women respectively (Janssen et al. 2000).

Regardless of the causes, sarcopenia, in essence, has been implicated as a risk factor for various adverse health outcomes associated with frailty such as physical weakness, falls and fractures, immobility, functional decline, disability and loss of independence among the elderly, and to a some extend is associated with increased mortality (Cruz-Jentoft et al. 2010; Batsis et al. 2014). Hence, understanding muscle anabolism and catabolism processes and awareness of factors that can affect muscle mass, particularly during the low nutritional state, is important.

1.4.1 Muscle protein synthesis

Muscle protein synthesis (MPS) is predominantly regulated by the mammalian target of rapamycin (mTOR) signalling cascade. The mTOR is a serine/threonine-protein kinase that integrates a multitude of signalling pathways in growth and proliferation processes via phosphatidylinositol 3-kinase (PI3K) activation on the cell membrane by various stimuli that phosphorylates protein kinase B (AKT), which then activate mTOR (Morita et al. 2015). mTOR comprises of two complexes,

mTORC1 and mTORC2, and as the name indicates, mTOR functions affected by rapamycin, with mTOR1 is potently inhibited by rapamycin acutely (Dos D. Sarbassov et al. 2004; Jacinto et al. 2004), and mTORC2 assembly is inhibited by prolonged rapamycin treatment (Sarbassov et al. 2006). Cascade function in muscle hypertrophy has been demonstrated where the blockade of mTORC1 signalling by rapamycin inhibited muscle hypertrophy with decreased rates of MPS, while the constitutive activation of AKT in vivo results in substantial increases in muscle mass, demonstrating that skeletal muscle is dependent on mTORC1 for cell growth (Bodine et al. 2001b; Ohanna et al. 2005; Drummond et al. 2009).

mTOR boosts cellular proliferation and growth by stimulating translation of specific mRNAs that encode proteins synthesis and acts as a major regulator of energy production in mitochondria by selectively modulating the synthesis of nuclear-encoded mitochondrial proteins. (Yecies and Manning 2011; Laplante and Sabatini 2012; Morita et al. 2013). Close coordination with cellular energy production via mitochondria activation is important as protein synthesis itself is one of the most energy-consuming processes in a cell (Buttgereit and Brand 1995; Rolfe and Brown 1997; Topisirovic and Sonenberg 2011). mTORC1 downstream stimulation of protein synthesis involves

phosphorylation of a plethora of substrates, but the two main effectors of this signalling pathway are the eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and ribosomal protein S6 Kinase (P70S6K) (Morita et al. 2015). Suppression of 4EBP1 and activation of P70S6K cause further cascade reaction that stimulates mRNA translation for protein synthesis that contributes to building block and mitochondria activation.

Whilst both mTORC1 and mTORC2 respond to growth factors, only mTORC1 is controlled by nutrients, such as glucose and amino acids (Jewell and Guan 2013). mTORC1 is activated under nutrient-rich conditions, and its function is blocked under nutrient limiting conditions such as VLCD. Besides protein synthesis, mTORC1 is also involved in lipid synthesis, insulin action, ribosome biosynthesis and autophagy (Laplante and Sabatini 2012). In the presence of nutrient sufficiency, activated mTORC1 suppresses autophagy via phosphorylation of Unc-51 like kinase-1 (ULK1) and autophagy-related protein 13 (ATG13). Both are subunits of the ULK1·ATG13·FIP200 complex, which acts as a node for integrating incoming autophagy signals into autophagosome biogenesis (Ganley et al. 2009; Jung et al. 2009).

Glucose availability and the fluctuation of energy is sensed by AMP-activated protein kinase (AMPK), a crucial cellular energy sensor found in all eukaryotes. Under nutrient deprivation, AMPK activation causes phosphorylating of substrates to inhibit anabolic processes and promote catabolic processes to conserve cell energy (Jewell and Guan 2013). This so-called "metabolic checkpoint" action of AMPK works by blocking mTORC1 activation and signalling through direct phosphorylation of tumour suppressor tuberous sclerosis complexes (TSC1/TSC2) to inhibit cell growth and preserve energy (Mihaylova and Shaw 2011). During the postprandial phase, when the insulin level is increased due to the rise in glucose level, suppression of muscle protein breakdown (MPB) was observed (Wilkes et al. 2009). Insulin and homolog insulin, insulin-like growth factor 1 (IGF-1) stimulate MPS through activation of PI3K-AKT pathway and reduce MPB by suppressing the synthesis of atrogin-1, a protein that plays a key role in muscle atrophy (Sacheck et al. 2004). A meta-analysis by Abdulla et al. found that insulin has a permissive role in MPS in the presence of elevated amino acids (AA) and plays a major role in reducing MPB independent of amino acid AA availability (Abdulla et al. 2016).

Different AA activates mTORC1 at different extent, and withdrawal of most amino acids separately for one to two hours causes inactivation of

mTORC1 signalling at a different degree (Avruch et al. 2009). Interestingly, withdrawal of the AAs leucine and arginine is as efficient as withdrawing total AAs in downregulating mTOR signalling (Avruch et al. 2009). This suggests that leucine and arginine are pre-requisite for mTOR activation by AAs. Besides, AA glutamine is required for extracellular leucine to activate mTOR (Nicklin et al. 2009). The exact mechanism by which AAs affect mTORC1 appears very complex and remains incomplete at present (Manifava et al. 2016). This includes a phenomenon known as 'anabolic' resistance that occurs in older individuals, where the activity of mTOR in response to AA feeding is impaired (Cuthbertson et al. 2004), which could be possibly due to decreased intramuscular expression and activation of AA sensing/signalling proteins in mTOR pathway.

It has been proposed that a greater amplitude of the response of MPS to whey protein ingestion may be due to a greater increase in the concentration of essential amino acid (EAA), in particular, leucine within the blood circulation. West et al. studied whey protein administration as a single bolus or as small pulsed doses every 20 min for three hours and found blood EAA concentrations were greatest in the bolus group shortly after consumption but averaged over the subsequent four hours of examination, with total blood EAA concentrations were equivalent

between the groups (West et al. 2011). Bolus administration was found to be superior in eliciting gains from the increase in MPS. Additionally, bolus administration resulted in greater ribosomal protein S6 (rpS6) phosphorylation, despite similar increases in AKT and mTOR phosphorylation status between the groups. rpS6 is a substrate of the mTORC1-S6K1 axis that regulates cell signalling in glucose homoeostasis, adipocyte metabolism, body mass and energy balance (Magnuson et al. 2012). It was shown previously that a bolus dose of micellar casein (similar digestion kinetics as whey but with a lower leucine concentration) improves MPS to a lesser degree than that of whey (Tang et al. 2009). These findings lead to the conclusion that some degree of leucaemia is important in the stimulation of MPS.

Deptor, a constituent of the mTOR complex, was shown to have a negative regulator of mTOR function (Kazi et al. 2011). When deptor was knocked down, phosphorylation of downstream mTOR targets was increased. Conversely, under catabolic conditions (e.g. sepsis), increased deptor protein levels in association with decreased mTORC1 activity have been observed, suggesting its role in part for the decrease in MPS rate. In keeping with this observation, a 50% decrease in deptor mRNA (deptor knockdown mouse) has been shown to prevent the loss

of muscle mass in immobilised muscle mainly by the preservation of MPS (Kazi et al. 2011).

In addition to nutrients, exercises have also been shown to affect molecular protein synthesis activity. mTOR co-localises with the lysosome in basal conditions and translocate to the cell periphery in response to resistance exercise and, at the same time, TSC2 dissociation from Rheb, which leads to a reduction in TSC2 abundance at the cell membrane, which enhances mTOR kinase activity (Song et al. 2017). Eccentric contractions led to 2 to 8-fold increases in the phosphorylation SK6 (a product of a downstream cascade of mTORC1 protein synthesis pathway) that persisted for two hours into recovery (Eliasson et al. 2006). Cuthbertson et al. reported that plasma amino acids, AKT and S6K phosphorylation could be sustained for up to 24 h following low-intensity exercise (Cuthbertson et al. 2006).

1.4.2 Muscle protein breakdown

The ubiquitin-proteasome system appears to be the main principle for the muscle protein breakdown (MPB); however, the proteasome is incapable of degrading intact myofibrils (Koohmaraie 1992), suggesting

that the myofibril has to be "prepared" before proteasome-mediated breakdown. It has been proposed that the proteolysis of myofibril protein is preceded by the calpain-dependent degradation of proteins composing the z-discs and releasing myofibres from the sarcomere for subsequent degradation by the ubiquitin-proteasome system. This theory is supported by observations that the use of calpain-inhibitors can prevent sepsis-induced MPB despite activation of the ubiquitin-proteasome system (Fareed et al. 2006).

In skeletal muscle, muscle atrophy box (MAFbx; also known as atrogin-1) and muscle RING finger-1 (MuRF-1) are two muscle-specific ubiquitin ligases that regulate protein ubiquitination and degradation. Knockout of either of these ligases has been shown to blunt the loss of muscle mass following denervation-induced atrophy (Bodine et al. 2001a). This is further supported by the findings that atrogin-1 and MuRF1 are upregulated in several muscle atrophy states (Lecker et al. 2004). Interestingly, unlike atrogin-1, MuRF1 may also play some degree in negative regulation of MPS when MuRF1 knockout animals do not exhibit the normal decrease of MPS observed after synthetic glucocorticoid administration (Baehr et al. 2011). Atrogin-1 and MuRF1 are transcriptionally regulated by the Forkhead box class O (FoxO) transcription factors which are sequestered in the cytoplasm and

rendered transcriptionally inactive after phosphorylation by AKT (Sandri et al. 2004; Stitt et al. 2004). This suggested that AKT not only serves as a regulator of MPS but also acts as a point of convergence between MPS and MPB.

Reactive oxygen species (ROS) is known to cause endothelial dysfunction as well as muscle loss. Excessive production of ROS accelerates muscle proteolysis (Powers et al. 2012), and the exposure of ROS to myotubes results in muscle atrophy (Gilliam et al. 2012). During muscle inactivity, mitochondrial ROS production serves as a signalling step to induce mitochondrial dysfunction leading to muscle atrophy (Min et al. 2011; Powers et al. 2011; Talbert et al. 2013). ROS can influence proteolytic pathways via regulation of FoxO transcriptional factors (Dodd et al. 2010), activating the autophagy-lysosome and the ubiquitin-proteasome systems (Levine et al. 2008; Andrianjafiniony et al. 2010; Brocca et al. 2012).

1.4.3 Schematic summary of MPS and MPB

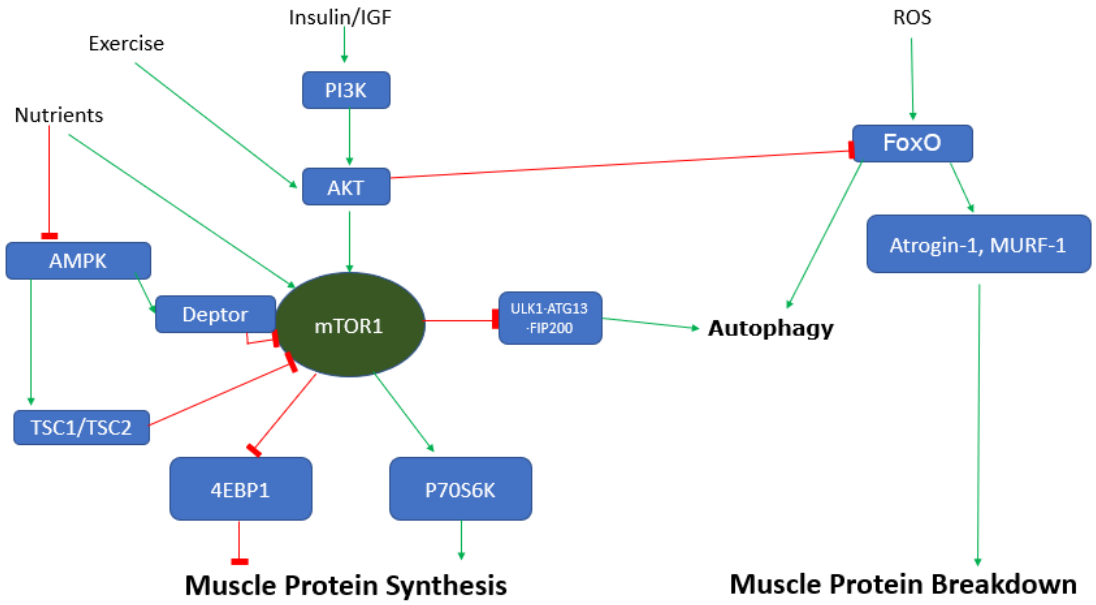


Figure 1.1 - Schematic summary of MPS and MPB. Mammalian target of rapamycin (mTOR), phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT), eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), Ribosomal protein S6 Kinase (P70S6K), Autophagy-related protein 13 (ATG13), Unc-51 like kinase-1 (ULK1), focal adhesion kinase family interacting protein of 200 kD (FIP200), AMP-activated protein kinase (AMPK), tuberous sclerosis complexes (TSC1/TSC2), forkhead box class O (FoxO), muscle atrophy box (atrogin-1), Muscle RING finger-1 (MuRF-1). → represents activation or positive feedback, —▮ represents deactivation or negative feedback.

1.4.4 Minimising muscle loss during the weight-loss period

The complexity of muscle synthesis and breakdown following various stimulants, including diet and exercises, requires careful attention and planning during the weight-loss period. Whilst we recognised the importance of weight loss, we are also aware that weight loss also comes with the risk of muscle loss as part of LM loss (Chaston et al. 2007; Garthe et al. 2011). Therefore, it is crucial to devise plans to preserve muscle mass or reduced muscle loss during the weight-loss period.

Incorporation of RET while on low-calorie intake has been shown to reduce proportion LM loss from 25% to as low as 17% from overall weight loss (Garrow and Summerbell 1995; Weinheimer et al. 2010; Washburn et al. 2014). A more focused study on VLCD showed additional an intensive, high volume resistance training program resulted in the preservation of LM (Bryner et al. 1999). A study by Donnelly et al. also showed a similar proportion of LM loss following VLCD despite additional RET, however, they demonstrated significant muscle fibre hypertrophy in RET group compared to diet only group (Donnelly et al. 1993).

Besides exercise, high protein content in the diet has also been shown to help to minimise LM losses (Barrows and Snook 1987; Layman et al. 2003; Bopp et al. 2008; Mettler et al. 2010). Longland et al. demonstrated that during a marked energy deficit, exercises (aerobic and anaerobic) in combination with high protein supplement at 2.4g $kg^{-1}d^{-1}$ is more effective than 1.2g $kg^{-1}d^{-1}$ in promoting increases in LM (Longland et al. 2016).

1.4.5 Literature on muscle MPS and VLCD

Taking into account the thesis interest in VLCD and the effect of weight loss on muscle, a literature search was performed in January 2017 using a systematic approach to find any research on MPS measurement related to VLCD, using the database from Embase, Medline and PubMed, for all available dates inclusive January 2017. Search terms used were (("very low calor* diet" OR "very-low calor* diet" OR "very-low-calor* diet" OR VLCD OR "very-low-energy diet" OR "very-low energy diet" OR "very low energy diet" OR VLED)) AND (("muscle protein synthesis" OR "muscle synthesis" OR "fractional synthesis rate" OR "muscle anabolism" OR "myofibrillar synthesis")). The search strategy yielded no result from Medline or Embase. PubMed gave nine results; however, none of these was relevant due to the following: studies involving high

protein or amino acid supplement, studies involving exercises, studies on rats, a paper on narrative review on muscle catabolism in sepsis.

1.5 Resistance exercise training

Resistance exercise training (RET) is a potent stimulator of MPS contributing to muscle protein accretion and gains of muscle mass. Studies have been conducted to assess the effect of different loading and repetition of the RET. From assessment on one set versus three sets RET of unilateral leg-extension exercise performed at an intensity of 70% of 1-RM, MPS was found to be significantly greater 5 hours after exercise with multiple sets and remained increased 24 hours later, demonstrating that high-volume exercise can have long-lasting effects on MPS, while a single set of the exercise was only sufficient to increase muscle protein synthesis at 5 hours (at a lesser degree than multiple sets), and MPS rate was found to have returned to basal levels at 24 hours (Burd et al. 2010a).

Further study was conducted to compare intensity and volume of exercise towards MPS, using different groups performing unilateral knee extension exercise at 90% of 1-RM until volitional failure (90FAIL), 30% 1-RM work-matched to 90%FAIL (30WM), and 30% 1-RM performed

until volitional failure (30FAIL) (Burd et al. 2010b). They found that four hours after exercise, mixed MPS was equivalent between 90FAIL and 30FAIL, however, only 30FAIL (reflecting high volume exercise) leading to an increase of myofibrillar protein (represents approximately 70% of mixed muscle protein content) synthesis 24 hours after exercise. MPS at five hours post-exercise in the 30WM group was lower than the other two groups suggesting that immediate MPS is receptive to exercise intensity. Increased in 4EBP1 phosphorylation 24 hours after exercise was observed only in exercise modalities that were performed to failure. Exercise training also increases skeletal muscle mitochondrial enzyme content and activity (Little et al. 2011) and improve mitochondrial function to reverse the sarcopenic process (Jubrias et al. 2001; Melov et al. 2007)

1.5.1 Muscle and LM preservation with RET under general caloric restriction

In the absence of caloric restriction, a meta-analysis demonstrated that consuming supplemental protein during RET can result in an accretion of LM (Cermak et al. 2012). Frontera et al. demonstrated an average 10% increase in cross-sectional area of thigh muscle following 12-week of RET intervention (Frontera et al. 1988). Their study involved elderly age 60-72 years old, performing three sets of eight repetitions at 80%

1-RM, with weekly load adjustment. The strength increases 100% for knee extensor and 200% for knee flexor on isokinetic dynamometer assessment. At a molecular level, Glynn et al. demonstrated a minor suppressive effect on leg MPB from increase serum insulin levels stimulated by carbohydrate and essential amino acid provision after RET (Glynn et al. 2010). Another study showed that rates of MPS increase >200% when protein is added to RET, while protein provision alone only enhanced MPS by ~150% (Biolo et al. 1997).

Under an energy deficit condition, Areta et al. showed that consuming 30 g protein after resistance exercise resulted in greater stimulation of MPS than did the consumption of 15 g protein (Areta et al. 2014). Pasiakos et al. reported that daily protein at twice (1.6 g kg^{-1} d^{-1}) the recommended daily allowance (RDA) (0.8 g kg^{-1} d^{-1}) attenuated the loss of LM during an energy deficit with both aerobic and resistance exercise. Interestingly, three times (2.4 kg^{-1} d^{-1}) the RDA did not have as much impact in minimising relative LM loss when compared to double the RDA protein (1.6 g kg^{-1} d^{-1}). Relative LM losses were 56%, 30% vs 36% for standard RDA, double the RDA and triple the RDA of daily protein, respectively (Pasiakos et al. 2013). Contrary to Pasiakos et al., Longland et al. showed a better outcome using higher protein (2.4 g kg^{-1} d^{-1}) supplement compared to slightly lower protein (1.2 kg^{-1} d^{-1}) supplement

during 4-week 40% calorie reduction (Longland et al. 2016). These differences are primarily due to the intensity of exercise activity and measurement methods, i.e. DXA vs multicomponent body composition models (Lohman and Going 1993).

Additional resistance or endurance training has been shown to be able to attenuate LM loss down to as low as 3% of overall weight loss after a 12-week intervention, compared to diet only group that recorded 30% weight loss in the form of LM (Kraemer et al. 1999). The maintenance of LM also comes with a bonus of an increase in strength of up to 30%. The protocol is described as alternating between heavy day (5-7 RM) and moderate day (8-10 RM) loads, with progressively increasing load and number of sets throughout the 12-week intervention.

Contrary to the above findings, Brochu et al. did not find any significant difference in LM loss between on a diet alone group or diet with RET of 6-month intervention among more than 100 postmenopausal women. Their RET protocol involved a progressive increase in resistance, two to four sets of 10-15 repetitions or 65-80% 1-RM max (Brochu et al. 2009). They divided their training program into 4 phases, where the progress of each phase means progressing in the total workload in the

form of percentage 1-RM, number of repetitions and number of the set (Figure 1.2).

Phase 1:	Introduction to training, 3 weeks, 15repetitions or~65% of maximum, two to three sets per exercise, 90–120 seconds between sets
Phase 2:	5 weeks, 12 repetitions or~70% of maximum, two to three sets per exercise, 90 seconds between sets
Phase 3:	9 weeks, eight to 10 repetitions or ~75–80% of maximum, two to four sets per exercise, 120–180 seconds between sets
Phase 4:	8 weeks, 10–12 repetitions or ~70–75% of maximum, three to four sets per exercise, 60–90 seconds between sets

Figure 1.2 - Progressive training phase protocol (Brochu et al. 2009).

1.5.2 Literature review on VLCD and RET using systematic approach

A systematic review was initially performed to look into the effect of RET incorporation with VLCD at the beginning of the PhD research stage (January 2017). However, due to scarcity of available studies on the subject, this was then changed into a narrative literature review performed using a systematic approach.

A literature search was done on Embase, Medline, PubMed and Cochrane databases for all available dates inclusive January 2017. Search terms used were: ("resistan* exercise*" OR "resistan* training" OR "weight

training" OR "weight exercise*") **AND** ("very low calor* diet" OR "very-low calor* diet" OR "very-low-calor* diet" OR VLCD OR "very-low-energy diet" OR "very-low energy diet" OR "very low energy diet" OR VLED). Search using EMBASE and MEDLINE database was combined via the Healthcare Databases Advanced Search (HDAS), example for the search strategy and results are as in Figure 1.3

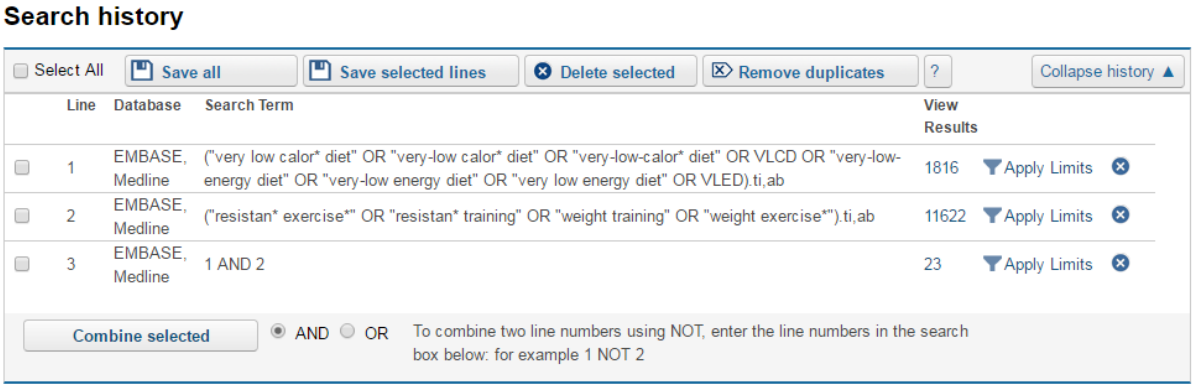


Figure 1.3 - Search strategy and results for RET and VLCD using HDAS.

Studies on VLCD and RET were very limited. Only 23 results came up from EMBASE and MEDLINE, 19 from PUBMED and 9 from Cochrane searches.

Most of the results were excluded due to 1) resistance exercise in a combination of aerobic exercise 2) resistance exercise was done after VLCD interventional period for weight maintenance purpose 3) study involving bariatric surgery 4) study on resistance exercise with modified VLCD (900 – 1100 kcal/d) 5) study involving LCD instead of VLCD 6) follow-up VLCD with behaviour weight therapy

Only one paper available as a full text online (Bryner et al. 1999), while three papers available only in abstracts with no full articles were found despite extensive internet search (Lemons et al. 1989; Pronk et al. 1992; Donnelly et al. 1994). These papers are only available in paper copy and were obtained from The British Library (ordered via University of Nottingham library).

Bryner et al. study on 'Effects of resistance vs aerobic training combined with an 800-calorie liquid diet on lean body mass and resting metabolic rate' involved 20 subjects (17 women and 3 men) incorporated resistance exercise with VLCD in the interventional group, were compared with the control group performing aerobic exercise while on VLCD. The resistance exercise training involved three times per week session with an off day in between each session, involving four lower body and six upper body muscle groups, with progressive weight

increment throughout the interventional period, and subject to maintain a minimal rest period between sets to incorporate a circuit type workout. The diet used in the study provided a total of 800kcal/d, was composed of 40% or approximately 80 g/day protein. The study found that there was no reduction in LM in the intervention group while the control group lost on average 4kg of LM, besides the exercise group showed increasing RMR and improvement in overall muscle strength up to 48% in some muscle groups measured by 1-RM tests.

Donnelly et al. compared between concurrent, delayed and sequential exercise and found that the delayed or sequential use of exercise during VLCD provided only small differences between groups, and any clinical significance is unknown (Donnelly et al. 1994). A study by Pronk et al. suggested that the combination of RET and VLCD increased strength despite a loss of fat-free mass (FFM), unlike the other groups (VLCD alone, VLCD + endurance, VLCD + resistance + endurance). The increase in strength is likely due to improved central neuromuscular function rather than muscular hypertrophy in light of reduced FFM (Pronk et al. 1992).

Lemons et al. demonstrated similar findings with weight loss and body composition patterns in diet and diet plus isotonic RET groups, but the

efficiency of residual FFM increased only in the exercise groups as shown from strength gains/kg body weight (Lemons et al. 1989). The summary of these studies is tabulated on the next page (Table 1.2).

In conclusion, although very limited, there are pieces of evidence to support the use of RET as a strategy to attenuate LM loss during VLCD intervention, as well as improving muscle strength during the weight-loss period with VLCD.

Table 1.2 - Summary of literature review on VLCD with RET

Study	N, gend	Age (y)	Daily cal, duration	Inclusion criteria	Groups and protocol	Outcomes
Bryner et al. 1999	17 F 3 M	Mean 38	800 kcal/d 12 weeks	No involvement in a regular exercise or weight loss program for at least 6 months	Randomised into control+VLCD (C+D) or resistance exercise plus VLCD(R+D) C+D group exercised 1 hour four times/week by walking, biking or stair climbing R+D group performed resistance training 3 days/week at 10 stations, increasing from two sets of 8 to 12 reps of estimated 8RM to four sets by 12 weeks.	Significant VO_2max increased, equally in both groups. Total weight decreased significantly more in C+D group. C+D group lost a significant amount of LM (51 to 47 kg). No LM decrease in R+D group. RMR increased in R+D, decreased in C+D.
Donnelly et al, 1994	115 F	NA	520kcal/d 12 weeks	Obese female	Non randomised into 6 groups: control (C), endurance exercise (EE), weight training (WT), endurance plus weight (EEWT), control 4 weeks, then EE (C4EE), WT for 4 weeks then EE (WT4EE). EE for 4d/w progressed from 20 to 60 mins/d using treadmill walking and stationary bicycling. WT for 3d/w using 5 weight lifting exercises 6-8 reps at 70-80% 1-RM.	Weight loss did not differ between the group. WT4EE has the lowest FFM loss (8.1%, 9.7%, 11.4% vs EE, C4EE and C, respectively). Significant increase in strength index for WT, EEWT, WT4EE.

Pronk et al. 1992	109 F	Avr 41	520kcal/d 90 days	Severely obese women Age range 19-70	4 groups: VLCD alone (LC), VLCD + endurance exercise (EE), VLCD + endurance + resistance training (EERST), VLCD+ resistance training (RST). Exercise sessions 4 times/week. EE using treadmill walking, stationary cycling, stationary rowing, aiming RPE 13 and 70% heart rate reserve, duration from 20min at the beginning and 60min at the end of the 90-day programme. RST 2 set of 6-8 reps of 1-RM, gradually increased to 3 sets of 6-8 reps of 80% 1-RM.	EERST group has the lowest FFM loss 3.26kg vs 4.79kg in LC vs 4.64kg in RST. RST was the only treatment that increased upper and lower body strength. No differences between groups were found for body mass losses, decreases in per cent fat and fat mass. VO_2max increased by 2,5,8 and 18% for LC, RST, EE and EERST, respectively.
Lemons et al. 1989	60 F	19-73	402kcal/d for 8w + 1500kcal/d for 8w	Women age 19-73 year	6 groups: Diet only (D), diet+ isotonic resistance (DW), diet+ bicycle ergometer (DB), isotonic resistance only (W), bicycle ergometer only (B), a group undertaking diet and 6w bicycle followed by 8w resistance (DB/W). Bicycle ergometer- 3/week, 20mins/session, at 18kph with HR target 150bpm. Isotonic resistance – 3/week, 6 muscle groups, started with 3x 10reps 60-70% 1-RM, then gradually to 4x 2reps 95% 1-RM.	Greatest mean weight loss is in DB/W group 13.1kg. Mean weight gain 2.6kg in W group and 0.1kg in B group. Increase in % of weight as FFM (due to more fat loss), no significant difference between the group. Significant increase in strength/kg body weight in DW and DB/W (37 and 41% respectively).

1.5.3 RET and VLCD summary

Although the findings of RET with VLCD on LM are somewhat variable, mainly due to different protocols between different studies, there are pieces of evidence to generally support the safety of the process and incorporation of the training regime to help mitigate LM or muscle loss during the caloric deficient period.

1.6 High-intensity interval training

High-intensity interval training (HIIT), as the name suggests, involves intense work periods ranging from 5 seconds to 8 minutes long, aiming to reach 80% to 95% of a person's estimated maximal heart rate, with recovery periods may last longer than the work periods, usually aiming for 40% to 50% of a person's estimated maximal heart rate, the workout continues with the alternating work and recovery periods totalling 20 to 60 minutes (Kravitz 2011). HIIT training can be tailored to fit people with different fitness levels using various exercise modes, including running, cycling, swimming, cross-training, and in various exercises classes. HIIT workouts provide similar fitness benefits as continuous endurance workouts but in shorter periods (Kravitz 2011; Kong et al. 2016).

HIIT has been shown to be better and more effective in reducing central obesity compared to moderate-intensity continuous training (MICT) –thrice the fat mass loss with half the time for exercises, i.e. 20 minutes for HIIT and 40 minutes for MICT for each session, two sessions per week for 16 weeks (Maillard et al. 2016). A similar benefit was also observed by Sabia et al. that found 3% fat mass reduction after 16 week HIIT compared to 2% fat mass reduction in low-intensity aerobic predominance exercise, with a similar 2% increase of FFM in both groups (Sabia et al. 2004). Following 15-week HIIT training vs 20-week endurance training (ET), Tremblay et al. found that the HIIT program induced a more pronounced reduction in subcutaneous fat despite shorter time intervals and much lesser total energy cost; 120.4 MJ for ET and 57.9 MJ for HIIT (Tremblay et al. 1994). When corrected for energy cost, the conclusion is that the HIIT program induced nine times greater subcutaneous fat loss than the ET program.

Various studies have also shown improvement in the maximum rate of oxygen consumption (VO_2max) in HIIT compared to MICT (Hwang et al. 2011; Bacon et al. 2013). Mean decreases in systolic blood pressure and diastolic blood pressure was recorded with HIIT of 12

and 8 mmHg, respectively, in comparison with continuous training subjects, who obtained non-significant decreases of 4.5 and 3.5 mmHg (Molmen-Hansen et al. 2012). Other studies have also demonstrated benefits of HIIT in the form of improvement in the overall fat profiles and fat oxidation (Smith-Ryan et al. 2016; Lazzer et al. 2017), glycaemic control, pancreatic β-cell function (Madsen et al. 2015), insulin sensitivity (Tjønna et al. 2008; Babraj et al. 2009), eNOS expression in cardiac muscle (Fallahi et al. 2016), antioxidant enzyme activity (Criswell et al. 1993), stimulation of mitochondrial biogenesis and GLUT4 expression (Ojuka 2004), exercise compliance (Jung et al. 2015) as well as mental health (Wu et al. 2015).

1.6.1 Literature review on VLCD and HIIT using a systematic approach

A literature search using a systematic approach was performed at the beginning of the PhD research stage (January 2017) on Embase, Medline, PubMed and Cochrane databases for all available dates inclusive January 2017. Search terms used were ("high-intensity interval training" OR "high intensity interval training" OR "high-intensity interval exercise*" OR "high intensity interval exercise*" OR HIIT OR HIT Or HIIE OR HIE OR "anaerobic exercise" OR

"anaerobic training") **AND** ("very low calor* diet" OR "very-low calor* diet" OR "very-low-calor* diet" OR VLCD OR "very-low-energy diet" OR "very-low energy diet" OR "very low energy diet" OR VLED). Search using EMBASE, and MEDLINE databases was combined via the Healthcare Databases Advanced Search (HDAS). Examples of the search strategy and results are as in Figure 1.4**:**

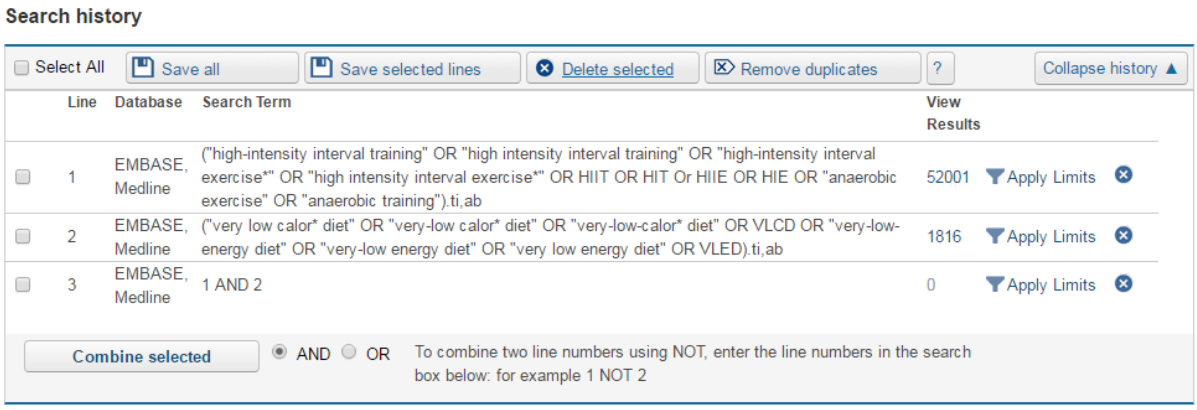


Figure 1.4 - Search strategy and results for HIIT and VLCD using HDAS.

Unfortunately, no result came up from EMBASE, MEDLINE and Cochrane. A similar outcome when "Aerobic Interval Exercise" OR "Aerobic Interval Training" were used instead of HIIT. PUBMED returned 295 results, down to 163 when restricted to humans. However, after screening through the titles and abstract, none are

related to HIIT and VLCD. All were excluded as these were studies involving moderate-intensity exercise instead of HIIT, in combination with aerobic exercise, vitamin D trial, Mediterranean diet, β-Hydroxy-β-Methylbutyric free acid supplementation.

1.6.2 HIIT under general caloric restriction

Whilst there is no study incorporating HIIT with VLCD, there were several studies for HIIT under general caloric restriction. Under a moderate calorie restriction, Lazzer et al. found that additional HIIT helped with weight loss, but no benefit was seen in retaining LM among adolescents (Lazzer et al. 2017). Under a 14-day carbohydrate caloric-reduced diet setting, the group on a diet combined with HIIT showed increased aerobic capacity, preserved LM when compared to a diet-only group, with both groups showed improvement in insulin sensitivity (Sartor et al. 2010). For LM preservation, a diet with the HIIT had an improvement of 48 kg baseline to 49 kg post-intervention, while diet only group showed LM reduction from 47 kg to 46 kg.

1.6.3 HIIT and VLCD summary

In conclusion, at the beginning of the research stage in January 2017, there was no study combining VLCD and HIIT. Studies incorporating HIIT with other general hypocaloric setting was very limited and showed the possibility that incorporation of HIIT could benefit LM retention during VLCD.

1.7 Chapter conclusion

VLCD can help to induce significant weight loss, with approximately a quarter of the loss comes from LM, which includes muscle mass. A decline in muscle mass is an ongoing ageing process but can get worse under inappropriate low nutrition settings. On a molecular level, a low nutrient can affect the mTOR pathway causing impairment in MPS, while exercises help to increase mTOR signalling pathway. Up until the start of the research project, there was no study looking into MPS rate in the VLCD setting. A few studies have been done to find strategies to mitigate muscle loss during VLCD, including RET and endurance training. However, we found no study incorporating HIIT with VLCD. With the scarcity of research on this topic, we believe this research project comparing VLCD with or without concomitant RET or HIIT could provide valuable insights and

add new knowledge on how MPS rate, LM (which includes muscle mass), cardiovascular status and metabolic markers evolve following 6-week interventions. The rationale for the duration of the study is discussed in Chapter 2 under the subheading '2.3.6.1 Rationale for the duration of intervention'.

We recognised that the different interventions would cause a degree of differences in caloric deficit due to different amounts of energy expenditure from exercises (RET or HIIT) compared to diet alone, which means daily net energy availability would not be similar between the groups. Hence it is important to highlight that the measurement of the intervention outcomes has taken into account this factor, i.e. the overall effect of diet with or without exercises on extra calorie burn towards physical and physiological changes. The rationale for this is to assess the overall effects of the diet and exercises, including the differences in the caloric deficit, instead of the effect of the exercises alone, which means daily net calories should be balanced between the groups.

1.7.1.1 Research questions and hypotheses

Primary research question:

1) Are there differences in MPS rate between VLCD, VCLD with RET and VLCD with HIIT groups?

   We hypothesised that groups with exercise integration would have a statistically higher MPS rate compared to diet-only group, with no difference in MPS rate between the exercise groups (VLCD with RET or HIIT). Study design and results on this theme can be found in Chapter 3 under the 'Muscle protein synthesis' subheading.

Secondary research questions:

1) Are there any significant changes in total weight, fat mass (FM) or LM following interventions within each group? Are there any differences in these parameters between the groups?

   We hypothesised that every group would have a significant loss in total weight, FM and LM following interventions. Diet groups with concomitant exercises would have statistically higher total weight and FM loss, with less LM loss compared to the diet-only group, with no difference between the exercise

groups. Study design and results on this theme can be found in Chapter 3 under the 'DXA' subheading.

2) Are there any significant changes in muscle structure and function following interventions within each group? Are there any differences in these parameters between the groups?

   We hypothesised that diet only group would have reduced muscle thickness on ultrasound scan (USS) of the vastus lateralis (VL) muscle, while groups with concomitant exercises would maintain muscle thickness, which is statistically different compared to the diet-only group. We hypothesised that groups on diet-only and on diet with HIIT would not have a change in muscle strength in the form of 1-RM, while the group on diet with RET would gain strength with significant difference compared with the other two groups. Study design and results on this theme can be found in Chapter 3 under the 'Vastus lateralis USS thickness', '1-RM' and 'Maximum voluntary contraction' subheadings.

3) Are there any significant changes in insulin function and lipid profiles following interventions within each group? Are there any differences in these parameters between the groups?

   We hypothesised that every group would have an improvement in insulin indices, HbA1c and lipid profiles. Diet

with exercise groups would have better improvement compared to the diet-only group in these parameters, with no difference between the exercise groups. Study design and results on this theme can be found in Chapter 4.

4) Are there any significant changes in cardiovascular function and cardiopulmonary fitness following interventions within each group? Are there any differences in these parameters between the groups?

   We hypothesised that every group would gain improvement in blood pressure, flow-mediated dilatation, microvascular flow, and cardiopulmonary exercise test following interventions. We hypothesised that diet with concomitant exercise groups would have better outcomes in these parameters compared to the diet-only group, with no difference between the exercise groups. Study design and results on this theme can be found in Chapter 5.

