In vivo and In vitro
Regulation of Skeletal Muscle Lipid Metabolism

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Declaration

I, the undersigned, declare that this thesis has been composed entirely by myself and that it has not been accepted in any previous application for a Doctor of Philosophy degree. The work, of which it is a record, except where specifically acknowledged, has been done entirely by myself. All sources of information have been specifically acknowledged by reference.

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Summary

Cardiovascular Disease (CVD) is the leading cause of death worldwide, a disease which has atherosclerosis at the centre of its pathology and is also linked to obesity. Atherosclerosis is known to be a chronic inflammatory disease with immune system activation. This immune activation is linked to increased postprandial triglyceride (TG) concentrations, which are an independent risk factor for the development of CVD. This thesis has demonstrated that a single bout of high intensity interval exercise (HIIE) is a low energy-expenditure (EE) and relatively time-efficient method of attenuating postprandial TG after high fat meals (HFMs). The mechanism underlying this reduction in postprandial TG is likely to be lipoprotein lipase (LPL) mediated, rather than via altered hepatic metabolism. To support this assertion the current thesis demonstrated that plasma β-hydroxybutyrate concentrations were unchanged while LPL dependent TG rich lipoprotein (TRL)-TG hydrolysis (LTTH) was increased the day after HIIE was performed. The beneficial effects do not remain when HFMs are consumed on a second day after HIIE. Furthermore HIIE does not alter select markers of postprandial immune cell activation, assessed via
flow cytometric measurement of leukocyte CD11b and CD36 expression. Similarly HIIE had no effect on postprandial soluble adhesion molecule expression (sICAM-1 and sVCAM-1). On the other hand, HIIE was shown to attenuate the postprandial rise in markers of oxidative stress (plasma TBARS and protein carbonyls). In a separate study, substrate metabolism was studied in vitro in C2C12 muscle cells. Recent work has, in several mouse models, shown that reduced citrate synthase (CS) activity may be important in obesity resistance and also lower plasma TG levels, via an increase in fatty acid (FA) oxidation. However, in the current thesis, when CS activity was reduced in C2C12 muscle cells via lentiviral transduction, FA oxidation was in fact reduced when cells were incubated with glucose (5mM) and palmitate (0.8mM), for both 2 and 24 hours. These findings highlight the importance and ambiguity of CS’s role as an intracellular metabolic modulator in muscle. This also warrants further research into how muscle citrate metabolism may be manipulated to improve substrate metabolism in diseases such as obesity, diabetes and CVD.
List of abbreviations

3'UTR, 3' untranslated region, 167
α-KD, α-Ketoglutarate dehydrogenase, 128
ACADM, acyl-Coenzyme A (CoA) dehydrogenase, 119
ACC, acetyl-CoA carboxylase, 129
ACC2, acetyl CoA carboxylase 2, 132
ACL, ATP-citrate lyase, 128
ACN, aconitase, 128
ADH, antidiuretic hormone, 59
ANOVA, analysis of variance, 178
Apo, apolipoprotein, 37
ASCM, American College of Sports Medicine, 109
ATGL, adipose triglyceride lipase, 54
AUC, area under the curve, 93
β-Ox, β-Oxidation, 128
CD36, cluster of differentiation 36, 66
CDS, coding DNA sequence, 167
CHD, coronary heart disease, 21
CI, confidence interval, 102
CiC, citrate carrier, 130
CoASH, coenzyme A, 139
Complex III, ubiquinone-cytochrome b-c1 region, 68
Complex I, NADH dehydrogenase, 68
COX, cytochrome c oxidase, 107
CPT1, carnitine palmitoyltransferase 1, 122
CS, citrate synthase, 8
CS-KD, citrate synthase knockdown, 166
CV, coefficient of variation, 153
CVD, cardiovascular disease, 21
DEXA, dual-energy X-ray absorptiometry, 116
EDHF, Endothelium derived hyperpolarizing factor, 59
EE, energy expenditure, 73
eNOS, endothelial nitric oxide synthase, 62
ET, endurance training, 81
EV, empty construct, 166
FA, fatty acid, 8
FCR, fractional catabolic rate, 99
FMR, fumarase, 128
Fructose 1,6-BP, fructose 1,6-Bi-phosphate, 128
Fructose-6-P, fructose-6-Phosphate, 128
GATM, glycine amidinotransferase, 119
Glucose-6-P, glucose-6-Phosphate, 128
HBS, HEPES buffered saline solution, 153
HDL, high density lipoprotein, 29
HFM, high fat meal, 7
HIIE, sub-maximal HIIT or high-intensity interval exercise, aerobic HIIT/HIIE, 81
HIIT, high-intensity interval training, 80
HSL, hormone-sensitive lipase, 54
HSPG, heparan sulphate proteoglycan surface receptors, 39
iAUC, incremental area under the curve, 93
ICAM-1, intracellular adhesion molecule-1, 63
ICD, isocitrate-dehydrogenase, 128
IDH₃β, isocitrate dehydrogenase [NAD+]₃β, 119
IL-1, interleukin-1, 23
IL-6, interleukin-6, 66
IRS1, insulin receptor 1, 118
LCF, long-chain fatty, 128
LCFA , long chain FA, 129
LCSFA , long chain saturated fatty acids, 117
LDL, low density lipoprotein, 35
LDL-C, low density lipoprotein cholesterol, 28
LFA-1, CD11a/CD18, leukocyte function-associated antigen-1, 63
LPL , lipoprotein lipase, 38
LTTH, LPL dependant TG rich lipoprotein (TRL)-TG hydrolysis, 7
Mac-1, CD11b/CD18, macrophage-1 antigen, 63
MCD, malonyl-CoA decaboxylase, 135
MCFA , medium chain FA, 37
MCKAT, medium-chain ketoacyl-CoA thiolase, 273
MCP-1, monocyte chemoattractant protein-1, 64
MDA, malondialdehyde, 152
MDH, malate dehydrogenase, 242
MI, myocardial infarction, 32
mtDNA, mitochondrial DNA, 283
NEFAs , non-esterified free fatty acids, 35

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NF-kB, nuclear factor-kappaB, 62
NMR, nuclear magnetic resonance spectroscopy, 116
NO, nitric oxide, 58
Non-target, NT, scrambled shRNA sequence, 166
NPY, neuropeptide Y, 59
O$_2^-$, superoxide, 68
ONOO-, peryoxynitrite, 69
oxLDL, oxidized LDL, 49
P, puromycin resistance marker, 166
PAF, platelet-activating factor, 59
PDC, pyruvate dehydrogenase complex, 125
PDH, pyruvate dehydrogenase, 85
PDK, PDH kinase, 125
PDP, PDH phosphatase, 125
PEP, phosphoenolpyruvic acid, 126
PFK-1, 6-phosphofructo-1-kinase, 125
PGC1-α, peroxisome proliferator-activated receptor-γ coactivator-1-α, 85
Phospho-AMPK, phosphorylation of AMPK at threonine 172, 248
PKC, atypical protein kinase C, 118
PKCδ, protein kinase Cδ, 118
PKG, protein kinase G, 59
pLKO.1-puro, empty plasmid vector – A.K.A. Empty Vector (EV), 166
PPAR, peroxisome proliferator-activated receptor, 56
PS, pyruvate shuttle, 128
Risk factor score, total number of risk factors, 60
RNS , reactive nitrogen species, 65
ROS, reactive oxygen species, 22
SD, standard deviation, 215
SDH, succinate dehydrogenase, 128
SDS, succinyl CoA synthetase, 128
shRNA1, short-hairpin RNA-1, CS-KD, 166
shRNA2, short-hairpin RNA-2, CS-KD 2, 166
SkM glycogen, skeletal muscle glycogen, 85
SR Ca2+ uptake, sarcoplasmic reticulum calcium 2+ uptake, 85
SR-B1, scavenger receptor B1, 43
TBARS, thiobarbituric acid reactive substances, 152
TCA, trichloroacetic acid solution, 153
TG, triglyceride, 31
TLac, lactate threshold, 85
TLR4, toll-like receptor4, 286
TNF-α, tumour necrosis factor-α, 23
TRL, TG rich lipoprotein, 7
VCAM-1, vascular cell adhesion molecule 1, 63
VIP, vasoactive intestinal peptide, 59
VLA-4, very late activation antigen 4, 63
VLDL, very low-density lipoproteins, 35
$\dot{V}O_{2\text{max}}$, maximal oxygen consumption, 73
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1. Introduction
1.1. Cardiovascular disease

Cardiovascular disease (CVD), defined as a group of diseases affecting the heart and blood vessels (Schoen 2005), is the leading cause of death worldwide and is becoming more prevalent (Fuster 2010). The most common CVD form is coronary heart disease (CHD), which accounts for approximately 73,000 (43%) of all CVD deaths per year, in the United Kingdom (World Health Organisation [WHO], 2011). On top of this mortality rate, the economic cost of CVD, to the UK healthcare system has been estimated at ~£8.7 billion per year (British Heart Foundation-CHD statistics, 2012).

1.2. Atherosclerosis

Atherosclerosis is the primary cause of CHD and is frequently the cause of a significant proportion (14-21%) of strokes as well (Madamanchi, Vendrov & Runge 2005). There are several stages in the pathology of atherosclerosis. Two well characterised and important stages that have been identified are the appearance of a lesion known as a ‘fatty streak’ and the development of fibrous plaques within major blood vessel walls (Hajjar, Nicholson 1995). The development of a fatty
streak requires an atherogenic environment, which can occur when the endothelial lining of a vessel exhibits dysfunction (endothelial cells normally control vascular tone, among other functions, and thus blood flow) or damage. This increases the extravasation of lipids and monocyte/macrophages from the blood (by activating proteins which mediate attachment and migration of lipid filled immune cells) to the intimal layer (*tunica intima*) leading to lipid deposition (Hajjar, Nicholson 1995), *i.e.* the ‘fatty streak’. The pathological progression of atherosclerosis includes the migration of immune cells such as monocytes to the site of the lesion where they differentiate into macrophages and digest lipid deposits. This digestion of lipid droplets includes some that have been modified by oxidising agents, such as reactive oxygen species (ROS). ROS modification of lipid droplets may lead to uncontrolled scavenging of oxidised lipid carrying particles from the local sub-endothelium by macrophages, and the eventual formation of foam cells and cholesterol deposition in the sub-endothelium (this will be covered in more detail in section 1.14). Following this is the development of fibrous plaques whereby smooth muscle cells migrate into the sub-endothelium near the ‘fatty streak’ and undergo hypertrophy. This can lead to the increased production of collagen from smooth
muscle cells within the lesion site (illustrated in Figure 1) (Wilson *et al.* 1998). Additionally, as smooth muscle cells and macrophages proliferate, the size of the atheroma is increased, which leads to a protrusion from the vessel wall altering local blood flow, and thereby producing a region of low pressure and reduced shear stress. This reduced shear stress can reduce local rolling speeds of leukocytes, allowing a longer interaction time with the inflamed endothelium and an increase in the expression of chemokines such as tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1), which act to further attract inflammatory cells to the area. This can result in an increased inflammatory environment, increased recruitment and extravasation of monocytes/macrophages and lipids and, ultimately, a larger fibrous plaque (Helderman *et al.* 2007). This combination of factors can eventually lead to the development of a layer of connective tissue over the atheroma that retains lipids and cells and forms a fibrous/atherosclerotic plaque (illustrated in Figure 1). Finally, a CHD or CVD event can occur when a vulnerable plaque becomes weakened and ruptures, releasing thrombotic and coagulant factors such as fibrinogen, factor VIII, factor IX, factor XI and prothrombin. While these factors are needed in a non-pathological setting for wound healing, release of these factors from a ruptured
atherosclerotic plaque can cause arterial thrombosis, i.e. an arterial blood clot, often resulting in CVD or stroke (Finn et al. 2010, Hansson 2005, Bertina 2003). Clearly atherosclerosis is a common condition which can have many deleterious effects and so understanding the risk factors for this condition are of great importance.
Figure 1 Diagram of the progression of atherosclerosis

An illustration of atherosclerotic plaque formation, labelling the arterial wall structure and demonstrating the long-term disease progression in atherosclerotic plaque formation from the formation of a ‘fatty-streak’ to the development of a fibrous plaque formed from the increased production of collagen and eventually leading to a protruding fibrous/atherosclerotic plaque which increases the local inflammatory response and is at risk of becoming weakened and rupturing (Based on the work of Hajjar, Nicholson 1995, Finn et al. 2010).
1.3. Risk factors for cardiovascular disease

Early epidemiological studies demonstrated strong relationships between several factors and CVD risk (Keys et al. 1984, Anderson, Castelli & Levy 1987, Stamler 1986). Several of these factors are non-modifiable such as sex, age and family history of CVD. For example, after 40 years of age the lifetime risk of developing CHD is 49% for men and 32% for women. In addition, more than four out of five people dying from CHD are over 64 years of age. It is unknown exactly why increasing age results in an increased disease risk but it is thought to be due to age-related changes in the structure and function of the cardiovascular system which are intertwined with disease-related processes and also altered by other modifiable risk factors such as hypertension, inflammation, smoking and obesity (Lakatta 2003). The Framingham Heart Study was key in identifying these other modifiable risk factors with 5,209 men and women between the ages of 30 and 62 from the town of Framingham, Massachusetts recruited and assessed by extensive physical examinations and lifestyle interviews. Four years after the beginning of the study and after 34 incidences of CVD, high blood pressure and high serum cholesterol were identified as being highly associated with CVD (Watt 1957).
Further work on the Framingham, and other studies, confirmed these risk factors (Anderson, Castelli & Levy 1987, Kagan et al. 1974, Kannel 1996, van den Hoogen et al. 2000, Marmot et al. 1975). For example van den Hoogen et al. (2000) looked at six different populations from around the world and examined baseline blood pressure in 12,031 men (40-59 years old). The unadjusted relative risk of death due to CHD was 1.17 per 10 mm Hg increase in systolic blood pressure and 1.13 per 5 mm Hg increased in diastolic blood pressure. Regarding cholesterol, Anderson et al. (1987) assessed data from the Framingham heart study after 30 years follow-up and demonstrated that under the age of 50 years the risk of death increased by 5% and risk of CVD death by 9% for each 0.26 mmol/l rise in cholesterol. Further follow-up studies identified other risk factors with strong associations for CVD including smoking (2-4 fold increase in CHD (O'Donnell & Elosua 2008, Ockene & Miller 1997, Lakier 1992), diabetes (2-3 fold increase (Fox et al. 2004) in CVD risk), obesity [1.5 fold (men)-1.6 fold (women) increased risk of CVD, demonstrated in a 44 year follow-up of the Framingham Heart Study (Wilson et al. 2002, Hubert et al. 1983)] (Figure 2) and physical inactivity (Kendrick, Williamson & Caspersen 1991). Furthermore, there are several meta-analyses and other studies which demonstrate...
an increased risk of developing CVD in physically inactive individuals (Sofi et al. 2008, Fagard & Cornelissen 2007, Kelley, Kelley & Tran 2005, Schuler, Adams & Goto 2013) (Figure 2). This will be discussed in more detail in section 1.16.