Chapter 2: General methods

2.1 Chapter synopsis

This chapter focuses on the choices of investigation or tests modalities to aid in the assessment of various aspects of outcomes following a 6-week very low-calorie diet (VLCD) with or without with and without concomitant resistance exercise training (RET) or high-intensity interval training (HIIT). We discussed in detail the rationale for each investigation being chosen for this research project. With the understanding of how the investigations or tests work, we then explained the study protocol to ensure correct timing and techniques were used for the data collection before and after interventions to allow for comparison to be made between the groups.

2.2 Investigation modalities and rationale of choice

2.2.1 Fractional synthesis rate for muscle protein synthesis and muscle protein breakdown

Direct incorporation of tracers such as labelled-leucine or labelled-phenylalanine has traditionally been used to measure protein synthesis. The underlying principle is that by providing a known amount of labelled-tracer and unlabelled amino acid (tracee), the tracer and tracee will mix with the endogenous pool and become incorporated into protein over time, and the rate of new protein synthesis can be calculated. The tracer and tracee are generally administered as a bolus followed by continuous infusion to maintain stable enrichment or as a flooding method where a supraphysiological dose of the bolus of tracer and tracee is provided over seconds to minutes. Protein synthesis is calculated by measuring the enrichment (tracer/tracee) of the protein against the enrichment of the precursor (i.e., precursor: product labelling ratios) (Gasier et al. 2010).

Whilst the above method is rather invasive, requiring intravenous administration of tracer, an alternative approach to calculate protein synthesis without the need for continuous infusion is available by using deuterium oxide (D_2O). This was first described by Hans Ussing back in 1941(Ussing 1941). Ussing demonstrated that by providing D_2O to mice and rats, the rate of protein renewal could be determined by the incorporation of D atoms, as D-labelled amino acids into protein in various organs, including the liver, kidney, skeletal muscle, as well as specific proteins such as myosin. When the D_2O was provided to an animal and the concentration was maintained, then the D would accumulate into protein over time, allowing for the calculation of the protein synthesis rates. This occurred by the transfer of D with the H of amino acids through transamination reactions, which would then be incorporated into protein (Ussing 1941).

While labelled amino acids must gain entry into the cell via transporters, D-labelling of carbon-bound hydrogens of amino acids (e.g., alanine) occurs intracellularly (Previs et al. 2004; Dufner et al. 2005). While all amino acids can become D-labelled at their α-carbon positions (Busch et al. 2006), alanine undergoes rapid turnover (Yang et al. 1984) and possesses four potential sites (α - and β-

hydrogens) for D-labelling to occur via transamination (Oshima and Tamiya 1961), thus allowing for better detection. Moreover, the D-labelling of the α-hydrogen is approximately equal to that of D-labelled water, and the labelling of total hydrogen is ~3.7 times that of D-labelled water (Dufner and Previs 2003; Katanik et al. 2003). The carbon-bound hydrogens on alanine do not undergo exchange during protein hydrolysis hence minimising labelling gradients between D-labelling of body water and alanine (Previs et al. 2004; Dufner et al. 2005). As a result, the ratio of the precursor (D-labelling of body water) to the product (either the D-labelling of the α-hydrogen or total hydrogens of protein-bound alanine divided by "n", the number of incorporated deuteriums) labelling can be calculated. As there may be differences between species, it is recommended for direct measurement of "n" when examining the rate of protein renewal in different species, i.e. number of incorporated D_2O is ~3.7 in mice (Bederman et al. 2006), rats (Dufner et al. 2005) and humans (Previs et al. 2004), and ~3.8 in fish (Gasier et al. 2009a).

Another important point is the lack of influence that feeding has on D-labelling gradients. For instance, provision of a supraphysiological bolus of unlabelled alanine (2 g·kg body mass-1) to rats following 90

min of D_2O administration showed that the labelling of the α - and the total hydrogens stable for hours (Dufner et al. 2005). (Belloto et al. 2007) et al. reported that feeding did not affect the kinetics of normal plasma protein or plasma protein bounded to D-labelled alanine in either the portal or peripheral circulation, suggesting that feeding does not result in dilution of the precursor and that the D-labelling of body water can be used to as an accurate representation of D-labelled protein-free alanine. Additionally, exercise was reported not to influence D-labelling gradients, as seen in D-labelling of body water between exercised and sedentary cage control animals (Gasier et al. 2009b). Even in a disease state, such as chronic kidney disease where fluid retention commonly occurs, no differences between the D-labelling of the α-hydrogen of alanine and that of body water were observed (Previs et al. 2004).

Our research unit had previously validated the use of stable D_2O as a stable isotope tracer to monitor changes in MPS (Wilkinson et al. 2014). This involved collection of saliva samples following D_2O consumption, blood samples collection and serial muscle biopsies (at known time frame). The samples were then processed accordingly as previously described in the paper to help in calculating body water and plasma D_2O enrichment, measurement of incorporation of D into

protein-bound alanine of the muscle. These values then can be used to calculate the fractional synthesis rate (FSR) of various proteins using the following equation:

$$FSR(\%/day) = [(APEAla)]/[(APEP) \times t] \times 100$$

where APEAla = deuterium enrichment of protein-bound alanine, APEP = mean precursor enrichment over time, and t is the time between biopsies.

FSR would be a valuable method to assess changes in muscle protein synthesis following weight loss from 6-week VLCD and allow comparison between groups. A more detailed technical and practical aspect of muscle biopsy and FSR measurement is available in Chapter 3 under the 'Study design' subheading.

2.2.2 Dual-energy X-ray absorptiometry

Dual-energy X-ray absorptiometry (DXA) is a special imaging modality that utilises two x-rays beams filtering and near-perfect spatial registration of the two attenuations (Shepherd et al. 2017a). The whole body can be scanned to measure bone mass and as well as whole-body tissue composition, including FM and LM which

includes muscles (Laskey 1996; Kelly et al. 1998). DXA differentiates fat and lean mass (LM) by detecting the differences in attenuation factors between these tissues (Segal et al. 2009). While computer tomography (CT) and magnetic resonance imaging (MRI) provide better accuracy for muscle or fat assessment, they are not as cheap and accessible in comparison to DXA, which run at a much lower cost than MRI and involves minimal radiation exposure compared to CT (Heymsfield et al. 2014).

There are also other advantages to using DXA as a modality for the assessment of body composition. Firstly, using simple algebra and the physical properties of the body materials that the two X-ray attenuations pass through, the mass of different materials can be calculated (Wahner et al. 1999). Secondly, DXA can assess regional body composition by dividing the body into specific areas, e.g., legs or arms, using specific lines generated using dedicated software. Thirdly, DXA is stable for many years, where the measurement of stability and accuracy in body composition assessment can be verified using phantoms, a quality control and cross-calibration (Shepherd et al. 2017a). Fourthly, DXA has also been shown to have excellent repeatability of whole-body scan in supine position with Pearson r values of 0.99 to 1.00 (Lohman et al. 2009).

There are a few limitations of DXA that needed to be mentioned. DXA is unable to differentiate between glycogen, water and lipid molecules stored within the muscle with muscle tissue itself, causing DXA to misinterpret loss of glycogen, water and inter/intramuscular fat as part of LM loss, hence overestimated the loss of LM or muscle mass following caloric restriction (Levine et al. 2000; Toomey et al. 2017). When analysing fat changes in the body (after a weight-loss period), it is important to make a distinction between steatosis within the myocytes (intramyocellular or intramuscular lipids) and the lipids in between the myocytes (extramyocellular or intermuscular lipids) (Miljkovic and Zmuda 2010). Whilst this difference can be made with both CT and MRI (Larson-Meyer et al. 2006), DXA has the disadvantage to overestimate the total muscle mass as intramuscular adipose tissues are mistakenly calculated as muscle (Levine et al. 2000).

Acute change in hydration status could also lead to whole-body miscalculation of body LM (Levine et al. 2000); hence adequate hydration status needs to be appropriately assessed during DXA measurement.

Large body habitus causes a few issues for DXA scanning: The length, width and rated load capacity might be exceeded. Table dimensions initially were designed to fit the primary market for DXA systems: bone density measurements of the spine and hip, where it is not necessary to fit the entire body on the table. Steadily over the years, the systems were improved to support higher weights and size with current specifications for widely used systems able to accommodate more than 200kg. For the assessment of body composition, ideally, the entire body should be scanned. However, in the event that the subject whole body does not fit the table width, instead of increasing the table size, which would make the unit more expensive, clever vertical and horizontal offset scanning techniques were devised using matching analysis techniques to piece together the whole complete body from partial scans. For example, a very wide participant will have offset horizontally such that in one scan, and all of the body can be estimated even though one of the arms, legs, or both were not acquired. This method is called offset scanning in general. On Hologic systems, it is known as reflection mode, and on GE systems, it is known as mirror mode. Rothney et al. demonstrated that mirrored scanning showed no significant difference in whole-body results when compared to standard scans

(Rothney et al. 2009). Shepherd et al. showed the reflection method could be used to exclude regions with motion artefacts in infant scanning (Shepherd et al. 2017b). For a tall subject that cannot fit in the scan field, a combination of two scans can be acquired: one where the feet are included at the expense of excluding the head, and a second partial scan from the head down through the torso. The head values then can be manually substituted into the first scan (Silva et al. 2013).

Another disadvantage of DXA is the exposure of subject and operator to ionizing radiation, but the dose is very small to both; the effective radiation dose from a single whole-body DXA is about 0.0084 mSv (Damilakis et al. 2010) which is less than the background from a transatlantic flight (Public Health England 2011).

Taking into consideration the above advantages and disadvantages, DXA would serve as a valuable modality to assess body composition changes. Hence, we opted to utilise this modality in our 6-week VLCD with and without concomitant resistance exercise training (RET) or high-intensity interval training (HIIT). The practical aspect of DXA measurement for the study is discussed in great detail in Chapter 3 under the 'Study Design' and 'DXA' subheadings.

2.2.3 Oral glucose tolerance test for insulin sensitivity and resistance

Insulin is a key regulator of glucose homeostasis and promotes efficient glucose utilization (Accili 2004). Impaired glucose tolerance is related to insulin resistance (IR), primarily due to genetic and environmental factors (Singh et al. 2010). IR has been described as a condition where a greater than normal amount of insulin is required to obtain a quantitatively normal glucose level (Berson and Yalow 1970). IR is known to play a major role in the pathophysiology of T2DM and is a hallmark of obesity, dyslipidaemias, hypertension, and other components of the metabolic syndrome (Olefsky and Saltiel 2000; Grundy et al. 2004). IR has also been associated with subclinical or clinical cardiovascular disease in both people without T2DM (Laakso et al. 1991; Bressler et al. 1996; Howard et al. 1996) and people with T2DM (Inchiostro et al. 1994; Bonora et al. 1997).

IR preceded many disease manifestations; hence it is important to identify and treat individuals with insulin resistance as early as possible, as this can remain undiagnosed for a long period (Wilcox

2005; Courtney and Olefsky 2007). Compensatory hyperinsulinemia is associated with early IR, and chronic hyperinsulinemia increases the risk of the development of other components of the metabolic syndrome and consequent diseases (Yeni-Komshian et al. 2000). Prompt recognition and management of this risk of metabolic syndrome offer important preventive measures before the disease process becomes apparent, leading to detrimental complications (Rao 2001).

Among the tools to measure whole-body insulin action and IR, the hyperinsulinemic-euglycemic clamp technique remains the gold standard for assessing IR in humans (Tam et al. 2012). As this technique is invasive and requires insulin infusion and repeated blood sampling, there is a need for simple, accessible measures for the evaluation of insulin sensitivity.

A mathematical model of the normal physiological dynamics of insulin and glucose produced the homeostasis model assessment to estimate IR (HOMA-IR) and β-cell function from simultaneous fasting measures of insulin and glucose levels (Matthews et al. 1985). The quantitative insulin sensitivity check index (QUICKI), derived from logarithmically-transformed fasting plasma glucose and insulin

levels, have a good correlation with insulin clamp (r=0.78) for insulin sensitivity assessment (Katz et al. 2000; Mather et al. 2001). The area under the curve for insulin and glucose concentration from the oral glucose tolerance test (OGTT) is of particular importance as this measures the variability in the amount of insulin required to maintain a certain blood glucose concentration and is easily understood to reflect insulin sensitivity (Potteiger et al. 2002). Matsuda and DeFronzo proposed an index that estimates hepatic and muscle insulin sensitivity and correlated well to clamp-derived insulin sensitivity (r = 0.73) (Matsuda and Defronzo 1999).

Cederholm and Wibell came up with an insulin sensitivity index that represents mainly peripheral insulin sensitivity and muscle glucose uptake, reflecting the dominant role of peripheral tissues in glucose disposal after an oral glucose load (Cederholm and Wibell 1990).

Following a 6-week VLCD with or without concomitant RET or HIIT, assessment using different insulin indices would offer valuable information to compare benefits in different groups in regards to improvement in insulin function. The technical and practical aspect of blood collection for OGTT and insulin indices calculation is discussed in great detail in Chapter 4 under the 'Study design' and

'Oral glucose tolerance test using arterialised venous sample' subheading.

2.2.4 Contrast-enhanced ultrasound

"Microvascular unit" is defined as a terminal arteriole and the group of capillaries it supplies (Emerson and Segal 1997). It is a functional organisation of muscle microvasculature where a single terminal arteriole giving rise to 12-20 capillaries, each capillary of 0.5-1 mm long runs along toward collecting vessels, which means the microvascular flow is axial with capillaries and parallel to muscle fibres (Bloch and Iberall 1982; Delashaw and Duling 1988). Two-thirds of capillaries are empty while the skeletal muscle is at rest, with blood flow around 3 mL min^{-1} 100 g^{-1}, which is equivalent to only 3–5% of the flow in visceral organs (Honig et al. 1982; Sweeney and Sarelius 1989; Frame and Sarelius 1993; Sarelius 1993). In response to hyperaemic stimuli such as feeding or exercise, terminal arterioles dilate, leading to a rapid increase in capillary perfusion and thus the fraction of tissue volume filled with blood within the microvasculature, which reflects microvascular blood volume (MBV) (Segal 2005).

Postprandial insulinaemia, for example, stimulates capillary muscle recruitment, doubling MBV (Rattigan et al. 1997; Vincent et al. 2004; Sjøberg et al. 2011) and increased surface area for insulin, glucose, and amino acids (AA) to diffuse out of the circulation into contact with myocytes, aiding in glucose disposal and protein anabolism (Wilkes et al. 2009). Remarkably, changes in MBV can also occur with only a minor change in total limb blood flow (Krogh 1919; Honig et al. 1980; Sweeney and Sarelius 1989). However, while the intensity of the hyperaemic stimulus increases, blood velocity and limb flow increase but only a little change in capillary density perfusion (Segal 2005).

Contrast-enhanced ultrasound (CEUS) is a technique that involves the measurement of the acoustic backscatter of intravascular capsule-stabilized gas-filled microbubbles and enables the assessment of the MBV in real-time, as well as the velocity of blood flow within the microvasculature known as microvascular flow velocity (MFV) and the product of both MBV and MFV which is microvascular blood flow (MBF) that represent units of volume of blood per volume of tissue per unit time (Wei et al. 1998). While CUES is generally used in diagnostic imaging, its use in a research setting is starting to gain attention in the past few decades (Quaia

2005). Several recent research has utilised CEUS to address aspects of muscle microvascular function in response to exercise (Dawson et al. 2002; Krix et al. 2010), insulin (Vincent et al. 2002; Clerk et al. 2004) and peripheral vascular disease (Womack et al. 2009; Amarteifio et al. 2013).

A range of microbubbles techniques have been used, with most reporting only included MBV measurement. Sjøberg et al. recently described a method combining intermittent high mechanical index (MI) pulses to achieve microbubble disruption to aid in the measurement of MFV, with real-time, continuous low MI recording that allowed precise assessment of MBV (Sjøberg et al. 2011). Mitchell et al. applied Sonovue™ to assess changes in human muscle MBF, demonstrating a biphasic response to amino acid ingestion and reveal an early postprandial capillary recruitment phase that is followed by later increases in limb muscle bulk flow (Mitchell et al. 2013). Similarly, Dawson et al. demonstrated that microvascular flow precedes the increase in total muscle blood flow following hyperinsulinaemia (Vincent et al. 2002).

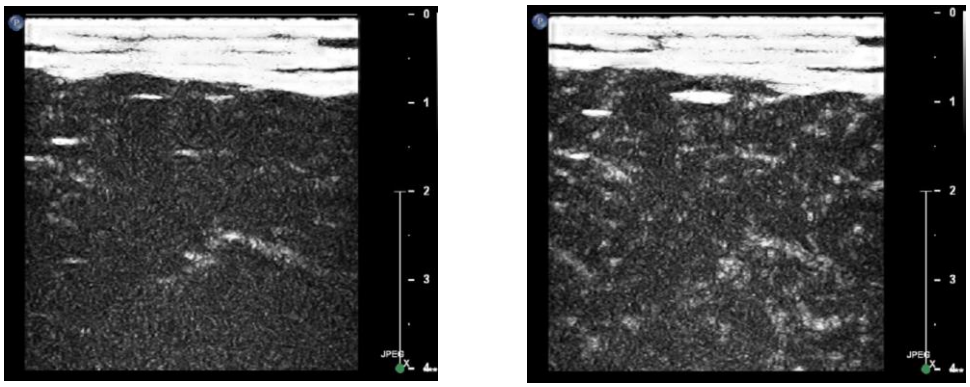


Figure 2.1 – CEUS images. The left image shows microbubbles concentration at rest, while the right image shows microbubbles concentration following acute stimulation- (in this study, it was knee extension exercise.

A recognised limitation of the CEUS technique is the poor reproducibility, which may be due to changes in transducer orientation and day-to-day physiological variation with measurements on different individuals' skeletal muscle or adipose tissue may reach 100-fold differences within the same study (Emanuel et al. 2020). Moreover, USS techniques are operator-dependent, which can contribute to intra- and inter- researcher variation (Emanuel et al. 2020).

Taking into account advantages and bearing in mind the limitations of CEUS, we planned to adopt the technique to assess changes in

MBV, MFV and MBF in the participants following weight loss from VLCD, with or without concomitant HIT or RET, and allow between groups comparison. A more detailed technical and practical aspect of CEUS measurement is discussed in Chapter 5 under the 'Study design' and 'Contrast-enhanced ultrasound' subheading.

2.2.5 Flow-mediated dilation

Vascular endothelial cell plays a crucial role in cardiovascular health, particularly as a structural component of the vasculature by producing components of the extracellular matrix such as glycosaminoglycans and fibronectin (Sato et al. 1987; Alley et al. 2014), as well as in the regulation of vessel tone, inflammatory processes, antithrombosis, and anticoagulation. Endothelial cells cause vasoconstriction mainly through endothelin effect, while vasodilation is mediated by nitric oxide (NO), prostacyclin, and endothelial-derived hyperpolarizing factor (EDHF) (Moncada et al. 1977; Ignarro et al. 1987; Yanagisawa et al. 1988; Ozkor et al. 2011). Chemicals that directly affect the endothelium to cause vasodilatation are termed endothelium-dependent vasodilators. Acetylcholine, for example, stimulates muscarinic receptors on endothelial cells, leading to the opening of ion channels and increase

in intracellular calcium concentration, activation of nitric oxide synthase, which contributes to the synthesis of NO, which cause vasodilation. Other chemicals that affect vasodilation without the involvement of the endothelium are called endothelium-independent agents. For example, nitroglycerin activates soluble guanyl cyclase and cyclic guanosine-3',-5'-monophasphate (cGMP), which affect protein kinases regulating intracellular calcium concentrations and causes vasodilation in the vessel wall (Linke et al. 2006).

NO is one of the key molecules in maintaining adequate arterial dilatations (Loscalzo and Welch 1995). Multiple recognised factors can reduce the bioavailability of vascular NO, including obesity (Frisbee and Stepp 2001; Naderali et al. 2001) and oxidative stress from hyperglycaemia (Ruderman et al. 1992; Bohlen and Lash 1993; Bagi et al. 2004) and hypertension (Jaap et al. 1994; Ungvari et al. 2003). Insulin resistance and obesity are also known to be associated with an increased vascular production of reactive oxygen species (ROS), which normally contribute to the inactivation of endothelial NO (Erdös et al. 2002, 2004).

Long term blood flow abnormality can lead to injury to the artery and causes endothelial damage and dysfunction. Endothelial

dysfunction is associated with the impairment of any of these mediators and is an important step in atherosclerosis. Endothelial dysfunction is associated with various clinically important conditions such as coronary artery disease, hypertension and diabetes mellitus (Williams et al. 1996; Neunteufl et al. 1997; Taddei et al. 1997; Suwaidi et al. 2000; Perticone et al. 2001b). Importantly, even in individuals without diagnosed cardiovascular disease, endothelial dysfunction serves as a predictive factor of future cardiovascular events (Suwaidi et al. 2000; Halcox et al. 2002; Schindler et al. 2003).

As endothelial cells play a significant role in clinical health, having a method to assess their functions would be very useful. A non-invasive in vivo method, called flow-mediated vasodilation (FMD), was developed by Celermajer et al. for quantifying endothelial dysfunction (Celermajer et al. 1992). In essence, changes to arterial blood flow cause shear stress that triggers ion channels in the endothelium via a second-messenger cascade and activates endothelial nitric oxide synthase (eNOS) which generate NO. NO diffuses across the cell membrane to the neighbouring smooth muscle cells (SMC), activating signals within the SMC that lower intracellular calcium concentration and affecting vasodilatation

(Thijssen et al. 2011). Following vasodilatation, the diameter of the artery lumen increases, leading to an increase in blood flow. FMD, however, does not demonstrate the maximum vasodilation of the vessel, which normally involved the use of nitroglycerin (Anderson et al. 1995). The effect of FMD may be abolished with the administration of an NO synthase inhibitor such as mono-methylarginine (L-NMMA) (Doshi et al. 2001).

Celermajer et al.'s work involved the use of high-resolution B-mode ultrasound to assess the change in artery diameter before (baseline) and after ischaemia period (reactive hyperaemia). For the procedure, a human subject rests supine, and the diameter of the brachial artery is measured in a longitudinal plane. A blood-pressure cuff is used to produce ischemia in the limb for 4-5 minutes, followed by the release of the blood pressure cuff, and the diameter of the artery is measured again. The rapid change in shear stress stimulates NO, which leads to vasodilation. The increase in the brachial artery diameter after the period of ischaemia signify the endothelial function. Various studies show that percentage changes in FMD following reactive hyperaemia can be used to predict cardiovascular ischaemia or cardiovascular events in patients with

known cardiovascular disease (Anderson et al. 1995; Neunteufl et al. 2000; Gokce et al. 2002, 2003; Brevetti et al. 2003).

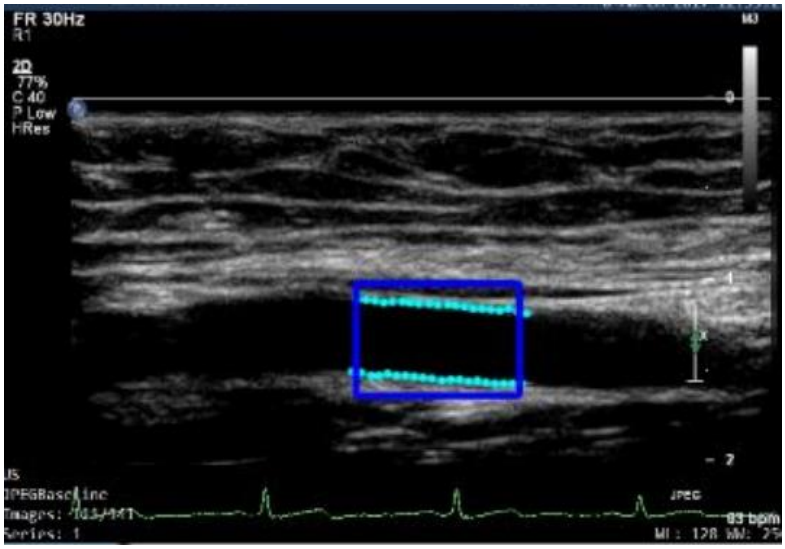


Figure 2.2 – Example of FMD, the diameter of the artery is measured before and after a few minutes of ischaemia induced using the blood pressure cuff.

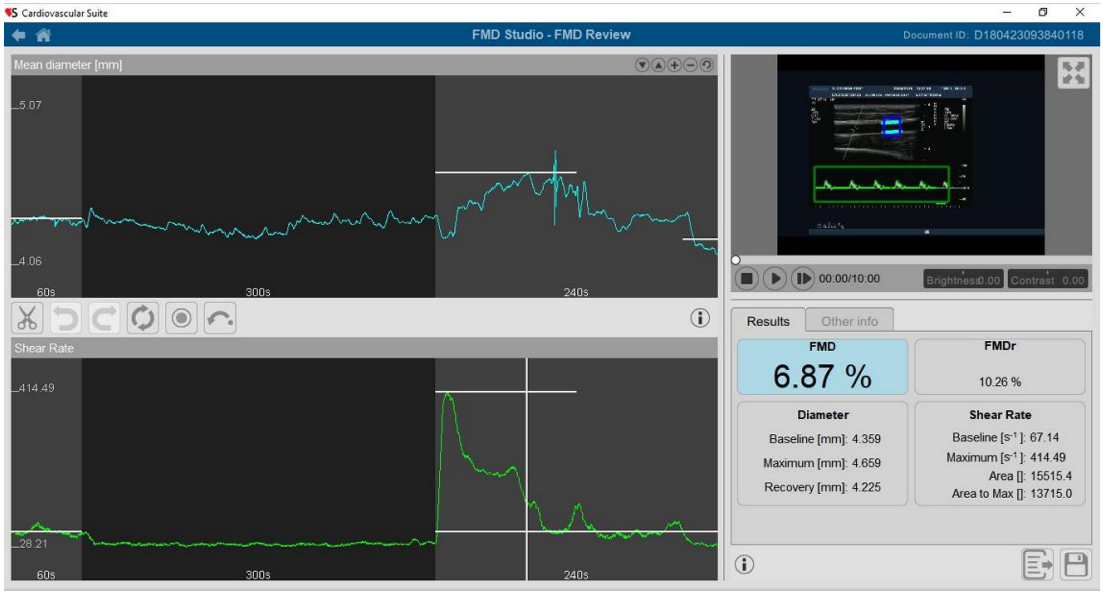


Figure 2.3 – Example of measurement of the change in arterial diameter using the real-time recording for the FMD calculation.

There are limitations in the measurement of percentage FMD changes, particularly a high degree of within-subject variation and inter-operator variability (De Roos et al. 2003). Other factors such as cigarette smoking, antihypertensive medications, time of day, and fasting state can affect the FMD changes. Additionally, technical factors such as cuff position in relation to the site of measurement and the duration of occlusion have been shown to affect the measurement (Berry et al. 2000; Betik et al. 2004).

Reproducibility of FMD is relatively good, with the intra-session coefficient of variation around 9.9% and the inter-session coefficient of variation around 12.9% (Ghiadoni et al. 2012). For standardisation between different research units, guidelines have been published that describe the current consensus (Corretti et al. 2002; Thijssen et al. 2011).

Taking into consideration the advantages and limitations that FMD has, we planned to utilise the modality to help to assess changes in endothelial function within and between groups following a 6-week VLCD with or without exercises. The technical and practical aspect of FMD is discussed in great detail in Chapter 5 under the 'Study design' and 'Blood pressure and flow-mediated dilatation' subheadings.

2.2.6 Ultrasound scan of vastus lateralis muscle

CT and MRI are known to facilitate the accurate quantification of muscle masses (Heymsfield et al. 2014). MRI is regarded as the gold standard in clinical and research setting for skeletal muscle imaging to accurately assess muscle mass (Ross et al. 1995, 1996; Cruz-Jentoft et al. 2010); however, these modalities are expensive, often inaccessible and, in the case of CT, involves radiation. With these in

mind, ultrasound scan (USS) has been advocated as a potential reliable tool for the quantification of skeletal muscle mass in young and older healthy volunteers (Narici 1999; Reeves et al. 2004; Abe et al. 2015) as well as in clinical setting, such as in intensive care patients (Bunnell et al. 2015; Parry et al. 2015; Cho et al. 2017; Dias et al. 2017). Previous studies reported a positive relationship between muscle thickness (MT) measured by USS and lean mass measured by DXA (Takai et al. 2013, 2014), MT and anatomical cross-sectional area (ACSA) measured by MRI (Abe et al. 1997), and MT and muscle volume (MV) measured by MRI (Miyatani et al. 2002, 2004; Sanada et al. 2006) at a single time point. MT derived from USS has also been found to be a useful marker of muscle growth following resistant training, demonstrated as a positive correlation between MT and myofibrillar protein synthesis (expressed in terms of fractional synthesis rates) after just three weeks (Brook et al. 2015) and four weeks (Franchi et al. 2015) of training. Although USS repeatability has often been questioned, with appropriate operator training, measures can be highly reproducible, with intraclass correlation coefficient (ICC) ranging between 0.85 and 0.99 (Reeves et al. 2004; Strasser et al. 2013; Franchi et al. 2018). It should be acknowledged that, despite USS reproducibility, when comparing MT measurements obtained from a single plane to MV measurements,

change in MT might not fully explain the changes in MV. Earlier cross-sectional studies comparing ultrasound-based measurements of muscle size to MRI derived muscle volumes showed that MT is related to MV with ~80% variance (Miyatani et al. 2002, 2004).

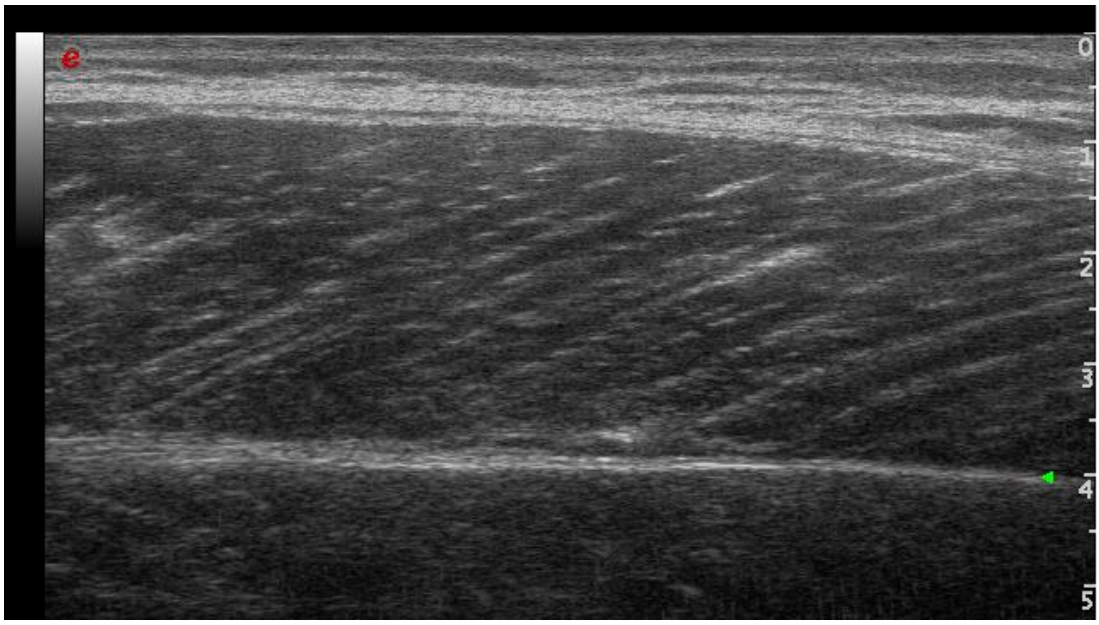


Figure 2.4 – Example of an ultrasound image of vastus lateralis muscle for muscle thickness measurement.

With the above factors taking into account, we utilised USS of the vastus lateralis (VL) muscle to assess changes in muscle thickness pre and post VLCD with or without exercises. The technical and practical aspect of the USS is discussed in great detail in Chapter 3

under the 'Study design' and 'Vastus lateralis muscle USS' subheadings.

2.2.7 Cardiac echocardiography

Two-dimensional (2D) echocardiography (ECHO), M-mode ECHO, Doppler ECHO, and 3D ECHO are the techniques used to assess cardiac function, particularly left ventricular (LV) function. Early validation studies in LV systolic function were mostly done using transthoracic echocardiography (TTE), whilst transoesophageal echocardiography (TEE) has gained more attention only in the past few decades.

LV systolic function can be assessed using changes in the LV dimensions and volumes between the systole and diastole phases. The normally used calculations include Fractional shortening (FS), Fractional area change (FAC), Ejection fraction (EF), stroke volume (SV) and cardiac output (CO).

Of all, FS is the simplest way to measure LV function. Using M-mode, aligning the cursor just at the tip of mitral leaflets, perpendicular to

the inferior wall and passing through the centre of the LV cavity will give an M-mode trace. The measurement of LV diameter is taken just below or at the tip of the mitral valve leaflets, either basal or mid-papillary level, during diastole and systole. This will help us to calculate the FS of LV at that plane by the following equation.

FS = LVIDd – LVIDs/LVIDd × 100%.

Where LVIDd = LV internal diameter at end-diastole and LVIDs = LV internal diameter at end-systole.

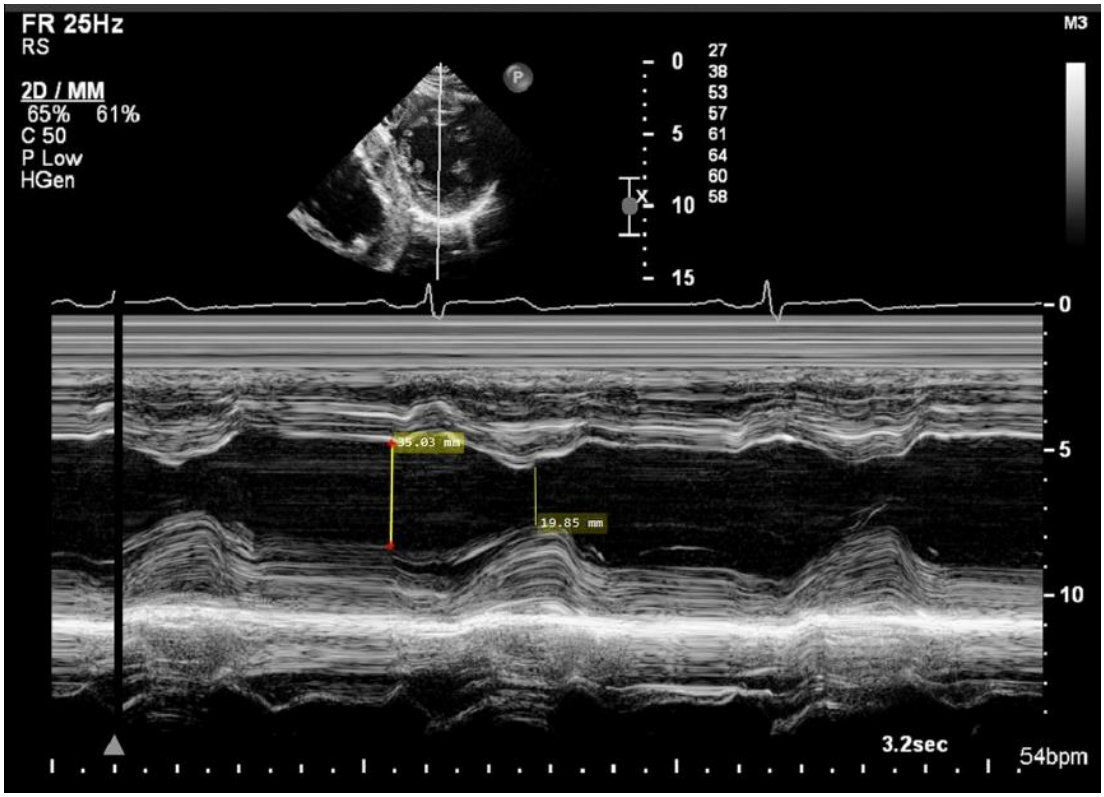


Figure 2.5 – Example of ECHO image for the fractional shortening calculation.

Mechanical systole usually lags behind the electrical systole, hence the measurement of LV dimension ideally is done at or immediately before the peak of the R-wave in QRS complex for LVIDd, and the LV dimension at the end of T-wave in is taken for LVIDs. This is applicable for all 2D and M-mode measurements (Chengode 2016).

Data on repeatability and reproducibility FS is limited. One paper suggested that intra- and interobserver variability for assessing cardiac systolic function using longitudinal FS were 6.2±3.5% 7.8±5.6%.

ECHO would help us to assess basic cardiac function pre- and post-intervention, mainly from the left ventricular ejection fraction aspect, to reflect the safety of VLCD. The technical and practical aspect of the ECHO is discussed in great detail in Chapter 5 under the 'Study design' and 'Echocardiogram' subheadings.

2.2.8 Cardiopulmonary exercise test

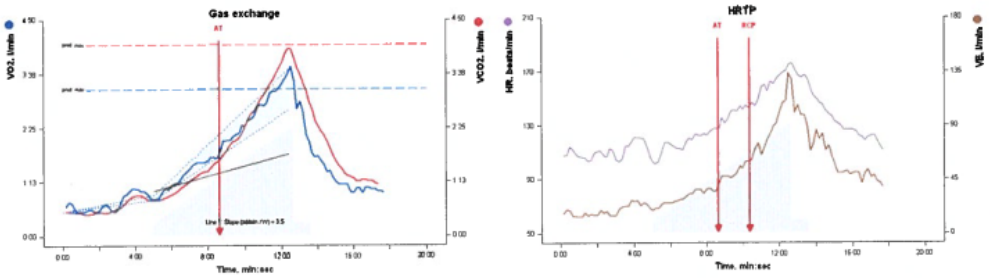
Cardiopulmonary exercise testing (CPET) provides an assessment of the fitness level involving the pulmonary, cardiovascular, and

skeletal muscle functions (Albouaini et al. 2007). It is increasingly being used in a wide spectrum of clinical applications to help with assessment exercise intolerance and for the objective determination of functional capacity and impairment, as well as in research settings to assess changes in overall fitness level following a period of intervention (Balady et al. 2010).

Both bicycle and treadmill protocols have been used for CPET. Optimal exercise duration for fitness assessment on the bicycle is usually aimed between 8 and 17 minutes (Buchfuhrer et al. 1983). Bicycle work is quantified in watts (W). The rate of workload progression is somewhat not informed, depending on the clinical unit or research settings. The initial workload can vary between 20–25 W, with a 15–25 W increment every minute until maximal exertion is reached.

Figure 2.6 –Ergometer bike for CPET.

CPET involves measurements of respiratory oxygen uptake (VO_2), carbon dioxide production (VCO_2), and ventilatory volume. Ventilation and respiratory gas parameters measurement rely on breath-by-breath analysis techniques using a non-rebreathing valve that is connected to a mouthpiece at the end of an airtight mask to prevent mixing of inspired and expired air. The mask with the expired breath is connected to oxygen and carbon dioxide gas analysers. Respiratory volumes are calculated by measuring the airflow signals over the time of inspiration and expiration, and average minute volumes are derived from the breath-by-breath data multiplied by the respiratory rate.