1.4. Lipids/lipoproteins and the risk of cardiovascular disease

As mentioned previously, increased serum total cholesterol was among the first risk factors to be associated with an increased risk of CVD (Anderson, Castelli & Levy 1987). Following this, further epidemiological studies sought to elucidate the role of other lipids in CVD risk. In particular, fasting low-density lipoprotein cholesterol (LDL-C), (the metabolic roles of lipoproteins will be discussed in the next section), was also directly associated with increased CVD risk, with a 0.26 mmol/l increase in LDL cholesterol associated with a 12% increase in CVD risk (Gofman, Young & Tandy 1966). Moreover LDL-C levels in young adults were also associated with increased risk of CVD in later life (Klag et al. 1993, Stamler et al. 2000). This supports the hypothesis that CVD progresses with increased length of exposure to elevated LDL-C levels. Simultaneously, increased serum high density lipoprotein cholesterol (HDL-C) concentrations have been demonstrated to have a protective
effect on CVD risk (Gofman, Young & Tandy 1966, Law, Wald & Thompson 1994). In these studies, men in the lowest quintile of HDL-C concentration (less than 0.93 mmol/l) had 2.0 times the risk of developing ischaemic heart disease than men in the highest quintile (greater than or equal to 1.33 mmol/l) (Pocock, Shaper & Phillips 1989) (Figure 2).
Figure 2 Modifiable risk factors and mean increased-fold risk of CVD

Modifiable risk factors of cardiovascular disease (CVD) and their associated increased-fold risk of developing CVD or mortality from CVD. Risk factors are defined below. Smoking (vs non-smoking, self-assessed) in males and females in meta-analysis from Huxley & Woodward 2011, diabetes (clinically assessed) assessing both males and females from a meta-analysis by Huxley, Barzi & Woodward 2005, physical inactivity - direct effects only - (leisure time, self-assessed) using both males and females from a meta-analysis by Sofi et al. 2008, obesity (>30 BMI, metabolically healthy) - both males and females - from a meta-analysis by Fan et al. 2013, high total cholesterol in middle-aged men and women from a meta-analysis by the Prospective Studies Collaboration 2007 (7.5 mmol/l vs 5.5 mmol/l), high low-density lipoprotein cholesterol (LDL-C) (4.2 mmol/l vs 6.2 mmol/l) from a meta-analysis by Sniderman et al. 2011, high high-density lipoprotein cholesterol (HDL-C) (1.33 mmol/l vs 0.93mmol/l) from Pocock, Shaper & Phillips 1989, high fasting triglyceride (TG) men and women (2.8 mmol/l vs 1.3 mmol/l) from a meta-analysis Hokanson & Austin 1996, and high postprandial triglyceride (TG) in women (1.7 mmol/l vs 1 mmol/l) from Bansal et al. 2007.
Several studies have demonstrated that increased (fasting and postprandial) plasma triglyceride (TG) is also associated with an increased risk of CVD. However, although this relationship is strong (Figure 2) (Nordestgaard et al. 2007, Emerging Risk Factors Collaboration 2007) there are some doubts as to the independent nature of this relationship. For example, the aforementioned study from The Emerging Risk Factors Collaboration (2007) assessed the records of 165,544 participants from 37 prospective cohorts between 1968-2007. These participants (equal numbers of males and females) were without baseline CVD and the study included a follow-up of ~10.4 years with 10132 CHD and 4994 stroke outcomes over 11.7 million person years. This study assessed the associated risk of CVD of several lipid-related markers (HDL-C, fasting TG, LDL-C, apolipoproteins A1 and B) compared to conventional risk factors such as age, systolic blood pressure, smoking status, history of diabetes, and total cholesterol. TG levels did not improve the risk prediction power when added to these conventional risk factors, indicating that fasting TG is not an independent risk factor.

Subsequently, Mora et al. (2008) assessed 26,330 healthy women with 19,983
fasting and 5,347 postprandial measurements taken, TG was the only lipid (measurements included HDL-C, LDL-C and total cholesterol) which was consistently elevated (over 5% mean increase) in a postprandial state (Mora et al. 2008). This study (Mora et al. 2008) also assessed the predictive power of fasting vs non-fasting values of these lipids. TG was the only commonly used CVD marker which had a stronger association with CVD risk when measured in the non-fasting (P=0.007) state compared to the fasted state (P=0.66). Investigating this further, a recent study assessed postprandial TG in 140,790 male and female subjects from baseline and followed participants for a duration of 6-15 years. A total of 3219 (4.8%) of men and 1434 (1.9%) women developed an acute myocardial infarction (MI) (Egeland et al. 2014), and it was demonstrated that non-fasting TG predicted (P<0.001) MI risk in groups which were classified as low-risk by their HDL-C levels. This study demonstrated that non-fasting TG is an independent predictor of MI, although it did not account for total cholesterol which may reduce the independent prediction power of non-fasting TG. However, one study which did account for total cholesterol assessed 13,981 participants (6394) male and (7587) female individuals (20-93 years old) who were followed for 26-28 years and developed 1793 cases of
MI. When adjusted for age, total cholesterol, BMI, hypertension, diabetes, smoking, alcohol consumption, physical inactivity and lipid-lowering therapy, non-fasting TG was still an independent predictor (P<0.001) of MI in both males and females (Nordestgaard et al. 2007).

Also, during The Women’s Health Study (Bansal et al. 2007), 26,509 healthy American women were assessed over 11 years for CVD development and mortality. The findings demonstrated that both fasting and non-fasting TG were associated (P<0.001) with future CVD after adjustments for age, blood pressure, smoking and HRT. Consistent with the previously mentioned studies the predictive power of fasting TG was abolished (P=0.9) when adjusted for factors such as HDL-C. However, non-fasting TG maintained a strong independent relationship with future CVD in fully-adjusted analyses (P=0.006). Furthermore, the highest quintile of TG measured 4 hours after a meal (the actual time of meal was self-reported) yielded the strongest (4.5-fold) relationship with CVD events compared with the lowest quintile (Figure 2).

Postprandial TG concentration is therefore an important predictor of CVD and the
overlap between the duration of postprandial lipaemia and frequency of meals means that plasma TG levels do not return to baseline levels for 8-10 hours following the last meal of the day (Groot et al. 1991). Thus, an individual following the classic western pattern lifestyle of repeated high fat meals (Cordain et al. 2005) may be exposed to a metabolic state, \textit{i.e.} elevated TG, with the potential for increased risk of CVD, for the majority of the day. As postprandial TG, and other fasting lipids are associated with CVD it is prudent to discuss the mechanisms underlying this association beginning with a general overview of lipid and lipoprotein metabolism.

**1.5. Overview of lipid/lipoprotein metabolism**

As lipids are insoluble in water they must be combined with water-soluble proteins for transport in particles named lipoprotems, with non-esterified free fatty acids (NEFAs) bound to albumin. Lipoprotein particles contain a core of TG and cholesterol esters surrounded by a surface of phospholipids, free cholesterol and proteins (Ginsberg, Zhang & Hernandez-Ono 2005). Lipoproteins are commonly separated into different density particles termed: chylomicrons, very low-density...
lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) (Table 1). However, within these lipoprotein classes there are still variations in particle density and size. These particles may also be categorised according to the TG and cholesterol content with chylomicrons and VLDL being triglyceride rich lipoproteins (TRL) and LDL and HDL being cholesterol rich lipoproteins (Sparks & Sparks 1994) (Table 1). Lipoprotein metabolic pathways can broadly be sub-divided into exogenous and endogenous lipid transport pathways. The exogenous pathway is responsible for the packaging, transport and delivery of dietary lipids such as cholesterol and TG from the small intestine to the liver and other peripheral tissues. On the other hand, the endogenous pathway is responsible for the transport of hepatically derived lipids to peripheral tissues (Figure 3). Both these pathways will be described in detail within the following sections.
Table 1 Lipoprotein classes and properties

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Density range (g/ml)</th>
<th>Diameter (nm)</th>
<th>Major Lipids</th>
<th>Apolipoproteins</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Protein</td>
<td>TG</td>
</tr>
<tr>
<td>Chylomicrons</td>
<td>&lt;0.950</td>
<td>80-1000</td>
<td>Dietary TG</td>
<td>B48, A1, A2, C1, C2, C3, E</td>
<td>2</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.950-1.006</td>
<td>30-80</td>
<td>Endogenous TG</td>
<td>B100, C1, C2, C3, E</td>
<td>8</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006-1.019</td>
<td>23-35</td>
<td>Cholesterol and cholesterol ester</td>
<td>E, B100, C1, C2, C3, E</td>
<td>19</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019-1.063</td>
<td>20-22</td>
<td>Cholesterol and cholesterol ester</td>
<td>B100</td>
<td>22</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.090</td>
<td>9-15</td>
<td>Cholesterol and phospholipid</td>
<td>A1, A2, A4 C1, C2, C3, D, E</td>
<td>40</td>
</tr>
</tbody>
</table>

A table of lipoprotein classes and properties, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglyceride (TG), cholesterol (chol), phospholipids (PL). Adapted from Frayn (2010).
1.6. Exogenous lipoprotein metabolism

Upon consumption of lipids, TG is broken down in the small intestine by pancreatic lipase into glycerol and NEFAs (Freie et al. 2006). The NEFAs then form mixed micelles and are absorbed by enterocytes in the small intestine (Frayn 2010). The NEFAs taken up are re-esterified within the enterocytes and packaged into chylomicrons which move into the blood-stream via the lymphatic ducts and enter the circulation via the subclavian vein (Schaefer, Jenkins & Brewer 1978). Chylomicrons are ~1000 nM diameter particles and are the largest of the lipoproteins and along with TG they also contain dietary cholesterol (2-7%) (Table 1). However, not all NEFAs are re-esterified within the enterocytes to form TG with shorter chain length FAs (<C_{12}-C_{14}) being poor substrates for esterification. Short-chain (<C_{8}) FAs enter the capillary system directly as NEFAs and medium chain FAs (MCFA) (C_{8}-C_{12}) enter the portal vein as NEFAs many of which are absorbed directly by the liver (Frayn 2010). Exogenous long-chain FAs esterified and packaged into nascent chylomicrons pick up apolipoprotein (apo) C-II (Table 1), from circulating HDL particles. Lipoprotein surface expression of apoC-II allows the formation of a high-
affinity complex of apoC-II and lipoprotein lipase (LPL), while activation of LPL occurs via direct helix-helix interactions between apoC-II amino acid residues 39-62 and the loop covering the active site of LPL (Bertina 2003). The enzyme LPL is situated on the luminal surface endothelium of blood vessels within peripheral tissues such as adipose tissue and skeletal muscle (Wang et al. 2013). LPL is water soluble and hydrolyses TGs in lipoproteins, such as chylomicrons and VLDL (Mead, Irvine & Ramji 2002). Once in the circulation, the majority of the TG contained in the chylomicrons is hydrolysed by LPL and the released NEFAs are taken up by local peripheral tissues. The resulting glycerol is returned to the liver via the circulation and metabolised as demonstrated in Figure 3a. The remains of the chylomicron particles after the majority of TG has been hydrolysed are termed chylomicron remnants. Chylomicron remnants are enriched in cholesterol esters with extra cholesterol obtained from both dietary and HDL-derived sources [(chylomicron remnant TG and cholesterol composition: 9-15.8 mmol/l TG, 0.7-1.5 mmol/l total cholesterol (De Pascale et al. 2006)]. HDL accepts TG from chylomicrons and chylomicron remnants in exchange for cholesterol esters, a process mediated by cholesterol ester transfer protein (CETP) (Chung et al. 2004). Approximately 44% of
chylomicron remnants remain in the circulation until they reach a small enough size (30-50nm) to act as ligands for heparan sulphate proteoglycan surface receptors (HSPG) in the liver (Willnow 1997), which fulfils a critical role in the sequestration of remnant particles by hepatocytes (Mahley & Ji 1999). The normal physiological function of chylomicron remnants is the transport of bile cholesterol to the liver, while the remaining TG content of the remnants is a major source of hepatic fatty acids, accounting for ~73% of newly synthesised VLDL in mice (Jung et al. 1999).
Figure 3 The metabolic pathway of (A) exogenous and (B) endogenous lipoproteins.