Parameter	Unit	Rest	AT	AT % Pred	AT % MAX	MAX	MAX % Pred	Pred.	Status
Time	min:sec	0:00 - 5:03	8:38	-	-	12:04 - 12:37	-	-	-
Load	W	0	132	61	52	252	116	217	High
HR	beats/min	110	130	75	75	172	99	174	Normal
HRR	beats/min	64	44	-	2428	2	-	< 10	Normal
O_2Puls	ml/beat	6	15	86	74	20	116	17	-
SBP	mmHg	151	179	94	86	209	110	170 - 210	Normal
DBP	mmHg	109	96	113	81	119	140	80 - 90	High
V_{O_2}	l/min	0.67	1.89	62	56	3.40	111	3.05	High
V_{O_2}/kg	ml/(kg*min)	6.2	17.5	52	56	31.5	93	33.8	Normal
V_{CO_2}	l/min	0.58	1.69	43	43	3.95	99	3.98	Normal
RER	-	0.86	0.90	77	77	1.16	100	1.07 - 1.25	Normal
V_E/V_{O_2}	l/l	24.8	22.6	60	66	34.2	91	37.6	Normal
V_E/V_{CO_2}	l/l	28.8	25.3	87	86	29.4	102	28.9	Normal
V_E	l/min	18	44	28	37	120	74	161	Low
BR	l/min	142.4	116.3	-	284	41.0	-	> 15.0	Normal
Bf	1/min	15	15	36	46	32	77	42	Low

Figure 2.7 - Example of CPET summary.

VO_2 is determined by cellular O_2 demand up to a level that equates to the maximum rate of O_2 transport. When VO_2 increases following increasing workload, one or more of the VO_2 transport mechanism approach limitations (e.g., stroke volume or heart rate), and VO_2 rate may begin to plateau, this plateau in VO_2 has traditionally been used as VO_2max. VO_2 can increase from a resting value of about 3.5

$ml.kg^{-1}.min^{-1}$ to around 30–50 $ml.kg^{-1}.min^{-1}$ with exercise, and in some athletes, the value can be over 20 times their resting values, i.e. up to 80 $ml.kg^{-1}.min^{-1}$ (Albouaini et al. 2007).

Anaerobic threshold (AT) is an index used to estimate fitness in cardiopulmonary exercise capacity. During the initial phase of CPET, when it is around 50–60% of VO_2max, expired ventilation (VE) increases linearly with VO_2, which reflects aerobic production CO_2 in the muscles. Blood lactate levels do not change significantly during this phase since muscle lactic acid production is minimal due to aerobic respiration. At the later stage of the exercise, anaerobic metabolism kicks in as O_2 supply cannot match with the increased metabolic requirements of exercising muscles. Anaerobic respiration causes a significant increase in lactic acid production in the muscles hence the blood lactate concentration. The VO_2 at the onset of the blood lactate accumulation is called the lactate threshold or the AT. This is when minute ventilation and VCO_2 rate increases disproportionately more relative to VO_2; a response normally occurs at 60–70% of VO_2max. A commonly used method to assess AT is the V-slope method, where the AT can be determined at the point at which the slope of the relative rate of increase in VCO_2 is more than the rate of increase in VO_2 rate (Schneider et al. 1993).

CPET offers a reasonable repeatability value with a coefficient of variation (CV) for VO_2max between 5 to 13% and 4 to 11% for $Watt_{max}$ with ICC over 0.86 (Bingisser et al. 1997; Skinner et al. 1999; Decato et al. 2018).

In our research project involving VLCD with or without RET or HIIT, we CPET would enable us to compare changes in cardiopulmonary fitness following the different interventions and allow comparison between groups. The technical and practical aspect of the CPET is discussed in detail in Chapter 5 under 'Study design' and 'Cardiopulmonary exercise test' subheading.

2.3 Study design

2.3.1 Ethical approval and consent

All volunteers gave written informed consent to participate in the study. The study was approved by the Faculty of Medicine and Health Science University of Nottingham Research Ethics Committee (Ethics Reference No B12092016) and conformed to the Declaration of Helsinki.

2.3.2 Sample size calculation

Based on previous research data on MPS rate differences between groups (Wilkinson et al. 2014; Brook et al. 2015, 2016; Franchi et al. 2015), a power calculation to detect α of 0.05 and β of 0.85 with a minimum detectable effect of 0.2, suggested that we need ten per group. We thus aimed to recruit 36 individuals (12 per group), taking into account possible drop-outs.

2.3.3 Recruitment

The study was aimed to recruit healthy, overweight or obese males with a BMI 27-50 $kg.m^{-2}$, aged 30-60y (inclusion criteria).

Advertisements were made via local directories (Mickleover Directories), posters at GP surgeries around Derby, local libraries and gyms, as well as Facebook advertisements, targeted to the defined group within 15 miles Derby radius. Interested respondents were invited to attend our research unit for a detailed in-person explanation regarding the study and were provided with a participant information leaflet (PIL). Once agreed to proceed, they were further screened for suitability for the study, which involved updated height

and weight measurement, medical history, physical examination, fasting blood tests (full blood count, urea electrolytes, liver function test, thyroid function test, glucose, HbA1c, lipid profiles and coagulation) and electrocardiogram (ECG). Respondents that have abnormal clinical or laboratory tests were excluded (see 2.3.3.1 Exclusion criteria), and those who were suitable were recruited as participants for the research programme. They were contacted a few days afterwards once the blood test results were ready to decide on the start date.

Overall, 96 people contacted our team to discuss research participation. 46 were excluded as they did not fit inclusion criteria or fell into exclusion criteria, 19 were unable to proceed due to time and work commitment. Overall, 31 participants fit the inclusion criteria and were recruited, but only 26 participants completed the programme. One participant changed his mind and did not start the baseline study day at all, and four participants dropped out after two to three weeks starting the intervention. Of these four, one was from the diet with RET, and three were from the diet-only group. The main reason was the inability to cope with the intense VLCD regime.

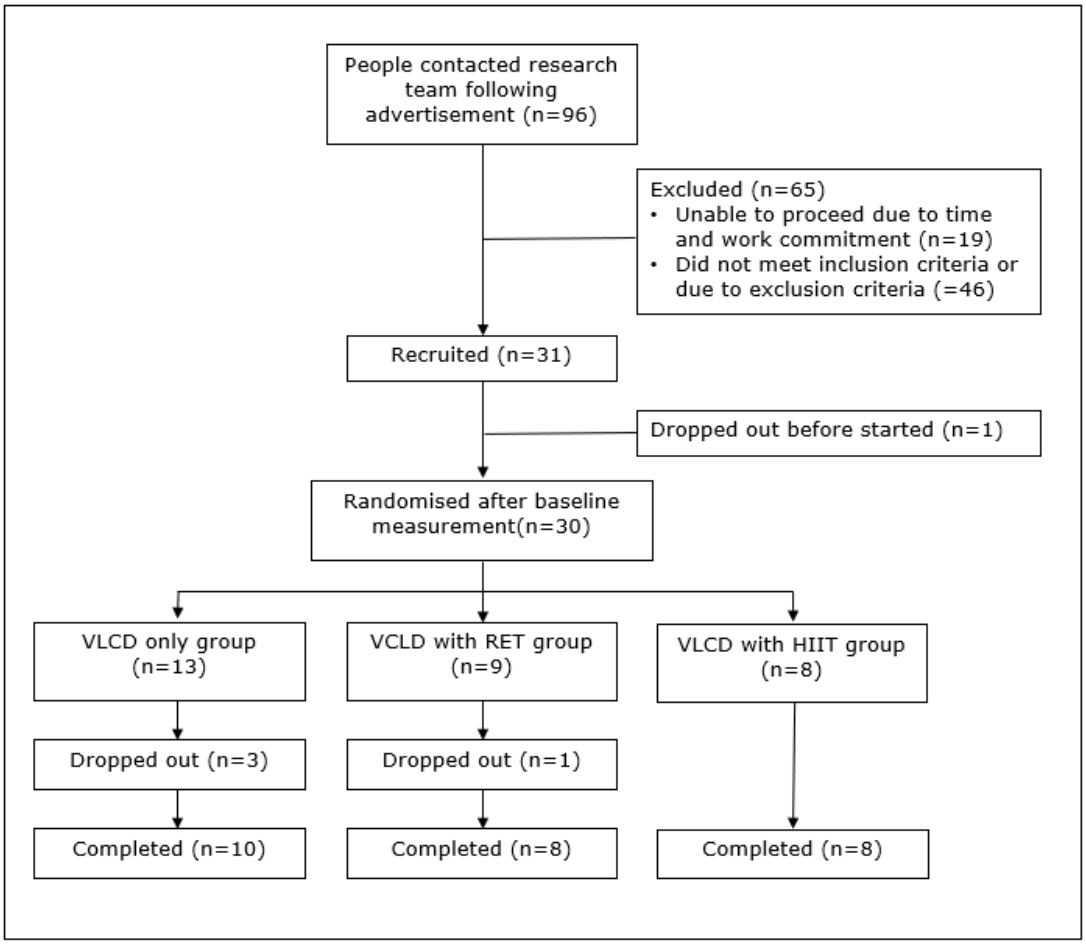


Figure 2.8 – Consort diagram of recruitment number, from initial contact until completion.

2.3.3.1 Exclusion criteria

- Participation in a formal exercise regime (2 hours, 2 times or more per week).
- Active cardiovascular disease: uncontrolled hypertension (BP > 180/110), angina, heart failure (class III/IV), arrhythmia, right to left cardiac shunt, recent cardiac event. Also include family history of early (<55y) death from cardiovascular disease.
- Cerebrovascular disease: previous stroke, aneurysm (large vessel or intracranial), epilepsy.
- Respiratory disease: pulmonary hypertension, COPD, severe uncontrolled asthma.
- Diabetes mellitus.
- Active inflammatory bowel or renal disease.
- Malignancy.
- Clotting dysfunction.
- Musculoskeletal or neurological disorders.
- Known sensitivity/ allergy to contrast Sonovue.
- Any other ongoing acute or significant chronic medical condition that is not mentioned above.
- Weight >120kg (weight limit for DXA scan)

2.3.4 Randomisation

List of groups; 1) VLCD only (VLCD+O); 2) VLCD with resistance exercise training (VLCD+R); 3) VLCD with high-intensity interval training (VLCD+H) were generated by our research assistant using an online tool Sealedenvelope™ https://www.sealedenvelope.com/.

The list was kept away from the researchers involved in the recruitment process to avoid bias in group allocation, which can be done by changing the order of the start date for a different participant. The participants will be allocated to the group on the list right after completing all the pre-intervention investigations. The reason that the allocation was done at this stage is to avoid bias on the baseline investigation as the intervention could not be blinded once group allocation is done.

2.3.4.1 Baseline demographics

The total number of participants were 10 in VLCD+O, 8 in VLCD+R and 8 in VLCD+H. Ages at baseline were 46y±3, 40y±3, 46y±4 for VLCD+O, VLCD+R, VLCD+H, respectively (mean±SEM). Mean baseline weights were 103.8kg±3.9, 98.4kg±3.4, 101.0kg±4.3, and

mean BMI were 32kg.m^{-2}±1, 32kg.m^{-2}±1, 33kg.m^{-2}±1 in similar respective order.

From ethnicity aspect, VLCD only (VLCD+O) had eight Caucasians and two Asians participants; VLCD with resistance exercise training (VLCD+R) had three Caucasians, four Asians, and one Hispanic participant; VLCD with high-intensity interval training (VLCD+H) had six Caucasians and two Asians participants.

2.3.5 Study day visits

Participants were asked to fast from midnight (except clear water) before attending STUDY DAY-1. On this day, baseline investigations including fasting specimen (blood glucose, saliva, muscle and fat) samples collection, flow-mediated dilation (FMD), oral glucose tolerance test (OGTT), DXA scan, contrast-enhanced ultrasound (CEUS), leg blood flow (LBF), and cardiac echocardiogram (ECHO) were performed. A standard lunch was provided in the afternoon before a cardiopulmonary exercise test (CPET) was carried out to assess cardiopulmonary fitness and to determine wattage level for the HIIT training (for participants in diet with HIIT group). $Watt_{max,}$ i.e., maximum wattage a participant was able to reach during the CPET, was determined during this session, using gradual increment

in wattage over a period of time as described in detail in section 5.3.5 Cardiopulmonary exercise test. More detailed technical and practical aspects of all the investigations are available in separate chapters corresponding to the outcome measured (Chapter 3, Chapter 4 and Chapter 5 under 'Study Design' subheading).

A loading dose of D_2O and D3-creatine tracer solutions were then given for participants to drink on-site and began 24h-urine collection. Before leaving, participants were supplied with 3-Methylhistidine (3MH) tracer solution to be taken two days later, plus daily 'top-ups' of D_2O, which is based on body weight. Saliva bottles for daily saliva collection (3 hours after daily D_2O top-up, to be stored in the fridge) and containers for urine collection for D3-creatine (daily urine sample collection for three days in containers, started with 24-hour urine collection, then spot urine at 30h, 48h and 72h) for muscle mass assessment were provided.

After consuming 3MH on the designated day, the subject then re-attended our unit the following day for STUDY DAY-2 in the morning, fasted from midnight (can only drink clear water). During this study day visit, hourly blood was taken for 6 hours to measure 3MH level (first sample to be taken after 18 hours 3MH ingestion). Urine

collection every two hours were also collected. An ultrasound scan (US) of the vastus lateralis (VL) of the dominant leg was performed, and baseline muscles strength were assessed. This involved maximal voluntary contraction (MVC) of knee extension and one-repetition maximum (1-RM) assessment for supine chest press, latissimus pull-down, seated row, leg curl, knee extension and leg press. A more detailed technical and practical aspect of 1-RM and MVC assessment is discussed in Chapter 3 under the 'Study Design' subheading.

3MH tracer, muscle and fat biopsies will be repeated mid intervention (end of week three or early week four). All investigations performed at the baseline were repeated after completion of the 6-week intervention.

These measurements will allow us to study the temporal aspects of adaptation to VLCD and training, both in terms of metabolism, cardiovascular function and muscle structure, architecture and glucose uptake. The blood samples will allow us to explore the systemic features of physiological changes following our VLCD with or without exercises.

For an overview of the study protocol, please see Figure 2.9.

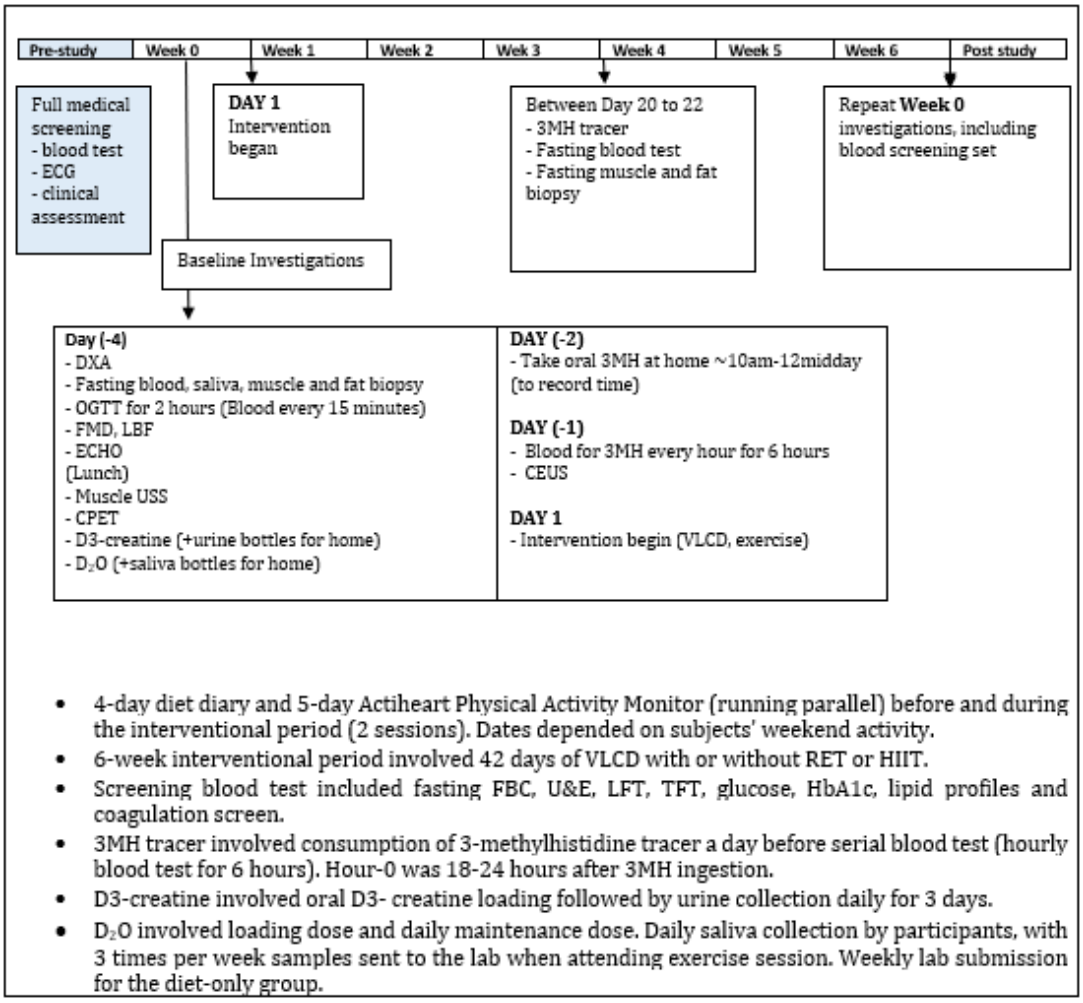


Figure 2.9 - Schematic protocol of study investigations.

2.3.6 Interventions

2.3.6.1 Rationale for the duration of intervention

As previously discussed in the introduction chapter, the duration of VLCD is deemed to be safe if used less than three months (which is roughly equivalent to 12 weeks). We also learned from previous experience that changes in MPS could be seen within six weeks

period (Brook et al. 2015, 2016). Taking into account the above factors, and considering the intensity of the interventions with the target age group that are mostly going to be busy with work and family commitment, we decided that a 6-week of interventions would be tolerated by most participants and give us enough time to see any significant changes if any to be seen.

2.3.6.2 VLCD diet

We used meal replacement formulas from the LighterLife® (LighterLife), which has previously shown good outcomes for weight loss (Rolland et al. 2009). The meal replacement formulas comprised of a powder mix, ready-to-drink blend, and instant soup, all of which contained roughly balanced macronutrients (35% protein, 50% carbohydrate, 15% fat) and micronutrient composition per product, providing about 150 to 160 kcals per product. Participants consumed four products a day, giving a total of 600 kcals, which consist of 55 g of protein, 75 g of carbohydrates, 12 g of fat, and more importantly, 100% of the daily requirement for vitamins and minerals. Participants also were allowed another 200kcal in the form of fruits, vegetables or hot drinks. The daily calorie was aimed to be less than 800kcal as per VLCD widely accepted definition.

LighterLife meal replacements that were available for participants in this study were: Banana shake, Chocolate shake, Porridge, Salted Caramel shake, Strawberry shake, Vanilla shake, Scotch Broth soup, Shepherd's Pie, Spaghetti Bolognese, Vegetable soup, Cranberry & Raspberry, Crispy Peanut, Nut Fudge, Toffee.

Figure 2.10 – Examples of LighterLife meal replacement products.

2.3.6.3 Exercises protocol

All participants received a 6-week intervention period of VLCD, and those randomised into exercise groups also had additional RET or HIIT depending on the group they were in. Diet only group (VLCD+O) continued on the diet alone intervention for the 6-week duration while maintaining the same level of physical activity they normally would have had if not on the research programme.

VLCD with RET group (VLCD+R) had resistance training to include supine chest press, latissimus pull-down, seated row, leg curl, knee extension, and leg press. Each exercise type comprised of two sets of 12 repetitions at 70% 1-RM for all muscle groups, with two minutes rest between sets after a prescribed warm-up, for three sessions per week (American College of Sports Medicine 2009; Phillips et al. 2012). Muscles strength were reassessed every two weeks to ensure that the intensity of training remains constant relative to the progressive increase in strength throughout the 6-week program.

In the group with diet and HIIT (VLD+H), there were three sessions per week, involving 60-sec high-intensity cycling at 95-125% $Watt_{max}$ ($Watt_{max}$ was determined during the study day visit as per

section 2.3.5), with 90 seconds recovery, repeated for five times, started with a two-minute warm-up and ended with a two-to-three-minute cooldown. Wattage was gradually increased throughout the six weeks period when heart rate failed to achieve 85% of the predicted maximum heart rate during the intensity interval, or when participants reported a value of ≤8 on a modified Borg Rating of Perceived Exertion (RPE) Scale [0-10] (Borg 1998) after completing the 5th interval. This protocol has previously shown significant improvement in cardiometabolic markers following six weeks of intervention (Phillips et al. 2017).

As previously discussed in the introduction chapter, we recognised the effect of additional exercise towards total energy expenditure, meaning more caloric deficit among participants in the exercise groups compared to the diet-only group. It is our intention to assess the overall effect of exercises, including extra deficit in daily calories toward physical and physiological changes among participants.

2.3.6.4 Safety measurement during exercises

All RET sessions were directly supervised by one of the research team members (could be a different person on a different day) that are fully trained with the exercises and competent with basic life-

support. For the HIIT session, there was additional clinician cover (medically trained professional) due to the intensity of the exercise, requiring continuous ECG interpretation, oxygen monitoring and regular blood pressure check. A second member of staff (in addition to the direct supervision) was always in close proximity to the exercise training facility, and a direct-access phone was available in the exercise training room so that additional staff could be contacted if needed. Due to the nature of the building in the Derby Hospital, all staff members of the Clinical Physiology group could reach the exercise training facility within one minute from their standard place of work (office or lab). A full resuscitation trolley, an automated defibrillator and oxygen were always readily available if needed. Exercise training was to be terminated immediately if a subject complains of:

- Chest pain or tightness,
- Faintness,
- Sudden pallor,
- Loss of co-ordination,
- Confusion,
- Significant dizziness,
- Signs of respiratory failure, or

- Excessive palpitations

In addition, for HIIT, the session would be terminated if blood pressures drop by greater than 20mmHg from the previous reading, or systolic reading more than 250mmHg, or diastolic reading more than 120mmHg.

2.3.6.5 Visits and compliance monitoring

Baseline nutritional intake was recorded in a 4-day diet diary, and physical activity (5-day including weekend) were monitored using Actiheart physical activity monitor before the start of interventions. Unfortunately, we found that the details being recorded on the baseline 4-day diet diary were less reliable than we were hoping for (some participants forgot to enter the food intake, illegible handwriting).

Another Actiheart monitor was used midway through the intervention. The date for the Actiheart monitor depended on the subject's weekend activity as the monitoring should include routine weekend activities.

To help to maintain diet compliance among participants in the VLCD control group (who had no regular exercise visits), they were contacted via the phone one to two times per week to remind them of the diet and saliva collection.

Participants in the exercise groups had three times per week interaction with the research team when attending the exercise sessions, while participants in the VLCD group were attending our research unit weekly for face-to-face general review.

To aid in diet compliance and for monitoring purposes, participants were asked to record everything that they had on a daily basis on a printed paper food diary supplied by our research team. Halfway through the research project, we started to use a calorie monitor app call Nutracheck, available on iPhone or Android smartphones. Nutracheck provided the most comprehensive food product coverage in the UK with a high score for customers' feedback (NutraTech; Review Centre). Participants who joined in later, which is about half of the total number of participants, got to use the app to record their daily dietary intake. Recorded diet on the printed food diary or Nutracheck app were reviewed on a weekly basis when participants attended the unit, and any issue or concern was discussed.

Participants also had the option to change and choose which meal replacement products that they like, as this would help them to comply with the diet.

2.3.7 Statistical analyses

Unless specifically mentioned in the separate chapter, all statistical analyses were done with the following approach:

Descriptive statistics were performed for all data sets to check for normal distribution (accepted if $P<0.05$) using a Kolmogorov-Smirnov test. Changes following intervention within-group and differences between groups were analysed using two-way ANOVA with repeated measure ANOVA (time) and between-subject factor (group). Where significant differences were found using repeated measure ANOVA, a Bonferroni post-hoc test was applied for multiple comparisons. Correlations were assessed using Pearson's product-moment correlation coefficient. Data are expressed as mean ± SEM. The significance level was defined as $P\leq0.05$. All of the statistical analyses were performed using Prism Graphpad Version 8.4 (Graphpad Software Inc., San Diego, California, USA).

Chapter 3: Change in body composition, muscle architecture and function

3.1 Chapter synopsis

Background: A very low-calorie diet (VLCD) helps with significant weight loss, unfortunately, at the cost of lean mass loss, which also includes muscle mass. As muscles play crucial roles in body metabolism, motor function and physical fitness, loss in muscle mass are of major concern.

Objectives: To assess changes muscle protein synthesis (MPS), body composition including fat mass (FM), lean mass (LM), muscle thickness, and muscle function following a 6-week of VLCD alone (VLCD+O) or with concomitant resistance exercise training (VLCD+R) or high-intensity interval training (VLCD+H).

Study design: 26 overweight/obese men were randomly allocated into VLCD+O (n=10; age 46±3y; weight 104±4kg; BMI 32±1, mean±SEM), VLCD+R (n=8; 40±3y; 98±3kg; BMI 32±1) or VLCD+H (n=8; 46±4y; 101±4kg; BMI 33±1). Participants were given deuterium oxide (D_2O) loading and maintenance dose, with regular saliva collection and muscle biopsy pre- and post-intervention to allow for muscle protein synthesis (MPS) calculation. Whole-body DXA scan, ultrasound of vastus lateralis (VL) muscle thickness, a one-repetitive maximum of upper limbs (chest press, latissimus pull-down, seated lever row) and lower limbs (leg curl, knee extension, leg press) exercises, and maximum voluntary

contraction (MVC) of the knee extension of the dominant leg were assessed pre and post 6-week intervention. All participants received VLCD 800 kcal/d. VLCD+R had additional three times per week six type of resistance training, each type comprises of two sets of 12 reps at 70% 1-RM, while VLCD+H had three times per week HIIT cycle ergometry which consists of five cycles of 95 to 125% Max_{Watt} at 60 seconds interval with 90 seconds rest in between.

Results: VLCD+H had the highest MPS rate, which is significantly different to VLCD+O (1.24%.d^{-1}±0.09 vs 0.84%.d^{-1}±0.07, P=0.004), while MPS for VLCD+R has no different with any other group (0.95%.d^{-1}±0.08, P>0.05).Significant changes in total weight (-11.1kg±1.1, -10.8kg±1.2, -11.7kg±1.3), total FM (-6.8kg±0.7, -6.9±0.8kg, -7.7±0.9kg) and total LM (-4.3±0.6kg, -3.9±0.6kg, -4.0±0.5kg) after intervention in all groups (VLCD+O, VCLD+R, VCLD+H respectively, mean±SEM). No statistically significant difference between the groups for the changes in body composition. No statistically significant changes in VL thickness between pre and post, nor between the group, although mean changes for VLCD+R and VLCD+H showed marginal increment while the VLCD+O group showed reduced in thickness (-1.1±0.7mm, 0.2±0.3mm, 0. 5±1.0mm; VLCD+O, VCLD+R, VCLD+H respectively, P≥0.2). This is contrary to DXA results showing a significant loss in leg LM (-

1.5±0.3kg, -2.0±0.3kg, -1.4±0.3kg). A significant increase in strength (1-RM) for all exercise types was only observed in the VLCD+R group, despite loss in LM. No changes in MVC torque between baseline and post-intervention, nor between groups analysis.

Conclusion: VLCD+H showed a significantly better MPS rate than VLCD+O, with no difference between VLCD+R and the other groups. No significant differences between groups for LM changes nor VL muscle thickness. Different findings from other studies were likely due to the intensity and duration of the exercise protocol or protein content during VLCD. High LM loss could be due to DXA inability to differentiate between inter/intramuscular fat, glycogen and water content inside the muscle with muscle tissue itself. Regardless of this, there were significant increases in 1-RM in VLCD+R, with stable or no decline in 1-RM in the other two groups, suggesting improved and preserved muscle function despite possible small LM or muscle loss.

3.2 Chapter introduction

Weight loss, in addition to primarily losing fat mass (FM), is also known to cause lean mass (LM) loss, which includes skeletal muscle tissues as a predominant component (Chomentowski et al. 2009). A larger caloric deficit is associated with larger LM loss (Chaston et al. 2007; Garthe et al. 2011), and VLCD being one of the most intense caloric restrictions, would carry more risk of LM loss alongside FM loss.

Skeletal muscles serve as the largest reserve of protein in the body, and contribute to up to 85% of glucose uptake as well as play a major role in insulin resistance, is of particular interest in the context of LM loss during caloric restriction setting (DeFronzo et al. 1981; Rennie et al. 2004; Bouzakri et al. 2005). Realising the importance of muscles in metabolic function, various studies have been conducted to find the best way to preserve muscles and their functions during the weight-loss period.

Resistance exercise training (RET) has been shown to attenuate LM and skeletal muscle loss while under the general calorie deficit

condition (Garrow and Summerbell 1995; Weinheimer et al. 2010; Washburn et al. 2014). A study more focused on VLCD showed that the addition of an intensive resistance exercise training (RET) program resulted in the preservation of LM (Bryner et al. 1999). On the other hand, other studies did not find any difference in FFM loss between VLCD or VLCD with RET, although additional RET may benefit in the form of strength improvement (Lemons et al. 1989; Donnelly et al. 1993).

HIIT workout has been under the spotlight for the past decades as it provides similar fitness benefits when compared to continuous endurance workouts, but in a shorter time means more time efficiency (Kravitz 2011; Kong et al. 2016). Under normal calorie intake, HIIT has been shown to be better and more effective in reducing central obesity, contributed to thrice the FM loss with half the time for exercises compared to continuous aerobic training (Maillard et al. 2016). Under a moderate calorie deficit setting, Sartor et al. found that HIIT, in addition to diet control, helps to prevent LM loss (Sartor et al. 2010). Unfortunately, at the start of this research project, there was no known study incorporating HIIT with VLCD, mainly to due safety concerns due to its intense caloric

restriction in combination with the intense nature of the VLCD (Gilden Tsai and Wadden 2006).

With adequate monitoring equipment and qualified clinicians within our research unit, plus previous evidence to support the safe use of VLCD (Moyer et al. 1989; NIH 1993), we, therefore, planned for a study design to look into VLCD alone in comparison to VLCD with HIIT or RET, towards changes in muscle protein synthesis (MPS), body composition, muscle architecture and muscle function.

3.3 Study design

3.3.1 Recruitment and interventions

Ethics study approval and participants recruitment are as per detailed description in section 2.3 Study design**.**

The total number of participants were 10 in VLCD+O, 8 in VLCD+R and 8 in VLCD+H. Ages at baseline were 46y±3, 40y±3, 46y±4 for VLCD+O, VLCD+R, VLCD+H, respectively. Mean baseline weights were 103.8kg±3.9, 98.4kg±3.4, 101.0kg±4.3, and mean BMI were 32kg.m^{-2}±1, 32kg.m^{-2}±1, 33kg.m^{-2}±1 in similar respective order.

3.3.2 Muscle protein synthesis calculation using fractional synthesis rate

We utilise D_2O as a stable isotope tracer to monitor changes in MPS (Wilkinson et al. 2014). This involved collection of saliva samples following D_2O consumption and muscle biopsies at baseline before the intervention and the week after completion of the 6-week interventions (week-7). Participants were provided with a loading dose of D_2O at the start of the research program, based on body weight (2ml/kg body weight), then provided with daily D_2O "top-ups" (~10% initial bolus dose). Saliva collections were done two to three days per week for the rest of the study period to help confirm the water enrichment level. Muscle biopsy of the mid-belly of vastus lateralis was done pre- and post-intervention, using conchotome technique under a sterile setting.

Detailed laboratory processes for the saliva and muscle preparation were extensively explained by Wilkinson et al. (Wilkinson et al. 2014). In brief, 80-90 µl of saliva was heated in inverted 2 ml vials for four hours at 90-100°C to purify fractions of the body water, then

cooled on ice before the condensed water was transferred to a clean autosampler vial for injection. A high-temperature conversion elemental analyser (Thermo Finnigan, Thermo Scientific, Hemel Hempstead, UK) that is connected to an isotope ratio mass spectrometer (Delta V Advantage, Thermo Scientific) was used to measure deuterium in saliva (0.1 µl). For protein-bound alanine muscle fraction enrichment, 30-50 mg of muscle was homogenised in ice-cold homogenization buffer to isolate myofibrillar proteins. 10 min rotary mixing was followed by centrifugation at 11,000 g for five minutes at 4°C. The myofibrillar pellet was solubilised in 0.3 M NaOH before being separated from the insoluble collagen by centrifugation, and myofibrillar protein was precipitated with 1 M perchloric acid. The sarcoplasmic proteins were precipitated using 1 M perchloric acid and separated by centrifugation. Following overnight hydrolysis at 110°C in a 0.1 M HCl and Dowex H^+ resin slurry, the amino acids were eluted with 2 M NH_4OH and then dried-down. Dried samples then were suspended in 60 µl of distilled water and 32 µl of methanol, followed by a brief vortex, and 10 µl of pyridine and 8 µl of methyl chloroformate were added. Samples then were vortexed again for 30 seconds and left to react at room temperature for five minutes. The n-methoxycarbonyl methyl esters of the amino acids were extracted after adding 100 µl chloroform. The remaining water

content was removed from the sample with the addition of a molecular sieve. The incorporation of deuterium into the protein-bound alanine was determined by gas chromatography-pyrolysis-isotope ratio mass spectrometry (Delta V Advantage, Thermo, Hemel Hempstead, UK) (Wilkinson et al. 2014).

Myofibrillar MPS was calculated from the deuterium enrichment (APE) in alanine in myofibrillar proteins, using the body water enrichment (APE, corrected for the mean number of deuterium moieties incorporated per alanine=3.7) as the precursor labelling between biopsies. The fractional synthesis rate (FSR) was calculated using the following formula:

$$FSR(\%/day) = [(APE_{Ala})]/[(APE_{P}) \times t] \times 100$$

where APE_{Ala} = deuterium enrichment of protein-bound alanine, APE_{P} = mean precursor enrichment over time, and t is the time between biopsies.

3.3.3 DXA

We utilised a fan-beam dual-energy X-ray absorptiometry (DXA) scan (Lunar Prodigy II, GE Healthcare) for assessment of body composition. To avoid dehydration, participants were asked to drink an adequate amount of water the night before and in the morning of the investigation to avoid feeling thirsty. Clinical assessment of hydration status, including mucous membrane and peripheral capillary refill time were also performed by a trained clinician. Participants were asked to empty their bladder and wear minimal cloth (e.g. t-shirt and shorts) with all jewellery removed before the DXA scanning. For the scanning, participants were lying down supine with their body fitted in the box outline on the DXA table, a position that has been shown to give a high repeatability value (Lohman et al. 2009). Participants were asked to avoid any movement during the six to seven minutes scanning period to minimise artefacts.

Whole-body was scanned, and total mass/ total weight (TW), FM, and LM were calculated automatically by the installed DEXA software (Prodigy enCORE, GE Healthcare). If participants were wider than the scan boundaries, half-body scans were taken of the right-hand side, and left-hand measures were assumed equal (Levitt et al. 2010). DXA was calibrated weekly by a standard calibration

phantom. LM reported in this protocol is related to FFM excluding bone mass (BM), i.e. LM = FFM – BM.

3.3.4 Vastus lateralis muscle USS

Vastus lateralis (VL) muscle thickness was assessed at rest using B-mode ultrasonography (Mylab 25; Esaote Biomedica, Genova, Italy), with a 50 mm, 7.5 MHz, linear-array probe. Images were taken while participants were lying supine on a bed with a relaxed leg and full knee extension. The images were taken at 50% of femur length, identified as a middle point between palpable lateral epicondyle of the femur bone and greater trochanter, and on the midsagittal line of the muscle, identified by the USS. This position is set to standardise the assessment between pre- and post-intervention. The transducer was then aligned in the fascicle plane to capture an optimal portion of fascicles (Franchi et al. 2015). Images were collected and digitally analysed by the same unblinded operator. Quantification of muscle architectural adaptations was done for muscle thickness (MT), measured as the perpendicular distance between the superficial and the deep tendon aponeurosis, were performed by using ImageJ 1.42q software (National Institutes of Health, Bethesda, MD). MT was measured as it was previously

established as an indicator of muscle mass (Miyatani et al. 2002; Takai et al. 2013; Abe et al. 2015)

3.3.5 1-RM assessment

The one-repetitive maximum (1-RM) was assessed as previously established (Dohoney et al. 2002) for three types of upper limbs exercises and three types of lower limbs exercises: supine chest press, latissimus pull-down, seated row, leg curl, knee extension, leg press. Participants began with a light warm-up to ensure familiarity while avoiding fatigue; 1-RM was aimed to be achieved (20 on Borg's rating of perceived exertion scale) in as few repetitions as possible with a maximum of 5 repetitions. The first repetition was estimated at 50% perceived 1-RM and then increased gradually until participants could not complete the full range of motion in any exercise type. Between attempts after each weight change, there was three minutes rest in-between. If the total weight was limited by the availability of the machine weight capacity, the assessments were extended to 10 or 20 repetitions (Reynolds et al. 2006), or changed into one limb assessment, i.e. right and left side separately.

3.3.6 MVC

After at least 30 minutes of rest, MVC for knee extension of the dominant leg was tested using an isokinetic dynamometer (Cybex norm, New York, NY, USA). Once participants sat comfortably in the right position, their body was fastened with a body belt connected to the dynamometer chair to provide extra stability, and participants were asked to keep their back straight and keep the arms by the side of the body holding to handgrips on each side. The dominant leg was connected to the dynamometer via a lever and secured with a Velcro strap above ankle level, as well as an extra strap around the thigh. Once ready, the software in the computer was activated, and the software would instruct the participants to move the knee to the desired angle. Maximal isometric knee extension contractions were assessed at three knee joint angles in order of 70°, 80° and 60°. Two attempts were made for each angle, with 60 seconds rest between each attempt and 90 seconds rest before the change of the angles. The software displayed a countdown before each measurement was taken so that participants and the assessor would be ready. The assessor gave loud verbal encouragement once the measurement started to encourage all-out attempts by the participant to reach their MVC. Each measurement phase lasted for

four seconds. The highest peak torque value was recorded for each angle.

3.3.7 Statistical analyses

Statistical analyses were performed as described in Chapter 2, section 2.3.6. The only difference is in MPS analysis, where one-way ANOVA instead of two-way ANOVA was used to assess the differences in FSR between the groups.

3.4 Results

3.4.1 Muscle protein synthesis

VLCD+H had the highest MPS rate at 1.24%.d^{-1}±0.09, followed by VLCD+R at 0.95%.d^{-1}±0.08 ,and VLCD+O at 0.84%.d^{-1}±0.07 (Figure 3.1). Following one-way ANOVA, a statistically significant difference was only seen between VLCD+H and VLCD+O (P=0.004). No significant difference was seen between VLCD+R with any other group.

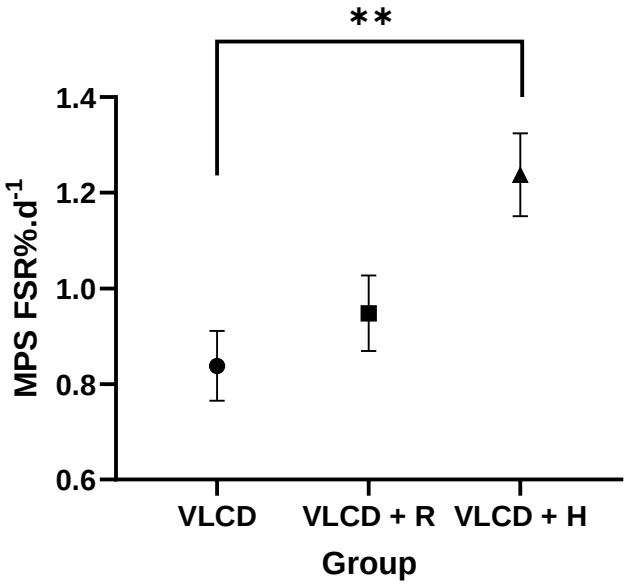


Figure 3.1 – MPS rate between groups. **P=0.004

3.4.2 DXA

3.4.2.1 Total weight loss

All groups showed significant TW loss from baseline. VLCD+H had the most TW loss with a mean weight loss of 11.7kg±1.3, followed by VLCD+O with loss of 11.1kg±1.1 and VLCD+R of 10.8kg±1.2, all P<0.0001 (Figure 3.2 and Figure 3.2). These translate slightly different in terms of ranking when calculated from relative loss as following; VLCD+H, followed by VLCD+R, then VLCD+O with 11.6%±1.2, 11.0%±1.2, 10.5%±0.8 respectively, all P<0.001

(Figure 3.3). No statistically significant difference was seen between the groups.