Continuous cycles of VLDL-TG hydrolysis (dashed lines) by LPL leads to the re-uptake of VLDL by the liver or the formation of LDL particles. Fatty acid (FA), high-density lipoprotein (HDL), lipoprotein lipase (LPL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), apolipoprotein CII (Apo CII), apolipoprotein B100 (Apo B100). Figures from Frayn (2010), pg. 282 and 284. Image rights obtained from Wiley-Blackwell 2015.
1.7. Endogenous lipoprotein metabolism

The majority of endogenous plasma TG is contained in VLDL particles, which are continuously synthesised and taken up hepatically (either as nascent, mature or remnant particles) (demonstrated in Figure 3b) (Frayn 2010). When synthesized hepatically, nascent VLDL contain TG, cholesteryl ester, apolipoprotein B100, and small amounts of apolipoproteins E and C. VLDL particles can again be sub-divided into two major classes: large TG-rich VLDL1, and smaller, more dense VLDL2, which contain a greater amount of cholesterol than VLDL1 (Packard, Shepherd 1997). VLDL particles are ~25-90 nM and in normolipidaemic individuals ~75% of plasma TG variation is determined by VLDL1 concentration (Tan et al. 1995). LPL dependent hydrolysis removes TG from VLDL1 particles as does the action of CETP (Chung et al. 2004). Further depletion of TG from VLDL1 particles results in a phenotype similar to VLDL2 or to formation of IDL, dependent on the rate of lipolysis (if the rate of lipolysis is high the ratio of IDL/VLDL2 formation is increased and vice versa for a lower rate of lipolysis). Further depletion of TG from IDL, by hepatic lipase and LPL, eventually leads to formation of LDL particles (Frayn 2010). Only ~10% of VLDL1 particles have sufficient lipid removed to form the terminal
particle LDL, while the remaining VLDL1 particles form either VLDL2-like particles or IDL remnant particles that remain in circulation before being removed from the plasma hepatically (Packard & Shepherd 1997). VLDL2 particles are readily delipidated (by peripheral lipases such as LPL) to LDL (Gaw et al. 1995). LDL contains the majority (~65%) of plasma cholesterol, and it functions primarily as a transporter of cholesterol to peripheral tissues (Wang et al. 1985) such as the liver with uptake mediated by the LDL receptor (Frayn 2010) (Figure 3b and Figure 4).

Cholesterol, while having pathological roles, is also required for many physiological functions including the synthesis and maintenance of cell membranes, synthesis of vitamin D and steroid hormones (Frayn 2010).

1.8. High density lipoprotein metabolism

As discussed, VLDL, IDL and LDL supply peripheral tissues with cholesterol and FA. While the delivery of FA is normally balanced by catabolic oxidation in peripheral tissues (depending of the delivery rate and oxidative capacity of peripheral tissues), cholesterol requires HDL to provide a method of removal from peripheral cells (Frayn 2010). At the start of the cycle, HDL particles are secreted from the liver and
intestine as nascent pre-β HDL particles and as they acquire phospholipids and cholesterol they form discoidal HDL particles that are able to acquire cholesterol in two ways. Firstly, HDL acquires cholesterol by interaction with peripheral cells and uptake of excess cellular cholesterol via the action of ATP-binding cassette (ABC)-A1. The action of the enzyme Lecithin-cholesterol acyltransferase (LCAT) is essential to this process since it esterifies cholesterol within HDL and thus maintains the concentration gradient required for HDL uptake of cellular cholesterol. Secondly, HDL acquires cholesterol by sequestration of excess surface material released during lipolysis of TRLs by LPL, LCAT again esterifies the cholesterol taken up by HDL particles in this process. As HDL particles continue their uptake and esterification of cholesterol, they eventually form mature spherical cholesterol-rich particles. These can then transfer their cholesterol to the liver, either directly via scavenger receptor B1 (SR-B1) for excretion in bile or for recycling [around 50% of excreted cholesterol is reabsorbed by the small intestine (Trigatti, Krieger & Rigotti 2003)] or by transferring cholesterol ester to TRLs as these eventually return to the liver. The cholesterol-depleted HDL particles are now ready to accept further cholesterol from peripheral tissues. Thus, there is a constant recycling of cholesterol-
rich HDL particles and cholesterol-depleted HDL which are engaged in sequestration of cholesterol from peripheral tissues from the hydrolysis of TRLs. By these mechanisms cholesterol is transferred from peripheral tissues to the liver, from where it can be excreted as cholesterol and bile salts or reabsorbed. This process is known as ‘reverse cholesterol transport’ (Figure 4) and is the opposite of LDL delivery of cholesterol to peripheral tissues (Frayn 2010).

As described previously CETP facilitates the exchange of cholesterol esters and TG between TRLs, LDL and HDL. When the plasma concentration of TG is increased, particularly when this is caused by increased numbers of VLDL particles, CETP will catalyse the movement of cholesterol ester from HDL to the TRLs, with TG moving to the HDL particles. This often happens in a postprandial environment when TRL particles are in relative abundance. The cholesteryl esters remain with TRL particles until they are taken up by the liver as a remnant. The HDL particles thus become TG enriched and can be hydrolysed by hepatic lipase and recycled to cholesterol-depleted particles (Frayn 2010) (Figure 4). This partly illustrates the close relationship between TG and HDL-C, the next section will discuss this further.
Figure 4 Cholesterol metabolism

*Illustration of forward and reverse cholesterol transport and the action of CETP in the transport of cholesterol between HDL and triglyceride rich lipoproteins. Low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), cholesterol ester (CE), free cholesterol (FC) and ATP-binding cassette transporter A1 (ABC A1). From Frayn (2010), image rights obtained from Wiley-Blackwell 2015.*
1.9. Relationship between plasma triglyceride and high-density lipoprotein-cholesterol concentration

As described earlier, elevated HDL-C is associated with a protective effect on CVD (Gofman, Young & Tandy 1966, Law, Wald & Thompson 1994). Epidemiological evidence also demonstrates a strong inverse relationship between plasma TG and HDL-C concentrations (Austin 1991). The mechanism behind this effect appears to be in part because the hydrolysis of TRLs by LPL is accompanied by the transfer of cholesterol and other components to HDL. Thus an increased activity of LPL increases HDL-C concentration alongside reduced TG serum concentration and reduced VLDL-TG (due to increased TG hydrolysis), this relationship is demonstrated by subjects that have a low fasting TG concentration. For example, a study (Lamarche et al. 1993) of men aged 20-42 years (mean=34) with either normal HDL-C and TG levels (n=61, fasting TG <2.0 mmol/l, HDL-C >0.9 mmol/l) or high TG and low HDL-C (n=13 fasting TG >2.0 mmol/l, HDL-C <0.9 mmol/l) demonstrated that men with normal TG and HDL-C had lower VLDL-TG (0.63±0.36) compared to the latter group (2.11±0.47). There was also an inverse association (p<0.05) between LPL activity and VLDL-TG levels in these men, demonstrating that subjects...
with higher HDL-C and lower fasting TG had reduced VLDL-TG and higher LPL activity (Lamarche et al. 1993). Conversely, if plasma TG is high there will be an increased opportunity for lipid exchange via the action of CETP. HDL will have cholesterol ester depletion and TRL remnants will become cholesterol enriched, reducing the amount of HDL mediated removal of cholesterol from tissues and facilitating the buildup of endothelial cholesterol deposits and the development of the fatty streak. This goes some way to explaining the association between TG levels and CVD (Frayn 2010).

1.10. Atherogenic lipoprotein phenotype

Although elevated fasting and non-fasting LDL-C remains an important independent risk factor for CVD (Stamler 1986, Gofman, Young & Tandy 1966, Klag et al. 1993, Stamler et al. 2000), it is also true that many CVD events [~56% (Stamler 1986)] will occur in the absence of elevated LDL-C concentrations. In terms of total risk in the population factors other than LDL-C are more important (see Figure 2) such as the combination of low HDL-C and elevated postprandial TG levels (Kastelein et al. 2008). There are several mechanisms which have been hypothesised to explain this
relationship, firstly, HDL-C may be a marker of the efficiency of reverse-cholesterol transport and thus reduce cholesterol deposition. Also, low HDL-C may indicate defective lipolysis of TG from TRLs. A slower rate of lipolysis may allow TRL remnants to remain in the circulation for a longer period as they have TG hydrolysed at a slower rate before they achieve a sufficiently small size for receptor-mediated uptake. TRLs and TRL remnants being in the circulation for a longer period allows for a greater number of interactions with other lipoproteins and thus the CETP facilitated uptake of cholesterol esters will be increased allowing the remnants to become cholesterol-enriched. This may lead to cholesterol deposition in the endothelium in an atherogenic state (Frayn 2010) (Figure 4).

In addition to these phenomena, low HDL-C and elevated TG are closely associated with a change in the LDL phenotype, leading them to become smaller and more dense than normal. This happens again as a result of increased VLDL-TG secretion and/or decreased LPL activity (both of which cause increased VLDL-TG concentrations). Again an increased VLDL-TG allows for greater exchange of TG for cholesterol with LDL particles via CETP. The excess exchange of TG is part of an
atherogenic pathology as TG rich/cholesterol depleted LDL particles are then further
delipidated by hepatic lipase and form smaller denser LDL particles (Figure 5). This
combination of events *i.e.* cholesterol depleted HDL, cholesterol enriched TRL
remnants, increased plasma TG and smaller, denser LDL is termed the ‘atherogenic
lipoprotein phenotype’ (Figure 5), and can be driven by increased postprandial TG
concentrations. Small dense LDL particles are more likely to penetrate the
endothelial lining and enter the sub-endothelial space, where they are prone to
damage by free radicals and thus form oxidized LDL (oxLDL). These small dense
particles are also at an increased risk of oxidative damage, as they tend to have less
fat-soluble antioxidant vitamins which protect against oxidative damage. As will be
described in detail later, oxidation of sub-endothelial LDL particles is a crucial step in
the development of atherosclerosis (Frayn 2010).
Figure 5 Atherogenic lipoprotein phenotype

Illustration of the metabolism involved in the atherogenic lipoprotein phenotype. The formation of smaller, denser LDL is central as these are more prone to oxidation in the sub-endothelium. Low-density lipoprotein particles (LDL), triglyceride (TG), cholesterol ester transport protein (CETP), lipoprotein lipase (LPL). From Frayn (2010), image rights obtained from Wiley-Blackwell 2015.

1.11. Integrated regulation of postprandial lipoprotein metabolism

As a vital metabolic pathway, lipoprotein metabolism is regulated at several stages. LPL is activated in adipose tissues (but slightly down regulated in skeletal muscle) by insulin, which is increased in the postprandial environment (Kessler 1963). Therefore, the removal of TRL-TG from the circulation is increased postprandially (Figure 6). This is, however, a saturable process, reflecting the limited activity of
LPL. In normolipidaemic men, a meal with fat content as low as 30-50g can saturate
the removal of TG from the plasma and cause an increase in plasma TG levels
(Cohen, Noakes & Benade 1988, Dubois et al. 1994, Murphy et al. 1995). After a
high fat meal (HFM) (>50g fat), the plasma concentration of both chylomicrons and
VLDL is increased (hepatic VLDL secretion is increased) and these lipoproteins
compete for hydrolysis by LPL. As LPL has a higher affinity for larger particles (Frayn
2010), chylomicron particles are hydrolysed with greater rapidity over VLDL.
Therefore, VLDL-TG concentration is increased after a HFM as its clearance is
decreased, and plasma TG is increased to a greater extent than accounted for by
the appearance of only chylomicron-TG. Although VLDL-TG concentration is
increased after a HFM, the effects of insulin somewhat counteract the reduced
clearance of VLDL-TG as insulin can inhibit postprandial hepatic VLDL secretion,
dependent upon the physiological state of the individual (Pavlic et al. 2010, Lewis &
Steiner 1996). Quantifying this attenuation studies have shown a 50-60% insulin
induced attenuation of both VLDL-TG and VLDL apoB (reflecting VLDL secretion) in
lean insulin-sensitive individuals compared to insulin resistant subjects (Lewis &
Steiner 1996). Furthermore insulin infusion can cause reduced production of VLDL,

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particularly of larger TG-rich VLDL1 particles (Malmstrom et al. 1998). This was
demonstrated by a study of healthy male subjects who underwent insulin infusion
with VLDL kinetics being monitored. VLDL1 apo B production was reduced after
insulin infusion suggesting reduced VLDL1 production, while VLDL2 production and
fractional catabolic rates were unchanged (Malmstrom et al. 1998). This attenuation
of VLDL secretion is reversed when individuals are infused with Intralipid (a lipid
emulsion containing linoleic acid and alpha-linolenic acid). This overrides the effect
of insulin by saturating the lipolysis of TG, a similar effect is seen with heparin
infusion, which removes endothelial LPL and reduces the lipolysis of TG. These
effects result in an increase in NEFA plasma concentration and an increase in
hepatic VLDL particle production in the fed state (Pavlic et al. 2010, Duez et al.
2008). This indicates that the effects of insulin are attenuated when the lipolysis of
TG either has decreased capacity or is saturated. Additionally, the rate of VLDL-TG
secretion is dependent on the delivery of NEFA to the liver, as a substrate for TG
synthesis, and NEFA release is suppressed postprandially due to the inhibition of
adipose tissue lipolysis by insulin (Frayn 2010) (Figure 6). Thus VLDL and VLDL-TG
secretion is increased postprandially, which gives rise to increased plasma
concentrations of VLDL/VLDL-TG.

Figure 6 Insulin’s postprandial actions on lipid metabolism

A diagram of insulin’s postprandial actions on lipid metabolism. Thin arrows indicate pathways downregulated in response to insulin signalling, while thicker arrows indicate pathways upregulated. Insulin directly and indirectly inhibits hepatic very low-density lipoprotein (VLDL) secretion. This indirect inhibition of hepatic VLDL secretion is achieved by inhibition of non-esterified fatty-acids (NEFA) formation from lipolysis through an inhibition of hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). This causes a reduction of NEFA conversion to triglyceride (TG) in the liver via inhibition of non-esterified fatty acid (NEFA) secretion from adipose tissue. A reduction of VLDL, particularly triglyceride (TG) rich VLDL1, reduces cholesterol exchange with high-density lipoprotein (HDL) for TG. This reduces the small-dense low-density lipoprotein (LDL) seen in the atherogenic lipoprotein phenotype, which is more at risk of being oxidatively modified to form oxidised LDL (oxLDL), an atherogenic molecule. Insulin also slightly reduces LPL activity on the endothelium of muscle tissue. Muscle (skeletal muscle), adipose (adipose tissue), based on the work of Frayn 2010, Choi & Ginsberg 2011).
Looking further into the role of insulin in lipid metabolism it is known that in most tissues, insulin promotes the synthesis of lipids and inhibits lipolysis. This happens with great magnitude in adipocytes, where insulin acts to inhibit hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), which are the lipolytic enzymes that break down TG (Kastelein et al. 2008, Kershaw 2006) (Figure 6). In addition insulin stimulates the re-esterification of any NEFAs produced within adipocytes from TG lipolysis (Frayn 2003). These phenomena demonstrate the importance of insulin in the postprandial state and so it is clear that dysregulation of insulin secretion or sensitivity will interrupt these complex metabolic interactions and lead to perturbations in postprandial lipid metabolism (DeFronzo & Tripathy 2009).

Therefore, in insulin resistant states, VLDL secretion will be greater postprandially due to the reduced effects of insulin on NEFA secretion from adipose tissue. An increased concentration of hepatic NEFA will result in increased hepatic TG to act as a substrate for VLDL synthesis. Additionally, insulin mediated attenuation of TG-rich VLDL1 secretion will be reduced, thus increasing postprandial VLDL-TG. Increased VLDL-TG allows increased TG exchange with HDL for cholesterol, leading to
cholesterol depleted HDL (Figure 6) (Kannel & Vasan 2009). This is partly why increased TG and decreased HDL-C are key metabolic abnormalities in insulin resistant states, such as type 2 diabetes, and are central to the generation of an atherogenic state. Furthermore this highlights why strategies to reduce lipids may be useful in the prevention and treatment of CVD

1.12. Interventions to reduce triglyceride

One of the most common therapies to reduce CVD risk via the lowering of lipids is the use of statins, which inhibit HMG CoA reductase and thus reduce endogenous cholesterol production. Whilst this is an effective strategy, other lipid risk factors may also be appropriate, or potentially more appropriate, treatment targets (Kastelein et al. 2008). In one study, a post-hoc analysis of two clinical trials (Kastelein et al. 2008), in which 10,001 and 8,888 patients with established CHD were assigned to either usual-dose or high-dose statin treatment LDL-C was lowered in both patient groups, although the risk of CVD was no longer associated with LDL-C. Instead non-HDL cholesterol, TRL and TRL remnant specific apolipoproteins were associated with CVD events, indicating that the CVD risk was only partially attenuated with LDL-
C lowering drugs because of TRL remaining elevated (Kastelein et al. 2008). Therefore, it appears that lowering the concentration of TRL and TRL remnants is imperative in many people taking LDL-C lowering drugs to reduce their risk of CVD. Importantly as postprandial TG is a strong independent CVD risk factor targets to lower this would be desirable as it will reduce TRL and TRL remnant concentration and thus counteract many of the negative effects of the atherogenic lipoprotein phenotype. For example, fibrates are particularly effective at reducing both fasting (Table 2) and postprandial TG levels [34-67% 3.5 h and 8 h postprandially, respectively in 55 subjects with hypertriglyceridemia >1.7 mmol/L fasting (Rosenson, Helenowski & Tangney 2010)], and these have been shown to reduce the risk of CVD in the Helsinki Heart Study in middle-aged men by up to 71% (Manninen et al. 1992). Fibrates increase lipolysis by activation of peroxisome proliferator-activated receptors (PPAR), particularly PPARα. Additionally, fibrate treatment reduced fasting TG levels by 21% and raised HDL-C levels by 18% with a reduction in CVD 7.3% in CHD patients with low HDL-C and high fasting TG [Bezafibrate Infarction Prevention (BIP) study 2000]. These findings again indicate the positive effects (increased HDL-C and reduced risk of CVD) of lowering both fasting and postprandial TG.
concentrations in patients at risk of CVD. This is further supported by 2 meta-analyses which showed that Fibrates significantly (P<0.001 and P<0.0001, respectively) reduced the risk of CVD in patients with atherogenic dyslipidaemia (Lee et al. 2011) and patients at risk of CVD (Jun et al. 2010). On the other hand, Sandeep and Rohit (2011) found that although treatment with fibrates reduces the amount of CVD development in patients (with an already high risk of developing CVD), it does not appear to reduce overall mortality. Other drugs that are effective at reducing fasting TG are immediate-release and extended-release niacin, omega-3, statins and ezetimibe (Table 2)

Table 2 Effect of lipid-lowering therapies on fasting triglyceride reduction

<table>
<thead>
<tr>
<th>Drug</th>
<th>% Triglyceride Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrates</td>
<td>30–50</td>
</tr>
<tr>
<td>Immediate-release niacin</td>
<td>20–50</td>
</tr>
<tr>
<td>Omega-3</td>
<td>20–50</td>
</tr>
<tr>
<td>Extended-release niacin</td>
<td>10–30</td>
</tr>
<tr>
<td>Statins</td>
<td>10–30</td>
</tr>
<tr>
<td>Ezetimibe</td>
<td>5–10</td>
</tr>
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</table>

(Miller et al. 2011).
1.13. **Endothelial function**

As has been previously mentioned, the elevation in plasma lipid levels results in endothelial uptake of lipids and immune cells such as monocytes/macrophages and is key in the pathology of atherosclerosis. It is prudent, therefore, at this point to discuss the biology of the endothelium. The endothelium is a monolayer of endothelial cells, which constitutes the inner cellular lining of the blood vessels and the lymphatic system. Thus it is in direct contact with blood/lymph and circulating cells. As such, the endothelium is central to the control of blood fluidity, platelet aggregation, vascular tone and plays a major role in the regulation of immune activation and inflammation. The endothelial cells control vascular tone and thus blood flow, by synthesising and releasing relaxing and contracting factors such as nitric oxide (NO) – a major relaxing factor, metabolites of arachidonic acid (*e.g.* prostaglandin E2), various peptides, adenosine, purines and ROS (for a full list and details see Table 3). Endothelial dysfunction is described as an imbalance in the synthesis and/or the release of these endothelial factors, in favour of vasoconstriction and production of inflammatory factors. It is also linked to the pathology of atherosclerosis and CVD as it allows the extravasation of
Table 3 Endogenous vasoconstricting and vasodilating substances

<table>
<thead>
<tr>
<th>Endogenous Vasoconstricting Substances or Mechanisms</th>
<th>Endogenous Vasodilating Substances or Mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>Adenosine</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>ADP</td>
</tr>
<tr>
<td>Antidiuretic hormone (ADH)</td>
<td>ATP (tissue/location dependant)</td>
</tr>
<tr>
<td>Asymmetric dimethylarginin</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>ATP (tissue/location dependant)</td>
<td>Carbon Dioxide (CO₂)</td>
</tr>
<tr>
<td>Dopamine</td>
<td>Endothelium derived hyperpolarizing factor (EDHF)</td>
</tr>
<tr>
<td>Endothelin</td>
<td>Epinephrine (tissue/location dependant)</td>
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<td>Endotoxin</td>
<td>Heparin</td>
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<td>Epinephrine (tissue/location dependant)</td>
<td>High Shear stress</td>
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<td>hypoxia</td>
<td>Histamine</td>
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<td>Insulin</td>
<td>L-arginine</td>
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<td>Low shear stress</td>
<td>Muscular contraction</td>
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<td>Mechanical stretch</td>
<td>Natriuretic peptides</td>
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<tr>
<td>Neuropeptide Y (NPY)</td>
<td>Niacin</td>
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<td>Norepinephrine</td>
<td>Nitric Oxide</td>
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<td>Thrombin</td>
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<td>Thromboxane</td>
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<td>Prostaglandin I2</td>
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<td>Protein kinase G (PKG) activity</td>
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<td>Substance P</td>
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<td>Vasoactive intestinal peptide (VIP)</td>
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*(Boron 2012).*
Vita et al. (1992) were among the first to show that endothelial dysfunction preceded and was linked to atherosclerotic plaque formation by demonstrating that patients with evidence of coronary endothelial dysfunction (as assessed by acetylcholine infusion) demonstrated a marked increase in sensitivity to the constrictor effects of catecholamines. Further research has confirmed that endothelial dysfunction is an independent risk factor for CVD and one of the first clinical indicators of atherosclerosis (Vogel 1997). This was shown by studies which demonstrated that traditional risk factors which predict CHD are also associated with endothelial dysfunction (e.g. Davignon 2004). Additionally, John et al. (1998) demonstrated the reduced availability of NO in hypercholesterolemic patients, there is also decreased acetylcholine response (a measure of endothelial dysfunction) in the same population (Vita et al. 1990). A decreased acetylcholine response is also demonstrated in familial CHD (Schachinger et al. 1999). Additionally, the total number of risk factors (hypercholesterolemia, cigarette smoking, higher blood pressure, male gender, older age, family history of premature vascular disease and larger vessel size) in a given CVD patient is a potent independent predictor of endothelial dysfunction as measured by flow mediated dilation (Celemajer et al.)
1994) and the acetylcholine test (Vita et al. 1990). Endothelial dysfunction is known to result from postprandial lipaemia (explained in more depth subsequently) (Bae et al. 2001, Gaenzer et al. 2001). This effect is mediated by remnant lipoprotein particles increasing the permeability of the endothelium causing a disruption of normal endothelial function and allowing increased lipoprotein migration across the endothelium. Furthermore, lipoproteins, particularly TRL, promote the expression of endothelial membrane proteins [low-density lipoprotein receptor-related protein, endothelial nitric oxide synthase (eNOS), and caveolin-1] that elicit an increased inflammatory response from local immune cells. Furthermore, TRL also increase the production or release of several bioactive substances, many of these being ROS, from immune cells and the local endothelial cells (Jagla & Schrezenmeir 2001). This process is known as acute immune activation, inflammation and oxidative stress.

1.14. Immune activation and inflammation

Acute inflammation is characterised by vasodilation and increased permeability of the endothelium which are induced by the actions of various inflammatory mediator
molecules. As mentioned previously this increased permeability of the endothelium leads to an accumulation of harmful TRL and the disruption of endothelial function (Jagla & Schrezenmeir 2001). Moreover, various leukocytes are centrally involved in the inflammatory response and the extravasation of these cells across the endothelium into the sub-endothelium is a key step in the propagation of an inflammatory response. Atherosclerosis is now known to involve an ongoing inflammatory response, which mediates all stages of the disease from initiation, progression and ultimately plaque formation and detachment. Many of the processes involved in the atherogenic inflammatory process are thought to originate from lipid derived signalling, particularly in the postprandial environment. Indeed, VLDL particles themselves may activate inflammatory pathways of endothelial cells such as nuclear factor-kappaB (NF-kB) (Dichtl et al. 1999), promoting a local atherogenic environment. Furthermore, TRL remnants and LDL are central in the inflammatory atherogenic state. The increased permeability of the endothelium during endothelial dysfunction leads to increased penetration by TRLs/TRL remnants, with the TG carried by the TRLs contributing directly to a pro-inflammatory state by activating signalling pathways on macrophages and Brendan Gabriel
endothelial cells (Libby 2002, Gower et al. 2011).

Recruitment of leukocytes to an inflammatory site is receptor-mediated, and products of inflammation such as histamine promote the expression of P-selectin on endothelial cell surfaces (Yang, Furie & Furie 1999). Initially, leukocytes are bound to the endothelium by weak bonds that P-selectin forms with carbohydrate ligands (Yang, Furie & Furie 1999) (Figure 7). The inflammatory response also promotes the release of vascular cellular adhesion molecule 1 (VCAM-1) and intra-cellular adhesion molecule 1 (ICAM-1) on endothelial cells and ICAM-1 on leukocytes which further slow leukocytes. Specifically, monocyte and neutrophils express ICAM-1 and VCAM-1. ICAM-1 and VCAM-1 then mediate monocyte and neutrophil extravasation to the sub-intima in a cycle of events resulting in immune cell activation, binding, extravasation and eventual differentiation into macrophages (Takashiba et al. 1999) (Figure 7). Indeed these adhesion molecules appear to be central in facilitating the progression of atherosclerosis and resultant CVD (Ley & Huo 2001, Nakashima et al. 1998, Motawi et al. 2012). VCAM-1 is involved mainly in the early adhesion of monocytes to the endothelium and its primary receptor is very late activation
antigen 4 (VLA-4) (Elices et al. 1990). ICAM-1 is involved in both monocyte and lymphocyte adhesion to the endothelium (Guray et al. 2004) and its main receptors are leukocyte function-associated antigen (LFA-1, CD11a/CD18) (Simmons, Makgoba & Seed 1988) and macrophage-1 antigen (Mac-1, CD11b/CD18) (Diamond et al. 1993). ICAM-1 is expressed on basal vascular endothelial, macrophage and lymphocyte cells, and its cell surface expression is increased by proinflammatory cytokines such as IL-1 and tumour necrosis factor-α (TNF-α) (Carlos & Harlan 1994). VCAM-1 is expressed on endothelial cells at very low levels basally, with its expression upregulated in response to the same proinflammatory stimuli as ICAM-1 (Vonderheide et al. 1994, Chen & Massague 2012). Thus, during atherogenic states, leukocytes first adhere to the endothelium, and then are firmly attached at which time ICAM-1 and VCAM-1 are expressed, mediating the progression to firm attachment. As the adherence progresses to firm attachment and finally migration through the endothelium, other members of the immunoglobin family are expressed on the surface of leukocytes and the endothelium (Figure 7). This process is central to the progression of atherosclerosis and occurs predominantly during a state of endothelial dysfunction (Deanfield et al. 2005). The
process of TG induced immune activation and inflammation can start with the migration of serum and sub-endothelial monocytes attracted by chemotaxic chemicals produced from activated macrophages such as monocyte chemoattractant protein-1 (MCP-1), a central event in an atherogenic state (Kusano et al. 2004). Migration is often initiated by the activation of the monocyte toll-like receptor pathway after contact with TG TRLs and their remnants in the plasma. Subsequently, CD36 internalises lipids as a direct result of toll-like receptor activation (Silverstein & Febbraio 2000). This results in activation of a signalling cascade via the increased activation of NF-κB that generates pro-inflammatory cytokines such as TNF-α and interleukin-6 (IL-6). Following the penetration of the sub-endothelium by TRLs/TRL remnants, many may be retained, possibly through proteoglycan complex formation with apo-B (Tabas, Williams & Boren 2007, Tabas 1999). LDL particles also cross the endothelium from the blood in increased numbers during an atherogenic state and again many are retained via a similar method as TRLs (Tabas, Williams & Boren 2007, Tabas 1999). During this retention, LDL particles appear to be particularly susceptible to modification by oxidising agents such as ROS and reactive nitrogen species (RNS) (Shimano et al.)
1991). As discussed in the previous section, ROS production is increased after a
HFM (Jagla & Schrezenmeir 2001). The degree of lipoprotein modification depends
on the size, charge and composition of the lipoprotein. Small, dense, TG rich and
cholesterol depleted LDL particles with reduced positive charge are more prone to
modification (Chang et al. 2001) and are known collectively as oxLDL (Hansson,
2005). oxLDL does not activate LDL receptors but scavenger receptors such as
cluster of differentiation 36 (CD36) on macrophages bind and internalise oxLDL to
remove the particle from the vessel wall in an apparent attempt to minimize the
proatherogenic effects on the local vascular tissue (Zaman et al. 2000).
Macrophages then become burdened with TG and cholesterol, escalating the local
atherogenic state, rather than reducing it and forming foam cells. This escalation
occurs via TG induced activation of inflammatory pathways such as toll-like
receptors (Witztum & Steinberg 2001, Feingold et al. 2012). This can further
activate monocytes and macrophages and cause apoptosis and lead to further
foam cell formation (Haddy et al. 2003). Overall the inflammatory response to a
HFM, particularly in response to TG contributes to the development of an
atherogenic state. Oxidative stress also plays a key role in this process and will be
explored further in the next section.