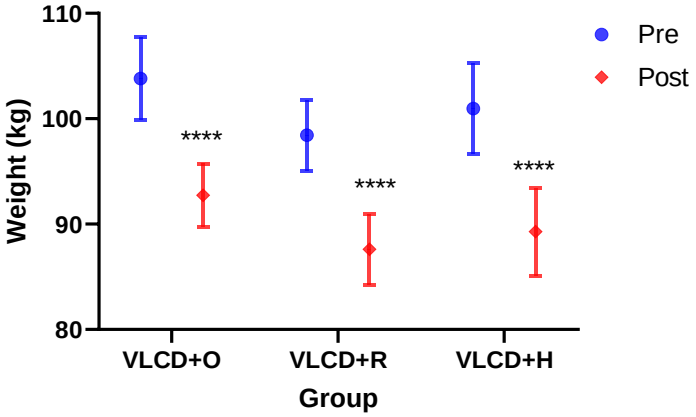


Figure 3.2 – Total weight pre- and post-intervention for all groups. ****P<0.0001

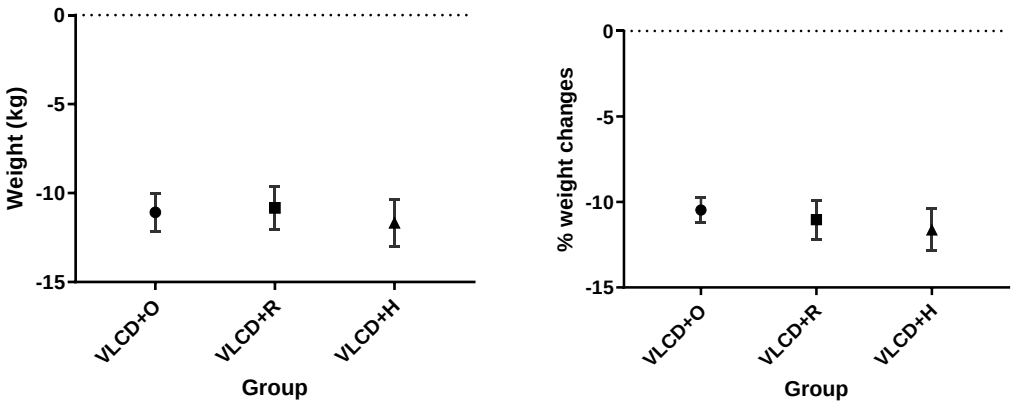


Figure 3.3 – Absolute (right) and relative (left) total weight changes from baseline.

3.4.2.2 Regional weight loss

The table below summarises absolute and relative regional weight changes from the baseline, divided into arms, legs and trunk:

Table 3.1 - Regional weight change from the baseline.

	VLCD+O	VLCD+R	VLCD+H
Arms	-1.5kg±0.3 -12.0%±2.2	-1.5kg±0.3 -12.0%±2.4	-1.8kg±0.4 -14.2%±2.7
Legs	-2.9kg±0.5 -8.6%±1.1	-3.7kg±0.4 -11.7%±1.4	-3.2kg±0.4 -10.0%±1.4
Trunk	-6.4kg±0.5 -11.7%±0.6	-5.4kg±0.7 -11.0%±1.3	-6.4kg±0.7 -12.6%±1.4

Comparing between the regions, the highest absolute weight loss was from the trunk with more than 5.4kg differences from baseline for all the groups. Arms appeared to lose the most relative weight from baseline with more than 12% mass changes. Between-group analyses showed that within the arm and trunk region, VLCD+H had the highest relative mass loss at 14.2% and 12.6%, respectively, while for the leg region, VLCD+R had the highest weight loss for both absolute (3.7kg) and relative (11.7%) value.

3.4.2.3 Fat mass loss

We observed significant FM loss in all groups, with most changes in VLCD+H with a mean of 7.7kg±0.9, followed by VLCD+R at 6.9kg±0.8 and VLCD+O 6.8kg±0.7, all P<0.0001 (Figure 3.4 and Figure 3.5). Similar order in terms of percentage fat mass loss compared to baseline, leading by VLCD+H, VLCD+R then VCLD+O with the mean percentage of 21.4%±3.0, 21.1%±3.0 and 19.7%±1.8 respectively (Figure 3.5). No statistically significant difference was seen between the groups.

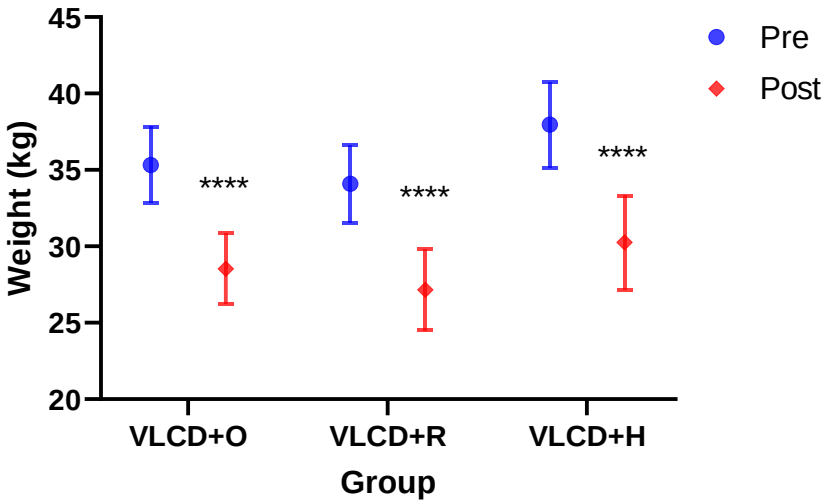


Figure 3.4 - Fat mass pre- and post-intervention for all groups. ****P<0.0001

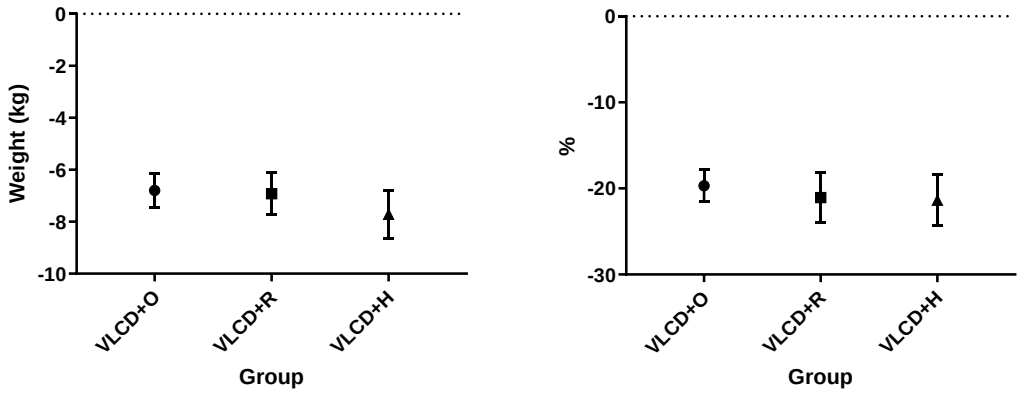


Figure 3.5 – Absolute (left) and relative (right) fat mass changes from baseline.

3.4.2.4 Regional fat mass changes

The table below summarises absolute and relative regional FM changes from the baseline, divided into arms, legs and trunk (mean±SEM):

Table 3.2 – Regional FM change from baseline.

	VLCD+O	VLCD+R	VLCD+H
Arms	-0.7kg±0.1 -19.8%±3.5	-0.7kg±0.2 -22.0%±3.9	-0.9kg±0.2 -22.2%±3.9
Legs	-1.4kg±0.2 -15.8%±1.4	-1.7kg±0.2 -19.5%±3.0	-1.8kg±0.2 -18.3%±2.6
Trunk	-4.6kg±0.4 -22.0%±2.2	-4.4kg±0.5 -22.3%±3.1	-4.9kg±0.6 -23.2%±3.5

Within-group analysis, most FM changes occurred from the trunk with more than 22% mass loss from baseline for all the groups. The regional analysis showed that within the arm and trunk region, VLCD+H had the highest relative FM loss, while within the leg region VLCD+R had the highest FM loss.

3.4.2.5 Android fat mass changes

In line with a general interest for overall health benefits from weight loss, we also assessed changes in android fat mass (AFM) as android fat has been strongly linked with metabolic syndrome as discussed in section 1.3.2 (Min and Min 2015; Sari et al. 2019).

All groups had a significant AFM loss, with VLCD+H showing most loss with mean at 1.2kg±0.1, and VLCD+O at 1.1kg±0.1 and VLCD+R at 1.0kg±0.1, all P≤0.0001 (Figure 3.6 and Figure 3.7). However, when translated into relative AFM loss from baseline, VLCD+R had more mass loss than VLCD+O. AFM loss at 28.9%±3.6, 27.9%±3.7, 27.2%±2.5 for VLCD+H, VLCD+R, VLCD+O respectively (Figure 3.7). No statistically significant difference was seen between the groups.

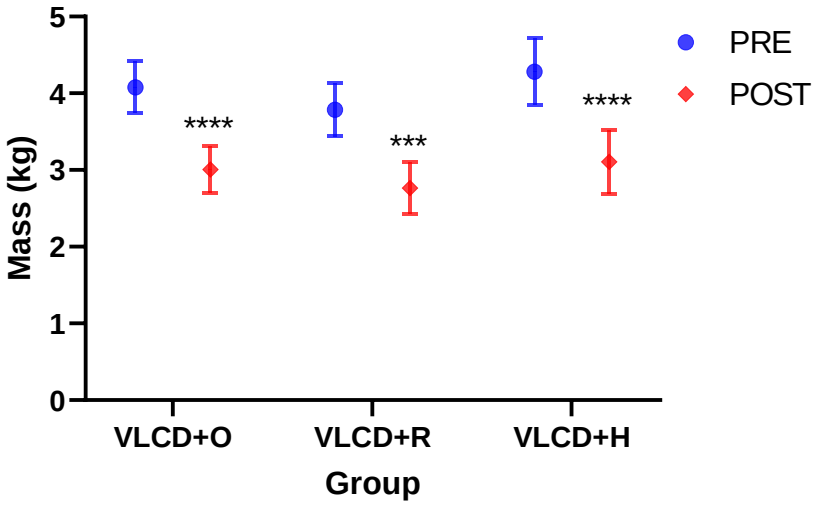


Figure 3.6 - Android fat mass pre and post-intervention for all groups. ***P=0.0001, ****P<0.0001.

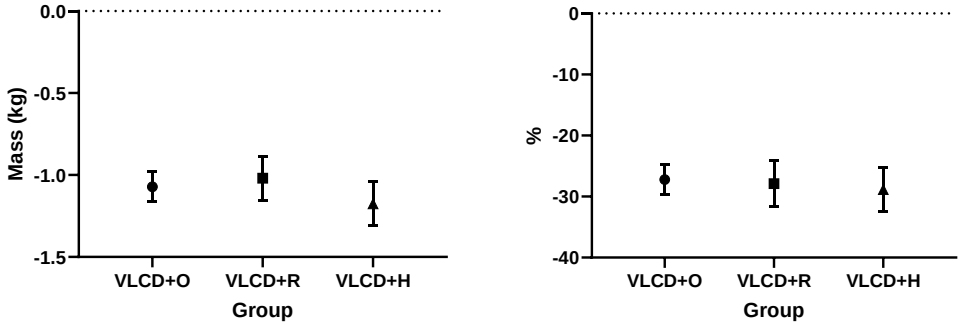


Figure 3.7 – Absolute (left) and relative (right) android FM changes from baseline.

3.4.2.6 Total lean mass changes

Following the 6-week intervention, all groups showed a significant LM loss, with the least loss in VLCD+R at a mean of 3.9kg±0.6, followed by VLCD+H at 4.0kg±0.5 and VLCD+O at 4.3kg±0.6, all $P<0.001$ (Figure 3.8 and Figure 3.9). Interestingly, relative LM loss from baseline showed that VLCD+H had more loss at 6.5%±0.6 compared to VLCD+O at 6.4%±0.8, while VLCD+R remains the least loss at 6.3%±1.0 (Figure 3.9). Regardless of this order, no statistically significant difference was seen between the groups.

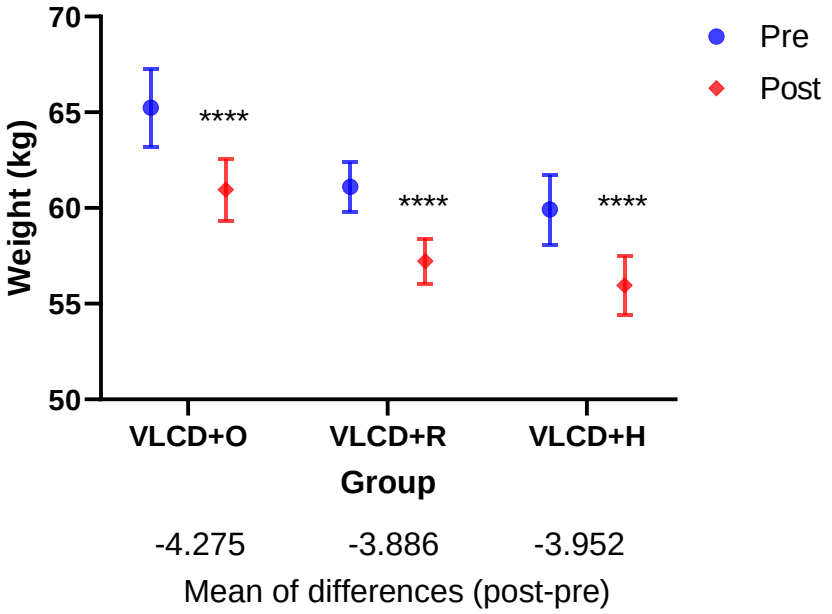


Figure 3.8 - Lean mass pre and post-intervention for all groups. ****P<0.0001

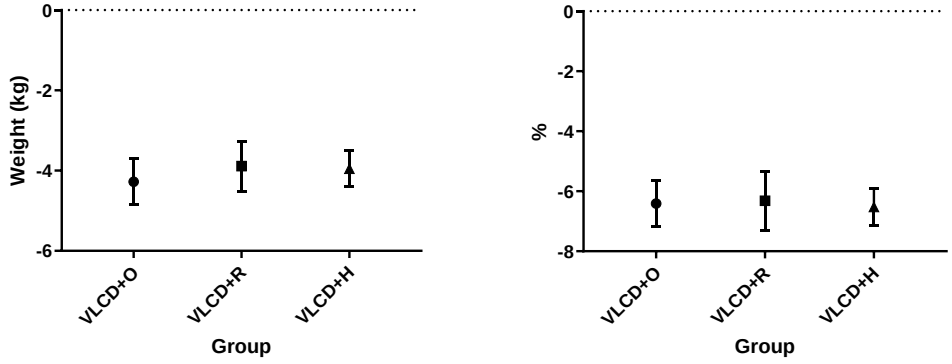


Figure 3.9 – Absolute (left) and relative (right) lean mass changes from baseline.

Comparing the relative LM loss from total weight loss, VLCD+R has the lowest lean mass lost at 34%±5.5, followed by VLCD+H at

35%±3.1 and as expected VLCD+O with the most proportion of the LM loss, which is at 37%±3.4 (Figure 3.10). No statistically significant difference was seen between the groups.

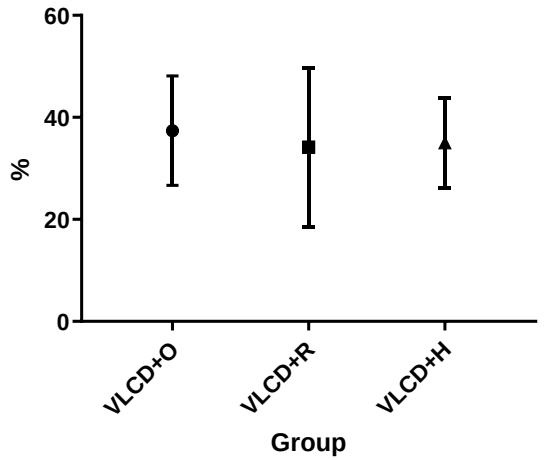


Figure 3.10 – Relative LM loss from total weight loss in all groups.

3.4.2.7 Regional lean mass loss

The table below summarises absolute and relative regional LM changes from the baseline, divided into arms, legs and trunk:

Table 3.3 - Regional LM changes from baseline for all groups.

	VLCD+O	VLCD+R	VLCD+H
Arms	-0.8kg±0.2 -9.7%±2.0	-0.7kg±0.2 -8.5%±2.4	-0.9kg±0.2 -11.3%±2.3
Legs	-1.5kg±0.3 -6.2%±1.1	-2.0kg±0.3 -9.1%±1.5	-1.4kg±0.3 -6.5%±1.1
Trunk	-1.8kg±0.4 -5.5%±1.2	-1.1kg±0.5 -3.7%±1.5	-1.5kg±0.3 -5.2%±1.0

Within the group analysis, VLCD+O and VLCD+H showed the highest relative LM loss from the arm region, with the trunk had the highest absolute LM loss. In VLCD+R, the leg region appeared to have the most absolute and relative LM loss from the baseline. Within the regional analysis, the highest LM loss from the arms was in VLCD+H, the highest LM loss from the legs was in VLCD+R, and VLCD+O had the highest LM loss for the trunk region.

3.4.2.8 Leg lean mass

Interesting to note that VLCD+R had the most leg mass loss in the form of FM and LM. We also looked at the LM changes on the leg for comparison with other assessment findings, such as USS muscle thickness and 1-RM. Whilst VLCD+R showed the least loss of whole-body LM, DXA showed that they have the highest LM loss in the leg region at 2.0kg±0.3, followed by VLCD+O at 1.5kg±0.3 and VLCD+H at 1.4kg±0.3, all P<0.01 (Table 3.3, Figure 3.11 and Figure 3.12). However, when this is translated into the percentage LM loss from baseline, VLCD+H showed more loss than VLCD+O. Relative LM losses were 9.1%±1.5, 6.5%±1.1, 6.2%±1.1 for VLCD+R, VCLD+H, VLCD+O, respectively (Table 3.3 and Figure 3.12). No statistically significant difference was seen between the groups.

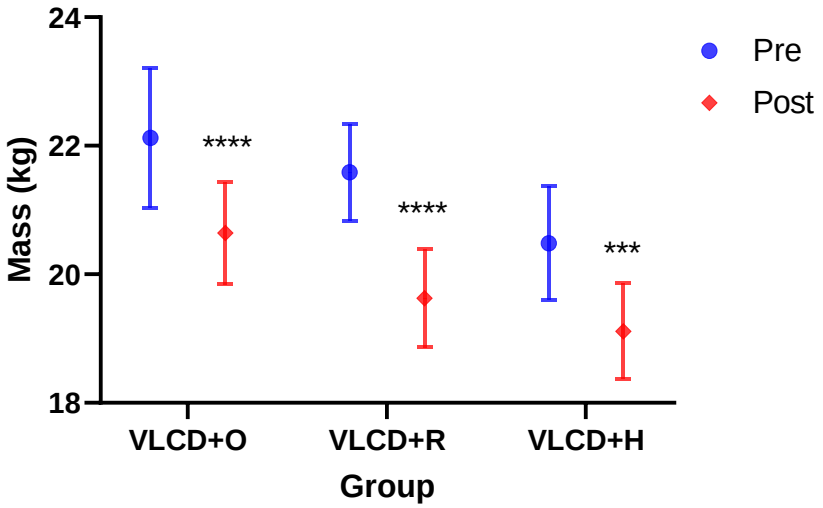


Figure 3.11 - Leg lean mass pre- and post-intervention for all groups. ***P=0.0009, ****P<0.0001.

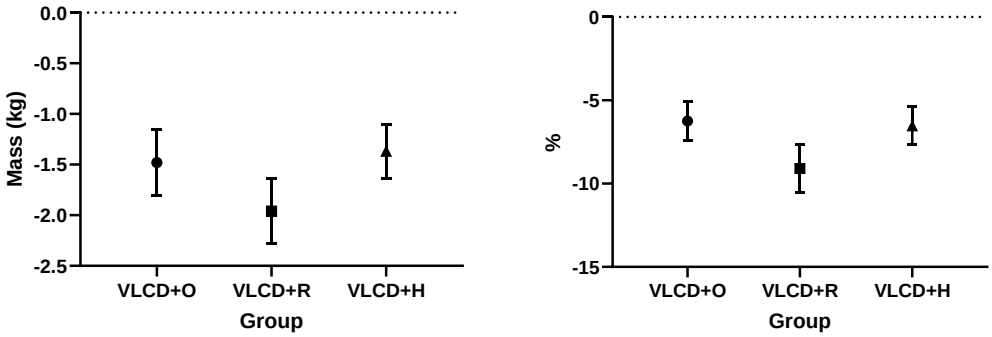


Figure 3.12 – Absolute (left) and relative (right) leg lean mass changes from baseline.

3.4.3 Vastus lateralis USS thickness

Despite DXA showing a significant loss in leg LM post-intervention, the USS of VL muscle thickness showed no statistically significant changes from baseline in all groups. VLCD+R had only a marginal increase in mean at 0.2mm±0.3 (P=0.6), VLCD+H at 0.5mm±1.0 (P=0.6), while VLCD+O showed a decrease in thickness means of 1.1mm±0.7 (P=0.2) (Figure 3.13 and Figure 3.14). Also, there was no statistically significant difference between the groups.

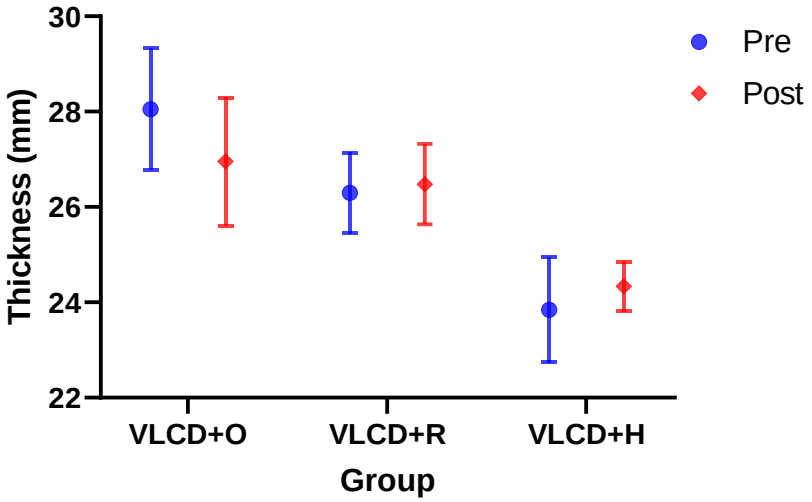


Figure 3.13 – Vastus lateralis muscle thickness pre- and post-intervention for all groups.

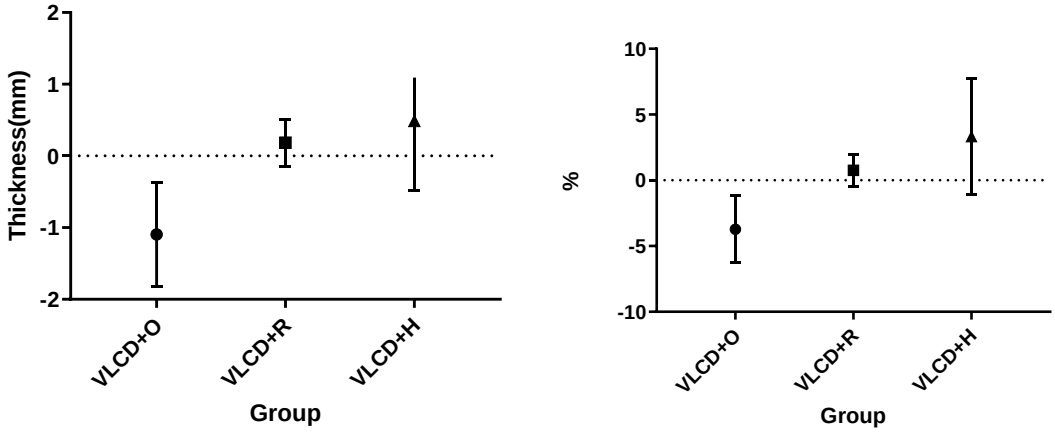


Figure 3.14 - Absolute (left) and relative (right) vastus lateralis muscle thickness changes from baseline.

3.4.4 1-RM

Only VLCD+R show a significant one-repetition maximum (1-RM) increase across all exercise types. We also observed significant improvement in the VLCD+H group for leg press exercise.

Following is the table for the 1-RM change (in kg) following intervention (post-intervention minus baseline):

Table 3.4 - Within-group analysis for 1-RM changes (in kg) from baseline following intervention for the different types of exercises.

	VLCD+O	VLCD+R	VLCD+H
Chest press	1.6±3.6	18.5±7.3**	3.4±2.9
Lat pull down	0.5±2.2	9.7±1.6**	-1.1±3.5
Seated row	5.8±1.8*	17.5±3.8****	2.1±3.2
Leg curl	2.8±1.8	18.6±5.5***	4.1±2.9
Knee extension	0.9±8.6	38.9±8.8***	9.0±5.2
Leg press	16.8±4.3	39.8±8.3***	28.5±13.9*

*Indicates *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Using two-way ANOVA, significant differences in 1-RM improvement were seen between VLCD+R and the other two groups only in chest press exercise (Figure 3.15). Significant differences in 1-RM improvement were seen between VLCD+R and VLCD+H in latissimus pull-down, seated row and leg curl exercises (Figure 3.16, Figure 3.17, Figure 3.18). No significant difference between the group in knee extension and leg press exercises (Figure 3.19 and Figure 3.20).

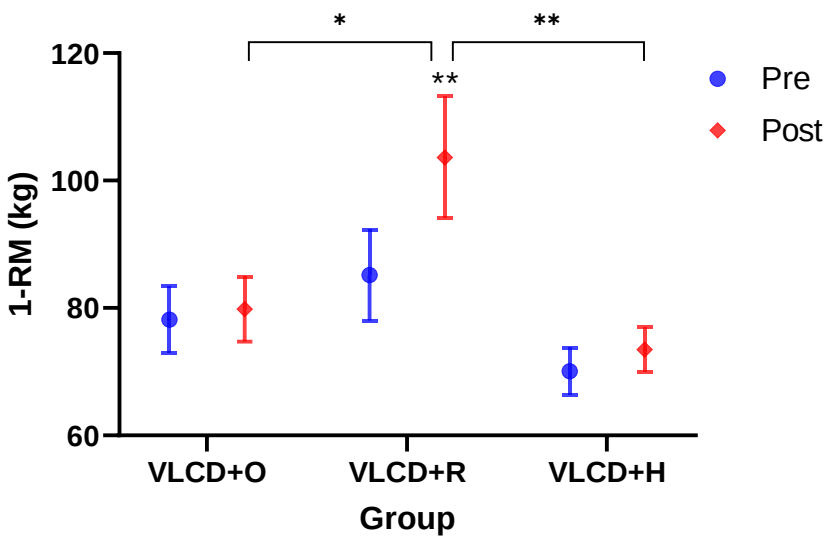


Figure 3.15 – Chest press 1-RM changes from baseline. *P<0.05 **P<0.01.

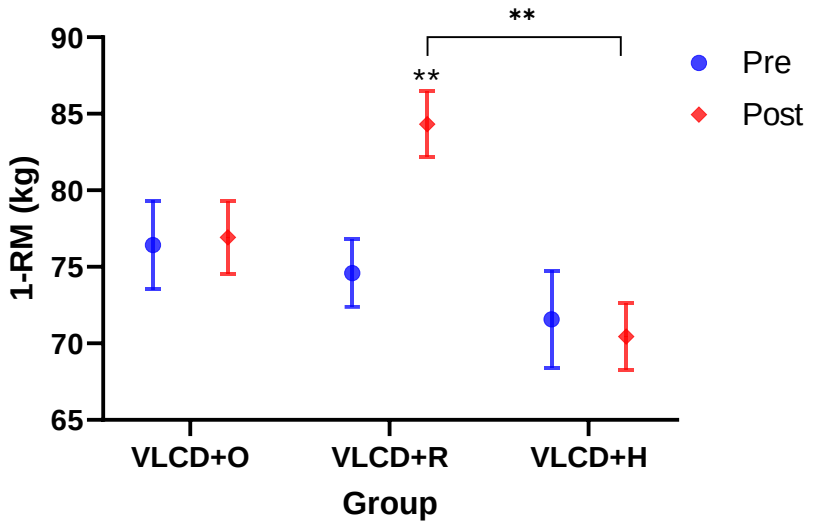


Figure 3.16 – Latissimus pull-down 1-RM changes from baseline. **P<0.01.

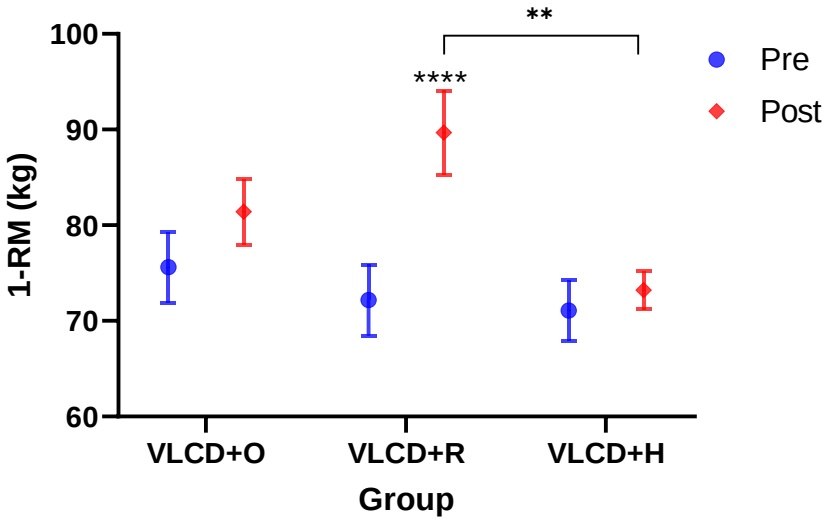


Figure 3.17 - Seated row 1-RM changes from baseline. **P<0.01, ***P<0.001.

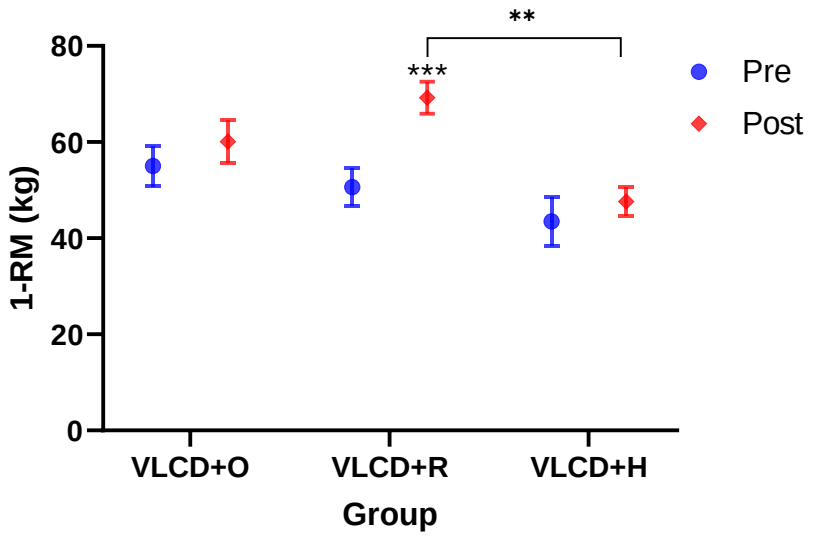


Figure 3.18 - Leg curl 1-RM changes from baseline. **P<0.01, ***P<0.001.

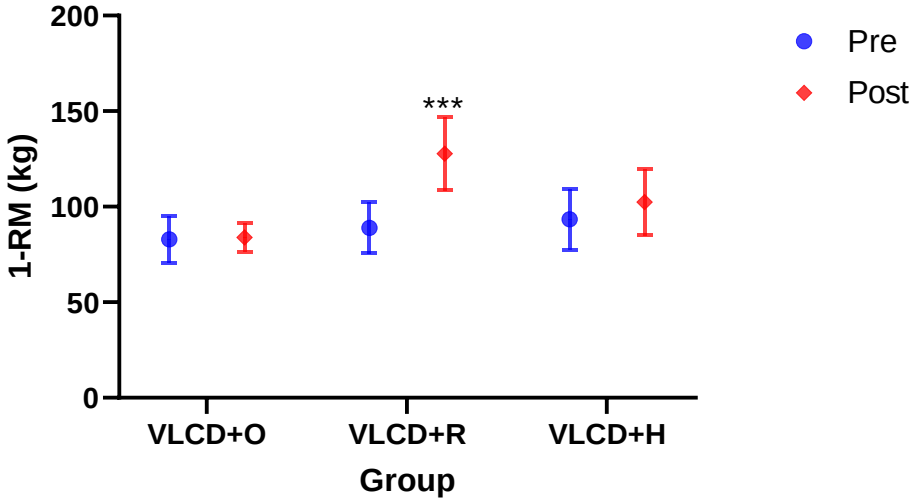


Figure 3.19 - Knee extension 1-RM changes from baseline. ***P<0.001.

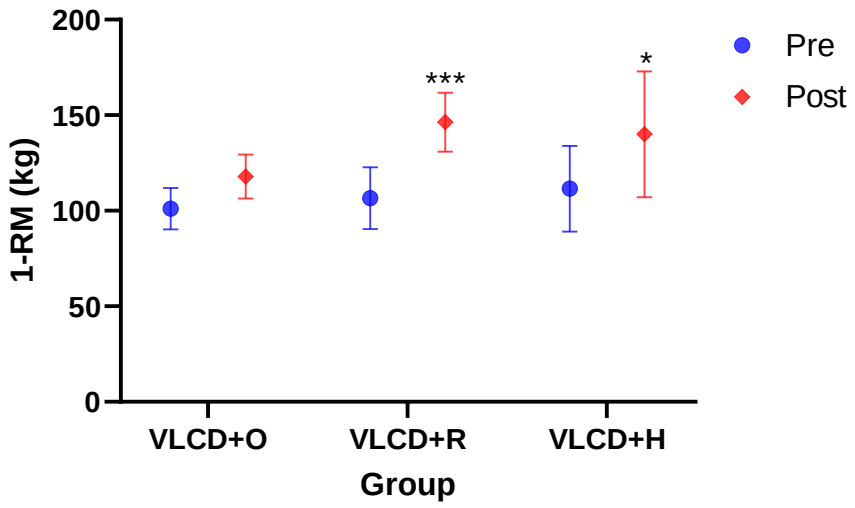


Figure 3.20 - Leg press 1-RM changes from baseline. *P<0.05, ***P<0.001.

3.4.5 Maximum voluntary contraction

Maximum voluntary contraction (MVC) did not show any significant torque changes pre- and post-intervention at any angle tested. No significant difference between groups either.

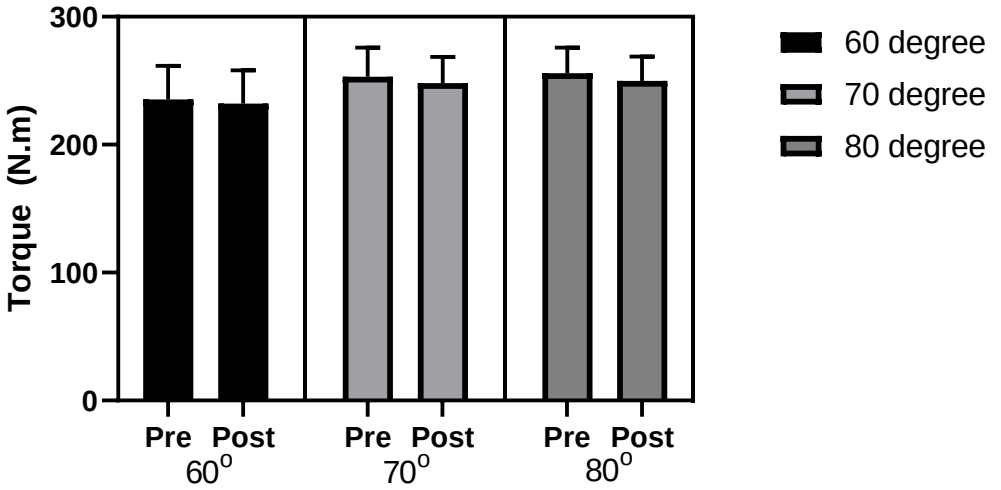


Figure 3.21 - Maximum voluntary contraction pre- and post-intervention all angles for VLCD+O group. No significant change was seen from the baseline.

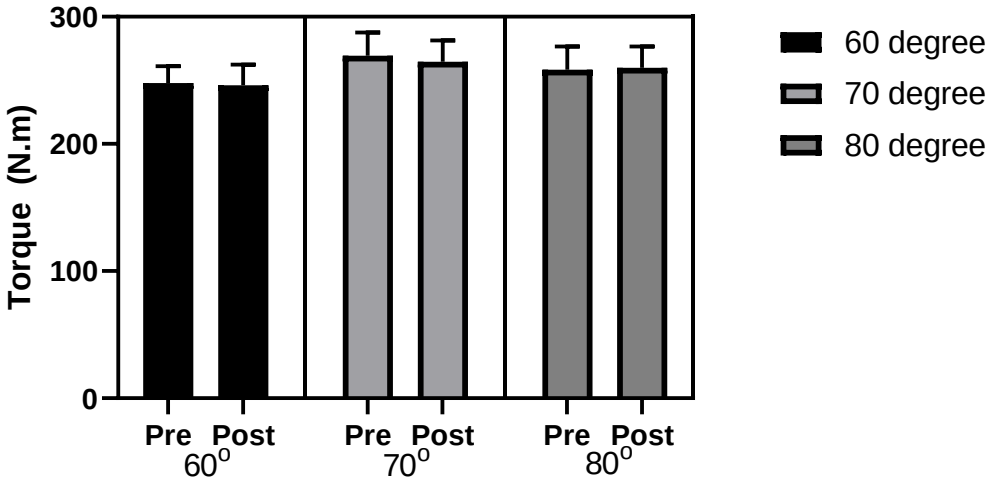


Figure 3.22 - Maximum voluntary contraction pre- and post-intervention all angles for VLCD+R group. No significant change was seen from the baseline.