**Basal State**

![Figure 7 Diagram of leukocyte adhesion and migration to the sub endothelium](image)

*Illustration of leukocyte activation, adhesion and migration to the sub-endothelium in response to an inflammatory stimulus. These stages are mediated, respectively, by selectin expression, intracellular adhesion molecules – 1 (ICAM-1) and vascular adhesion molecule -1 (VCAM-1) (based on the work of Libby 2002, Yang, Furie & Furie 1999, Elices et al. 1990, Guray et al. 2004).*

1.15. **Oxidative stress**

Oxidative stress is defined as an imbalance in the production and removal of free radicals and other reactive species, including, RNS and ROS and the damage
resulting from their interaction with local bio-molecules, such as NO, and cells.

Although, a certain level of ROS production is tolerated and perhaps even necessary during/for normal homeostasis (Ristow & Schmeisser 2011), once production exceeds antioxidant defences sufficiently it can lead to damaged or dysfunctional cells (Styskal et al. 2012).

Free radicals are atoms or molecules with one or more unpaired electrons (which are highly reactive), and are products of many different processes. Some of the particularly problematic free radicals in the postprandial environment are ROS, particularly superoxide (O$_2^-$). Superoxide is a by-product of mitochondrial metabolism, although there are other sources, and often results from a ‘leak’ in the electron transport chain from NADH dehydrogenase (complex I) and ubiquinone-cytochrome b-c1 region (complex III) (Forstermann 2010). Steady state concentrations of O$_2^-$ within the mitochondrion are $\sim$1x10$^{-4}$ µM, whereas O$_2$ concentration ranges from $\sim$0.08-.57 µM, i.e. <0.2% of mitochondrial O$_2$ is converted to O$_2^-$ during steady state laboratory conditions (Turrens 2003, Gnaiger et al. 1998). O$_2^-$ production from the mitochondria is upregulated during
postprandial lipaemia and this may be due to an increase in mitochondrial flux resulting from an increased cellular uptake of FA (Brownlee 2005). Other sources of O$_2^-$ include NADPH oxidase –which synthesises O$_2^-$ in activated monocytes and granulocytes- and also eNOS (Forstermann 2010).

A state of oxidative stress often culminates in an imbalance of NO and O$_2^-$, which can ultimately lead to endothelial dysfunction (reviewed by (Forstermann 2010) as NO acts as a quencher to O$_2^-$ with the reaction forming peroxynitrite (ONOO$^-$). Peryoxynitrite can cause eNOS uncoupling resulting in a lower production rate of NO and increased production of O$_2^-$ (Pritchard et al. 1995). In this case O$_2^-$ is generated from the oxygenase domain by dissociation of the ferrous-dioxygen complex (Vasquez-Vivar et al. 1998). Additionally, the endothelial cells themselves may contribute to ROS production directly resulting from hyperlipidaemia as remnant lipoproteins have been demonstrated to increase endothelial intracellular ROS production, mainly via NADPH oxidase (Singh & Jialal 2006). However, in endothelial cells there is a far lower volume of O$_2^-$ produced in comparison to the immune cells (Li 2003). In spite of this lower volume of O$_2^-$, both vascular and
monocyte/macrophage NADPH oxidase \( \text{O}_2^- \) production play critical roles in atherogenesis (Vendrov et al. 2007).

Oxidative stress is important in the atherosclerotic process as leukocytes and endothelial cells activated by TG can produce both inflammatory cytokines and ROS escalating the inflammatory and atherogenic state. This process is a series of linked, often simultaneous events. In support of this link, impairment of endothelial dysfunction can be prevented when antioxidant vitamins (vitamin C and E) are co-ingested with a HFM in healthy subjects (Anderson et al. 2006, Vogel, Corretti & Plotnick 1997). The protective effect of vitamin C ingestion on endothelial function and oxidative stress was also observed in patients with type 2 diabetes after a HFM (Evans et al. 2003). Thus, there is a clear link between oxidative stress, endothelial dysfunction and postprandial lipaemia. From all of the aforementioned information it is clear that atherosclerosis is a multifactorial disease with a complex pathology, the mechanisms of which complement and propagate each other, often in the presence of postprandial lipaemia and the atherogenic lipoprotein phenotype. On top of pharmaceutical interventions to reduce the risk of cardiovascular disease it has been
known for many years that regular physical exercise is of clear benefit to one’s health, particularly the health of the cardiovascular system.

1.16. Benefits of physical activity and current guidelines

1.16.1. Evidence for physical activity benefits on general health

As mentioned, regular physical exercise is known to provide a range of health benefits, and the absence of physical activity can be detrimental to health. Early evidence of the protective effect of exercise on CVD was shown in a study, which demonstrated that males (N~31,000, age 35-64) in more physically active jobs, such as bus conductors compared to bus drivers, were less likely (2.3% compared to 0.49% rate of CVD, respectively) to develop CVD before the age of 70 (Morris et al. 1953). Regular physical activity is associated with reduced risk of CHD, obesity, type 2 diabetes and other chronic diseases (Blair et al. 1989, Sandvik et al. 1993, Slattery & Jacobs 1988, Blair et al. 1996, Blair et al. 1995, Paffenbarger et al. 1986, Paffenbarger et al. 1993). Looking at physical inactivity, it is estimated that ill-health as a result of physical inactivity costs the UK health service more than 1.06 billion per year (Allender et al. 2007). This study (Allender et al. 2007) used WHO
collected statistics to assess physical activity and mortality in the UK. When the 2003-2004 mortality figures were assessed they found that 287,206 deaths out of 484,367 in the UK occurred due to diseases which were linked to physical inactivity, while ~35,429 deaths were estimated to be directly attributable to physical inactivity, with 64% of these deaths due to CHD (Department of Health 2004, O'Donovan et al. 2010).

The Aerobics Center Longitudinal Study has been responsible for much of the pioneering research on fitness and health. Within a study in 1989 10,244 men and 3120 women had cardiorespiratory physical fitness assessed by a maximal treadmill exercise test and a follow up of 110, 482 person-years of observation. There were 240 deaths in men and 43 deaths in women. Adjusted for other risk factors such as age, smoking habit, cholesterol level, systolic blood pressure, fasting blood glucose level, parental history of CHD, and follow-up interval, low physical fitness was an independent risk factor for all-cause mortality, combined site cancer and CVD (Blair et al. 1989). In other work, physical fitness has been shown to be highly associated with high levels of physical activity (Stofan et al. 1998).
Another study from the same centre demonstrated the relationship between physical activity, fitness and CVD by assessing 13,444 men and 3972 women from 20 to 87 years old who underwent at least one comprehensive medical test (blood pressure, maximal oxygen consumption (\(\bar{V}O_{2\text{max}}\)), cholesterol, LDL-C, HDL-C, skin-fold callipers, BMI). Leisure time activity was assessed by an activity questionnaire with a 3 month call-back. The findings of this study were that leisure time activity energy expenditure (EE) of 7-22 kcal/kg/week for men or 7-21 kcal/kg/week for women was associated with a moderate to high level of cardiorespiratory fitness as assessed by maximal treadmill exercise test. Further studies from this centre under the ‘Behavioural Risk Factor Surveillance Study’ back up this relationship between increased physical activity, increased cardiorespiratory fitness and reduced all-cause mortality which, is primarily due to lowered rates of CVD and cancer (Blair et al. 1989, Caspersen, Merritt 1995, American College of Sports Medicine 2014). A meta-analysis by Nocon et al. (2008) supports the relationship between high physical activity and reduced CVD rate and all-cause mortality rate. This meta-analysis assessed 33 studies with 883372 participants. Follow-up ranged from 4 years to over 20 years. Concerning cardiovascular mortality, high physical activity
levels were associated with a CVD risk reduction of 35%, while all-cause mortality was reduced by 33%.

There also appears to be a dose-dependent nature of physical activity, whereby increased levels of physical activity confer a greater protective effect on health. For example, a study of over 250,000 middle-aged adults found that CVD risk and all-cause mortality risk were reduced by ~40% in those who met either of the ‘conventional’ recommendations (at least 30 minutes of moderate-intensity exercise on ~5 days a week or at least 20 min of vigorous-intensity activity 3 times per week – discussed in detail in section 1.16.2). However, this risk was reduced by 50% when an individual met both of the exercise recommendations (Leitzmann et al. 2007). In terms of frequency of exercise, although one or two bouts of vigorous exercise a week can reduce the risk of chronic diseases and premature death (Leitzmann et al. 2007), there appear to be additional benefits to exercising more regularly. Firstly, exercising more regularly often reduces physical inactivity which is known to increase the risk of obesity and all-cause mortality (Stamatakis, Hirani & Rennie 2009, Katzmarzyk et al. 2009) and secondly single bouts of aerobic activity

As little as 3-10 minute bouts of moderate or vigorous intensity exercise are beneficial, so long as they are accumulated throughout the day to equate to ~30 mins (Miyashita & Tokuyama 2008, Altena et al. 2006, Altena et al. 2004, Murphy, Blair & Murtagh 2009, Strath et al. 2008). Lastly, the intensity of physical activity seems to confer a dose-dependent protection against CVD and other chronic diseases. Exercise intensity over 6 metabolic equivalents (METs) is associated with a lower risk of CVD than physical activities of 3-6 METs, especially in men (Blair 1989).

As stated earlier, physical activity is known to reduce the risk of CVD primarily through the reduction of other known modifiable CVD risk factors, (Sofi et al. 2008, Fagard & Cornelissen 2007, Kelley, Kelley & Tran 2005, Schuler, Adams & Goto 2013) and this will now be explored further. A recent meta-analysis assessed 513,472 individuals who suffered 20,666 CHD events and were followed for 4-25
years. As hypothesised by the authors, individuals who reported a high level of leisure time physical activity had lower (17%, \(P<0.0001\)) CHD rates, whereas individuals who practiced a moderate level also had reduced (12%, \(P<0.0001\)) risk (Sofi et al. 2008). Several large epidemiological studies have reported an inverse relationship between physical activity and blood pressure, while controlling for age and anthropometric characteristics (Fagard 2005). This was also supported by a meta-analysis of exercise training trials which demonstrated that exercise training induced significant reductions in resting and daytime ambulatory blood pressure of, respectively, 3.0/2.4 mmHg \((P<0.001)\) and 3.3/3.5 mmHg \((P<0.01)\) (Fagard & Cornelissen 2007).

Due to this clear evidence linking activity with reductions in disease rates several governmental and non-governmental bodies have generated recommendations for the amount and form of activity that individuals should participate in.
1.16.2. **Activity guidelines, adherence and reported barriers**

The UK government has set out guidelines for the amount, intensity and volume of exercise for various populations in the UK (Department of Health, 2011). These guidelines are based on the evidence presented above amongst other studies and the recommended physical activity for overall health and protection against CVD for previously active adults is to:

- engage in 150 mins or more of moderate-intensity aerobic exercise per week in bouts of at least 10 minutes.
- Or 75 mins or more of vigorous-intensity aerobic activity per week.
- Or an equivalent combination of moderate and vigorous aerobic activities.
- Perform resistance training of 8–10 sets of 8–12 repetitions on two or more days each week (the evidence behind resistance training is beyond the scope of this thesis).
- Minimise sedentary time.
These guidelines are similar to those from other contemporary organisations (WHO 2014, Haskell et al. 2007, Department of Health 2011, O'Donovan et al. 2010).

Despite the wealth of evidence supporting the health benefits of exercise and the protective effects against CVD, the numbers of people meeting these recommendations in the UK is low. In England, only 29% of women and 39% of men reported participating in sufficient activity (Health and Social Care Information Centre, Lifestyle Statistics 2008), while only 38% of adults reported meeting the recommended levels of activity in Scotland (Scottish Government 2006), although this increased to 62% in 2012 (Scottish Government 2012). Self-reported measures are often inaccurate so a sub-section of adults (in the previously mentioned survey of English subjects) wore an accelerometer for a week following the survey. According to this data only 6% of men and 4% of women met the Chief Medical Officer’s recommendations for physical activity (Health and Social Care Information Centre, Lifestyle Statistics 2008). The most common barriers, stated by the working age population, were time related and refer to work commitments, lack of leisure time and caring responsibilities (Trost et al. 2002, Health and Social Care
Information Centre, Lifestyle Statistics 2007). In the Scottish Government report on Sport, Exercise and Physical Activity: Public Participation, Barriers and Attitudes from 2006, similarly to the aforementioned studies, the major barriers preventing adults from doing more physical activity in this investigation were time related, with 45% of men and 34% of women reporting work commitments and 38% of men and 37% of women reporting a lack of leisure time as barriers to exercise. Furthermore, caring responsibilities were cited by 25% of women and 13% of men as a barrier to performing more exercise. This study (Scottish Government 2006) also reported that two thirds of adults surveyed who wanted to exercise more cited lack of time as one of the main barriers to exercise. Furthermore, the change most wanted by respondents was to be able to fit activity in around their usual routine (Scottish Government 2006). Surprisingly, 94% of participants did not know the current recommendations for physical activity (69% stated a level of intensity and time below the current recommendations) in a survey of English adults (Health and Social Care Information Centre, Lifestyle Statistics 2007). Furthermore, in the same survey approximately two thirds of the surveyed subjects reported that they wanted to do more physical activity.
1.16.3. High intensity interval exercise and modification of cardiovascular disease risk factors

Given that a major reason cited for not meeting the recommended exercise levels was a lack of time, and adults reported being more likely to exercise if they were able to fit it in around their normal routine (Scottish Government 2006), strategies have been identified to develop exercise protocols which take less time, but elicit similar or greater health benefits compared to traditional exercise recommendations.

One of these is high intensity interval training (HIIT). This is any physical exercise that contains brief, intermittent bursts of vigorous activity, interspersed by periods of rest or low-intensity exercise (Gibala et al. 2012). There appear to be two distinct definitions of HIIT in the literature: shorter, higher intensity exercise such as the Wingate test i.e. 30 seconds of maximal effort followed by 4 mins rest with 4/5 repetitions (Freese, Gist & Cureton 2013, Little et al. 2011a, Little et al. 2011b, Little et al. 2010, Gibala et al. 2009, Tabata et al. 1996, Burgomaster et al. 2008, Burgomaster et al. 2005) and also longer duration ‘HIIT’ with longer intervals of around 4 mins at sub-maximal intensity with a shorter rest period and ~4 repetitions.
(Tyldum et al. 2009, Tjonna et al. 2013, Rognmo et al. 2012, Tjonna et al. 2008, Wisloff et al. 2007). For the purposes of this thesis, the sub-maximal HIIT or acute exercise (as opposed to training) will be defined as aerobic HIIT/HIIE.

Aerobic HIIT is well established as an effective alternative to traditional long duration, moderate intensity endurance training (ET), eliciting similar or even greater training effects in improving aerobic capacity, improving endothelial function, improving insulin sensitivity, lowering blood pressure, promoting skeletal muscle biogenesis, lowering blood glucose and increasing peak O$_2$ consumption (Tjonna et al. 2008, Wisloff et al. 2007, Hwang, Wu & Chou 2011). The findings of studies investigating the benefits of HIIT and aerobic HIIT are summarised in Table 4. Indeed, Hwang Wu & Chou (2011) conducted a meta-analysis and found that 6 randomised control trials with 153 participants demonstrated that aerobic HIIT is superior to moderate intensity training in terms of improving $\dot{V}O_2$max, and has a similar effect as moderate intensity exercise on most metabolic factors, including a trend (-0.4 mmol/L; 95% confidence interval (CI), -0.9 to 0.2, $P = 0.18$) for being more effective at reducing fasting glucose levels (Hwang, Wu & Chou 2011).
Aerobic HIIT may also be more enjoyable for CHD patients as 20 patients with stable CHD were assigned to either HIIE or isocaloric moderate intensity exercise. All participants preferred HIIE mainly because the perceived exertion measured by the Borg scale was lower (P<0.05) (Guiraud et al. 2011). Another study (Bartlett et al. 2011) also found that aerobic HIIE was more enjoyable for healthy, active men (n=8). In this study, these subjects performed either (6 × 3 min at 90% \( \dot{V}O_{2\text{max}} \) interspersed with 6 × 3 min active recovery at 50% \( \dot{V}O_{2\text{max}} \) with a 7-min warm-up and cool down at 70% \( \dot{V}O_{2\text{max}} \)) or 50 min moderate-intensity continuous running at 70% \( \dot{V}O_{2\text{max}} \). Ratings of perceived enjoyment after exercise were higher (P < 0.05) following interval running compared with continuous running (88 ± 6 vs. 61 ± 12) despite higher (P < 0.05) ratings of perceived exertion (14 ± 1 vs. 13 ± 1) (Bartlett et al. 2011).

Specifically relating to the postprandial environment, a recent study using 8 healthy males (42±4 years old) participated in an acute, randomised control cross-over study, performing either control, aerobic HIIE or moderate intensity exercise. Subjects then consumed a HFM for breakfast the day after exercise. Aerobic HIIE
acutely improve flow mediated dilation following the high-fat breakfast and improved antioxidant status, both to a greater extent than moderate intensity exercise, but did not affect postprandial TG levels (Tyldum et al. 2009) (shown in Table 4).
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<th>Rest duration</th>
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<tr>
<td>Hazell et al. 2014</td>
<td>15 F Active, (Healthy, young)</td>
<td>3</td>
<td>6</td>
<td>4-6</td>
<td>All-out</td>
<td>30 sec</td>
<td>4 min</td>
<td>Fat mass↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Waist circumference↓</td>
</tr>
<tr>
<td>MacDougall et al. 1998</td>
<td>12 M</td>
<td>3</td>
<td>7</td>
<td>4-10</td>
<td>All-out</td>
<td>30 sec</td>
<td>2.5-4 min</td>
<td>PFK↑</td>
</tr>
<tr>
<td></td>
<td>(Healthy, young)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CS↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VO2max↑</td>
</tr>
<tr>
<td>Parra et al. 2000</td>
<td>5 M</td>
<td>2</td>
<td>6</td>
<td>4-7</td>
<td>All-out</td>
<td>15-30 sec</td>
<td>45 sec-12 min</td>
<td>PFK↑</td>
</tr>
<tr>
<td></td>
<td>(Healthy, young)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CS↑</td>
</tr>
<tr>
<td>Rakowchuk et al. 2008</td>
<td>5/5 M/F</td>
<td>3</td>
<td>6</td>
<td>4-6</td>
<td>All-out</td>
<td>30 sec</td>
<td>4.5 min</td>
<td>endothelial function↑</td>
</tr>
<tr>
<td></td>
<td>(Healthy, young)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rodas et al. 2000</td>
<td>5 M</td>
<td>2</td>
<td>6</td>
<td>4-7</td>
<td>All-out</td>
<td>15-30 sec</td>
<td>45 sec-12 min</td>
<td>PCK↑</td>
</tr>
<tr>
<td></td>
<td>(Healthy, young)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SkM Glycogen↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CK↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PFK↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CS↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VO2max↑</td>
</tr>
<tr>
<td>Tabata et al. 1996</td>
<td>7 M</td>
<td>5</td>
<td>6</td>
<td>7-8</td>
<td>170% VO2max</td>
<td>20 sec</td>
<td>10 sec</td>
<td>VO2max↑</td>
</tr>
<tr>
<td></td>
<td>(Healthy, young)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Anaerobic work capacity↑</td>
</tr>
<tr>
<td>Study</td>
<td>N (subjects)</td>
<td>Frequency (d/wk)</td>
<td>Weeks</td>
<td>Reps</td>
<td>Intensity</td>
<td>Work duration</td>
<td>Rest duration</td>
<td>Results</td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------------</td>
<td>------------------</td>
<td>-------</td>
<td>------</td>
<td>------------------</td>
<td>---------------</td>
<td>---------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>Whyte et al. 2010</td>
<td>10 M (Overweight /Obese)</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>All-out</td>
<td>30 sec</td>
<td>4.5 min</td>
<td>ISI ↑ Lipid Oxidation ↑ Systolic BP ↓ CHO ↓</td>
</tr>
<tr>
<td>Whyte et al. 2013</td>
<td>10 M (Overweight /Obese)</td>
<td>acute</td>
<td>acute</td>
<td>4</td>
<td>All-out</td>
<td>30 sec</td>
<td>4.5 min</td>
<td>Lipid Oxidation ↑ CHO ↓</td>
</tr>
</tbody>
</table>

**Aerobic HIIT**

<table>
<thead>
<tr>
<th>Study</th>
<th>N (subjects)</th>
<th>Frequency (d/wk)</th>
<th>Weeks</th>
<th>Reps</th>
<th>Intensity</th>
<th>Work duration</th>
<th>Rest duration</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burke et al. 1994</td>
<td>10 F (Healthy, young)</td>
<td>4</td>
<td>7</td>
<td>NR</td>
<td>85-98% VO2max</td>
<td>30-120 sec</td>
<td>30-120 sec</td>
<td>VO2max ↑ Tlac ↑</td>
</tr>
<tr>
<td>Keith et al. 1992</td>
<td>7 M (Healthy, young)</td>
<td>2-4</td>
<td>8</td>
<td>2</td>
<td>Tlac + 30%</td>
<td>7.5 min</td>
<td>30 min</td>
<td>VO2max ↑ CS ↑ Tlac ↑ Systolic/Diastolic BP ↓ Body Fat %↓ Total-C ↓ OX-LDL ↓ LDL-C ↓ Fasting Glucose ↓ FMD ↑ Total antioxidant status ↑ PP Glucose ↓ PP TG ↓ PP HDL-C ↓</td>
</tr>
<tr>
<td>Tjonna et al. 2013</td>
<td>11 M (overweight, healthy)</td>
<td>3</td>
<td>10</td>
<td>4</td>
<td>90/95% peak HR</td>
<td>4 mins</td>
<td>3 mins</td>
<td>Total-C ↓ OX-LDL ↓ LDL-C ↓ Fasting Glucose ↓</td>
</tr>
<tr>
<td>Tyldum et al. 2009</td>
<td>8 M (Healthy)</td>
<td>acute</td>
<td>acute</td>
<td>4</td>
<td>90/95% peak HR</td>
<td>4 mins</td>
<td>3 mins</td>
<td>VO2max ↑ Endothelial function ↑ PGC-1α ↑ OX-LDL ↓ Antioxidant Status ↑ SR Ca2+ uptake ↑</td>
</tr>
<tr>
<td>Wisloff et al. 2007</td>
<td>7/2 M/F (post MI)</td>
<td>3</td>
<td>12</td>
<td>4</td>
<td>90/95% peak HR</td>
<td>4 mins</td>
<td>3 mins</td>
<td></td>
</tr>
</tbody>
</table>

**Summary evidence table of a selection of studies showing physiological changes after acute or chronic high intensity interval exercise (HIIE) or aerobic HIIE. Numbers (N) are displayed with subject information in brackets. Frequency of exercise bouts is displayed with days per week in brackets (d/wk). Repetitions (Reps). Work duration of the exercise protocol**
is displayed with the rest duration between work intervals. Results are measurable physiological outcomes. Changes are significant ($P<0.05$). Citrate Synthase (CS), Creatine Kinase (CK) lactate threshold (TLac), phosphocreatine (PCr), Skeletal muscle glycogen (SkM glycogen), phosphofructo kinase (PFK), lactate dehydrogenase (LDH), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), Pyruvate Dehydrogenase E1α (PDH E1α), Time trial (TT), Cytochondrome C Oxidase (COX), β-hydroxyacyl CoA dehydrogenase (βHDC), Carbohydrate (CHO), insulin sensitivity index (ISI), Flow-mediated dilation (FMD), Post-prandial (PP), high-density lipoprotein cholesterol (HDL-C), oxidised low-density lipoprotein (OX-LDL), sarcoplasmic reticulum calcium 2+ uptake (SR Ca2+ uptake), blood pressure (BP), total cholesterol (Total-C), low density lipoprotein cholesterol (LDL-C).

The most common exercise protocol used for HIIT is the ‘Wingate test’ which involves 30s of maximal cycling, on a friction braked cycle ergometer, at a resistance of 7.5% body mass. Subjects normally perform 4-6 bouts separated by 4 mins of recovery. As little as six sessions of this protocol over two weeks can increase skeletal muscle oxidative enzyme maximal capacity in healthy subjects [pyruvate dehydrogenase E1 (PDH E1), citrate synthase (CS) and peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC1-α)] in a similar manner to traditional ET despite large differences in training volume and time commitment (~90% and ~67% lower, respectively in HIIT) (Burgomaster et al. 2008) (shown in Table 4). For example, 16 active men (21±1 years old) were assigned to
either HIIT (Wingate protocol) or moderate intensity ET (90-120 mins) protocols and performed 6 sessions over 14 days. Training improved cycling time trial performance in both groups over 50 and 750 kJ time trials. Training also improved muscle oxidative capacity as reflected by an increase in the maximal activity of cytochrome c oxidase (COX). Furthermore, training in both groups induced increases in muscle buffering capacity and glycogen content (Gibala et al. 2006) shown in Table 4. This study, therefore, demonstrates that HIIT can induce training adaptions in a similar, but more time-efficient, manner to ET. As well as increased skeletal muscle oxidative capacity, other adaptations have been shown which were once thought to be the specific domain of ET. Specifically, HIIT has been found to reduce the rate of glycogen utilization and lactate production during exercise, increase capacity for whole-body and skeletal muscle lipid oxidation, enhance peripheral vascular structure and function, increase $\dot{V}O_{2\text{max}}$, and increase performance in time-to-exhaustion tests (Gibala et al. 2006), (shown in Table 4). A study (Burgomaster et al. 2008) which exemplifies this used 10 healthy young males and females, who carried out 3 Wingate sessions per week over the course of 6 weeks compared to an ET programme (N=10) that consisted of 40-60 min of
continuous cycling 5 times per week. Despite the much lower time commitment and lower training volume in HIIT, both protocols elicited similar increases in mitochondrial markers for skeletal muscle carbohydrate oxidation (PDH E1α) and lipid oxidation (3-hydroxyacyl CoA dehydrogenase maximal activity) and protein content of PGC-1α. Glycogen and phosphocreatine utilisation during exercise were reduced after both training protocols, and calculated rates of whole-body carbohydrate oxidation and lipid oxidation were decreased and increased, respectively to a similar extent during both protocols (Table 4). Additionally, HIIT has been shown to improve health markers in overweight/obese sedentary subjects such as improved insulin sensitivity, decreased systolic blood pressure and resting carbohydrate oxidation (Whyte, Gill & Cathcart 2010) (shown in Table 4).
1.16.4. Potential risks associated with high intensity interval training

HIIT may not be immediately suitable for all populations, for example the American College of Sports Medicine (ASCM) (Kravitz 2014) advises that persons who have been living rather sedentary lifestyles or periods of physical inactivity may have an increased coronary disease risk to high intensity exercise. Family history (of CVD), cigarette smoking, hypertension, diabetes (or pre-diabetes), abnormal cholesterol levels and obesity may also increase this risk. However, there is little evidence suggesting HIIE directly provokes MI. Studies have been carried out to assess the safety and effectiveness of aerobic HIIT in different populations. One such study (Wisloff et al. 2007) demonstrated that aerobic HIIT seems to be particularly effective in promoting recovery for post MI patients compared to other forms of exercise training as it reversed left ventricle remodelling, improved aerobic capacity, endothelial function and mitochondrial function in post MI patients to a greater extent than ET after a 12 week program (Wisloff et al. 2007) (Table 4). Aerobic HIIT has been found to be safe and effective as 4846 patients with coronary heart disease engaged in either aerobic HIIE or moderate intensity exercise. The aerobic HIIE consisted of a warm-up for a minimum of 10 minutes at 60% to 70% of
HRpeak before working four 4-minute intervals at 85% to 95% of HRpeak. Each interval was separated by active pauses at 50% to 70% of HRpeak. Whereas moderate intensity consisted of an hour of continuous work, with intensity ≤70% of HRpeak. Both high- and moderate-intensity exercise sessions were conducted throughout a rehabilitation period containing, on average, 37 exercise sessions.