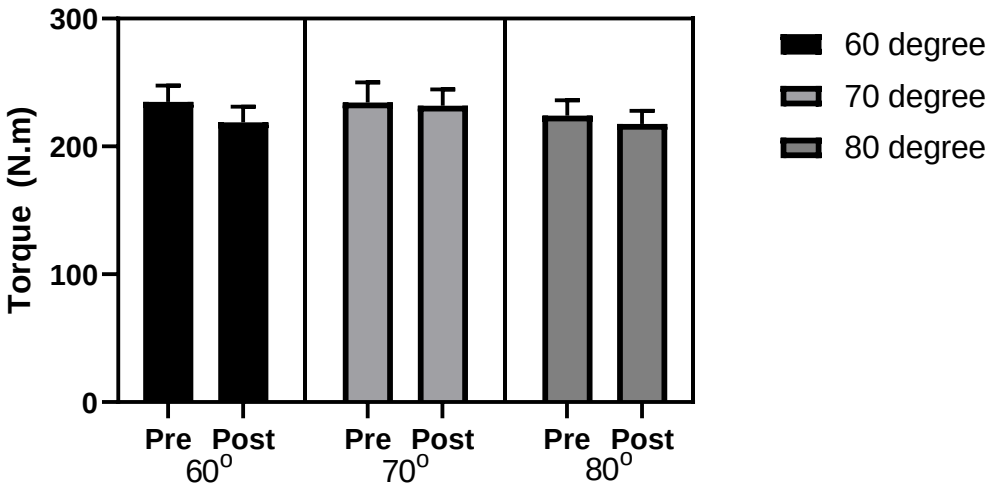


Figure 3.23 - Maximum voluntary contraction pre- and post-intervention all angles for VLCD+H group. No significant change was seen from the baseline.

3.5 Discussion

As expected, VLCD helped with significant weight loss across all groups, ranging between 10.5 to 11.6% from the baseline. This is important as even as low as 5% weight loss is associated with improvement in cardiometabolic health (Wing et al. 2011). As observed in many studies, LM makes up a relatively large percentage of total weight loss. In our study, we surprisingly found a huge range between 3% to 56% lean mass losses from the total weight loss across all the groups, with mean loss was highest in the VLCD+O

and least loss in VLCD+R (37% vs 34%; not statistically significant). This percentage range is a slightly wider range than observed in other studies, with LM loss between 17 to 55% of the total weight loss following VLCD (Donnelly et al. 1991a; Krotkiewski 2001; Chaston et al. 2007). Multiple factors could have contributed to the differences in these findings, namely the amount of protein content, the intensity of the training as well as the duration of the interventions, to name a few (Barrows and Snook 1987; Chaston et al. 2007; Jo et al. 2019). In our study, we supplied four meal replacement products per day from LighterLife to provide 600kcal/d energy, which contain carbohydrate:protein:fat ratio at the range around 44-48%, 39-43% and 12-13%, respectively. Our participants also consumed worth 200kcal/day in addition to the supplied meal replacement products in the form of coffee, tea, vegetables and fruits. Our RET protocol which consisted of two sets of 12 reps at 70% 1-RM for six different types of exercises that we adopted, was based on the recommendation by the American College of Sports Medicine for untrained individuals with no RET experience or who have not trained for several years (American College of Sports Medicine 2009). Moreover, it has been previously shown that only at the latter phase i.e. after week 6 of resistance training regimen, myofibre hypertrophy with enlarged fibre cross-sectional

area started to predominate the overall adaptive changes and contributed to a total increase in LM (Donnelly et al. 1993; Jo et al. 2019). A meta-analysis of the middle age and older population under different caloric restriction methods that were not specifically VLCD with exercises integration showed similar variation in LM loss between 3-50% of total weight loss (Weinheimer et al. 2010).

AFM has been linked to cardiovascular disease mainly due to impaired glucose and lipid profile and worsening endothelial function (Sari et al. 2019). In our study, we have seen significant AFM loss close to 30% changes from baseline in all groups. No statistically significant difference between groups was seen, suggesting VLCD alone suffice to achieve such AFM loss.

Interestingly, whilst whole body LM loss appeared to be the least in VLCD+R, leg LM loss was the highest in VLCD+R compared to the other two groups. VLCD+R had the highest total mass loss in the leg region, consisting of both LM and FM loss when compared to other groups. These are rather unexpected findings, as USS of VL muscle thickness did not show any significant change in VL muscle thickness, rather mean changes showing 0.2mm increase in thickness (statistically not significant P=0.6). VLCD+O, on the other

hand, had the least relative leg LM loss, which is rather intriguing, as in general, we would expect that exercises incorporation would help to attenuate muscle loss compared to diet only intervention.

The explanation for this might be that RET, while on intense caloric restriction (i.e. VLCD), promotes more inter and intramuscular fat loss, as well as a significant decrease in glycogen concentration in the muscle tissues after the first two weeks of weight reduction (Krotkiewski et al. 1990) causing glycogen-associated water loss hence less water content within the tissues (Eston et al. 1992; Kreitzman et al. 1992; Toomey et al. 2017). With the inability of DXA to differentiate between glycogen, water content as well inter/intramuscular lipids and muscle tissue, DXA could have misinterpreted loss of glycogen, water and inter/intramuscular fat as part of LM, hence overestimated LM or muscle mass loss in this VCD+R group (Levine et al. 2000; Toomey et al. 2017).

Additional analysis using the MPS rate showed that VLCD+H has the best outcome for MPS, with the rate at $1.24\%.d^{-1}\pm0.09$ following the 6-week intervention, which appears relatively at a similar level to the baseline MPS of untrained, normal diet muscle (Brook et al. 2015, 2016). With statistically significantly different from the MPS

rate in VLCD+O, HIIT appears to be beneficial compared to diet alone in terms of muscle anabolism. However, a question arises as the MPS result appears in disagreement with DXA finding and USS scan, as to what extent this result is reliable. As discussed earlier, DXA inability to differentiate inter and intramuscular fat, glycogen and water content could have contributed to the inconsistency. In comparison with USS VL muscle thickness, although there is no statistically significant change in the USS findings, we found a similar trend of VLCD+H showing the best outcomes with a mean increase of 0.5mm±2.8 (P=0.6), followed by VLCD+R 0.2mm±0.9 (P=0.6), while VLCD+O showed a decrease in thickness means of 1.1mm±2.3 (P=0.2). MPS rate has previously indicated a good correlation with muscle hypertrophy (Wilkinson et al. 2014; Brook et al. 2016). This finding would provide valuable insight into the future integration of HIIT with VLCD.

Despite the findings suggesting LM loss from DXA assessment, or lack of muscle gain from USS of VL muscle thickness, we have observed massive improvement in 1-RM among VLCD+R, showing the highest increase in all exercise types. While VLCD+O and VLCD+H did not show significant 1-RM improvement in most of the exercises, we found that there is no decline in the strength. The

explanation for this could be related to neural adaptation during the period of 6-week resistance training before delayed muscle hypertrophy, which usually follows a later period after the week six interventional period (Moritani and DeVries 1979; Jo et al. 2019). This is a very important finding as whilst we concern regarding muscle mass loss during intense caloric restriction, the motor function of muscles improved following adequate stimulation from resistance training and was preserved even in the diet-only condition.

Our isometric knee extension MVC assessment did not show any significant change. Data on the MVC in relation to muscle loss following intense calorie restriction are rather scarce. One study found no significant change in isometric knee extension MVC following caloric restriction despite significant improvement in 1-RM (Longland et al. 2016), which is rather similar to our findings. Among athletes on a normal diet, isometric MVC data did not help with performance improvement (Haycraft et al. 2016). Improvement in MVC is mainly associated with muscle hypertrophy (Reynolds et al. 2006). As our study showed a loss in leg LM with no significant change in muscle thickness, this might explain why we did not observe significant changes in MVC. The decrease in the isokinetic

muscle strength is normally seen in malnourished patients due to selective atrophy of type II muscle (fast-twitch) fibres and resulted in a greater proportion of type I fibres which contract slowly (Lopes et al. 1982; Russell et al. 1984; Shizgal et al. 1986). Unfortunately, in our study, we did not have data on muscle type to correlate to this finding.

3.6 Chapter conclusion

No statistically significant differences between groups were seen for LM changes nor VL muscle thickness. However, MPS showed promising data to suggest HIIT incorporation would be beneficial to improve muscle synthesis rate while on VLCD. While VLCD+R did not show any significant difference to other groups from LM preservation or muscle thickness change, we found a significant increase in 1-RM when RET was incorporated with VLCD, while the other two groups had a stable, more importantly, no decline 1-RM. The differences in intensity and duration of the exercise protocol or protein content during the VLCD period could affect the LM preservation in comparison with other studies. Higher LM loss than expected from DXA assessment are due to DXA inability to differentiate between inter/intramuscular fat, glycogen and water content with muscle tissue itself.

Chapter 4: Metabolic effect following 6-week interventions

4.1 Chapter synopsis

Background: A very low-calorie diet (VLCD) is one of the most intense dietary strategies, capable of inducing rapid weight loss among overweight/obese people and has been proven to significantly improve lipid profile and insulin sensitivity, hence reducing the risk of cardiovascular disease (CVD). Resistance exercise training (RET) and high-intensity interval training (HIIT) have also been shown to improve those markers. However, research incorporating RET or HIIT with VLCD and the impact on these parameters are limited in number. We carried out a study on the effect of 6-week VLCD only (VLCD+O) or with exercises; RET (VLCR+R) or HIIT (VLCD+H) towards lipid profiles and insulin indices.

Objectives: 1) To evaluate changes in lipid profiles which include total cholesterol (TC), high-density lipoprotein cholesterol (HDL), TC to HDL ratio (Chol/HDL) and triglyceride (TG) following interventions; 2) To evaluate changes in insulin indices which include Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), the ratio of area under the curve for insulin over the area under the curve for glucose from 120-minute oral glucose tolerance test ($AUC_{i/g120}$), Quantitative Insulin Sensitivity Check Index (QUICKI),

Matsuda Index and Cederholm Sensitivity Index following interventions.

Protocol: 26 overweight/obese men were randomly allocated into VLCD+O (n=10; age 46±3y; weight 104±4kg; BMI 32±1, mean±SEM), VLCD+R (n=8; 40±3y; 98±3kg; BMI 32±1) or VLCD+H (n=8; 46±4y; 101±4kg; BMI 33±1). Fasting blood samples were taken for lipid profiles and HbA1c. Arterialised blood sampling was taken at baseline before 75g oral glucose loading and every 15 minutes afterwards for 120 minutes. Glucose and insulin were measured, and insulin indices were calculated using established formulas.

Results: All groups had significant improvement in insulin indices (HOMA-IR, $AUC_{i/g120}$, QUICKI, Matsuda Index or Cederholm Sensitivity Index), but no significant difference between the group statistically. Significant improvement from baseline was seen in TC (21.2%±4.8, 20.4%±5.2, 27.6%±5.1), Chol/HDL (25.6%±3.6, 25.9%±6.4, 25.3%±6.1) and TG (42.4%±3.5, 31.9%±14.0, 38.6%±8.1) for all groups, but no significant changes in HDL. No statistically significant difference was seen between the groups in any of the lipid parameters.

Conclusion: We found significant improvement in most of the insulin indices and lipid profiles. However, no statistically significant

difference was seen between groups suggesting exercise incorporation with VLCD confer no additional benefit for insulin function and lipid profiles.

4.2 Introduction

Obesity is associated with elevated blood glucose and insulin levels, elevated triglycerides (TG), low level of high-density lipoprotein cholesterol (HDL), and high level of low-density lipoprotein cholesterol (LDL), all of which serve as prominent risk factors for cardiovascular disease (CVD) (Mooradian et al. 2008; Klop et al. 2013).

VLCD is known to contribute to mainly fat mass (FM) loss among overweight/obese people, as well as lean mass (LM) loss which includes muscle mass. With skeletal muscle accounts for up to 85% of glucose uptake under the influence of insulin and acts as a primary site of insulin resistance (DeFronzo et al. 1981; Rennie et al. 2004; Bouzakri et al. 2005), the ability of the body to maintain or improve glucose control is of concern following significant weight loss. It has been previously shown that weight-reduced individuals were significantly more insulin sensitive compared with others without weight loss. Foo et al. found that HOMA-IR improved significantly from 6.8 to 4.3 following 6-day VLCD (Foo et al. 2011). Similarly, Svendsen et al. found improvement in HOMA-IR significantly from 1.0 to 0.6 following 8-week VLCD (Svendsen et al. 2012).

In addition to benefit on glucose homeostasis, Rolland et al. found VLCD contributed to a significant reduction of CHOL, LDL, and fasting glucose, with increase HDL at three months compared to a low carb/high protein diet. Only at nine months, VLCD showed significant TG and HbA1c improvement compared to the other group (Rolland et al. 2009). Reduction in total cholesterol (TC), TG, LDL has also been seen in another study (Raitakari et al. 2004). Changes in these lipid profiles are important as lowering LDL by 1 mmol/l is associated with about 20% reduction in the 5-year incidence of major CVD events (Baigent et al. 2005), 1 mmol/L increase in TC is associated with a 20% increase CVD in women and 24% in men (Peters et al. 2016), and 1 mmol/L increase in TG has been associated with an increase in CVD risk by 12% in men and 37% in women (Abdel-Maksoud and Hokanson 2002). Excess cellular cholesterol also plays a direct role in pancreatic islet dysfunction and might be a key factor underlying the progression of type 2 diabetes mellitus (T2DM) (Hao et al. 2007). Visceral obesity has been associated with insulin resistance mediated by cell signalling proteins adipokines and free fatty acids (FA). Adipokines such as leptin and adiponectin improve insulin sensitivity, while resistin and retinol-binding protein 4 (RBP4) decrease insulin sensitivity (Flock et al. 2011).

Weight loss improves metabolic profile via an increased in adipose tissue capacity for glucose uptake and FA oxidation (Bouwman et al. 2014). Under normal condition adipose tissue relies on glucose as the primary energy source (Frayn et al. 1995), however, under a low-calorie environment such as in VLCD, adipose tissue switch into the oxidation of FA as the main source of energy (Magnusson et al. 2008; Franck et al. 2011). Using FA as an energy source reduces the FA load in the body, which contribute to fat loss and improvement in insulin sensitivity of peripheral tissues (Maassen et al. 2007). Interestingly, adipose tissue remains to favour energy from mitochondrial β-oxidation of FA instead of glucose as their major source of energy despite an increase in the glucose uptake even after 3-week post VLCD while on a maintenance diet (Bouwman et al. 2014). Hagström-Toft et al. demonstrated that following weight loss from VLCD, insulin began to significantly stimulate lipolysis rate in skeletal muscle up to five-fold the baseline value (Hagström-Toft et al. 2001).

Besides dietary intervention, various exercised regimes, including resistance exercise training (RET) and high-intensity interval training (HIIT), have been utilised to improve lipid profiles and insulin

function to minimise the risk of CVD (Prabhakaran et al. 1999; O'Donovan et al. 2005; Gordon et al. 2014; Mann et al. 2014; Fisher et al. 2015; Ouerghi et al. 2017; Khammassi et al. 2018), in which all undoubtedly proven to be beneficial. Despite this, research looking into the combination of VLCD with or without concomitant RET or HIIT and their effects on lipid profiles and insulin function remain scarce.

4.3 Study Design

4.3.1 Recruitment and interventions

Ethics study approval and participants recruitment are as per detailed description in Chapter 2, section 2.3 Study design**.**

The total number of participants were 10 in VLCD+O, 8 in VLCD+R and 8 in VLCD+H. Ages at baseline were 46y±3, 40y±3, 46y±4 for VLCD+O, VLCD+R, VLCD+H, respectively. Mean baseline weights were 103.8kg±3.9, 98.4kg±3.4, 101.0kg±4.3, and mean BMI were 32kg.m^{-2}±1, 32kg.m^{-2}±1, 33kg.m^{-2}±1 in similar respective order.

4.3.2 Fasting blood sampling for lipid profile and HbA1c

Participants attended our research unit fasted from midnight but was allowed to drink clear water to avoid dehydration. Blood samples were taken at 9 am by a competent research team member via a retrograde cannula inserted on the dorsal aspect of the hand. This cannula also was used for OGTT (explained below). Blood samples were taken from this cannula after insertion to avoid unnecessary repeated needle pain to the participants.

The blood samples that were taken for the lipid profiles and HbA1c were sent to the on-site pathology laboratory run by the Royal Derby Hospital. Results were normally available the following day within the hospital result database that was assessable to the clinician within the research team. Results were recorded into an electronic copy (cropped using Windows Snipping Tool or individually copied into excel) by ensuring the identifiable details were excluded and only linked to the research ID unique to each participant.

4.3.3 Oral glucose tolerance test using arterialised venous sample

Following initial fasting blood sampling, the hand with the cannula was rested in a handwarmer box (Figure 4.1) set to 50°C. The warming of the hand help to arterialise the venous circulation, which is an accepted surrogate for arterial blood at a significantly lower risk than the arterial puncture (Copeland et al. 1992). After about 20 minutes inside the hand warmer, a baseline OGTT blood sample was taken into a syringe from the retrograde cannula connected to a three-way tap via a long line outside the handwarmer box. The three-way tap was used to make the withdrawing blood procedure much easier with less blood leaked from the line. A blood sample (approximately 3ml) was transferred into an EDTA blood container, and only a small amount (about 0.5ml) was left in the syringe to be used for immediate glucose level measurement using a glucose analyser (YSI 2300 STAT Plus, Yellow Springs Inc., Ohio, USA). The glucose analyser was set for automatic calibration every three hours, as well as manual calibration check by trained researchers or research assistants every morning.

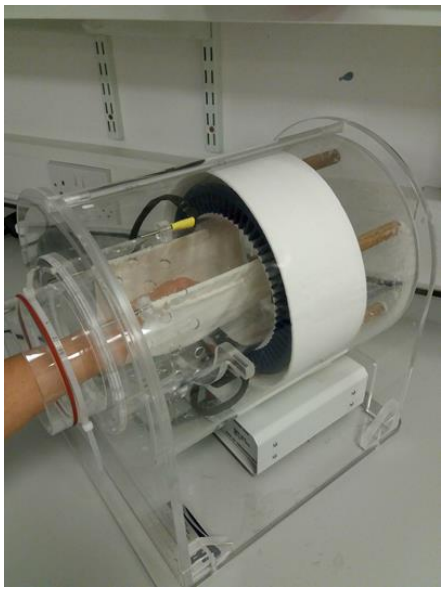


Figure 4.1 – Handwarmer box for arterialisation of the venous sampling.

Participants then drank 75g dextrose diluted in 200-350 mls of water (Pure Dextrose Powder, Bulk Powders™, Colchester, UK). After the glucose load, blood samples were taken every 15 minutes for 2 hours. Each sample was transferred into an EDTA blood container, and a small volume left in the syringe was immediately analysed for the glucose level. This would provide us with the glucose data every 15-minute interval for the 2-hour procedure.

At the end of the OGTT, the EDTA blood containers were centrifuged at 20000rpm for 20 minutes to separate the plasma from the blood

cells. Plasma samples then were aliquoted into Eppendorf tubes and stored in a -80^{c} freezer for analysis of insulin level at a later stage.

4.3.4 Insulin ELISA

Plasma from the -80^{c} freezer was defrosted completely, then spun at 12,000 rpm for 5-10 minutes to precipitate any debris. Plasma was then diluted in 1:13 with calibrator 0.1x Enzyme conjugate, and 1x washing buffer was made according to the kit protocol (Mercodia, Sweden). 25µl of each calibrator and samples were added into the appropriate pre-designed well. 100µl of 1x enzyme conjugate was added using a multichannel pipette to each well. The plate was then incubated at room temperature for 60 minutes on a plate shaker (700 rpm). The plate was then inverted to discard the solution and washed with 350µl of 1x washing buffer by using a multichannel pipette. The wash was repeated five times with firmly tapping against absorbent paper to remove any excess. By using the multichannel, 200µl of TMB substrate was added to each well and incubated at room temperature for 30 minutes. The reaction was then stopped with 50µl of stopping reagent. The plate was mixed shortly (10 seconds) before reading the optical density at 450 nm on the plate reader. Concentration was calculated from a standard

linear curve. The insulin levels from the calculation were recorded for the insulin indices calculation.

4.3.5 Calculation formula for insulin indices

HOMA-IR was used to assess insulin resistance (Matthews et al. 1985; Wallace et al. 2004):

HOMA-IR= (glucose in mmol/L x insulin in mIU/mL)/22.5

The quantitative insulin sensitivity check index (QUICKI) have a good correlation with insulin clamp (r=0.78) for insulin sensitivity assessment (Katz et al. 2000; Yeni-Komshian et al. 2000; Mather et al. 2001). QUICKI was calculated using the following formula:

QUICKI = 1/[log(I0) + log(G0)], where I0 is fasting insulin in microU/ml, and G0 is fasting glucose in mg/dL.

It has been shown that different methods used to calculate AUC may affect the interpretation of whether or not an intervention was effective. Incremental or positive incremental AUC methods will lead to similar conclusions, whilst the use of the total AUC method yields a different result and is preferred over other methods as it is not dependent upon an ever-changing 'baseline' level for glucose and

insulin (Potteiger et al. 2002). We applied the previously described trapezoidal method (Tai 1994; Curve 1995) to calculate the total area under the curve for both insulin and glucose over the 2-h OGTT, using 15-minute interval glucose and insulin result.

Area = $\frac{1}{2}\sum_{i=1}^{n} X_{I-1}(Y_{i-1} + Y_i)$ (Tai's formula)

When the curve passes the origin: $X_0 = Y_0 = 0$, $X_0 = X_1 - 0$;

When the curve intercepts Y-axis at Y_0: $X_0 = X_1 - 0$

When the curve neither passes the origin nor intercepts at y-axis: $X_0 = Y_0 = 0$

An index proposed by Matsuda and DeFronzo was correlated to clamp-derived insulin sensitivity (r = 0.73, P <0.0001), with parameters from the OGTT decrease when glucose tolerance worsening (Matsuda and Defronzo 1999). It is a composite of whole-body insulin sensitivity index that estimate hepatic and muscle insulin sensitivity. The following formula was applied:

Matsuda Index= $\frac{10{,}000}{\sqrt{(fasting\ glucose)x(fating\ insulin)x(mean\ glucose)x(mean\ insulin)}}$

Glucose unit is in mg/dl, and insulin unit is in microU/ml

Cederholm and Wibell proposed an insulin sensitivity index that represents mainly peripheral insulin sensitivity and muscle glucose uptake, reflecting the dominant role of peripheral tissues in glucose disposal after an oral glucose load. (Cederholm and Wibell 1990). The formula for the Cederholm-Wibell index is:

$$Cederholm = \frac{75000 + [(\mathrm{G0} - \mathrm{G120}) \times 1.15 \times 180 \times 0.19 \times \mathrm{m}]}{120 \times \mathrm{G}mean \times \log(\mathrm{I}mean)}$$

where 75,000 = oral glucose load in an OGTT in mg, G*0* = fasting plasma glucose concentration (mmol/l), G120 = plasma glucose concentration in the 120th min of OGTT (mmol/l), 1.15 = factor transforming whole venous blood glucose to plasma values, 180 = conversion factor to transform plasma glucose concentration from mmol/l into mg/dl, 0.19 = glucose space in litre per kg of body weight, m = body weight (kg), 120 = duration of OGTT (min), G*mean* = mean plasma glucose concentration during OGTT (mmol/l) and I*mean* = mean plasma insulin concentration during OGTT (mIU/l).

4.3.6 Statistical analyses

Statistical analyses were performed as described in Chapter 2, section 2.3.7.

4.4 Results

4.4.1 Insulin resistance and insulin sensitivities

Reduction in the values in HOMA-IR reflects a reduction in insulin resistance, hence improvement in overall body insulin function (Imamura et al. 2013). Low $AUC_{i/g120}$ means that less total amount of insulin is needed for a unit volume of glucose, hence reduction in this value means better whole-body response to insulin and less insulin resistance.

For insulin sensitivity, improvement in the QUICKI, Matsuda index and Cederholm values would signify an improvement in glucose homeostasis (Aloulou et al. 2006).

4.4.1.1 HOMA-IR

HOMA-IR changed from 1.2±0.2 to 0.5±0.1 for VLCD+O, 2.2±0.4 to 1.3±0.4 for VLCD+R and 1.9±0.5 to 1.0±0.2 for VLCD+H (Figure 4.2). No statistically significant difference was seen between the groups.

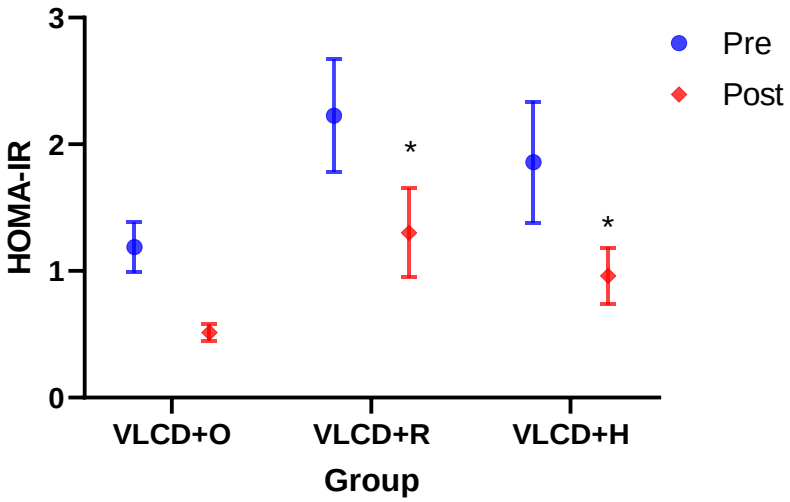


Figure 4.2 – HOMA-IR pre- and post-intervention value. *P<0.05.

4.4.1.2 $AUC_{i/g120}$

VLCD+R and VLCD+H groups showed statistically significant (P<0.01) improvement in $AUC_{i/g120}$ post-intervention; from 24.0±6.5 to 13.8±3.6, 13.4±3.5 to 6.7±1.3 respectively, while VLCD+O had

improvement from 7.3±1.6 to 4.5±0.7 but not statistically significant (Figure 4.3). No statistically significant difference was seen between the groups.

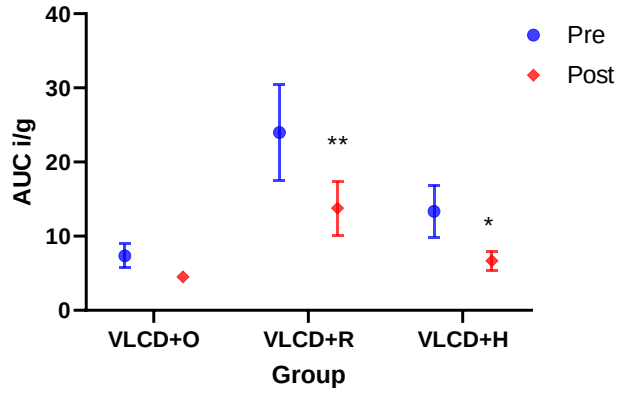


Figure 4.3 – $AUC_{i/g120}$ pre- and post-intervention value. $^{*}P<0.05$, $^{**}P<0.01$.

4.4.1.3 QUICKI

VLCD+O and VLCD+R showed statistically significant ($P<0.05$) improvement from the baseline with a mean of 0.38±0.01 to 0.44±0.01 and 0.35±0.01 to 0.39±0.02, respectively (Figure 4.4). Non-significant change in VLCD+H from 0.37±0.02 to 0.40±0.02. Despite this, no statistically significant difference between the groups was seen.

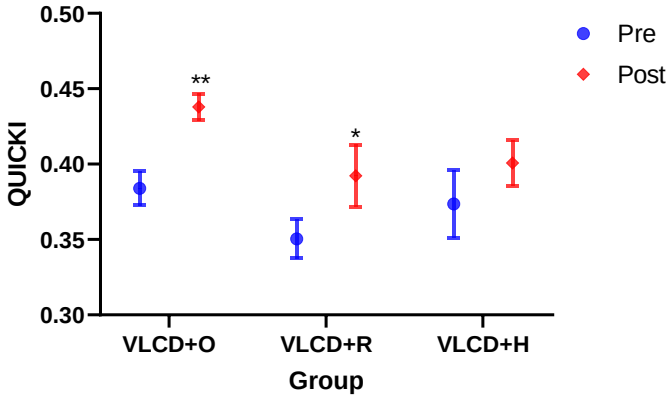


Figure 4.4 - QUICKI pre- and post-intervention value. *P<0.05, **P<0.01.

4.4.1.4 Matsuda index

Significant improvement from the baseline in Matsuda index was seen for VLCD+O and VLCD+R groups (Figure 4.5). Changes were from 7.1±1.0 to 12.5±1.4 and 3.5±1.0 to 7.1±1.9 respectively. Non-significant change in VLCD+H from 5.7±1.7 to 8.6±1.6 was seen. No statistically significant difference between the groups was seen.

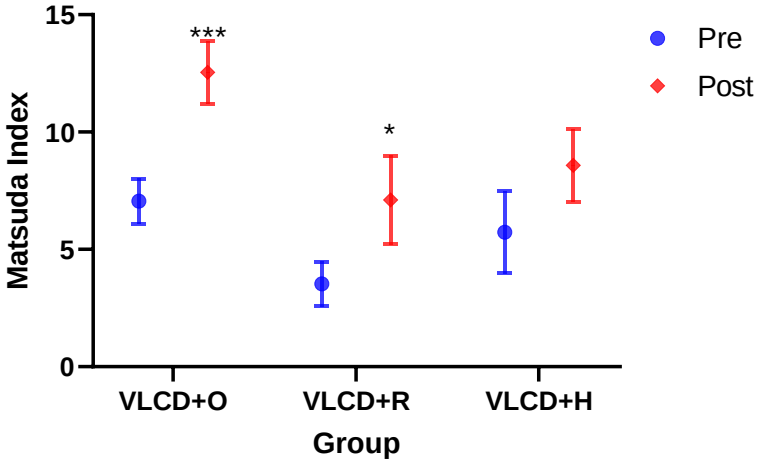


Figure 4.5 – Matsuda index pre- and post-intervention value. *P<0.05, ***P<0.001.

4.4.1.5 Cederholm Sensitivity Index

Statistically significant improvement in Cederholm Sensitivity Index was seen in VLCD+O and VLCD+R from 42.4±3.0 to 51.6±4.6 and 33.8±4.2 to 43.1±4.1, respectively (Figure 4.6). Improvement in the means was seen in VLCD+H groups but was not statistically significant, with changes from 39.3±3.9 to 41.5±3.3. No statistically significant difference between the groups was seen.

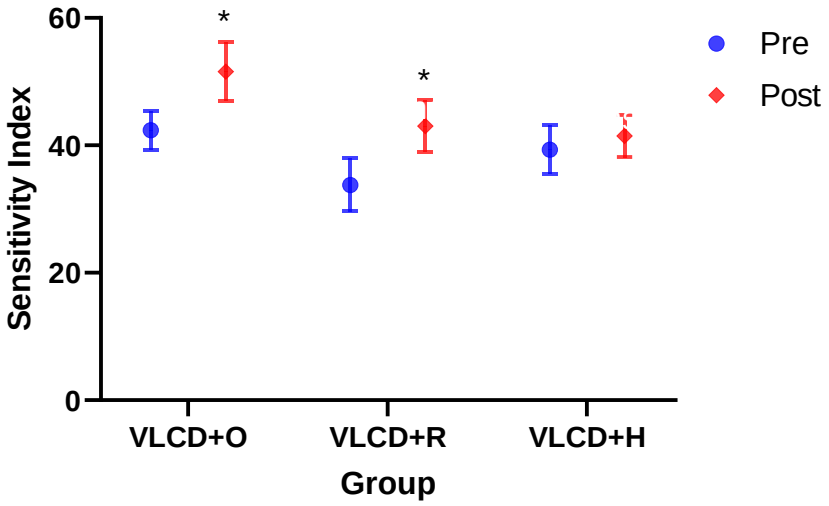


Figure 4.6 – Cederholm Sensitivity Index pre- and post-intervention value. *P<0.05.

4.4.1.6 Summary table for the changes in insulin indices

Table 4.1 - Insulin indices at baseline and post- interventions for all groups. No significant difference between the groups.

	VLCD+O	VLCD+R	VLCD+H
HOMA-IR			
Baseline	1.12±0.2	2.2±0.4	1.9±0.5
Post-intervention	0.5±0.1	1.3±0.4*	1.0±0.2*
$AUC_{i/g120}$			
Baseline	7.3±1.6	24.0±6.5	13.4±3.5
Post-intervention	4.5±0.7	13.8±3.6**	6.7±1.3*
QUICKI			
Baseline	0.38±0.01	0.35±0.01	0.37±0.02
Post-intervention	0.44±0.01**	0.39±0.02*	0.40±0.02
Matsuda Index			
Baseline	7.1±1.0	3.5±1.0	5.7±1.7
Post-intervention	12.5±1.4***	7.1±1.9*	8.6±1.6
Cederholm			
Baseline	42.4±3.0	33.8±4.2	39.3±3.9
Post-intervention	51.6±4.6*	43.1 ±4.1*	41.5±3.3

*Indicates P-value changes from baseline; *P<0.05, **P<0.01, ***P<0.001.

4.4.2 HBA1c

No significant changes in mean HbA1c from baseline in all groups. The values were 2.0mmol/mol±1.4, -1.8 mmol/mol±1.7, and -1.4 mmol.mol±1.0 for VLCD+O, VLCD+R, and VLCD+H respectively (Figure 4.7 and Figure 4.8). No significant difference was seen between the groups statistically.

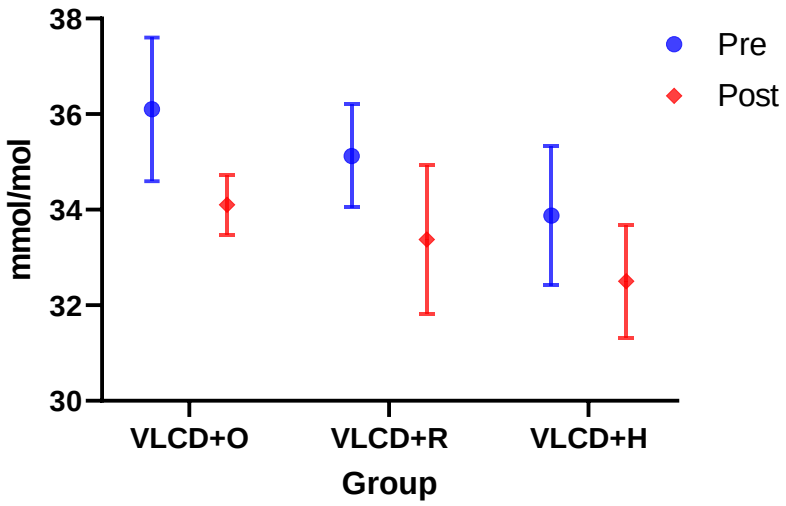


Figure 4.7 – HbA1c pre- and post-intervention for all groups.

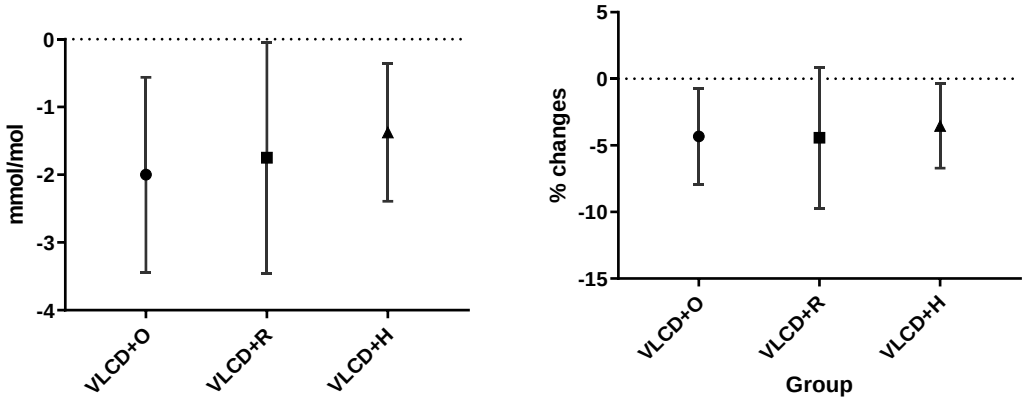


Figure 4.8 – Absolute (left) and relative (right) HbA1c changes from baseline for each group. No significant difference between the groups.

4.4.3 Lipid profiles

4.4.3.1 Total cholesterol

All groups had a statistically significant reduction in TC level ($P<0.05$). TC changes in VLCD+O from 5.3mmol/l±0.3 to 4.1mmol/l±0.3, VLCD+R from 5.3mmol/l±0.4 to 4.2mmol/l ±0.3 and VLCD+H from 5.8mmol/l±0.4 to 4.1mmol/l±0.3 (Figure 4.9). The relative drop in level was highest in the VLCD+H group at 27.6%±5.1, followed by VLCD+O at 21.2%±4.8 and VLCD+R at 20.4%±5.2. No statistically significant difference was seen between the groups.

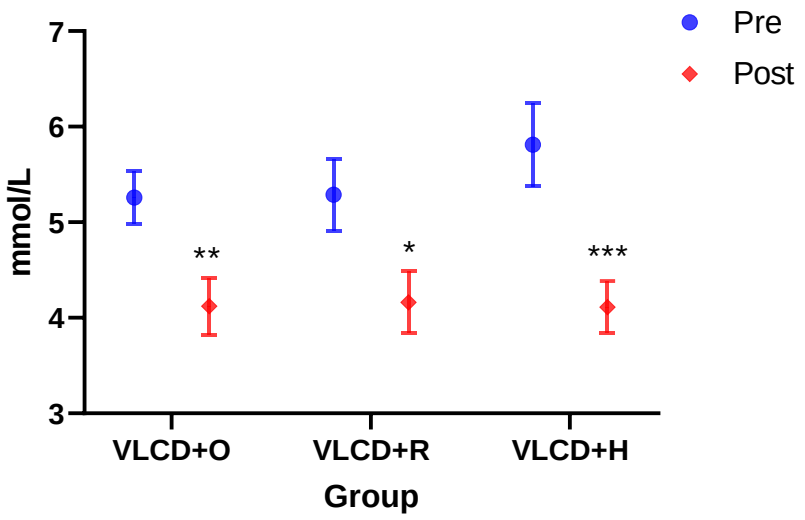


Figure 4.9 – Total cholesterol level pre- and post-intervention. *P<0.05 **P<0.01, ***P<0.001.

4.4.3.2 HDL cholesterol

No significant absolute or relative changes in the HDL level in all groups. Mean changes were 0.07mmol/l±0.08, 0.04mmol/l±0.14 and -0.04mmol/l±0.05 for VLCD+O, VLCD+R and VLCD+H respectively. Relative changes were 8.1%±6.8, 9.8%±11.8, and -2.0%±4.8, respectively. No statistically significant difference was seen between groups.

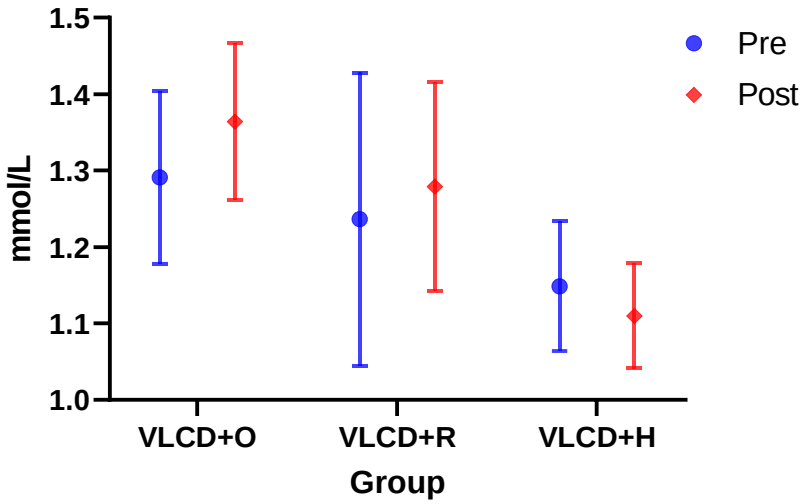


Figure 4.10 – HDL level pre- and post-intervention.