Overall, the incidences included 1 cardiac arrest with fatal outcome during moderate-intensity exercise and 2 nonfatal cardiac arrests during high-intensity exercise. Adverse events related to exercise training were defined as cardiac arrest or acute MI during exercise or within the first hour afterward. These results indicate that both types of exercise training are associated with low event rates (Rognmo et al. 2012). Nevertheless, in the absence of evidence supporting HIIE (rather than aerobic HIIE) as a safe exercise for high-risk groups medical clearance from a physician may be an appropriate safety measure; additionally achieving a foundation level of fitness before embarking on a vigorous exercise routine that includes HIIT may be wise. This foundation level of fitness is considered being able to sustain 3-5 times a week of 20-60 min sessions at a ‘somewhat hard’ intensity for several weeks (Kravitz 2014). However, HIIT/HIIE is still a useful tool for suitable
populations, particularly as a preventative treatment approach.

1.16.5. Exercise, cardiovascular disease and lipids

Regarding all forms of physical activity, a putative mechanism behind physical activity's ability to reduce the risk of CVD (section 1.16.1) is an alteration of lipid metabolism. For example, in a meta-analysis (Kelley, Kelley & Tran 2005) of exercise training studies of more than 8 weeks in adults >50 years old (1427 subjects: 806 exercise, 621 control), exercise training significantly reduced fasting total cholesterol/HDL-C (P<0.001, -0.8±0.2 mmol/l) and increased HDL-C (P=0.01, 0.06±0.03 mmol/l). An epidemiological study found that physically active (assessed by questionnaire and defined as 2-3 sessions of exercise outside of school hours per week) adolescents (1869 subjects) had increased HDL-C concentration and reduced postprandial TG concentrations compared to lesser active counterparts (Rangul et al. 2012). Additionally, regular exercise has been shown to reduce fasting total TG and VLDL-TG concentrations, increase HDL-C and increase LDL particle size in adult men and women (Kraus et al. 2002, Altena et al. 2006). Further improvements in lipid metabolism (i.e. decreased VLDL secretion and increased
catabolism of TG in VLDL) which could provide protection against CVD, result from exercises’ ability to improve insulin sensitivity. As improved insulin sensitivity reduces the secretion and increases the catabolism of VLDL-TG, this can reduce the atherogenic lipoprotein phenotype (Gill et al. 2002b, Houmard et al. 2004, Alam et al. 2004). On top of these effects of chronic exercise acute exercise also has a beneficial acute effect on postprandial TG and this will be discussed next.

1.17. Exercise and postprandial lipaemia

To summarise the points that have been discussed so far, a significant amount of time is spent in the postprandial state by individuals following a western diet. This often leads to longer periods of elevated TG concentrations. Elevated postprandial TG levels are associated with reduced HDL-C and increased LDL-C (Ford, Giles & Dietz 2002), impaired endothelial function (Vogel, Corretti & Plotnick 1997), and resultantly, increased atherosclerotic plaque formation (Zilversmit 1979). Therefore investigating the effects of a bout of exercise on postprandial TG levels would appear to be a sensible approach.

Previous studies which have assessed postprandial TG acutely after exercise were
included in a recent meta-analysis (Freese, Gist & Cureton 2013). In these studies, to differentiate between the effect on fasting and postprandial rise in TG, the incremental area under the curve (iAUC) is calculated (adjusted for fasting TG) with the total area under the curve (AUC) accounting for overall TG concentrations (i.e. not adjusted for fasting concentrations) (Equation 1).

\[
\begin{align*}
AUC &= (b - a) \left[ \frac{f(a) + f(b)}{2} \right] \\
iAUC &= (b - a) \left[ \frac{(f(a - a_1) + f(b - a_1))}{2} \right]
\end{align*}
\]

Equation 1: AUC and iAUC

Where \( b \) is the latter \( y \) value of an integral and \( a \) is the former, and \( f \) is the function of these which is approximated by dividing the graph into integrals, \( a_1 \) = the \( y \) value of the baseline.

This meta-analysis took into account 121 effects (i.e. an outcome from a specific intervention) from 76 studies (referenced beneath Table 5) which accounted for 1,365 total participants (Table 5). The inclusion criteria for this meta-analysis were:

- The dependant variable was a measure of TG response for a period of time taken after oral ingestion of a standardised meal.
• The independent variable was exercise performed before the meal and a repeated-measures design was used to limit variability.

• Postprandial response after exercise could be compared with a baseline or control measure in the absence of an exercise session.

• An effect could be expressed as a Cohen's d (Cohen's d is defined as the difference between two means divided by a standard deviation for the data).
Table 5 The effect of prior exercise on postprandial lipaemia

<table>
<thead>
<tr>
<th>Effect Moderator</th>
<th>N</th>
<th>Effects</th>
<th>Cohen's d (95%CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>iAUC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Type of Exercise</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td>574</td>
<td>54</td>
<td>-0.58 (-0.75,-0.40)*</td>
<td>0.01</td>
</tr>
<tr>
<td>HIIE</td>
<td>56</td>
<td>6</td>
<td>-1.49 (-2.03, -0.95)*</td>
<td></td>
</tr>
<tr>
<td>Resistance</td>
<td>115</td>
<td>10</td>
<td>-0.13 (-0.54,0.28)</td>
<td></td>
</tr>
<tr>
<td><strong>Timing of exercise</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.73</td>
</tr>
<tr>
<td>&lt;8 h</td>
<td>144</td>
<td>11</td>
<td>-0.32 (-0.73, 0.10)</td>
<td></td>
</tr>
<tr>
<td>8-24 h</td>
<td>592</td>
<td>58</td>
<td>-0.66 (-0.85, -0.48) *</td>
<td></td>
</tr>
<tr>
<td>&gt;24 h</td>
<td>9</td>
<td>1</td>
<td>0.56 (-0.85, 1.97)</td>
<td></td>
</tr>
<tr>
<td><strong>AUC</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Type of Exercise</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td>1,073</td>
<td>95</td>
<td>-0.59 (-0.7, -0.48)*</td>
<td></td>
</tr>
<tr>
<td>HIIE</td>
<td>91</td>
<td>8</td>
<td>-0.97 (-1.36, -0.59)*</td>
<td></td>
</tr>
<tr>
<td>Resistance</td>
<td>177</td>
<td>16</td>
<td>-0.43 (-0.7, -0.16)*</td>
<td></td>
</tr>
<tr>
<td><strong>Timing of Exercise</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.78</td>
</tr>
<tr>
<td>&lt;8 h</td>
<td>263</td>
<td>19</td>
<td>-0.44 (-0.68,-0.19)*</td>
<td></td>
</tr>
<tr>
<td>8-24 h</td>
<td>1,072</td>
<td>99</td>
<td>-0.64 (-0.75, -0.54)*</td>
<td></td>
</tr>
<tr>
<td>&gt;24 h</td>
<td>30</td>
<td>3</td>
<td>-0.15 (-0.78, 0.48)</td>
<td></td>
</tr>
</tbody>
</table>

Table adapted from met-analysis of 76 studies carried out by (Freese et al. 2013). Number of subjects (N), high intensity interval exercise (HIIE), area under the curve (AUC), incremental area under the curve (iAUC). It should be noted that this meta-analysis included studies of HIIE including work from this thesis, however at the start of this thesis HIIT/HIIE’s effect on

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Looking at moderate/aerobic intensity exercise from Table 5, we can see that the meta-analysis accounted for 574 subjects and that the effect size was a reduction of iAUC of -0.58 Cohen’s d value and a significance of P<0.05, whereas, resistance exercise had no effect postprandial TG iAUC. This meta-analysis also included studies of HIIE (including work from this thesis). At the start of this thesis HIIT/HIIE’s effect on postprandial TG had not been investigated. Several studies have, however, been published since then, and Table 5 demonstrates that HIIE reduced
(P<0.05) postprandial TG in both AUC and iAUC by -0.97 and -1.49, respectively. HIIE’s reduction of postprandial iAUC TGs was larger than moderate intensity and resistance exercise, thus HIIE may be a more effective method of attenuating postprandial TG compared to acute moderate intensity and resistance exercise. The table (5) also demonstrates that the most effective time to perform exercise in terms of reduction of iAUC postprandial TG was 8-24 hours before consumption of a HFM. The number of subjects was, however, low in the other timepoints and therefore this was not a significantly moderating variable. While assessing effects on postprandial TG total AUC, both prior aerobic/moderate intensity and resistance have a beneficial effect (both P<0.05 and -0.59, -0.43 d values, respectively). The time that exercise was performed before the meal seems to have no effect from <8 hours to >24. Overall, it is clear that moderate intensity exercise and HIIE can reduce either iAUC or AUC when performed 8-24 hours before a normal fat or a HFM.
1.18. The mechanisms underlying exercise induced attenuation of postprandial lipaemia

The mechanism behind the ability of exercise to acutely attenuate postprandial TG has not been entirely elucidated, although both increased LPL activation and decreased VLDL secretion and/or altered VLDL kinetics (i.e. decreased VLDL1 secretion) may be responsible (Plaisance & Fisher 2014, Sondergaard et al. 2014). Increased LPL activity could increase the lipolysis of TG in TRL which is taken up by peripheral tissues such as skeletal muscle or adipose tissue. A decrease in hepatic VLDL secretion would decrease the amount of VLDL-TG in circulation, thus reducing plasma TG.

Table 6 shows studies which have assessed LPL activity after moderate intensity exercise and measured HDL-C or TG concentrations after exercise, with no such studies for HIIE. Taken together these studies incorporate 153 subject results across 13 separate outcomes. Seven of these study outcomes show no change in LPL activity (in post-heparin and pre-heparin blood plasma or muscle enzyme activity/protein content) while demonstrating a positive change in either TG or HDL-C concentrations (Miyashita & Tokuyama 2008, Herd et al. 2001, Harrison et al.)
2009, Gill et al. 2003a, Katsanos et al. 2004a) (Table 6). While Ferguson et al. (1998) demonstrated increased post-heparin LPL activity and increased HDL-C (TG not measured), this observation only occurred after moderate exercise with over 1,300 kcal EE. Magkos et al. (2006) demonstrated no change in skeletal muscle protein LPL, but did observe an increase in plasma LPL (non-heparin) as measured by ELISA, in response to 2 h moderate cycling in 7 young healthy men, while also observing a decrease in VLDL and an increase in the fractional catabolic rate (FCR) of VLDL (Table 6). Lastly, (Katsanos, Grandjean & Moffatt 2004a) demonstrated increased post-heparin LPL activity after low-intensity exercise 25% \( \dot{V}O_{2\text{max}} \), while there was no change in LPL activity after moderate (65% \( \dot{V}O_{2\text{max}} \)) exercise, even though this exercise occurred alongside a reduced postprandial TG (Table 6). However due to the variety of measures used in these studies of LPL, e.g. pre-heparin, post-heparin, non-heparin and skeletal muscle enzyme activity it is hard to make definitive comparisons. Meanwhile, other studies which have assessed LPL activity without an assessment of postprandial lipid metabolism after moderate intensity exercise, using healthy young subjects, demonstrate an increase in skeletal muscle LPL activity (Greiwe, Holloszy & Semenkovich 2000), and LPL
expression (mRNA) (Catoire et al. 2014). Thus, increased LPL activity is often hypothesised to facilitate exercise induced attenuation of postprandial TG (Peddie, Rehrer & Perry 2012), however the evidence is clearly ambiguous.
Table 6 The Association between prior exercise, LPL activity and postprandial lipaemia

<table>
<thead>
<tr>
<th>Study</th>
<th>LPL change</th>
<th>N</th>
<th>Exercise</th>
<th>Cohort</th>
<th>Time of Measurement (h Post exercise)</th>
<th>EE (KCAL)</th>
<th>TG or Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferguson et al. 1998</td>
<td>PH – PH ↑ PH ↑ PH ↑ PH ↑</td>
<td>11</td>
<td>Treadmill 70% ( \dot{V}O_{2\text{max}} ) max</td>
<td>Healthy young males</td>
<td>24</td>
<td>800</td>
<td>HDL-C – 1,100 HDL-C – 1,300 HDL-C – 1,500 HDL-C ↑</td>
</tr>
<tr>
<td>Gill et al. 2003a</td>
<td>PH – PH –</td>
<td>9 and 10</td>
<td>2 h treadmill walk at 50% ( \dot{V}O_{2\text{max}} )</td>
<td>Pre-menopausal Women, Fasting and postprandial post exercise</td>
<td>18 18</td>
<td>812</td>
<td>Fasting TG ↓ Postprandial TG ↓</td>
</tr>
<tr>
<td>Harrison et al. 2012</td>
<td>Enzy. –</td>
<td>8</td>
<td>Cycle ergometer 90 mins 70% ( \dot{V}O_{2\text{max}} )</td>
<td>Active healthy young males</td>
<td>~15</td>
<td>1,251</td>
<td>Postprandial TG ↓</td>
</tr>
<tr>
<td>Herd et al. 2001</td>
<td>Enzy. –</td>
<td>8</td>
<td>Cycle ergometer 90 min 62.3% ( \dot{V}O_{2\text{max}} )</td>
<td>Active healthy young males</td>
<td>16</td>
<td>1,075</td>
<td>Postprandial TG ↓</td>
</tr>
<tr>
<td>Katsanos et al. 2004a</td>
<td>PH* ↑ PH –</td>
<td>13</td>
<td>238 mins 25% ( \dot{V}O_{2\text{max}} ) * 91 mins 65% ( \dot{V}O_{2\text{max}} ) Both treadmill</td>
<td>Active healthy young men</td>
<td>8* 8</td>
<td>1,446* 1,454</td>
<td>Postprandial TG* – Postprandial TG ↓</td>
</tr>
</tbody>
</table>

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**Summary evidence table showing studies which looked at prior exercise, LPL activity and postprandial lipaemia in non-insulin resistant human subjects.** Lipoprotein lipase (LPL), triglyceride (TG), enzyme (enzy.), post heparin (PH), pre heparin (PreH), no heparin (NH), number (N), high density lipoprotein – cholesterol (HDL-C), energy expenditure (EE).  \( \uparrow / \downarrow = \text{increase/decrease} \ (P<0.05) \), – = no change. For Katsanos et al. 2004a, * denotes results from the low intensity exercise trial (25% \( \bar{VO}_{2\text{max}} \)).

<table>
<thead>
<tr>
<th>Study</th>
<th>Protocol</th>
<th>Heparin</th>
<th>Duration</th>
<th>Interventions</th>
<th>Participants</th>
<th>Energy Expenditure</th>
<th>Postprandial TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magkos <em>et al.</em> 2006</td>
<td>Prot. –</td>
<td>NH ↑</td>
<td>7</td>
<td>2 h cycling 60% ( \bar{VO}_{2\text{max}} )</td>
<td>Active healthy young men</td>
<td>Intervals up to 17 h</td>
<td>1,980 VLDL ↓</td>
</tr>
<tr>
<td>Miyashita <em>et al.</em> 2008</td>
<td>PreH – PreH – PreH – PreH –</td>
<td>12</td>
<td>30 min 65% max HR</td>
<td>Healthy young males</td>
<td>11</td>
<td>13</td>
<td>15</td>
</tr>
</tbody>
</table>

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To further investigate the reduction in plasma postprandial TG after exercise several studies have investigated VLDL kinetics to determine whether exercise increases clearance, or decreases secretion, of VLDL or a combination of these mechanisms. Table 7 shows studies which have assessed VLDL kinetics after acute, non-resistance, exercise in subjects without insulin resistance. One of the studies in Table 7 demonstrated that VLDL-TG secretion rate is not changed after a moderate intensity cycling bout with EE of ~1,980 kcal EE, whereas the VLDL-TG clearance rate was increased (Magkos et al. 2006). A second study with 2 hr of moderate exercise (~1,130 kcal EE) supports these findings as a bolus dose of 0.1g/kg of 20% Intralipid followed immediately by an intravenous infusion of 0.1g/kg/h of 10% Intralipid for 75mins was used to demonstrate [via a previously described method (Al-Shayji et al. 2007)], that the exercise reduced plasma VLDL-TG via an increase in clearance rather than a decrease in secretion of VLDL-TG (Al-Shayji, Caslake & Gill 2012). Tsekouras et al. (2007) also demonstrated that a bout of moderate walking with ~945 kcal EE also did not induce a reduction in VLDL secretion, but again did result in an increased VLDL clearance (using a stable
isotopically labelled tracer infusion) which appeared to be the mechanism by which postprandial TG was reduced. An increase in VLDL clearance has also been shown after 45 min treadmill walking at 40% $\bar{VO}_2\text{max}$ (Morio et al. 2004) in healthy young subjects, both immediately (Morio et al. 2004) and up to 14 hours after exercise (Bellou et al. 2013a). However, conflicting evidence has been produced by Sondergaard et al. (2011) demonstrating that 90 mins of walking (~790 kcal-men,~467.1 kcal-women) suppressed VLDL-TG secretion while VLDL-TG clearance was not changed. In addition to the previous study, a decreased secretion rate of VLDL-TG was shown after 2 h walking at 60% $\bar{VO}_2\text{max}$ in sedentary young women, alongside an increased VLDL-TG clearance rate and decreased total and VLDL TG (Bellou et al. 2013b). Furthermore, 90 mins of walking at 30% $\bar{VO}_2\text{max}$ (healthy sedentary woman) or 1 h cycling at 60% of $\bar{VO}_2\text{max}$ (Healthy sedentary men) did not change VLDL clearance, secretion or VLDL-TG concentration (Magkos et al. 2006). When higher intensity exercise was used, an increase in VLDL clearance (using a stable isotopically labelled tracer infusion) is shown after exercise in studies using alternating 60% and 90% $\bar{VO}_2\text{max}$ cycling in 4 min intervals for a total of 32 min (Bellou et al. 2013a). Thus an increase in VLDL
clearance is the most common finding after moderate exercise in studies which also
demonstrated reduced VLDL-TG or TG (Magkos et al. 2006, Tsekouras et al. 2007,
Bellou et al. 2013a, Bellou et al. 2013b, Al-Shayji, Caslake & Gill 2012), although
not all studies confirm this association (Morio et al. 2004, Magkos et al. 2008,
Magkos et al. 2007, Magkos et al. 2009). Thus, increased VLDL clearance may play
a role in exercise induced attenuation of postprandial TG, however the evidence is
not clear. As stated earlier, increased VLDL clearance could be due to an increase
in LPL activity after exercise.

Alterations in VLDL kinetics are also demonstrated in the sub-fractions of VLDL, in
the previously mentioned study (Al-Shayji, Caslake & Gill, 2012) shown in Table 7.
In this study 12 overweight/obese men either walked for 2 h on a treadmill or
performed no exercise. These subjects had Intralipid infused and multiple blood
samples taken after exercise or the control rest. Exercise reduced fasting VLDL1-
TG concentration by 30% (P=0.007) and increased TG enrichment of VLDL1
particles. Furthermore, the increased clearance of VLDL1-apoB shown in this study
correlated strongly with the exercise induced changes in the compositional changes
in VLDL1 particles. Thus, this study provides some evidence that increased
clearance of VLDL1 particles may be partly mediated by a change in their
composition (Al-Shayji, Caslake & Gill, 2012), perhaps increasing their affinity for
TG lipases.
Table 7 The association between prior exercise, VLDL kinetics and postprandial lipaemia

<table>
<thead>
<tr>
<th>Study</th>
<th>VLDL-TG change</th>
<th>N</th>
<th>Exercise</th>
<th>Cohort</th>
<th>Time of Measurement (h Post exercise)</th>
<th>EE (KCAL) (approx. values)</th>
<th>TG or Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al-Shayji et al. 2012</td>
<td>↑</td>
<td>12</td>
<td>Treadmill walk for 2 h at ~50% ( \dot{V} \text{O}_{2\text{max}} )</td>
<td>Overweight/obese, age 44</td>
<td>16-18 hours</td>
<td>1,130</td>
<td>Fasting TG↓</td>
</tr>
<tr>
<td>Bellou et al. 2013a</td>
<td>↑</td>
<td>8</td>
<td>Alternating 60% and 90% of ( \dot{V} \text{O}_{2\text{max}} ) in 4 min intervals for a total of 32 min</td>
<td>Healthy sedentary men</td>
<td>14, 48</td>
<td>500</td>
<td>Fasting TG ↓</td>
</tr>
<tr>
<td>Bellou et al. 2013b</td>
<td>↑, ↓</td>
<td>11</td>
<td>2 h walking at 60% ( \dot{V} \text{O}_{2\text{max}} )</td>
<td>Healthy sedentary young women</td>
<td>Intervals up to ~18</td>
<td>604</td>
<td>VLDL-TG↓</td>
</tr>
<tr>
<td>Magkos et al. 2006</td>
<td>↑</td>
<td>7</td>
<td>2 h cycling 60% ( \dot{V} \text{O}_{2\text{max}} )</td>
<td>Active healthy young men</td>
<td>Intervals up to 17 h</td>
<td>1,980</td>
<td>VLDL↓</td>
</tr>
<tr>
<td>Magkos et al. 2007</td>
<td>–</td>
<td>7</td>
<td>1 h cycling at 60% ( \dot{V} \text{O}_{2\text{max}} )</td>
<td>Healthy sedentary young men</td>
<td>Intervals up to ~23</td>
<td>576</td>
<td>VLDL-TG –</td>
</tr>
<tr>
<td>Magkos et al. 2008</td>
<td>–</td>
<td>7</td>
<td>90 min walking at 30% ( \dot{V} \text{O}_{2\text{max}} )</td>
<td>Healthy sedentary young men</td>
<td>Intervals up to ~18</td>
<td>400</td>
<td>VLDL-TG –</td>
</tr>
<tr>
<td>Magkos et al. 2009</td>
<td>–</td>
<td>8</td>
<td>1 h cycling at 60% ( \dot{V} \text{O}_{2\text{max}} )</td>
<td>Healthy sedentary young women</td>
<td>Intervals up to ~23</td>
<td>364</td>
<td>VLDL-TG –</td>
</tr>
<tr>
<td>Morio et al. 2004</td>
<td>↑</td>
<td>8</td>
<td>45 min treadmill 40% ( \dot{V} \text{O}_{2\text{max}} )</td>
<td>4 male, 4 female, Healthy young</td>
<td>3</td>
<td>242</td>
<td>VLDL-TG –</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Study</th>
<th>Exercise Description</th>
<th>Triglycerides</th>
<th>Number</th>
<th>Energy Expenditure</th>
<th>Fractional Catabolic Rate of VLDL (FCR)</th>
<th>Hepatic Secretion Rate of VLDL (SR)</th>
<th>Intervals</th>
<th>Energy Expenditure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sondergaard et al. 2011</td>
<td>90 min of 50% VO2max cycling</td>
<td>–</td>
<td>8</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>790</td>
<td>–</td>
</tr>
<tr>
<td>Tsekouras et al. 2007</td>
<td>90 minutes at 60% VO2max</td>
<td>↑</td>
<td>10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>945</td>
<td>–</td>
</tr>
</tbody>
</table>

**Summary evidence table showing studies which looked at Prior Exercise, VLDL kinetics and postprandial Lipaemia in non-insulin resistant human subjects. Triglyceride (TG), number (N), energy expenditure (EE), fractional catabolic rate of VLDL (FCR), hepatic secretion rate of VLDL (SR), very low density lipoprotein (VLDL). ↑/↓ = increase/decrease (P<0.05), – = no change.**

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1.19. Exercise, postprandial lipaemia and energy expenditure

The EE of moderate intensity exercise has been postulated to be a major factor in determining the magnitude of postprandial TG attenuation. In support of this, Gill et al. (2002a) demonstrated that 1 hour of walking (~358 kcal) attenuated postprandial TG by 9.3%, compared to 22.8% for a 2 hour walk (~740 kcal) at the same intensity. Furthermore, a study (Tsetsonis & Hardman 1996) demonstrated that 9 healthy young normolipidemic subjects (5 men and 4 women), had a similar attenuation of postprandial TG after a HFM compared to control when they performed either a 3 hour walk at 32% $\dot{V}O_{2}\text{max}$ (~998 kcal) or a 1.5 hour walk at 63% $\dot{V}O_{2}\text{max}$ (~1022 kcal) which had a similar EE (Tsetsonis & Hardman 1996). This indicates that at moderate to low intensities of exercise the EE has a greater effect on the attenuation of postprandial TG than the intensity of exercise.

In a randomised crossover trial (Bellou et al. 2013b) shown in Table 7, eleven healthy young women performed a single exercise bout of brisk walking at 60% $\dot{V}O_{2}\text{max}$, with an EE of 492 kcal or an equivalent dietary energy restriction. The exercise trial reduced total (~14%) and VLDL TG (~30%) levels while there was no
change after the dietary restriction. In another study a 90 min walk reduced postprandial TG by ~20%, whereas isocaloric intake restriction only induced a 7% non-significant reduction when compared to a control trial (Gill & Hardman 2000) suggesting that exercise induced EE is more effective at reducing postprandial TG compared to an isocaloric dietary intake restriction, in other words it is not simply an energy deficit that is important for this effect.

However, an exercise induced energy deficit still appears to be important in attenuating postprandial TG levels. In a study (Burton et al. 2008) using 13 overweight/obese men who completed a treadmill walk for ~90 mins with an EE of ~669 kcal with and without equivalent dietary energy replacement (i.e. consumption of food with a caloric content equal to the EE of the exercise trial in addition to the control trial diet). The energy deficit created by the exercise was necessary to induce an attenuation of postprandial lipaemia as energy replacement saw similar postprandial TG to the control trial. This indicates that the EE of exercise is an important factor in attenuating postprandial TG, however the mechanism by which exercise attenuates postprandial TG is different from, and more effective than an
1.20. Aims of high intensity interval exercise studies

Although Tyldum et al. 2009 (shown in Table 5) had previously demonstrated that aerobic HIIE