4.4.3.3 Chol/HDL

Statistically significant changes were seen in Chol/HDL ratio following interventions in all groups. VLCD+O value improved from 4.3±0.3 to 3.2±0.3, VLCD+R from 4.7±0.5 to 3.4±0.4, and VLCD+H from 5.2±0.5 to 3.8±0.3 (Figure 4.11). Relative changes from baseline were 25.6%±3.6, 25.9%±6.4, 25.3%±6.1 for VLCD+O, VLCD+R and VLCD+H, respectively. However, no statistically significant difference between groups was seen.

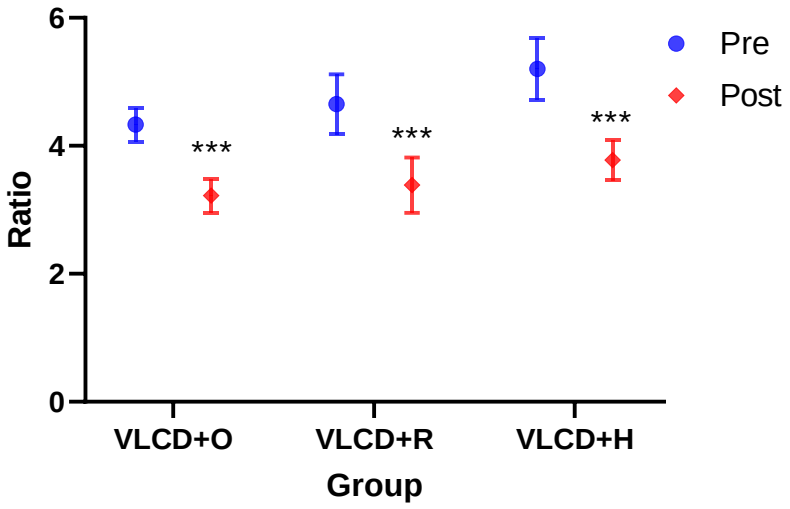


Figure 4.11 – Chol/HDL ratio pre- and post-intervention. ***P<0.001.

4.4.3.4 Triglyceride

All groups had significant reduction in TG level, with VLCD+O from mean of 1.5mmol/l±0.1 to 0.9mmol/l±0.1, VLCD+R from 2.0mmol/l±0.4 to 1.2mmol/l±0.2 and VLCD+H from 2.1mmol/l±0.3 to 1.2mmol/l±0.2 (Figure 4.12). The relative drop in TG level was highest in the VLCD+O group at 42.4%±3.5, followed by VLCD+H at 38.6%±8.1 and VLCD+R at 31.9%±14.0. No statistically significant difference between groups was seen.

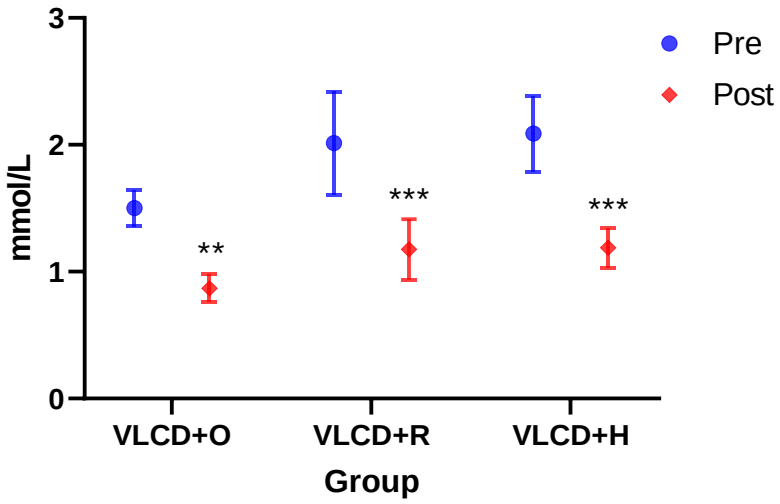


Figure 4.12 – TG level pre- and post-intervention. $^{**}P<0.01$, $^{***}P<0.001$.

4.4.3.5 Summary table for changes in lipid profiles

Table 4.2 – Lipid profile at baseline and post-6-week interventions for all groups.

	VLCD+O	VLCD+R	VLCD+H
TC (mmol/l)			
Baseline	5.3±0.3	5.3±0.4	5.8±0.4
Post-intervention	4.1±0.3**	4.2±0.3*	4.1±0.3***
HD (mmol/l)			
Baseline	1.3±0.1	1.2±0.2	1.1±0.1
Post-intervention	1.4±0.1	1.3±0.1	1.1±0.1
Chol/HD (mmol/l)			
Baseline	4.3±0.3	4.7±0.5	5.2±0.5
Post-intervention	3.2±0.3***	3.4±0.4***	3.8±0.3***
TG (mmol/l)			
Baseline	1.5±0.1	2.0±0.4	2.1±0.3
Post-intervention	0.9±0.1**	1.2±0.2***	1.2±0.2***

*Indicates *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. No significant difference between the groups.

Table 4.3 – Relative lipid profile changes from baseline in all groups.

	VLCD+O	VLCD+R	VLCD+H
TC	-21.2%±4.8**	-20.4%±5.2*	-27.6%±5.1**
HDL	8.1%±6.8	9.8%±11.8	-2.0%±4.8
Chol/HDL	-25.6%±3.6****	-25.9%±6.4**	-25.3%±6.1**
TG	-42.4%±3.5****	-31.9%±14.0**	-38.6%±8.1**

*Indicates *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. No significant difference between the groups.

4.5 Discussion

Central obesity is strongly related to metabolic syndrome, which includes a magnitude of conditions that are recognised risk factors for CVD, in particular insulin resistance and dyslipidaemia (Huang 2009; Boden 2011; Zalesin et al. 2011). Various studies have proven that weight loss helps to improve insulin function as well as lipid profiles hence reducing the risk of CVD (Melanson et al. 2003). We have observed a similar benefit from weight loss following our 6-week of VLCD with or without concomitant RET or HIIT.

In our study, various assessments in insulin sensitivity indices pointed to a significant improvement in insulin function, although some indices only showed significant changes within certain groups. HOMA-IR and $AUC_{i/g120,}$ for example, only show significant changes for VLCD+R and VLCD+H, while QUICKI, Matsuda index and Cederholm Sensitivity Index showed significant changes in VLCD+O and VLCR+R groups. Regardless of the presence or absence of significant changes from statistical analyses, all groups trending towards improvement in overall insulin function. With no statistically

significant difference between the groups, these findings suggest that no additional benefit from exercises towards insulin sensitivities.

The inconsistency in statistically significant changes could be owed to the fact that we had only a small number of samples in each group, i.e. 10 in VLCD+O, 8 in each VLCD+R and VLCD+H. We also had participants from various ethnicity, i.e. White, Asian, and Hispanic, with the majority of VLCD+O and VLCD+H participants were from the White ethnic groups, while the majority of VLCD+R participants were from Asian ethnic groups. Different ethnicity has been shown to have different normal cut-off value due to different degree of insulin sensitivity/resistance indices (Stern et al. 2005; Radikova et al. 2006; Kodama et al. 2013; Raygor et al. 2019).

$AUC_{i/g120}$ is a rather straightforward calculation involving the ratio of total insulin to the total glucose within the arterial circulation (sampling via arterialised venous sampling) for the total duration of 120-minute OGTT. The $AUC_{ins/glu}$ has been previously recognised as an index of insulin secretion (Kahn 2001; Charbonnel et al. 2006; Retnakaran et al. 2008; Lee et al. 2014). The lower the insulin required for a similar unit of glucose, i.e. low $AUC_{i/g120}$ value suggests better insulin sensitivity and less insulin resistance. In other words,

with improved insulin sensitivity, less insulin needed to be secreted for a particular amount of glucose in the circulation. Hence insulin secretion is conversely related to insulin sensitivity (Kahn 2001; Ahrén and Larsson 2002). We found that the relative reduction of $AUC_{i/g120}$ was nearly 40%, which is more than previously seen by Lee et al. at only 14% relative reduction (Lee et al. 2014). This is likely due to the differences in the degree of total weight loss as well as the integration of exercise regimes in our study.

In the context of HbA1c, we found that all groups showed reduced levels, although these were not statistically significant. This is because as our participants are non-diabetic, the HbA1c level was already within the normal range at the baseline. Hence further reduction in the HbA1c might not reflect benefit, but rather the risk of hypoglycaemia.

For lipid profiles, we found significant changes from baseline in all groups for TC, Chol/HDL ratio and TG level. No statistically significant difference was seen between the groups suggesting no added benefit from exercises in regards to these parameters. Reductions in the lipid profiles have been observed in other various weight loss studies highlighting the apparent benefit of weight reduction (Brook et al.

2004; Keogh et al. 2008; Mavri et al. 2011; Fayh et al. 2013; Mohler et al. 2013; Joris et al. 2017).

We have seen no significant changes in HLD level from baseline in any group. Interestingly, VLCD+H had a 2% reduction in the mean HDL compared to baseline, but not statistically significant. Under normal calorie settings, O'Donovan et al. and Fisher et al. found a similar pattern of a non-significance drop in HDL level by 0.7% and 3% respectively from the baseline within the HIIT group (O'Donovan et al. 2005; Fisher et al. 2015). Khammassi et al. and Ouerghi et al. found a non-significant increase in HDL from baseline in the HIIT group by 0.3% and 0.5% (Ouerghi et al. 2017; Khammassi et al. 2018). With weight loss, we would expect improvement in HDL level owing to the fact that adipose cells are known to bind to HDL (Fong et al. 1985), and losing weight means losing adipose tissue, hence less HDL uptake from circulation results in an increase in plasma HDL levels (Shoji et al. 1991). As a high level of HDL has been associated with decreased risk of coronary artery disease, a reduction in the level would be of concern (Toth 2005). HDL plays an important role in transporting cholesterol in peripheral tissues to the liver for recycle or for bile acid synthesis, reducing the accumulation of cholesterol in the arteries (Tall 1990), as well as serves as

cardioprotective due to its antioxidant activity (Mooradian et al. 2006). Overall, while HDL level reduced following weight loss, when compared to the significant drop in TC, the drop in HDL constitutes rather only a small relative drop in this cholesterol-HDL dynamic, as evidenced from a significant decrease in Chol/HDL ratio for all groups by around 25% from baseline.

VLCD+H had the highest reduction in TC by 28%, which might compensate for inadequate HDL level changes. To date, we have yet to understand if the lack in HDL level change is actually due to reciprocal response to dropping in TC level, given its role in clearing cholesterol from the arteries (Tall 1990). We also found a significant decrease in TG from baseline in all groups between 32% to 42% change. Our findings are consistent with others showing significant reduction in TC and TG among overweight/obese subjects following HIIT intervention while on normal calorie intake (O'Donovan et al. 2005; Fisher et al. 2015; Ouerghi et al. 2017; Khammassi et al. 2018). Our study had the biggest changes compared to the other HIIT study, likely due to our VLCD aspect of the intervention, although the different intensity of HIIT, age group distribution, age, baseline weight and BMI, to name a few, could have contributed to some degree of variation in the outcomes. Unfortunately, no other

HIIT in combination with VLCD has been carried out apart from ours for direct comparison.

4.6 Chapter conclusion

Insulin indices showed significant improvement from baseline in all three groups. Significant improvement in TC, TG and Chol/HDL were also seen in all groups. No significant changes in HbA1c level in any group, most likely because the level has been normal at baseline. From our findings, we conclude that VLCD leads to weight loss which in turn contributes to significant improvement in various insulin indices and lipid profiles. However, no statistically significant difference was seen with additional exercises suggest additional exercises confer no benefit for improvement in insulin function and lipid profiles.

Chapter 5: Cardiac, vascular and cardiorespiratory outcomes

5.1 Chapter synopsis

Background: Obesity is known to be associated with a multitude of health problems, including impairment in cardiovascular physiology and reduced cardiorespiratory fitness. Weight loss as the countermeasure has been proven to improve various parameters related to cardiovascular and cardiorespiratory health, namely blood pressure, endothelial function and $VO_{2,}$ to name a few. A very low-calorie diet (VLCD) is one of the most intense dietary strategies able to induce rapid weight loss among overweight/obese. We carried out a study on the effect of 6-week VLCD alone (VLCD+O) or with concomitant resistance exercise training (VLCD+R) or high-intensity interval training (VCD+H) on cardiorespiratory fitness.

Objectives: To investigate the effect of VLCD alone or with a combination of exercises on 1) blood pressure; 2) endothelial function using flow-mediated dilatation (FMD); 3) microvascular blood circulation using contrast-enhanced ultrasound (CEUS); 4) cardiorespiratory using cardiopulmonary exercise test (CPET).

Protocol: 26 overweight/obese men were randomly allocated into VLCD+O (n=10; age 46±3y; weight 104±4kg; BMI 32±1, mean±SEM), VLCD+R (n=8; 40±3y; 98±3kg; BMI 32±1) or VLCD+H (n=8; 46±4y; 101±4kg; BMI 33±1). Interventions were for 6 weeks, all participants received VLCD in the form of LighterLife®

meal replacement plus free form diet to maintain daily calories below 800 kcal, while VLCD+R had additional resistances exercise and VLCD+H had additional cycle ergometer high-intensity interval training alongside the VLCD, with all form of exercises performed three times per week.

Results: Improvement in systolic blood pressure for all groups, pre- and post-intervention readings (mean±SEM) for VLCD+O, VLCD+R and VLCD+H were 128.2mmHg±3.2 to 117.3mmHg±4.0, 126.5mmHg±3.6 to 114.9mmHg±2.5 and 126.5mmHg±3.6 to 114.9mmHg±2.5 respectively, $P<0.05$. Similar trend for diastolic blood pressure from 84.0mmHg±3.8 to 77.0mmHg±3.8, 83.0mmHg±2.3 to 72.8mmHg±2.9, and 85.3mmHg±2.6 to 77.4mmHg±3.1 respectively, $P<0.05$. No significant change in FMD in any group with results of 1.8%±0.5 to 2.2%±0.6, 3.2%±0.6 to 4.3%±1.8, and 3.0%±0.6 to 5.7%±2.2 respectively. No significant change following intervention in microvascular blood volume, microvascular flow volume, microvascular blood flow, or left ventricular ejection fraction in any group. For CPET parameter, all groups showed no change in absolute VO_2max, but improvement in relative VO_2max from 28±3 to 31±3, 30±2 to 33±3 and 25±2 to 30±3 (unit is in $ml.kg^{-1}.min^{-1}$) for VLCD+O, VLCD+R and VLCD+H respectively, $P<0.05$. VLCD+O had a small drop in $Watt_{max}$ 244W±25

to 235W±23, while VLCD+R and VLCD+H had a small increase from 239W±16 to 241W±17 and 187W±14 to 198W±12, respectively, however, these were not statistically significant. No significant change in anaerobic threshold (AT) with readings of 17.2±2.3 to 16.8±2.2, 13.7±1.5 to 13.6±1.1, and 11.4±0.4 to 12.7±1.0 for VLCD+O, VLCD+R and VLCD+H respectively (all unit in $ml.kg^{-1}.min^{-1}$). No statistically significant difference was seen between the groups for any of the outcomes.

Conclusion: All groups showed improvement in blood pressure. Improvement in relative VO_2max was mainly due to the reduction in weight. There was no statistically significant difference between the groups for any of the outcomes, suggesting concomitant exercises with VLCD does not confer any additional benefit in term of cardiovascular markers and cardiorespiratory fitness.

5.2 Chapter introduction

Central obesity is commonly associated with hypertension, as well as dyslipidaemia and insulin resistance, which are the hallmark of metabolic syndrome (Grundy and Barnett 1990; Grundy 2004). Obesity is also long known to be a critical risk factor for atherosclerotic cardiovascular disease (CVD), principally via the impairment of endothelial function (Hubert et al. 1983; Steinberg et al. 1996; Boulanger 2016). Various studies involving human (Perticone et al. 2001a) and animal models (Galili et al. 2007; Ungvari et al. 2010; Tucsek et al. 2014) demonstrated that obesity reduces the bioavailability of nitric oxide (NO), a key molecule in vasodilatation, by promoting oxidative stress in the endothelial cells, leading to hypertension. Flow-mediated dilatation (FMD) has been used for decades to assess endothelial dysfunction (Celermajer et al. 1992), and a 1% decrease in FMD has been associated with an 8% increased risk of future cardiovascular events (Inaba et al. 2010). Interestingly beyond physiological effect, FMD has also been shown to have an inverse association with depression, in a way that worsening FMD being the adverse effect from depression (Pizzi et al. 2009; Cooper et al. 2011).

Whilst obesity in itself is a recognised risk factor for CVD, and interestingly, this risk is significantly increased if hypertension is present in overweight or obese people (Thomas et al. 2005). Remarkably, obesity and hypertension work as a two-way street; while obese subjects are prone to hypertension, hypertensive subjects who are not obese also appear to be prone to weight gain, likely due to underlying sympathetic overactivity (Julius et al. 2000). Raised free-fatty acids (FFA) in obese hypertensive individuals have been identified as one of the factors that can enhance one of the sympathetic vascular pathways, α-adrenergic sensitivity and consequently, the increase of vessel tone (Stepniakowski et al. 1995). Alternatively, there has been a suggestion that FFA inhibit Na+, K+ ATPase and the sodium pump leading to an increase in vascular smooth muscle tone and resistance (Oishi et al. 1990). Excess visceral fat has also been indicated as an important source of cytokines and other factors that create a milieu of oxidative stress that leads to hypertension and atherosclerosis (Lyon et al. 2003). Increased visceral and retroperitoneal fat may also cause hypertension due to physical compression on the kidneys, with excess fat accumulation in and around the kidneys is associated with abnormal physiological compensation by the kidney that leads to hypertension (Hall et al. 2014). Regardless of various underlying

pathophysiological effects of obesity that can lead to hypertension, multiple studies have proven that weight loss by diet or exercise can significantly improve blood pressure (Neter et al. 2003; Hall et al. 2014).

In addition to obesity effect on arterial vasculature, which leads to hypertension, obesity also causes adverse structural and functional alterations in the microcirculation, involving heart, brain, kidneys, lungs, adipose tissue, and skeletal muscle, via pathological changes including inflammatory processes, metabolic alterations, impaired barrier and transport functions (Sorop et al. 2017). As a consequence, this global impairment of microcirculation impairs organ perfusion and leads to the pathogenesis of coronary microvascular angina, heart failure, pulmonary hypertension, chronic kidney disease, exercise intolerance and even dementia (Elias et al. 2005; Whitmer et al. 2008; Sorop et al. 2017). A study on a middle-aged man showed improvement in microvascular endothelial function following weight loss (Csipo et al. 2018). Hagström-Toft et al. demonstrated that insulin infusion following weight loss following VLCD caused a marked increase in skeletal muscle blood flow (four- to fivefold) hence increased in lipolysis rate, while before VLCD, insulin has no stimulation effect on the blood flow

to the skeletal muscle (Hagström-Toft et al. 2001). Defect in blood flow regulation in obesity itself has a profound effect on insulin delivery (Baron 1993; Czernichow et al. 2010). It has also been shown that obesity has a poor microvasculature response following mixed meal feeding (Vincent et al. 2006; Keske et al. 2009). In the absence of obesity, insulin plays a crucial part in skeletal microvascular capillaries recruitment and tissue perfusion, which contribute to glucose homeostasis and metabolism by muscle tissues (Coggins et al. 2001; Vincent et al. 2002). With all these findings in mind, we, therefore, planned to utilise the contrast enhances ultrasound scan (CEUS) technique to assess the changes in microvascular blood circulation following 6-week VLCD with and without exercise interventions. CEUS would enable us to assess microvascular blood volume (MBV) and microvascular flow velocity (MFV) that can reflect microvascular blood flow (MBF) (Wei et al. 1998; Quaia 2005; Mitchell et al. 2013).

Beyond cardiovascular abnormality, obese people have been generally subjected to poor fitness compared to lean people. Whether that is just a preconceived idea, studies utilising a cardiopulmonary exercise test (CPET) to assess fitness level among obese subjects and weight loss effect on cardiopulmonary markers

showed intriguing findings. Various studies demonstrated that obesity is associated with reduced exercise capacity in the form of submaximal exercise, despite normal $VO_2.kg^{-1}$ (McInnis et al. 1996; Serés et al. 2003; Glucksman et al. 2015; Königstein et al. 2018; Matheus et al. 2018). Among obese youth, relative VO_2max $ml.kg^{-1}.min^{-1}$ was significantly improved after significant weight loss, despite absolute VO_2max $L.min^{-1}$ remained unchanged (Sothern et al. 2000). Among bariatric patients, absolute VO_2max was reduced by 10% after surgery, however, when corrected for body weight, there was an improvement in the result (Dereppe et al. 2019). Nedeljkovic-Arsenovic et al. demonstrated a significant increase in peak oxygen uptake (VO_2 peak), duration of exercise testing, and peak O_2 pulse among patients who had lost more than 18% of initial weight following bariatric surgery (Nedeljkovic-Arsenovic et al. 2019). Similarly, Browning et al. found a significant overall improvement in exercise capacity, as the patients with significant weight loss following gastric bypass surgery were able to exercise longer due to significant reductions in body mass and a subsequent reduced metabolic requirement during exercise (Browning et al. 2017).

Studies assessing the effect of VLCD on cardiopulmonary function showed various results. Hakala et al. observed improvement in VO_2peak following VLCD alone, without exercise (Hakala et al. 1996). Some found improvement in $Watt_{Max}$ as well as VO_2peak following VLCD with endurance exercises (Bryner et al. 1999; Pasman et al. 1999). Walberg et al. found subjects who underwent VLCD with endurance training had significant improvement in exercise tolerance while maintaining their VO_2peak (Walberg et al. 1988). Davis et al. found different outcomes following VLCD, where there was a reduction in VO_2peak and exercise tolerance unless VLCD were given with higher protein content (Davis and Phinney 1990). To our knowledge, to date, there is no study incorporating VLCD with HIIT. We, therefore, performed a CPET as part of our cardiopulmonary fitness assessment following VLCD with or without RET or HIIT.

5.3 Study design

5.3.1 Recruitment and interventions

Ethics study approval and participants recruitment are as per detailed description in Chapter 2, section 2.3 Study design**.**

The total number of participants were 10 in VLCD+O, 8 in VLCD+R and 8 in VLCD+H. Ages at baseline were 46y±3, 40y±3, 46y±4 for VLCD+O, VLCD+R, VLCD+H, respectively. Mean baseline weights were 103.8kg±3.9, 98.4kg±3.4, 101.0kg±4.3, and mean BMI were 32kg.m^{-2}±1, 32kg.m^{-2}±1, 33kg.m^{-2}±1 in similar respective order.

5.3.2 Blood pressure and flow-mediated dilatation

Participants were asked to fast from midnight except for clear water and attended our research unit at approximately 0800h. They were rested at a supine position for at least 30 minutes in a 24 °C temperature-controlled room. After this resting period, blood pressure (BP) was measured and recorded in three readings, a minute apart from each reading. BP measurement was done using

oscillometry (Datascope trio patient monitor, Datascope, New Jersey, USA) and a blood pressure cuff (Welch Allyn, New York, USA) of appropriate size (British and Irish Hypertension society 2017) on the participant's non-dominant arm.

Following this resting period and baseline serial BP measurements, FMD was assessed according to the International Brachial Artery Reactivity Task Force guidelines (Corretti et al. 2002) in the subject's dominant arm. A linear array ultrasound transducer (17 – 5 MHz with Philips iU22 ultrasound machine; Philips Healthcare, Amsterdam, Netherlands) was used for the brachial artery imaging. The ultrasound machine was connected to the automated real-time arterial diameter measurements using a dedicated FMD software (Quipu Cardiovascular Suite FMD Studio version 3.2.0; Quipu, Pisa, Tuscany, March 2017) (Gemignani et al. 2008). The recording was started with a baseline measurement of brachial artery diameter for one minute, followed by arterial occlusion distal forearm using a BP cuff (Hokanson, Washington, USA) inflated to at least 50 mmHg above baseline BP for 5-min. The cuff was then deflated, and immediately dilatation of the brachial artery was assessed for a further 5-min. Changes in mean arterial diameter before and after

arterial occlusion was calculated automatically using the software and expressed in percentage (%).

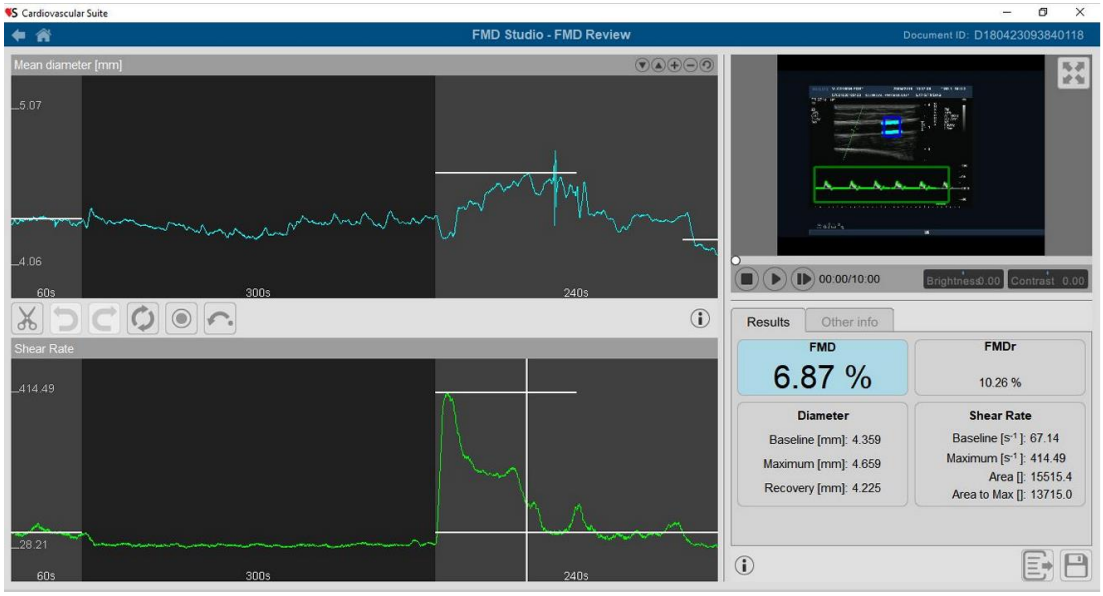


Figure 5.1 – Example of measurement of the change in arterial diameter using the real-time recording for the FMD calculation.

5.3.3 Contrast-enhanced ultrasound

Participants were seated on the knee-extension exercise machine seat while the ultrasound transducer was secured onto the thigh using a polyethene-housing unit (manufactured by the University of Nottingham's Medical Engineering) strapped onto the thigh circumference of the dominant leg using Velcro™ straps (Amsterdam, NL) to maintain constancy of position throughout the procedure. CEUS imaging of the quadriceps of the dominant leg was

performed using an L9-3 probe of Phillips iU22 scanner. Participants were rested for at least 30 minutes before recording began to eliminate any exertional effect that can mask the initial scan.

All recordings were made in contrast mode to minimise tissue-dependent signal while maximising signal from the microbubbles by a selective register of harmonics (Quaia 2005). A real-time recording was made using cine mode at 21 Hz with the following settings: low persistence (to maximize temporal resolution), contrast resolution C30 (simple harmonic registration), gain 95%, cursor gain constant throughout, depth 4-5 cm (depending on the thickness of the fatty tissue), focused 2–4 cm, and a working MI of 0.08 (MI is a unitless index proportional to peak negative pressure × transmit frequency$^{-0.5}$), causing microbubbles to resonate without destruction.

Sonovue™ powder was reconstituted into microbubbles with normal saline using a transfer system (mini-spike plus; Bracco) with a typical yield of 4.5 mL, which was increased to 5.5 mL using additional normal saline washed via the transfer system to increase yield. Prepared microbubbles were infused via a Vueject oscillating infusion pump (Bracco) connected to a low volume PVC connection

line (Acist, Eden Prarie, MN) and an 18GA (1.3 mmØ) cannula sited in the left median cubital vein. The infusion was primed at the rate of 2 mL min^{-1} for 1 min, followed by 1 mL min^{-1} until completion of the infusion, usually between 2 to 2 minutes and 15 seconds. Back-to-back 30-sec recordings were made from the start of the infusion, and high MI "flashes" (MI 1.12, duration 0.57 sec) were applied at the very beginning of every recording cycle beginning at the fourth recording cycle, allowing enough time for the microbubbles to reach a steady-state during the first three recordings (Mitchell et al. 2013). The high MI flashes destroyed all existing microbubbles and allowed for the recording of replenishment of the microbubbles, and enabled us to evaluate the changes in acoustic intensity (AI). AI that was generated from the resonating microbubbles is proportional to the microbubbles concentration in the area of interest (Sjøberg et al. 2011). The fourth and fifth recordings were made while participants were at rest. Immediately after the fifth recording, participants performed six reps of knee extension at 50% 1-RM, which was set as a standardised exertional stimulant for this study. The 1-RM was measured at an earlier time when participants arrived at the unit in the morning, at least 2 hours before CEUS measurement started. Following the knee extension, another two 30-second recordings were done to complete the procedure. This would allow us to

compare the effect of an exertional stimulant on the changes in the microvascular blood flow (MBF).

Recordings were exported to quantification software (Q-lab; Philips) for analysis. Regions of interest (ROI) were drawn to exclude connective tissue and large vessels, and property files for the ROI shape were copied to subsequent files (over the total ~2 minutes recordings) to ensure that ROIs were identical (i.e., same ROI shape for the same participant). A period immediately after high MI flash was used to calculate the acoustic AI. Using previous calculation by Sjøberg et al., the AI obtained the first 0.5s post flash in the rest period, and after the first 0.24s post flash following muscle contractions were averaged and subtracted from the AI recorded during the remaining seconds to eliminating background noise and from rapid-filling vessels, e.g. arteries, veins, arterioles or venules (Sjøberg et al. 2011).

Mean AI was expressed against time and, using the method previously described by (Wei et al. 1998), data were fitted to the exponential function of one phase association, $y = A(1-e^{-\beta t})$, where A is the plateau of the AI graph, and β is the rate constant that determines the rate of rising of the AI. A, therefore, represent

MBV proportionate to a unit volume of blood in a unit volume of tissue, and β is the rate constant of microvascular flow proportionate to MFV. The product of Aβ hence is proportional to MBF. MBF was calculated in each individual at rest and following muscle contraction.

5.3.4 Echocardiogram

Participants were rested at least 1 hour before a cardiac echocardiogram (ECHO) was done. ECHO was performed using an ultrasound transducer 5– 2 MHz of Philips iU22 ultrasound machine (Philips Healthcare, Amsterdam, Netherlands). Participants were lying in a supine position with their left arm raised and rested behind the head. Some participants were asked to lie on their left side to improve the imaging window. Heart chambers and mitral valve were identified from the parasternal long-axis plane, then using a short-axis view, and the array line was passed through the basal segment of the left ventricle between the mitral valve and the papillary muscle. The M-mode was then used to record the changes in interventricular size to reflect fractional shortening. Images from M-mode were analysed using ImageJ 1.42q software (National Institutes of Health, Bethesda, MD).

5.3.5 Cardiopulmonary exercise test

Participants were asked not to have any vigorous exercise for 48 hours before the CPET assessment. On the study day visit, CPET was performed with a Lode Corival cycle ergometer (Lode Corival, Lode, Groningen) and inline gas analysis system (ZAN 680, nSpire Health, Colorado, USA). Standard calibration of the gas analysis system was performed on a daily basis by a qualified research technician or trained PhD student. The assessment began with a 2-min period of unloaded cycling as part of warming up, followed by a gradual increase in ramp wattage. We used between 15 or 20 W/min ramp increments depending on participants' level of fitness. Participants were instructed to maintain a cadence of 50–60 revolutions per minute (rpm) and were given verbal encouragement to continue the exercise to reach 85% or more of predicted maximal heart rate and respiratory exchange ratio (VCO_2/VO_2) above 1.0. The test was complete when the participant indicated that they had reached their maximum possible effort (i.e., BORG score of 10) or when they were unable to maintain the 50-60 rpm cycle. During the CPET, participants were supervised by an advanced life support-trained clinician and monitored with a 12-lead ECG, pulse oximetry and non-invasive blood pressure monitoring sessions.

5.3.6 Statistical analyses

Statistical analyses were performed as described in Chapter 2, section 2.3.7.

5.4 Results

5.4.1 Blood pressure

Significant systolic blood pressure (SBP) reduction was seen after the 6-week interventions in all groups. Mean±SEM SBP reading pre and post for VLCD+O, VLCD+R and VLCD+H were 128.2mmHg±3.2 to 117.3mmHg±4.0, 126.5mmHg±3.6 to 114.9mmHg±2.5 and 126.5mmHg±3.6 to 114.9mmHg±2.5 respectively, all $P<0.05$ (Figure 5.2). The biggest change in SBP was in VLCD+H at 13.3mmHg±4.0, followed by VLCD+R at 11.6mmHg±4.1 and VLCD+O at 10.9mmHg±3.1. A similar trend was also seen when looked at relative changes from baseline (9.7%±2.9, 8.7%±2.9, and 8.5%±2.4, respectively). No statistically significant difference between groups was ween.

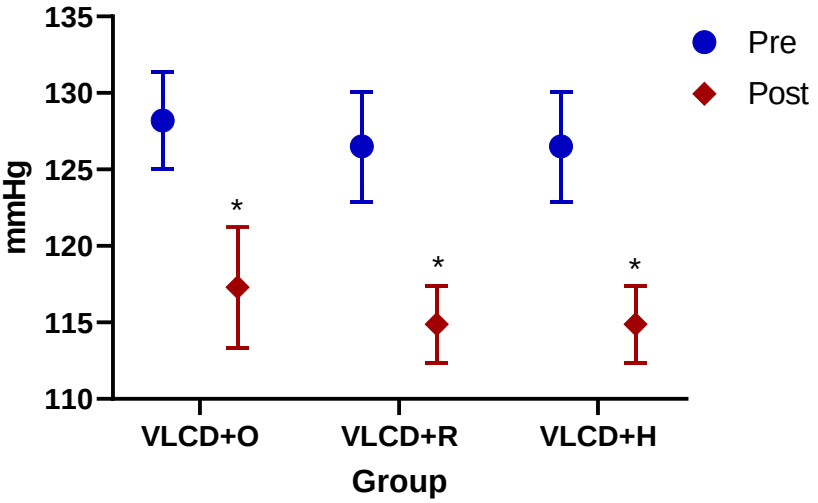


Figure 5.2 - Systolic BP pre- and post-intervention. *P<0.05.

Similar to SBP, there was significant diastolic blood pressure (DBP) reduction post interventions. Mean±SEM DBP reading pre and post for VLCD+O, VLCD+R and VLCD+H were 84.0mmHg±3.8 to 77.0mmHg±3.8, 83.0mmHg±2.3 to 72.8mmHg±2.9, and 85.3mmHg±2.6 to 77.4mmHg±3.1 respectively, P<0.05 (Figure 5.3). The biggest change in DBP was in VLCD+R at 10.1mmHg±3.2, followed by VLCD+H at 7.8mmHg±2.6 and VLCD+O at 7.1mmHg±1.8. A similar trend was also seen when looked at relative changes from baseline (11.9%±3.4, 9.0%±3.2, and 8.4%±2.3, respectively). No statistically significant difference between groups was seen.

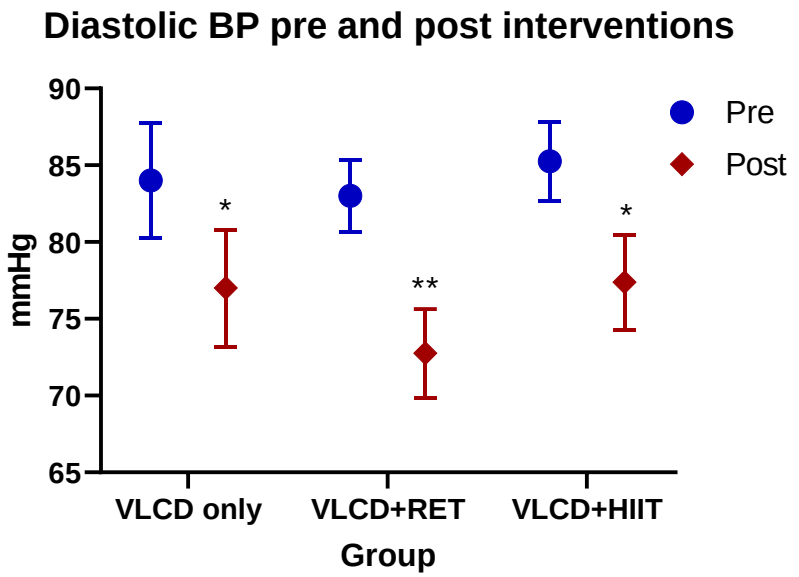


Figure 5.3 - Diastolic BP pre- and post-intervention. *P<0.05, **P<0.01.

5.4.2 Flow-mediated dilatation

For FMD analysis, we were only able to include the following number of participants in each group: VLCD+O=9, VLCD+R=6, VLCD+H=7. Incomplete FMD data was due to technical reasons i.e., software malfunction and license expiry.

Pre- and post-intervention FMD for VLCD+O, VLCD+R, VLCD+H were 1.8%±0.5 to 2.2%±0.6, 3.2%±0.6 to 4.3%±1.8, and 3.0%±0.6 to

5.7%±2.2 respectively, which were not statistically significant (Figure 5.4). In addition, differences between groups were also not significant.

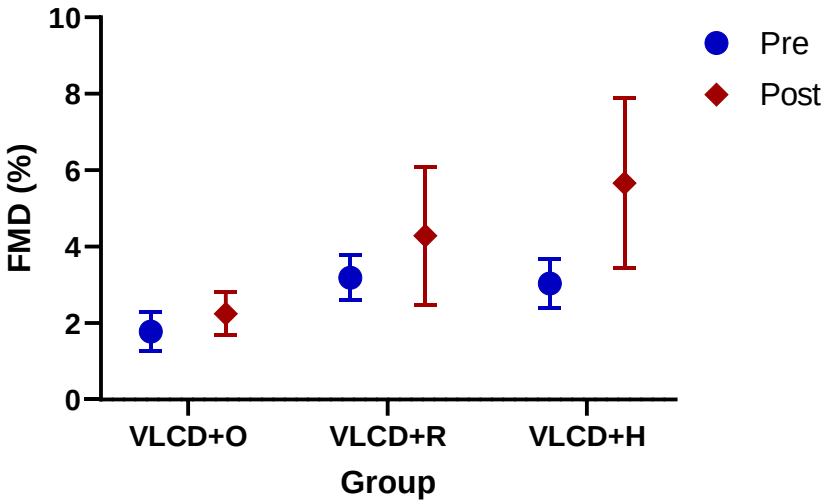


Figure 5.4 - FMD pre- and post-intervention.

5.4.3 Contrast-enhanced ultrasound

Below are graphs (Figure 5.5) representing the acoustic index (AI) of the CUES reperfusion curve for the dominant leg at rest (Week-0 Rest) followed by six reps of knee extension at 50% 1-RM (Week-0 Exercise) pre-intervention, as well as AI after the 6-week intervention (Week-7 Rest and Week-7 Exercise) separated between groups.

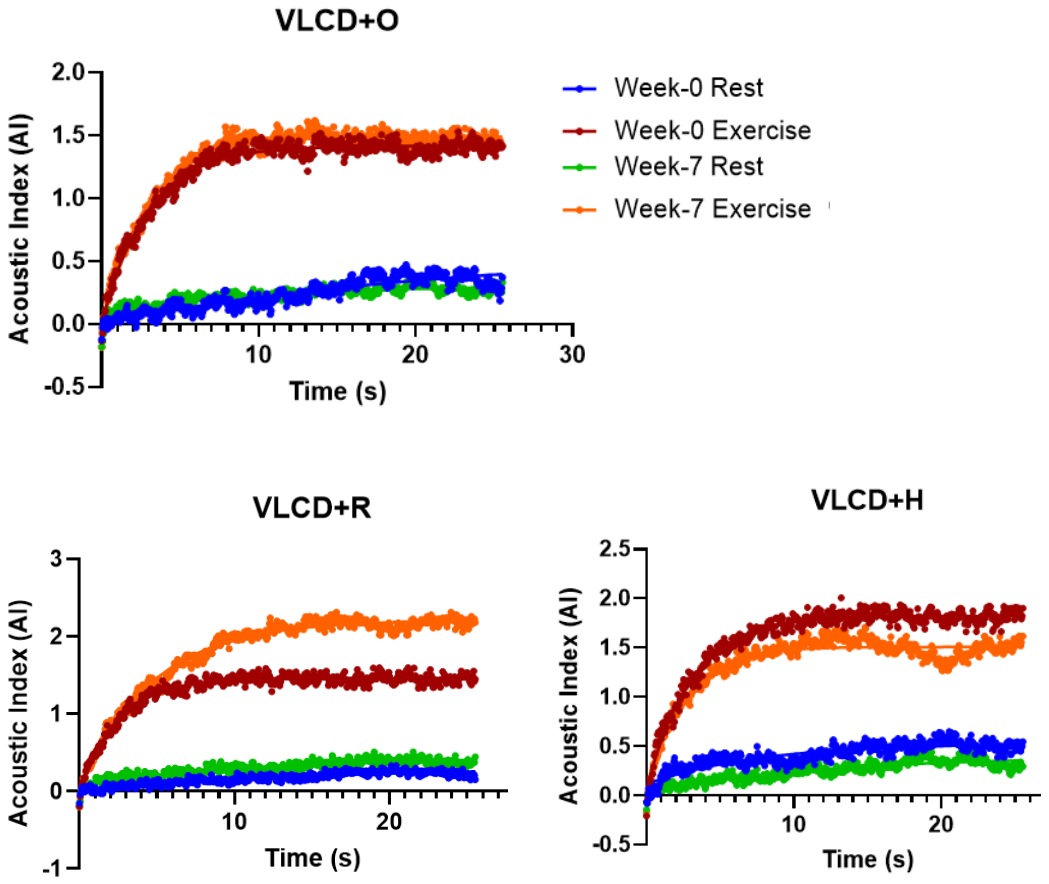


Figure 5.5 - Acoustic index (AI) at rest and following exercise stimulant of 6 reps 50% 1-RM knee extension, at baseline (week-0) and post-intervention (week-7) for all groups.

Table 5.1 - MBV, MFV, MBF changes following exercise stimulant (6 reps of 50% 1-RM knee extension) pre- and post-6-week VLCD intervention.

	VLCD+O	VLCD+R	VLCD+H
MBV			
Pre-intervention	0.94±0.27	1.12±0.26	1.09±0.39
Post-intervention	1.41±0.28	1.84±0.33	1.13±0.28
MFV			
Pre-intervention	0.15±0.11	0.34±0.12	0.11±0.17
Post-intervention	-0.14±0.31	-0.59±0.73	-0.41±0.72
MBF			
Pre-intervention	0.48±0.15	0.65±0.26	0.43±0.35
Post-intervention	0.25±0.22	0.39±0.13	0.56±0.14

A negative value signifies that the flow was reduced following an acute stimulation of knee extension (post-exercise flow minus resting flow rate), while a positive value signifies increasing flow or volume following acute stimulation vs rest.

5.4.3.1 MBV

MBV is represented by the plateau of nonlinear regression analysis of the CEUS reperfusion curve. All groups showed improvement in the MBV; 0.94±0.27 to 1.41±0.28, 1.12±0.26 to 1.84±0.33, and 1.09±0.39 to 1.13±0.28 for VLCD+O, VLCD+R and VLCD+H respectively, however, none were statistically significant (Figure 5.6). No statistically significant differences were seen between the groups either.

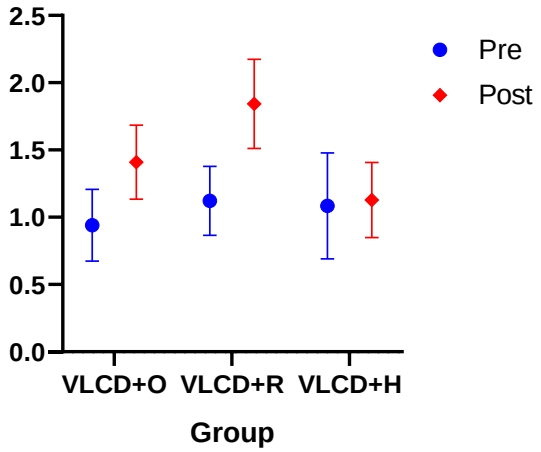


Figure 5.6 – Graph represents changes in MBV in response to acute exertion (MBV after knee extension minus MBV at rest) performed pre- and post-6-week intervention**.**

5.4.3.2 MFV

MFV is represented by the K value of nonlinear regression analysis of the CEUS reperfusion curve. All groups showed a reduction in the mean MFV value; 0.15±0.11 to -0.14±0.31, 0.34±0.12 to -0.59±0.731, and 0.11±0.17 to -0.41±0.72 for VLCD+O, VLCD+R and VLCD+H respectively, however, none were statistically significant (Figure 5.7). No statistically significant differences were seen between the groups either.

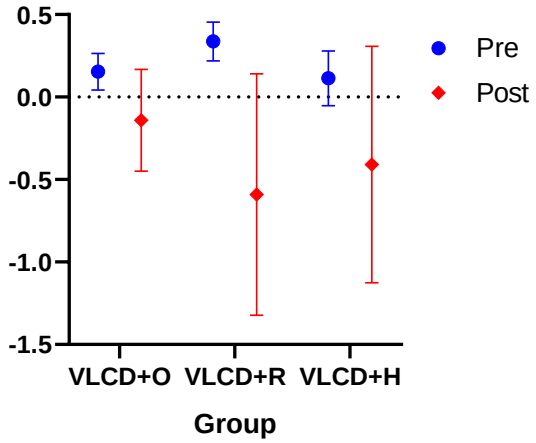


Figure 5.7 – Graph represents changes in MFV in response to acute exertion (MFV after knee extension minus MFV at rest) performed pre- and post-6-week intervention.

5.4.3.3 MBF

MBF is represented by the product of MBV multiplied with MFV. VCLD+O and VLCD+R showed a reduction in the mean MBF value; 0.48±0.15 to 0.25±0.22, 0.65±0.26 to 0.39±0.13, respectively, while VLCD+H showed some increase in the value 0.43±0.35 to 0.56±0.14 (Figure 5.8). These, however, do not represent any statistically significant changes between pre and post 6-week intervention within any of the groups, nor any significant differences between the groups.

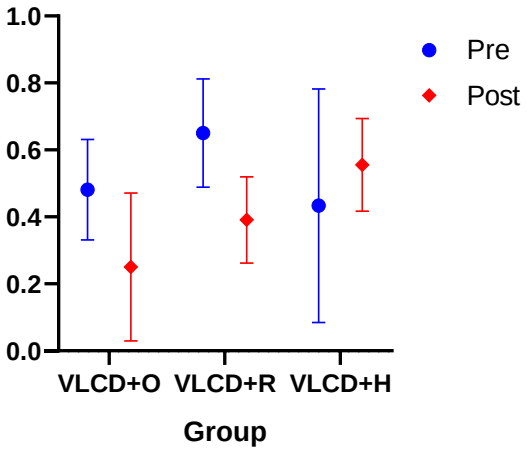


Figure 5.8 – Graph represents changes in MBF in response to acute exertion (MBF after knee extension minus MBF at rest) performed pre- and post-6-week intervention.

5.4.4 Echocardiogram

The normal left ventricular fractional shortening (LVFS) value is between 25 to 45% (Chengode 2016). Pre- and post-intervention ECHO mean±SEM for VLCD+O, VLCD+R, VLCD+H were 34.7%±2.2 to 36.5%±1.8, 34.4%±2.6 to 31.1%±2.0, and 39.6%±2.1 to 37.7.7%±2.8 respectively, which are not statistically significant (Figure 5.9). No statically significant difference between groups was seen.

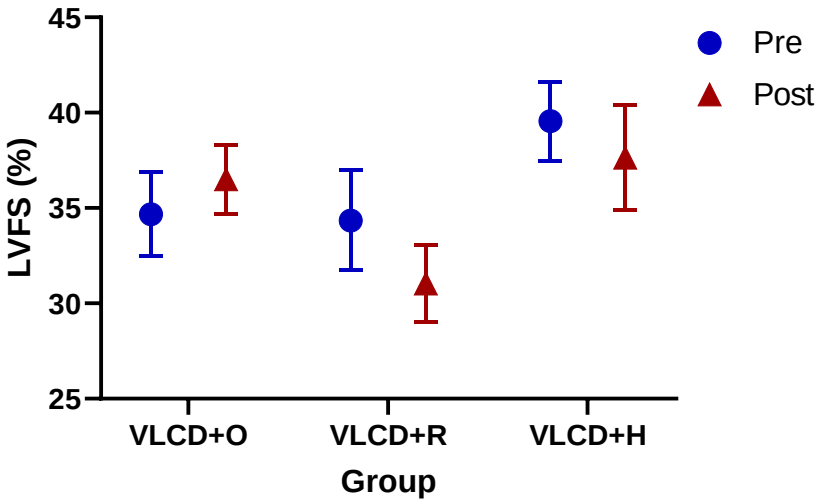


Figure 5.9 – Left ventricular fractional changes (LVFS) pre- and post-intervention.

5.4.5 Cardiopulmonary exercise test

For CPET analysis, unfortunately, we were only able to analyse six out of the eight participants in the VLCD+R group, as one of the tests had a leak in the gas measurement, and another participant had an unexpected leg pain hence unable to perform the post-intervention cycle ergometer CPET test. The number of participants in each group for the CPET analysis were VLCD+O=10, VLCD+R=6, and VLCD+H=8.

5.4.5.1 $Watt_{max}$

VLCD+O had a small reduction in maximum wattage ($Watt_{max}$) following the 6-week intervention, from 244W±25 to 235W±23 while VLCD+R and VLCD+H, on the other hand, had a small increase in from 239W±16 to 241W±17 and 187W±14 to 198W±12 respectively, however, these are not statistically significant (Figure 5.10). There was also no significant difference between the groups.

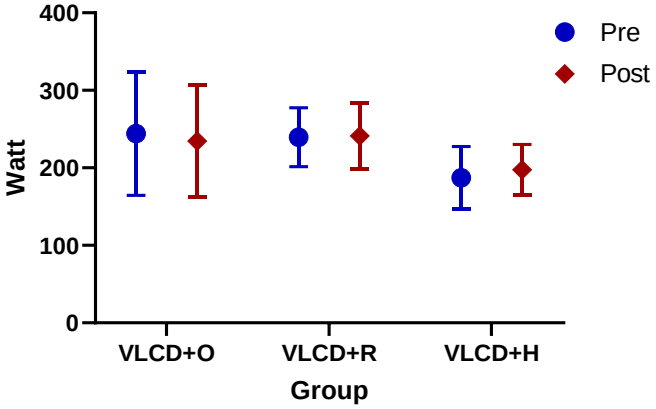


Figure 5.10 - $Watt_{max}$ pre- and post-intervention.

5.4.5.2 VO_2max

Absolute VO_2max ($l.min^{-1}$) showed a marginal reduction in VLCD+O and VLCD+R post-intervention, from 2.96±0.3 to 2.89±0.3, and 2.90±0.2 to 2.86±0.2 respectively, while VLCD+H showed a small increase in value from 2.46±0.2 to 2.61±0.2 (Figure 5.11). These, however, did not translate into statistically significant values. Similarly, no statistically significant difference between the groups was seen.

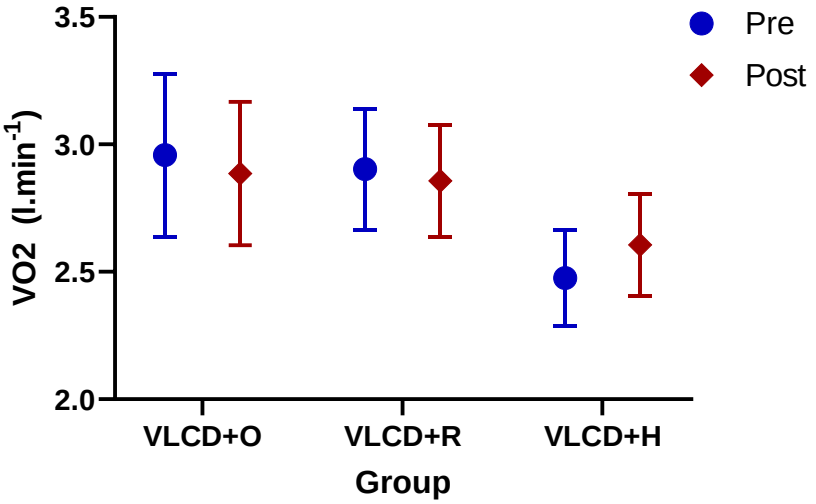


Figure 5.11 – Absolute VO_2max pre- and post-intervention.

When corrected for weight, all groups had significant improvement in VO_2max following interventions (Figure 5.12). This is mainly due to a significant reduction in the total body weight. The biggest improvement was in VLCD+H, followed by VLCD+R and VLCD+O (unit in $ml.kg^{-1}.min^{-1}$), at 25±2 to 30±3, 30±2 to 33±3 and 28±3 to 31±3 respectively. Statistically, no significant difference between the groups was seen.

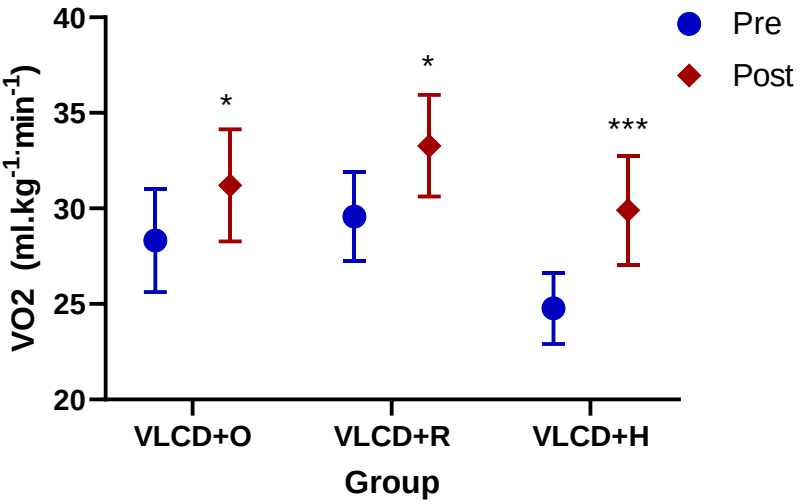


Figure 5.12 – Relative VO_2max pre- and post-intervention. $^{*}P<0.05$, $^{***}P=0.0007$.

5.4.5.3 Anaerobic threshold

No significant change statistically was seen in anaerobic threshold (AT) after the 6-week intervention in any group (Figure 5.13). Changes in VLCD+O, VLCD+R and VLCD+H groups were from 17.2±2.3 to 16.8±2.2, 13.7±1.5 to 13.6±1.1 and 11.4±0.4 to 12.7±1.0 respectively (all unit in $ml.kg^{-1}.min^{-1}$). Similarly, no statistically significant difference between the groups was seen.

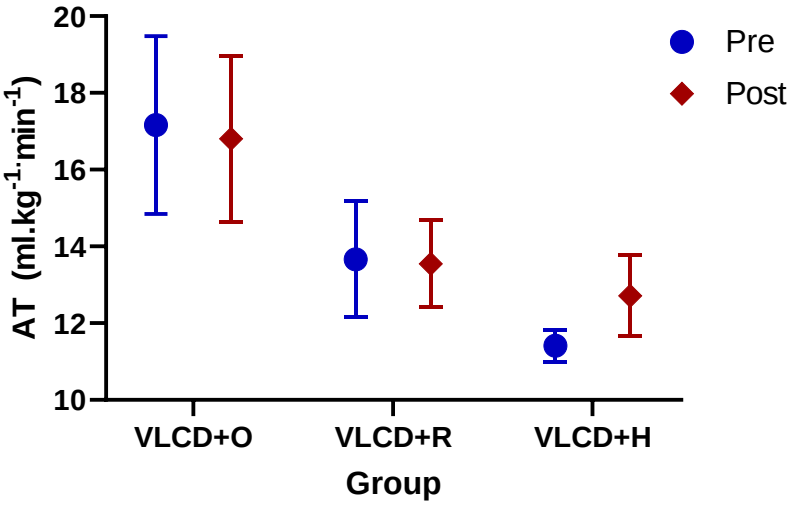


Figure 5.13 - Anaerobic threshold pre- and post-intervention**.**

5.4.5.4 Summary table for CPET parameters

Table 5.2 - CPET parameters at baseline and post-interventions for all groups.

	VLCD+O	VLCD+R	VLCD+H
$Watt_{max}$ (W)			
Baseline	244±25	239±16	187±14
Post-intervention	235±23	241±17	198±12
Absolute VO_2max ($l.min^{-1}$)			
Baseline	2.96±0.3	2.90±0.2	2.46±0.2
Post-intervention	2.89±0.3	2.86±0.2	2.61±0.2
Relative VO_2max ($ml.kg^{-1}.min^{-1}$)			
Baseline	28±3	29.6±5.7	25±2
Post-intervention	31.2±3*	33.3±6.5*	30±3***
AT ($ml.kg^{-1}.min^{-1}$)			
Baseline	17.2±2.3	13.7±1.5	11.4±0.4
Post-intervention	16.8±2.2	13.6±1.1	12.7±1.0

*Indicates *P<0.05, ***P<0.001. No significant difference between the groups.

5.5 Discussion

As expected, we found that significant weight loss contributed to improvement in blood pressure control, as observed by many (Clifton et al. 2005; Phillips et al. 2008; Wycherley et al. 2008; Blumenthal et al. 2010; Seligman et al. 2011). All groups showed a statistically significant reduction in systolic and diastolic BP following the 6-week interventions. We, however, did not see any correlation between the degree of weight loss and the degree of reduction in BP ($P=0.6$), as previously demonstrated in a meta-analysis finding (Neter et al. 2003).

Whilst there was a significant improvement in blood pressure, we did not see such an effect on FMD results. There was some degree of FMD changes following the 6-week interventions within each group, but these are not statistically significant ($P>0.1$), which seems similar to some other studies that showed no significant change in FMD following various weight loss programmes (Brook et al. 2004; Clifton et al. 2005; Keogh et al. 2008; Wycherley et al. 2008, 2016; Fayh et al. 2013; Mohler et al. 2013; Joris et al. 2017). Contrary to ours, other VLCD studies demonstrated significant improvement in

FMD following weight loss from the intense caloric restriction alone without concomitant exercises (Raitakari et al. 2004; Sun et al. 2020). Other weight loss studies using caloric restriction other than VLCD or with exercises have shown improvement in FMD, likely via the improvement in endothelial progenitor cell (EPC) function involving autophagy activation, reduced asymmetric dimethylarginine (ADMA) concentrations which is an inhibitor for endothelial nitric oxide synthase (eNOS) and increase of the expression of eNOS which is the enzyme responsible for the production of the majority of NO (Goodpaster et al. 1999; McLaughlin et al. 2006; Bigornia et al. 2010; Blumenthal et al. 2010; Egan 2010; Ades et al. 2011; Seligman et al. 2011; Mavri et al. 2011; Cotie et al. 2014; Sato et al. 2014; Barbosa-Yañez et al. 2018). Unfortunately, as our study did include these measurements, we are unable to directly compare the results to explain the differences in our findings. Quite possibly, our participant number was too small to give any significant FMD result.

While FMD is known to reflect the risk of hypertension via a pathway involving endothelial function, oxidative stress, endothelial NO synthase expression and NO bioavailability (Celermajer et al. 1992; Jaap et al. 1994; Ungvari et al. 2003; Förstermann and Münzel

2006; Cocks et al. 2013), the improvement in BP in this study in the absence of significant FMD change suggested multiple factors beyond endothelial function. In addition, protein content in the weight loss diet also has been shown to exert a degree of effect towards FMD changes (Petyaev et al. 2012; Ballard et al. 2013; Fekete et al. 2016). Hence weight loss alone might not be sufficient to account for FMD improvement. The technical variation could also play a major part in FMD results between different studies as it is known to have some degree of inter-operator variability (Jensen-Urstad and Rosfors 1997; Alley et al. 2014).

To our knowledge, whilst there are studies assessing MBV affect following exercise and nutrition (Timmerman et al. 2012; Phillips et al. 2015), this is the first study looking into MBV changes following weight loss from VLCD. Our study showed only minor improvement in MBV across all groups, but none were statistically significant. Lack of significant MBV improvement is likely due to net effect reduced in LM following weight loss despite increased skeletal muscle capillarisation following exercises which normally can be observed from exercises under normal caloric setting (Cocks et al. 2013). Contrary to MBV, there was a minor reduction in MFV mean across all groups, again not statistically significant. MFV assessment can be

tricky due to the inherently poor signal-to-noise ratio and is highly susceptible to variation depending on the subtle changes in probe angulation in respect to the direction of the muscle fibres and associated capillaries (Durham et al. 2010; Mitchell et al. 2013). We also assessed MBF, based on our previous group studies investigating the microvascular response to feeding stimuli, in which there appears to be a biphasic response whereby increases in MBV precede MFV (Mitchell et al. 2013). Again, in our study, there is no significant change in MBF across all groups following the 6-week intervention. Most of the microvascular improvement expected following exercise might have been diminished due to reduction in LM, including muscle mass from the intense 6-week VLCD.

It is also important to note that we assessed MBV response to acute muscle exercise, which is 6-reps of 50% 1-RM knee extension. Whilst we endeavoured to ensure exact positioning and angle of the USS probe following the leg exercise motion using a polyethene-housing unit strapped onto the thigh circumference and manually assessing the USS image on the screen, we can never guarantee this is the case. There is also a concern regarding the application of the probe at the exact region of the muscle before and after the 6-week intervention period. Even if the probe position was marked based on

static anatomical landmarks (i.e., distance in mm from the top of the patella bone), changes in the underlying tissue composition, particularly adipose loss after intense caloric restriction, would likely alter the measured region.

Various studies demonstrated that weight loss from VLCD leads to a decrease in endurance to exercise, which is measured by max power output and time to fatigue using cycle ergometer test (Phinney et al. 1980, 1988; Bogardus et al. 1981; Lemons et al. 1989; Davis and Phinney 1990; Donnelly et al. 1991b; Velthuis-te Wierik et al. 1994). Our study showed a similar trend of reduced endurance in the form of reduced $Watt_{max}$ in VLCD+O, however, this was not statistically significant. Reduced endurance in an energy deficit state is most likely associated with a decrease in muscle glycogen content (Eston et al. 1992) and phosphofructokinase, a rate-limiting enzyme of glycolysis together with slowing of pumps transferring free calcium into the sarcoplasmic reticulum due to increased intracellular calcium (Russell et al. 1984). Exercise groups, on the other hand, have been associated with improved strength (as discussed in Chapter 3 of this thesis), which would indirectly contribute to improved durability to exertion.

Our study showed improvement in relative VO_2max in all groups, which is in keeping with previous studies that involved exercises (not HIIT) under different caloric restrictions (not VLCD) (Shinkai et al. 1994; Penesova et al. 2018; Joseph et al. 2020), as well as VLCD (Hakala et al. 1996; Bryner et al. 1999), while some studies showed improvement limited only in the diet with exercise group, but and not in the diet-only group (Pavlou et al. 1985; Weiss et al. 2007; Silverman et al. 2009). We found that as there was no change in absolute VO_2max, the changes in relative VO_2max were mainly due to significant weight loss in all participants following VLCD.

To our knowledge, at the time of the research period, there was no other study combining HIIT with VLCD. We found that the VLCD+H group had the biggest relative VO_2max improvement, which translates into a 20% increase. Having said that, there was no statistically significant difference in VO_2max improvement between the groups, with VLCD+R had 13% and VLCD+O had 11% improvement in VO_2max following interventions.

5.6 Chapter conclusion

Our research is the first to perform HIIT in combination with VLCD, which enabled us to compare the outcomes with VLCD alone and VLCD with RET. We have confirmed the similar benefit of weight loss in terms of improvement in blood pressure and cardiorespiratory fitness in the form of VO_2max, as seen in various studies. Some other cardiovascular and cardiorespiratory parameters showed no improvement from the baseline, which include FMD, microvascular blood circulation, absolute VO_2max and AT. As expected, there was no change in cardiac ejection fraction, evidenced by the ECHO findings, supporting the safety of HIIT in combination with VLCD from a cardiac function perspective. In essence, we also found no significant benefit from additional exercises to VLCD. More studies with a larger sample size combining VLCD and different types of exercises, particularly HIIT, would help further comparison in these parameters in the future.

Chapter 6: Thesis discussion and conclusion

6.1 Thesis findings

A very low-calorie diet (VLCD) is one of the most intense diet regimes, has been used for rapid weight loss, including as a pre-surgical preparation to help with liver shrinkage (Colles et al. 2006). Concerns arise when about a quarter of weight loss is in the form of lean mass (LM), which also includes muscle mass as a predominant component (Chomentowski et al. 2009). Muscles play crucial roles in physical locomotor function as well as various physiological balance, including glucose homeostasis, and acts as a primary site of insulin resistance in the context of metabolic syndrome (DeFronzo et al. 1981; Rennie et al. 2004; Bouzakri et al. 2005).

I conducted a literature review at the beginning of the PhD period to better understand the benefit and risks of VLCD and to explore muscle metabolism at a molecular level. The complexity of the different signalling cascades involving hormones, nutrition and physical activity that affecting muscle protein synthesis (MPS) and muscle protein breakdown (MPB) remains a vast area for exploration (Kimball et al. 2002). Molecular information and understanding of muscle metabolism provide essential guidance on practicality for

muscle preservation under different circumstances, particularly during the period of energy deficit such as VLCD.

Exercises have been recognised as one of the ways to preserve LM during the weight-loss period. At the start of this PhD research phase, a literature search using a systematic approach was done to explore previous studies incorporating exercises with VLCD. Unfortunately, results on this subject were scarce mainly due to concern of safety issues surrounding intense physical work while on an extreme caloric deficit. Only four studies were available that include resistance exercise training (RET) with VLCD (Lemons et al. 1989; Pronk et al. 1992; Donnelly et al. 1994; Bryner et al. 1999), and none that involved high-intensity interval training (HIIT). Taking into account previous literature confirming the safety aspect of VLCD and available resources within our research team to provide safety monitoring the participants, we planned for a 6-week of VLCD alone and in combination with RET or HIIT to allow a comparison for various parameters that are essential for health outcomes.

It is important to acknowledge that due to additional exercises on top of VLCD, there would have been differences in total energy expenditure between the groups. Hence energy availability would

not be equivalent between the different arms of the study. However, it was the intention of the research project to assess the overall effect of the exercises with VLCD towards changes in physical and physiological elements, similar to many other previous studies involving diet with exercises (Lemons et al. 1989; Pronk et al. 1992; Donnelly et al. 1994; Sartor et al. 2010; Lazzer et al. 2017).

This study involving VLCD with rapid weight loss is the first of its kind within our research unit. It has been a rewarding experience to see participants enjoying the immediate outcome of weight loss, feeling better in themselves and more energetic than before the programme. All groups showed significant total weight (TW) loss from the baseline between 10 to 11%. This is a remarkable achievement as even 5% weight loss is associated with improved cardiometabolic health (Wing et al. 2011).

With the concern surrounding LM loss, we utilised the DXA scan to assess changes in body composition. Our findings on percentage LM loss from the total weight loss were similar, but with a slightly wider percentage range to previously documented VLCD studies (Donnelly et al. 1991a; Krotkiewski 2001; Chaston et al. 2007), likely owing to the measurement methods, i.e., hydrostatic weighing is some of the

studies, as well as due to the different protein content and exercise intensity. With us being the only one that conducted VLCD with HIIT, we found no significant difference statistically in the proportion of LM loss compared to VLCD alone or VLCD with RET.

Looking into regional LM loss, DXA showed a similar trend of significant LM loss within the legs area. The main question arises when the ultrasound (USS) of vastus lateralis (VL) did not show any statistically significant change in the muscle thickness. In fact, the exercise groups showed an increasing trend in thickness while the diet-only group showed decreasing trend. Muscle thickness has been shown to positively predict muscle volume measured by MRI (Miyatani et al. 2002, 2004; Sanada et al. 2006). Taking into account the possible inter-operator effect, USS would still reliably help with estimation in changes in muscle mass (Ticinesi et al. 2018). The frequent use of legs during intense RET and cycle HIIT might have contributed towards muscle build up around the thigh area, which could have contributed towards the finding in USS of VL. Extra muscle stimulation from exercises could have contributed towards the depletion of glycogen content within muscle tissue, and as the replenishment process depends heavily on the dietary intake, participants on VLCD could have had a significant reduction in

glycogen storage (Ivy 2004). Taking into account DXA inability to differentiate between intramuscular glycogen content with the muscle tissue itself, it is not a surprise that DXA overestimated LM loss, particularly in the exercise groups where glycogen storage would be depleted more. Assessment of change in glycogen content within the muscle tissue would provide useful information on this topic.

Interestingly, MPS data showed that VLCD+H has the highest MPS rate, with a statistically significant difference to VLCD+O following the 6-week interventions. To put this in the context of muscle volume, although there was no statistically significant change in USS of muscle thickness, VLCD+H showed an increasing trend in muscle thickness, while VLCD+O showed a decreasing trend. Being a novel study incorporating HIIT with VLCD, this provided a very important insight into molecular elements in preserving muscle anabolism while on severe caloric deficit states such as VLCD. Perhaps, if the study was prolonged to a few more weeks, we might be able to see a statistically significant increase in muscle thickness or improvement in LM preservation within the HIIT group or even in RET group, considering that it has been suggested that muscle hypertrophy predominantly takes place after six weeks of exercise

interventions (Moritani and DeVries 1979; Donnelly et al. 1993; Jo et al. 2019).

We had initially planned to utilise 3-methylhistidine (3MH) measurement for muscle protein breakdown (Pasiakos and Carbone 2014) and D3-creatine for muscle mass estimation (Clark et al. 2014). Unfortunately, due to unforeseen circumstances, i.e., issues with 3MH and D3-creatine supply at the start of the research, a backlog of lab works, as well as university closure due to the COVID-19 pandemic, we have to abandon these analyses for the thesis and will later continue for the publication purpose. Using 3MH data to analyse MPB could also help to explain if there is also an increase in MPB in the HIIT group that counter-balanced the high MPS value, leading to an insignificant difference in LM and muscle thickness compared to the other groups that had lower MPS rate. Perhaps, using D3-creatine data in total muscle mass estimation could also add value in assessing changes in muscle mass following the 6-week interventions.

Despite a significant loss in LM as seen on DXA results, the primary locomotor function of muscle in the form of strength was proven to be favourable, where significant strength improvement in all

exercise types was seen in the VLCD+R group, while VLCD+O and VLCD+H had no reduction in the strength. The increase in strength is likely due to improved central neuromuscular function rather than the muscular mass itself (Pronk et al. 1992).

Our study has confirmed the improvement in insulin indices, as well as a significant reduction in total cholesterol (TC), triglyceride (TG) as well as Chol/HDL ratio following weight loss. As android fat mass (AFM) has been linked to cardiovascular disease with associated abnormal lipid profiles and insulin function (Sari et al. 2019), we had further analysis on the change in AFM (in Chapter 3) to find any correlation with metabolic profiles (in Chapter 4). Unfortunately, statistical analysis using Pearson's correlation coefficient found no significant correlation between the degree in AFM loss and improvement in insulin indices nor lipid profiles (all $P>0.1$). With a significant AFM loss of close to 30% changes from baseline in all groups and no statistically significant differences between groups analysis, this suggests that VLCD alone suffice to achieve optimum AFM loss.

TG concentration was found to be positively associated with insulin resistance (Raygor et al. 2019). Our findings also support this, as we

found that the relative reduction in TG level is positively correlated with a relative improvement in insulin indices from the baseline: HOMA-IR (r=0.78, P<0.0001), $AUC_{i/g120}$ (r=0.39, P=0.05), QUICKI (r=0.49, P=0.01), Matsuda Index (r=0.53, P=0.0005). No significant correlation was seen between insulin indices with other lipid components, i.e., TC, HDL, or Chol/HDL.

From the cardiovascular aspect, all groups had a significant improvement in blood pressure but with no significant statistical difference between the group. No significant change was seen from other cardiovascular markers, possibly due to the small sample size. No significant change was seen in CET parameters, apart from improvement in the relative VO_2max, primarily due to change in weight, as the formula involved absolute VO_2max divided by the weight, hence lower weight post-intervention would surely give a higher relative VO_2max value.

6.2 Study limitations

This study was not able to complete n=10 for each group as per sample size calculation. Although we did see a significant difference in the primary outcome, i.e., MPS between VLCD+H and VLCD+O, a

complete number of participants in each group might have revealed possible differences with VCLD+R as well. There was difficulty in the recruitment process due to the target age between 30- to 60-year of age, which is a working age with work and family commitment. The intense VLCD regime had also affected participants leading to a total of four drops out.

As discussed earlier in this chapter, a 6-week duration might not be long enough to see exercised effect on muscle hypertrophy (Moritani and DeVries 1979; Donnelly et al. 1993; Jo et al. 2019). Hence this might have impaired the true potential of concomitant exercise while on VLCD. This, however, might be the compromise we had to take into consideration due to difficulty in the recruitment process as mentioned earlier. Longer study duration would require more time commitment, which might reduce the number of available volunteers.

Baseline dietary habit was not completely reliable. This was seen when some participants only started to fill the part of the '4-day diet diary' while attending the study day, instead of filling in the food details at home during the agreed time frame. A similar situation was also observed with the daily diet recording during the 6-week

intervention period on paper or apps, where a small number of participants only entering some of the diet detail during the weekly review instead of at home. With no reliable recording of the data, analysis of the baseline diet and daily diet during intervention would not be possible.

DXA inability to differentiate between the actual muscle mass with glycogen, water and lipid molecules stored within the muscle tissue itself caused DXA to overestimate LM loss. This, however the only imaging modality that is practical, accessible and cost-justified for our research project. We also did plan to use D3-creatine to estimate whole-body muscle mass to allow comparison between pre- and post-intervention, however, due to supply issues and time constraints, we were unable to analyse the samples and integrate the result for the thesis.

Whilst various measures were taken to ensure repeatability of the USS technique, with limited experience of research team members, including me that had only been doing the specific scan (USS for muscle thickness, FMD, CEUS and ECHO) for this research purpose, the reproducibility might have been affected as various USS scan

techniques have been shown to be affected by operator experience (Mikkonen et al. 1996; Moga et al. 2018; Guerriero et al. 2021).

Integration of HIIT with VLCD into real-life practice would be difficult considering the intensity of the exercise while on such an intense diet. Our research setting could help to ensure the safety of the participants through interval blood tests, regular review by the research team, and close monitoring of vital physiological signs during the exercise session by a medically trained team member. The data on benefit against the safety risk is still limited to justify the adoption of HIIT with VLCD for the public.

6.3 Recommendations for future research

It would be ideal to have completed the sample size or even a greater number of participants to allow any other significant results to be seen.

Having an extended duration of the intervention, e.g., 8 or 10 weeks, would also be ideal to be able to assess any potential muscle

hypertrophy after 6-week of exercise training while on VLCD with or without exercises.

Perhaps, target age group could include 18- to 30-year of age. In general, the younger age group have less work and family commitment. More importantly, the younger age group has higher muscle protein synthesis potential, so that any exercise stimulation would have more effect on muscle changes (Wall et al. 2015; Brook et al. 2016).

Analysis of D3-creatine to estimate whole-body muscle mass would be useful to add value in assessing the effect of VLCD with or without exercises on muscle mass.

A dedicated team member or research technician who is specialised in USS could ensure consistency of the techniques for muscle thickness, FMD, CEUS or ECHO.

Whilst the incorporation of HIIT with VLCD is still far from being practical to the public, the healthcare field could possibly benefit from this combination. VLCD is used for 2 to 12 weeks pre-

operatively to help with reduction in liver volume before bariatric operations (Colles et al. 2006; González-Pérez et al. 2013; Tan et al. 2020). With the potential of MPS rate preservation following HIIT incorporation into VLCD, more studies in this field could be beneficial, particularly in the preservation of muscle mass following initial VLCD diet and post-operation physiological changes.

6.4 Conclusion

Being the first group to combine VLCD with HIIT, this research has enabled us to compare the outcomes with VLCD only and VCLD with RET interventions. The main benefit that we found by integrating HIIT with VLCD, in comparison to diet alone, is in the form of a higher MPS rate which could translate into potential muscle preservation. Incorporating RET with VLCD, on the other hand, benefit from the strength aspect, evidenced by the significant improvement in 1-RM in the VLCD+R group compared to the other groups.

While lean mass loss remains a concern, all groups showed remarkable improvement in metabolic functions, for example, reduction in TG, TC and improvement in insulin indices to indicate

improved glucose homeostasis. TG is proven to correlate with insulin sensitivity following weight loss, which could serve as a useful marker in the actual practice. Essentially, although there was significant improvement within the groups, there was no statistically significant difference between the groups, which suggests integrating exercises to the intense VLCD regime offers no additional benefit for insulin sensitivity and lipid profiles.

Several limitations in this study, particularly sample size, study duration, and available investigations modalities, might have limited other potential significant findings. Perhaps more studies combining HIIT or RET with VLCD in the future could offer more insight into any uncovered benefits in this research.

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Appendices

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Appendix 1

Ethics Committee Approval Letter

Direct line/e-mail
+44 (0) 115 8232561
Louise.Sabir@nottingham.ac.uk

Faculty of Medicine and Health Sciences

Research Ethics Committee
C/o Faculty PVC Office
School of Medicine Education Centre
B Floor, Medical School
Queen's Medical Centre Campus
Nottingham University Hospitals
Nottingham
NG7 2UH

18th October 2016

Dr MH Abdul Aziz
Clinical Research Fellow/MRes Student
Division of Medical Sciences and Graduate Entry
School of Medicine
Royal Derby Hospital
Uttoexeter Road
Derby
DE22 3DT

Dear Dr Aziz

Ethics Reference No: B12092016 **– please always quote**
Study Title: The effect of very low calorie diet (VLCD) with and without concomitant resistance exercise training (RET) or high intensive interval training (HIIT) on muscle protein synthesis (MPS) in middle-aged overweight male.
Chief Investigator/Supervisor: Dr I Idris, Associate Professor in Diabetes and Vascular Medicine, Medical Sciences and Graduate Entry Medicine (MSGEM) Derby.
Lead Investigators/student: Dr MH Abdul Aziz, MRes Student, MSGEM, School of Medicine, University of Nottingham.
Other Key Investigators: Professor P J Atherton, Professor of Clinical, metabolic & Molecular Physiology Dr BE Phillips, Assistant Professor, Dr K Smith Principal Research Fellow, MSGEM, Derby Dr A Philp Senior Lecturer, School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham.
Type of Study: MRes Student, physiological, muscle biopsies, DXA scan
Proposed Start Date: 01/10/2016 **Proposed End Date:** 30/09/2019 36 mths
No of Subjects: 36 **Age:** 30-60 years
School: Medicine

Thank you for submitting the above application which was considered by the Committee at its meeting on 12th September 2016 and the following documents were received:

Short Title: VLCD Exercise

- FMHS Research Ethics Application form version 1.0 date 26.07.2016
- DEXA Scan sign off by Aline Nixon IRMER Radiation Practitioner dated 8/9/2016.
- Study Proposal version 1.0 Date: 26/07/2016
- Recruitment Poster version 1.0 Date 26/07/2016
- Healthy volunteers Information Sheet version1.0: 26/07/2016
- Consent Form version 1.0:26/07/2016
- DEXA information leaflet v1.1 dated 14 May 2014.

These have been reviewed and are satisfactory and the study is approved.

Approval is given on the understanding that the conditions set out below are followed:

1. You must follow the protocol agreed and inform the Committee of any changes using a notification of amendment form (please request a form).

2. You must notify the Chair of any serious or unexpected event.

3. This study is approved for the period of active recruitment requested. The Committee also provides a further 5 year approval for any necessary work to be performed on the study which may arise in the process of publication and peer review.

4. An End of Project Progress Report is completed and returned when the study has finished (Please request a form).

Yours sincerely

pp Lamagatni

Professor Ravi Mahajan
Chair, Faculty of Medicine & Health Sciences Research Ethics Committee

Appendix 2

Consent Form

Division of Medical Sciences and GEM
School of Medicine

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Human Tissue Act:
Healthy Volunteer Consent Form (HVCF)

Premises covered	MSGEM	This is version	7
Document Created	15.7.2009	Document issued in	May 2017
Review Period	Every two years	To be reviewed in	May 2019

Project Title: The effect of very low calorie diet (VLCD) with and without concomitant resistance exercise training (RET) or high intensity interval training (HIIT) on muscle protein synthesis (MPS) in middle-aged overweight male.

Name of Investigators:
Dr M Hariz A Aziz
Dr Iskandar Idris
Professor Philip Atherton
Dr Beth Phillips
Dr Kenneth Smith
Dr Andy Philp

REC REF: B12092016

Please read this form and sign it once the investigator above-named, or their designated representative, has explained fully the aims and procedures of the study to you.

	Please Initial Box
• I voluntarily agree to take part in this study.	
• I confirm that I have been given a full explanation of the study and that I have read and understand the information sheet (version number …… dated……) for the above study and have had the opportunity to ask questions.	
• I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing.	
• I agree to comply with the reasonable instructions of the research team and will notify them immediately of any unexpected unusual symptoms or deterioration of health.	
• I give permission to collect, store, analyse and publish information obtained from my participation in this study. I understand my personal details will be kept confidential.	
• I understand that data collected in the study may be looked at by responsible individuals from the University of Nottingham and regulatory authorities where it is relevant to my taking part in this study.	
• I understand that information about me recorded during the study will be kept in a secure database. If data is transferred to others it will be made anonymous. Data will be kept for 7 years after all results of this study have been published. After this time, it will be disposed of in line with University policy.	
• I understand that the tests or procedures are carried out for research only and not for clinical diagnostic purposes. However, if the study investigator should feel it necessary to inform my GP of my participation in the study, or of an adverse event or abnormal test result, I understand I am giving my consent to do so.	
• I confirm that I have disclosed relevant medical information before the study.	
• I shall receive an inconvenience allowance. If I withdraw from the study for medical reasons not associated with the study a payment will be made to me proportional to the length of the period of participation, but if I withdraw for any other reason, the payment to be made, if any, shall be at the discretion of the lead investigator.	

Human Tissue Act:
Healthy Volunteer Consent Form (HVCF)

- I have not been a volunteer in any other research study in the *last three months*, which involved: taking a drug, being paid an inconvenience allowance**,** having an invasive procedure (e.g. venepuncture >50ml, endoscopy) or exposure to ionising radiation. ☐
- I confirm that I have not been exposed to more than 5 mSv of ionising radiation in the *last 12 months* (to clarify, please discuss with a member of the research team). ☐
- **OPTIONAL** - I agree to my tissue/ blood samples being shared with other researchers including those working outside the University. I understand that I would not gain commercially. ☐
- **OPTIONAL** - Where tissue is removed from my body for the purposes of this study, but not used for this study, and when the ethical approval for this project expires, I agree to the future and continued storage and use of this tissue for future research into functioning of the human body and disease. Such tissue will fall under the University of Nottingham licence of the Human Tissue Act. As specified under the Licence tissue records will be maintained for 25 years. ☐

Personal Data:

- **OPTIONAL** - I agree to my contact details being stored for the purpose of being invited to participate in future research studies. ☐

Volunteer Name: ……

Signature: …… **Date:** ……..……..

I confirm that I (investigator) have fully explained the purpose of the study and what is involved to:

……..

I have given the above named a copy of this form together with the information sheet.

Investigator Name: ……

Signature: ……. **Date:** ……..……..

PI Signature: …….. **Date:** ……

Study Volunteer Number: ……

2 copies: 1 for the participant, 1 for the study file.

Appendix 3

Volunteers Information Sheet

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Division of Medical Sciences and Graduate Entry Medicine
School of Medicine

Healthy Volunteers Information Sheet

Study Title: The effect of very low calorie diet (VLCD) with and without concomitant resistance exercise training (RET) or high intensity interval training (HIIT) on muscle protein synthesis (MPS) in middle-aged overweight male.

Name of Researchers:
Dr M Hariz A Aziz
Professor Philip Atherton
Dr Kenneth Smith
Dr Iskandar Idris
Dr Beth Phillips
Dr Andy Philp

You have been invited to take part in a research study. Before you decide whether to take part it is important for you to understand about the research and what it will involve. Please take time to read the following information carefully and discuss it with friends or relatives if you wish to. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. If you decide to take part, you will be given a copy of your consent form and this leaflet.

Background:
It is well known that weight loss, in general, particularly among obese people provides good long-term health benefits, especially in term of lowering the risk of diabetes and heart disease. Diet control, which usually means a low calorie diet, is one way to achieve this weight loss. However, not many people are aware that with low calorie diets, there are also losses of muscle mass- up to 20-30% of total weight loss on average. Losing muscle mass is negatively associated with poor general health, as muscle plays a vital role in numerous body functions. As part of normal human physiological ageing, people undergo sarcopenia- the age-associated loss of muscle mass, which begins at the age of 40-years at approximately 0.5-1% muscle loss per year. The muscle weakness and reduced function of the muscle that accompanies sarcopenia increases the risk of falls (and complications such as fracture or head injury), lack of independence, poor quality of life and ultimately reduced life expectancy. One recognised way to slow down this loss of muscle is by performing sufficient exercise to stimulate muscle growth. Exercise has been shown to stimulate the building of muscle proteins and improve overall physiological function. Putting these factors together, we are planning to study on the effect of very low calorie diets (VLCD) with two different exercise regimes on muscle mass production and function, as well as overall body health. We hope that the findings from this study will inform a large number of people as to how best optimise the care plan for healthy weight loss.

Who can take part?
For the purpose of the study, we need male volunteers who fit the entry criteria and do not have significant medical problems. We are inviting a total of 36 participants aged between 30 to 60 years, with a BMI between 27 to 50 kg/m^2 (overweight and obese) to take part.

WEIGHT lbs	100	105	110	115	120	125	130	135	140	145	150	155	160	165	170	175	180	185	190	195	200	205	210	215
kgs	45.5	47.7	50.0	52.3	54.5	56.8	59.1	61.4	63.6	65.9	68.2	70.5	72.7	75.0	77.3	79.5	81.8	84.1	86.4	88.6	90.9	93.2	95.5	97.7
HEIGHT in/cm	Underweight					Healthy					Overweight					Obese				Extremely obese				
5'0" - 152.4	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
5'1" - 154.9	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	36	37	38	39	40
5'2" - 157.4	18	19	20	21	22	22	23	24	25	26	27	28	29	30	31	32	33	33	34	35	36	37	38	39
5'3" - 160.0	17	18	19	20	21	22	23	24	24	25	26	27	28	29	30	31	32	32	33	34	35	36	37	38
5'4" - 162.5	17	18	18	19	20	21	22	23	24	24	25	26	27	28	29	30	31	31	32	33	34	35	36	37
5'5" - 165.1	16	17	18	19	20	20	21	22	23	24	25	25	26	27	28	29	30	30	31	32	33	34	35	35
5'6" - 167.6	16	17	17	18	19	20	21	21	22	23	24	25	25	26	27	28	29	29	30	31	32	33	34	34
5'7" - 170.1	15	16	17	18	18	19	20	21	22	22	23	24	25	25	26	27	28	29	29	30	31	32	33	33
5'8" - 172.7	15	16	16	17	18	19	19	20	21	22	22	23	24	25	25	26	27	28	28	29	30	31	32	32
5'9" - 175.2	14	15	16	17	17	18	19	20	20	21	22	22	23	24	25	25	26	27	28	28	29	30	31	31
5'10" - 177.8	14	15	15	16	17	18	18	19	20	20	21	22	23	23	24	25	25	26	27	28	28	29	30	30
5'11" - 180.3	14	14	15	16	16	17	18	18	19	20	21	21	22	23	23	24	25	25	26	27	28	28	29	30
6'0" - 182.8	13	14	14	15	16	17	17	18	19	19	20	21	21	22	23	23	24	25	25	26	27	27	28	29
6'1" - 185.4	13	13	14	15	15	16	17	17	18	19	19	20	21	21	22	23	23	24	25	25	26	27	27	28
6'2" - 187.9	12	13	14	14	15	16	16	17	18	18	19	19	20	21	21	22	23	23	24	25	25	26	27	27
6'3" - 190.5	12	13	13	14	15	15	16	16	17	18	18	19	20	20	21	21	22	23	23	24	25	25	26	26
6'4" - 193.0	12	12	13	14	14	15	15	16	17	17	18	18	19	20	20	21	22	22	23	23	24	25	25	26

Do you have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and we will give you a consent form for you to sign. If you decided to take part, you are still free to withdraw at any time and do not need to give reason for your withdrawal.

Would an inconvenience allowance be paid?
You will be paid an inconvenience allowance of £100 upon completion of the study. If you withdraw at any time, for reasons not relating to the study any inconvenience allowance payable will be at the discretion of the principal investigator for this study.

What do I do if agree to participate as one of the volunteers?
If you agree to participate in this study, we will first ask you to attend for a health screening at The Medical School at the Royal Derby Hospital (where all of your study visits will take place). This session will allow you to meet some of the research team and ask any questions you many have about the study and also assess your suitability for the study. We ask you to attend this session following an overnight fast (except water). This session will last approximately 1 hour and will include:

i. A health assessment, including an ECG to monitor your heart, a blood pressure check, an abdominal examination and a medical history check
ii. Measurement of your height and weight
iii. A small blood sample to measure health parameters, including glucose, HbA1c, liver and kidney function, lipid profiles, thyroid function and full blood count (fasting blood sample)

Once we received the details and results from your screening session (within7 days) we will contact you to let you know if you are suitable for the study. If you are, then we (you and the research team together) will decide on the dates to start the programme.

All participants will be provided with a commercially available VLCD meal replacement plan for 6 weeks. There will be 4 products/day (in a form of bar, shakes, porridge, soup, spaghetti etc). Each product provide about 150kcal. This gives you a total of 600 kcal/day. As VLCD definition is <800kcal/day, you have the option to consume some additional intake eg coffee, fruits etc up to additional 200kcal/day. In addition to this diet programme, if you are in one of the exercise training groups you will also be required to attend 3 exercise-training sessions each week for the 6 weeks. All exercise-training sessions will take place at the Medical School at the Royal Derby Hospital. To motivate you and encourage compliance with the dietary intervention, those not in the exercise groups will receive 1-2 times/week phone calls in addition to weekly visit to our unit for the period of the 6-week intervention.

For those in the exercise groups, all exercise sessions will be fully supervised with the intensity of exercise personalised to you. Total duration per session would be would be ~20 min per session for the VLCD+HIIT group and ~40 min per session for the VLCD+RET group. The duration could be slightly longer depending on your recovery duration.

For the research purpose, the group allocation will be a random process. You will be randomly assigned to one of three groups:
Group 1- Very low calorie diet only (VLCD)
Group 2- Very low calorie diet plus resistance exercise training (weight training) (VLCD+RET)
Group 3- Very low calorie diet plus high intensity interval training (cycling) (VLCD+HIIT)

We recognise that some people might not be able to have flexibility in their working hour hence would not be able to commit to the exercise schedule three times/week. In this case, you can request to be allocated to the diet only group if you are keen to participate and can commit to the diet programme. You also can request to be in the exercise group if you have flexibility in your working hour. In this case, you will be randomised into either VLCD+HIIT or VLCD+ RET group.

You will be excluded from the study if you:
- Are unable to tolerate allocated exercise regime.
- Are unable to attend for more than 3 consecutive sessions.
- Miss more than 6 sessions in total.
- Fail to complete the set exercise regime on 6 sessions or more.

In addition to the intervention(s) you will also be asked to attend the Medical School for a number of study days as outlined below:

DAY(1)

For this day, we will ask you to attend after an overnight fast (except water).

We will take small samples of your saliva, blood, muscle and fat (fasting sample) to use as our baseline/pre-intervention measures. All of these samples will be taken by individuals fully trained in these techniques, with muscle and fit biopsies taken only by a fully qualified

medical practitioner. Both of the biopsies are performed as sterile procedures, with local anaesthetic. Details of both procedures are outlined below.

Muscle biopsies:
Muscle biopsies are taken from the thigh. Before the biopsy the area will be cleaned and anaesthetised with local anaesthetic, after which a small (approx. 1cm wide, 2-3cm deep) incision will be made through the skin. The muscle biopsy (smaller than the size of a pea) will then be removed from the thigh using conchotome forceps (essentially a sharp pair of tweezers), with 3-5 small pieces taken to ensure adequate sampling. The incision will then be stitched shut and covered with a waterproof adhesive dressing.

Fat biopsies:
Fat (adipose tissue) biopsies are taken few cm to the left or right of the belly button. Similar to a muscle biopsy, a fat biopsy is a sterile procedure, done under local anaesthetic. The method is called gun biopsy. The area will be lateral to the belly button (either to the left or right side of the abdomen) will be cleansed and surrounding area being covered with sterile drape, small amount of local anaesthetic will be applied. A needle will be introduced into the superficial fatty tissue layer to form a tract for the gun needle biopsy. The gun needle will be inserted via the tract and a snap sound will indicate a sample has been obtained. This will be painless, and the gun needle biopsy will be repeated 2-4 times depending on the amount of sample obtained. The wound will be closed with a medical strip and covered with water resistance dressing.

Biopsy aftercare:
You will be advised about care of the wounds at the end of each biopsy and given an information leaflet about this. Five to seven days after each muscle biopsy the stitches will be removed and the wounds for both types of biopsy will be checked. Five to seven days after the week-6 biopsy, you will need to attend for a final visit so that stitches from the final biopsy sites can be removed and the wounds checked. You will also be paid your inconvenience allowance at this final visit.

In addition above you will also have a series of non-/ minimally invasive tests to assess different aspects of your health/ metabolic status:

1. We will measure how well your body able to process glucose (sugar) by performing oral glucose tolerance test (OGTT). This test is usually use in the clinical setting to help doctors diagnosed diabetes or stratify the risk of diabetes in the future. For this test you will be given 75g of a sugary drink. Small blood samples will be taken prior to the drink and every 15 minutes (for 2 hours) from a cannula which will be sited in to a vein on the back of your hand. During this test, your hand will be placed in a warm box.

2. A series of ultrasound scans will be done to assess the delivery of blood to your arms and legs. One of these scans will involve a non-invasive scan in your groin area (called leg blood flow, LBF) while another will scan your arm after a blood pressure cuff has been inflated on the forearm (called Flow Mediated Dilatation, FMD).

3. We will perform another ultrasound scan on your leg to look at the structure of your muscle. As will the ultrasound to look at the flow of your arm and leg, this measurement is completely non-invasive.

4. Echocardiogram (ECHO) scan will be performed to check for your heart function. This is similar to ultrasound scan which is non-invasive and involves using a probe with gel application on the front to left side of your chest to view your heart structure and its contractility.

5. You will have a dual energy x-ray absorptiometry (DXA, or sometimes called DEXA) scan to measure your fat and muscle composition. More details on DXA scan can be found in enclosed leaflet.

6. Cardiopulmonary exercise test (CPET) will be performed. This is a non-invasive measurement of your heart and lung function during exercise to determine your exercise tolerance/ capacity. This is done on a static exercise bike. At the start, you will be attached to a 12 lead ECG to record your heart trace, blood pressure cuff to measure your blood pressure, saturation probe to monitor your oxygen saturation and a soft rubber face mask with a turbine to measure your expired gases. You then will start paddling at 50-60 rotations per minute (rpm) as a warm up followed by gradual increase in the bike resistance. You will continue pedaling at 50-60rpm until you reach your maximum effort, or if you experience chest pains, discomfort, or clinically unwell.

7. Before you leave to go home you will be given a 'heavy water' drink called Deuterium Oxide (D_2O). This is almost the same as normal water with a small chemical change that allows us to track this drink throughout the body and from this calculate how much new muscle your body is making. The amount of D_2O you are given will be calculated based upon your body weight. After this day you will be given small daily top-ups of D_2O and asked to collect your saliva 3 hours after taking your daily top-ups and keep the sample in the fridge/freezer. For those in the exercise group, you can bring the collection when you attend exercise session (3 times/ week). For those not in the exercise groups, you can send the collection on weekly basis for lab analysis.

8. You also will be given a D_3 creatine solution to drink. This solution allows us to estimate your lean muscle mass. After this drink you will be required to collect urine sample for the next 3 days- initially a 24-hour urine collection and then spot urine sample at 30h, 48h and 72h. You will be given containers for the urine collection and asked to return them when you come for your DAY(4) visit.

9. You will also be supplied with 3-Methylhistidine (3MH) tracer solution and for you to take on DAY(3) which is 2 days after this visit, this is another tracer drink which allows us to assess the breakdown rates of your muscle.

Light lunch will be provided midday.

DAY(2)

On this day, you will complete 24-hour urine collection, and will be collecting small amount urine sample at home (spot urine 30-hour after the D_3 creatine drink).

DAY(3)

On this day, you will be collecting small amount urine sample at home (spot urine 48-hour after the D_3 creatine drink.

You also will be taking 3MH solution between 10am and 12pm (midday) at home and record the time you take the solution.

DAY(4)

You will attend the unit in the morning, fasted from midnight (except clear water), and via a forearm cannula you will have hourly blood tests and 2 hourly urine collection for 6 hours to measure the level of 3MH (solution ingested on the previous day) in your blood. On this visit you will be returning all of your urine collection bottles/pots.

In the morning session, we will perform a scan to evaluate the delivery of blood to your thigh muscles, called contrast enhanced ultrasound scan (CEUS). For this scan we need to insert a cannula (a fine plastic tube) into a vein by your elbow area to deliver a contrast-agent (microbubbles that glow under ultrasound) to help us determine blood flow to your muscle. We will record the blood flow to your muscle at rest and after a light leg exercise.

In the afternoon, your baseline muscle strength will be assessed (involving repetition of muscle movement upper and lower body) on this day.

Light lunch will be provided midday.

END OF WEEK 3 AND WEEK 6

All of the procedures from DAY(1) to DAY(4) will be repeated after weeks 6. At the end of Week 3 (or early week 4), only 3MH blood and urine collection, muscle and fat biopsy will be done. You will be asked to take 3MH solution and fasted from midnight (except clear water) before attending study day at the end of week 3 (or early week 4).

DIETARY AND PHYSICAL ACTIVITY RECORD

During the course of the study (before intervention and during intervention), you will be provided with 4-day diary to record your normal dietary intake, and a monitor (Actiheart Physical Activity Monitor) with small sticky electrodes attached to your chest to record your physical activity for 5 days. This will be in 2 sessions. The date for this will be determined later which suit your normal weekend activity (one date has to be before intervention, and the other date has to be during the intervention).

What do I have to do?
With the exception of the study days above and compliance to the diet and exercise regimes (if applicable) we ask that you maintain your normal activities of daily living. You will be required to stick to the prescribed diet to ensure you are getting the amount of calories that we intend, and also getting adequated vitamins and minerals etc. to attend the exercise

What is the drug or procedure that is being tested?
This study assesses the effect of a VLCD with and without 2 different types of exercise on weight loss, muscle loss and muscle metabolism (protein building and breaking down and glucose uptake).

What are the side effects or risk of any treatment or procedures received when taking part?
Research studies often involve some risks, not all of which may be currently known.

In all cases of invasive procedures there is a slight risk of infection at incision sites (biopsies and venipuncture); this will be minimized by the use of sterile techniques in a designated clinical investigation room and suitable wound dressing and monitoring. In light of our 35-year experience, 11 years in Derby, biopsies are well tolerated by all subjects. Scarring is hardly perceptible and fades with time to near invisibility. Some swelling, tenderness, stiffness, or bruising may be felt for 2-3 days afterwards – these are normal sensations which not all people experience. If needed, painkillers can be prescribed by one of doctors to ease the discomfort. There is a slight risk of damaging skin nerves during the biopsy techniques. These nerves usually grow back with restoration of normal sensation without difficulty, although there is a small risk of this not happening.

What are the possible benefits of taking part?
We cannot promise the study will definitely help you, but if you are assigned to one of the exercise groups we would expect to see improvements in muscle mass, strength and function. From the tests that you are going to get involve in, we would be able to assess your general health condition, including any risk of future health problem (eg risk of diabetes, high cholesterol etc). This would help you to be more cautious of any unhealthy lifestyle. All participants in the study will receive meal replacement products for the 6 week intervention period worth nearly £60/week. In addition, the information we get from this study will give us a better understanding of the effects of VLCD on muscle function and overall metabolism. We hope this will benefit many people, in providing information to design a program that helps with weight loss among overweight/ obese people and at the same time would optimise muscle development and preservation.

What are the possible disadvantages and risks of taking part?
Healthy volunteers have been chosen for this study; however, as mentioned above, it is possible that the routine tests could detect any risk as well any undiagnosed health problems, eg diabetes and high blood pressure, as they are common and often undiagnosed. Should this be the case, you will be informed and advised to attend your GP practice for further management. Your GP will also be notified. Be advised, whilst detecting early medical problem

would be beneficial for early intervention, this will affect future insurance status e.g. for life insurance or private medical insurance.

What are the exclusion criteria for this study?
We are recruiting male subject between the ages of 30 to 60 years, who are overweight (BMI 27-50kg/m^2). Unfortunately, we would not be able to include those with weight more than 120kg due to weight limit of our DXA scan. If you have been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance; having an invasive procedure (e.g. blood sample >100ml), you would not be eligible to take part. You would also be unsuitable if you have particular medical conditions (particularly diabetes mellitus and serious heart problem etc), or are taking certain medications. If you are interested in this study, please discuss these further with the study doctor.

What if something goes wrong?
If you suffer any symptoms or side effects, you should report them immediately to the study team. If you have a concern about any aspect of this study, you should ask to speak to the study team who will do their best to answer your questions. The researchers contact details are given at the end of this information sheet. If you have a complaint on your treatment by a member of staff or anything to do with the study, you can initially approach the principal investigator for this study, Dr Iskandar Idris. If this achieves no satisfactory outcome, you should then contact the Ethics Committee Secretary, Mrs Louise Sabir, University of Nottingham, Faculty of Medicine & Health Sciences Research Ethics Committee c/o School of Medicine Education Centre, B Floor, Medical School, QMC Campus, Nottingham University Hospitals, NG7 2UH. E-mail: louise.sabir@nottingham.ac.uk. In the unlikely event that you suffer injury to yourself or damage to your property as a result in taking part in this research, the University does have an insurance policy to cover harm arising as a result of the defect in the design of the study. In addition, all medical practitioners taking part in the research have personal medical negligence cover.

Will my taking part in this study be kept confidential?
We respect your right to privacy and we will take measures to safeguard confidentiality. A single form, on which you are asked to sign to give consent for involvement will carry details of your name, but no health related details. This is kept securely in a locked cabinet within the Medical School. Access to this cabinet is restricted to personnel directly involved in the study and to University staff with direct responsibility for ensuring the study is conducted appropriately.

We will follow current ethical and legal practice and all information about you will be handled in confidence. If you join the study, some parts of the data collected about you will be looked at by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by people authorised to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

All information which is collected about you during the course of the research will be kept strictly confidential, stored in a secure and locked office, and on a password protected

database. Any information about you which leaves the hospital will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it, with the exception of a letter sent to your GP advising of your participation in this study.

Your personal data (address, telephone number) will be kept for 5 years after the end of the study so that we are able to contact you about the findings of the study and possible follow-up studies. All other data (research data) will be kept securely for 7 years. After this time your data will be disposed of securely. During this time precautions will be taken by all those involved to maintain your confidentiality and only direct members of the research team will have access to your personal data.

What will happen to any samples I give?
We will use the biopsy samples to see how your muscle and fat responds to your intervention (VLCD with/without exercise). We will also seek your consent for any samples remaining after analysis for this study has been completed, to be stored and used in future research. This is optional and you will be asked to give separate consent for this. The samples will be securely stored with a code unique to you at the University of Nottingham under the Universities Human Tissue Research Licence (No. 12265). Some samples will also be kept at the University of Birmingham for analysis at their laboratory.

Some of these future studies may be carried out by researchers other than the current research team. This may include researchers working for commercial companies. Any samples or data used will be anonymised so that you could not be identified in any way. If you do not agree to this, any remaining samples will be disposed of in accordance with the Human Tissue Authorities code of practice.

Will any genetic tests be done?
In the current study we will not be using your samples for genetic testing.

What will happen to the results of the research study?
Data collected during the study will be published in the scientific literature enabling other health professionals and scientists to use the information. You will not be identified in any publication. There is usually a delay of up to one year from the study completion before this occurs. Should you wish to be informed of publications resulting from this study, please inform the study team.

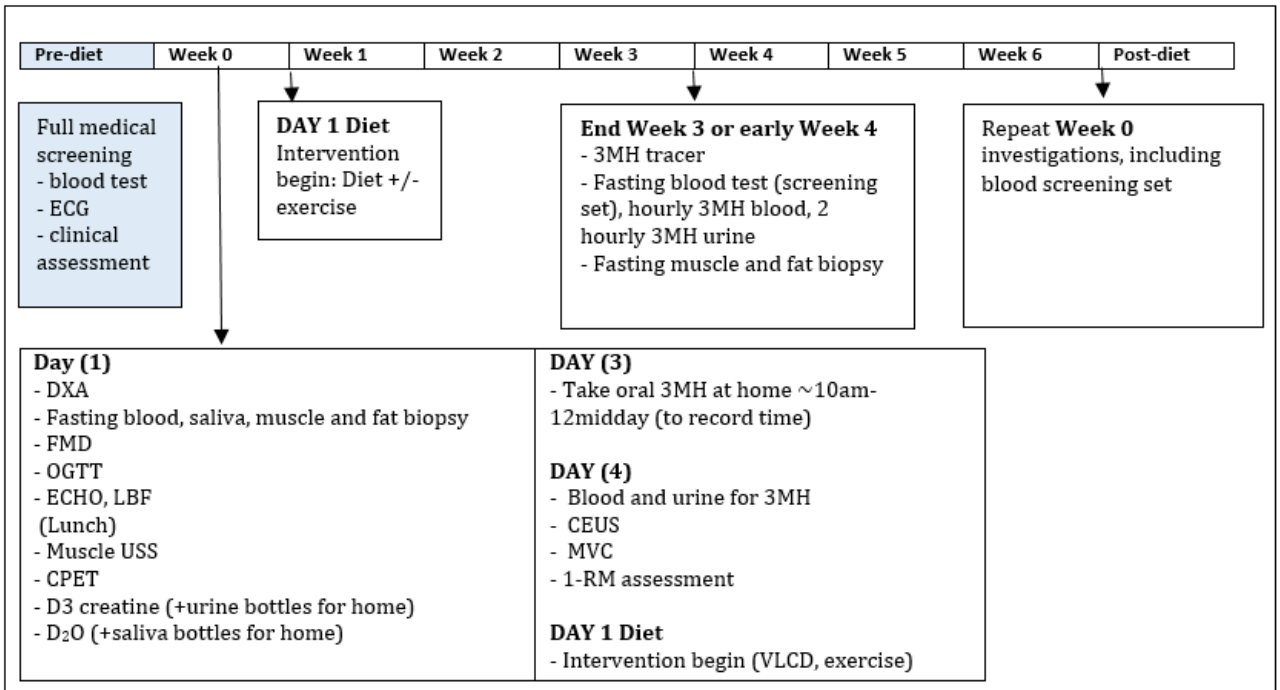
Who is funding the research?
This study is funded by the University of Nottingham.

Who has reviewed the study?
This study has been reviewed and approved by the University of Nottingham Medical School Ethics Committee.

Contact for Further Information

Thank you for your interest in this study. For further information, please contact Dr Hariz Aziz:

Email: stxmhab@nottingham.ac.uk
Office Tel: 01332724841
Mobile Tel: 07729182321
alternatively contactable at The University of Nottingham, Division of Medical Sciences and Graduate Entry Medicine, School of Medicine, Royal Derby Hospital, Derby, DE22 3DT.



- There will be 4-day diet diary and 5-day Actiheart Physical Activity Monitor (running parallel) before and during the interventional period (2 sessions). Dates depend on subjects' weekend activity.
- 6-week interventional period involves 42 days of VLCD, VLCD+RET, and VLCD+HIIT.
- Screening blood test includes fasting FBC, U&E, LFT, TFT, glucose, HbA1c, lipid profiles and coagulation screen.
- 3MH tracer involves consumption of 3-methylhistidine tracer a day before serial blood test (hourly blood test for 6 hours). Hour-0 is 20-24 hours after 3MH ingestion.
- D3 creatine involves oral D3 creatine loading followed by urine collection daily for 3 days.
- D_2O involves loading dose and daily maintenance dose. Daily saliva collection by subjects, with 3 times per week samples to be sent to the lab when attending exercise session. Weekly lab submission for controlled group.

Appendix 4

Study Questionnaires

VLCDex Physical Activity Questionnaire

ID:

PRE/ DURING/ POST Intervention

Date:

No	Activity	Response
Q1-Q6: Work nature		
Q1	Does your work involve vigorous-intensity activity that causes large increases in breathing or heart rate like [eg carrying or lifting heavy loads, digging or construction work] for at least 10 minutes continuously?	Yes [ ] Go to Q2 No [ ] If No, go to Q4
Q2	In a typical week, on how many days do you do vigorous intensity activities as part of your work?	Number of days: ……
Q3	How much time do you spend doing vigorous-intensity activities at work on a typical day?	Hours: Min:
Q4	Does your work involve moderate-intensity activity that causes small increases in breathing or heart rate such as brisk walking [or carrying light loads] for at least 10 minutes continuously (eg postmen, plumber etc) This also include job that require standing up or walking most of the working shift (eg theatre nurses, surgeon, train conductor, cleaner etc)	Yes [ ] Go to next question No [ ] If No, go to Q7
Q5	In a typical week, on how many days do you do moderate-intensity activities as part of your work?	Number of days: ……
Q6	How much time do you spend doing moderate-intensity activities at work on a typical day?	Hours: Min:
Q7-Q9: Travel to and from places		
Q7	Do you walk or use a bicycle (pedal cycle) for at least 10 minutes continuously to get to and from places?	Yes [ ] Go to next question No [ ] If No, go to Q10
Q8	In a typical week, on how many days do you walk or bicycle for at least 10 minutes continuously to get to and from places?	Number of days: ……
Q9	How much time do you spend walking or bicycling for travel on a typical day?	Hours: Min:
Q10-Q15: Recreational activities (Please exclude exercises from the research programme)		
Q10	Do you do any vigorous-intensity sports, fitness or recreational (leisure) activities that cause large increases in breathing or heart rate like [running or football] for at least 10 minutes continuously?	Yes [ ] Go to next question No [ ] If No, go to Q13
Q11	In a typical week, on how many days do you do vigorous intensity sports, fitness or recreational (leisure) activities?	Number of days: ……

VLCDex Physical Activity Questionnaire

ID:

Q12	How much time do you spend doing vigorous-intensity sports, fitness or recreational activities on a typical day?	Hours: Min:
Q13	Do you do any moderate-intensity sports, fitness or recreational (leisure) activities that cause a small increase in breathing or heart rate such as brisk walking, [cycling, fast walk, volleyball] for at least 10 minutes continuously?	Yes [ ] Go to next question No [ ] If No, go to Q16
Q14	In a typical week, on how many days do you do moderate intensity sports, fitness or recreational (leisure) activities?	Number of days: ……
Q15	How much time do you spend doing moderate-intensity sports, fitness or recreational (leisure) activities on a typical day?	Hours: Min:
Q16-17: Sitting or reclining at work, at home, getting to and from places, or with friends including time spent sitting at a desk, sitting with friends, traveling in car, bus, train, reading, playing cards or watching television, but do **not include time spent sleeping**		
Q16	How much time do you usually spend sitting or reclining on **a** typical weekday?	Hours: Min:
Q17	How much time do you usually spend sitting or reclining on **a** typical weekend day?	Hours: Min:

ID:
Date:

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VLCDex Symptoms Questionnaire

For WEEK:
Comparing to WEEK:

Please rate to following condition/symptom with scoring where:

-5: Symptoms worsening (very severe/significant)
-3: Symptoms worsening (moderate)
-1: Symptoms worsening (mild)
0: No changes
1: Symptom improving (mild)
3: Symptom improving (moderate)
5: Symptom improving (very significant)

No	Symptoms	-5	-4	-3	-2	-1	0	1	2	3	4	5
1	Hunger sensation											
2	Craving for food											
3	Mood/ General motivation											
4	Concentration											
5	Energy feeling tired/ lethargic											
6	Night sleep											
7	Interaction with others											
8	Participation in hobby/interest											
9	Bowel symptoms (constipation/ diarrhoea)											
10	Other physical symptoms (eg headache. Exclude muscle pain/ache from biopsy and exercise)											

Appendix 5

Data collection Forms

VLCDex

OGTT and 3MH data collection

PRE / POST INTERVENTION

Subject ID:

OGTT

Day / Date:

Blood (Glu) fasting Time ……. Level: ……

OGTT Glucose loading Time ……..

Intervals (min)	Time blood taken	Glucose level
15		
30		
45		
60		

Intervals (min)	Time blood taken	Glucose level
75		
90		
105		
120		

3MH

Day / Date: …….

Date+time 3MH taken: ……..

Baseline 0 hour Time……

*Pre MH urine sample = D3
Creatine urine @30hr

Blood Intervals (hour)	Time blood taken
1	
2	
3	

Blood Intervals (hour)	Time blood taken
4	
5	
6	

Urine collection (hour interval)	Time colected
0	
2	
4	
6	

6hr MH urine sample = D3
Creatine urine @~71-72hr

D3 creatinine

Date+time taken:

Total 24hr volume:

Sample	Date	Time
Completed 24h		
30h spot		
48h spot		
72h spot		

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VLCDex USS record

PRE / POST INTERVENTION

Subject ID:

FMD Date, Day, Time: Baseline BP 1: 2: 3:

Baseline		Min 6:10	
Min 5:15		Min 6:20	
Min 5:50		Min 6:30	
Min 6:00			

Distance from tip of thumb (cm) for 1)probe placement : 2)bottom cuff:
IV fluid total (if any):

LBF (on dominant leg)
Date, Day, Time:

(cm^3/sec)	Time 1	Time 2	Time 3
Baseline			
Post exercise (6 reps of 50% 1RM)			

ECHO
Date, Day, Time:

%FS (short axis)	
%FAC (short axis)	

CEUS (warm device >30mins on dominant thigh)
Date, Day, Time:
Top patella to bottom probe distance:

Timing	Procedure	Imaging
0	2ml/min infusion begin	
0:30	30s capture (30 sec)	
1:00	30s capture (and reduce rate to 1ml/min simultaneously)	
1:30	30s capture flash	
2:00	30s capture flash	
	Leg extention (6x 50%1RM) after above then	
3:00	30s capture flash	
3:30	30s capture flash	
4:00	30s capture flash	

Total time infusion run:

VLCDex baseline/ post intervention

Subject ID:

1-RM assessment
Day Date Time:

	Chest press	Latissimus-pull down	Seated lever row	Leg extension	Leg curl	Leg press
Weigh(kg) & reps						
1-RM equivalent						
70% 1-RM						

MVC
Day Date Time:

Angle	Max Load
70	
80	
60	

Chair Rotation:
Antero-posterior Slide:
From motor scale:

Motor rotation:
Motor height:

CPET
Day Date Time:
Seat height:
Ramp setting:
RPM -55-60

	Pre	Post	Peak
BP			
HR			

Time taken to back to baseline BP&HR:

Max Watt :
Max HR :
Total time :
VO2 max / VO2 peak:
Reason for exercise termination:
BORG score at termination:

ID:

VLCDex
Control Group weekly review

Wk/ Date	Weight / Update / Phone call etc
Week 1	
Week 2	
Week 3	
Week 4	
Week 5	
Week 6	

VLCDex RET training record

Session 1 Supervised by:
Date, Day, Time:

	Chest press	Latissimus-pull down	Seated lever row	Leg extension	Leg curl	Leg press
1-RM and 70%						
Training weight						
Rep set 1						
Rep set 2						

Session 2 Supervised by:
Date, Day, Time:

	Chest press	Latissimus-pull down	Seated lever row	Leg extension	Leg curl	Leg press
Training weight						
Rep set 1						
Rep set 2						

Session 3 Supervised by:
Date, Day, Time:

	Chest press	Latissimus-pull down	Seated lever row	Leg extension	Leg curl	Leg press
Training weight						
Rep set 1						
Rep set 2						

Subject ID:

Session 4 Supervised by:
Date, Day, Time:

	Chest press	Latissimus-pull down	Seated lever row	Leg extension	Leg curl	Leg press
1-RM and 70%						
Training weight						
Rep set 1						
Rep set 2						

Session 5 Supervised by:
Date, Day, Time:

	Chest press	Latissimus-pull down	Seated lever row	Leg extension	Leg curl	Leg press
Training weight						
Rep set 1						
Rep set 2						

Session 6 Supervised by:
Date, Day, Time:

	Chest press	Latissimus-pull down	Seated lever row	Leg extension	Leg curl	Leg press
Training weight						
Rep set 1						
Rep set 2						

Subject ID:

VLCDex HIIT

Seat height:
Max Watt:

Session 1 Supervised by:
Date, Day, Time:

Pre BP and HR:

%watt	Watt	Notes

Post BP and HR:

Session 2 Supervised by:
Date, Day, Time:

Watt:	Pre BP HR	Post BP HR	Notes

Time end:

Session 3 Supervised by:
Date, Day, Time:

Watt:	Pre BP HR	Post BP HR	Notes

Subject ID:

Session 4 Supervised by:

Date, Day, Time:

Watt:	Pre BP HR	Post BP HR	Notes

Time end:

Session 5 Supervised by:

Date, Day, Time:

Watt:	Pre BP HR	Post BP HR	Notes

Time end:

Session 6 Supervised by:

Date, Day, Time:

Watt:	Pre BP HR	Post BP HR	Notes

Time end:

Session 7 Supervised by:

Date, Day, Time:

Watt:	Pre BP HR	Post BP HR	Notes

Time end:

Session 8 Supervised by:

Date, Day, Time:

Watt:	Pre BP HR	Post BP HR	Notes

Time end: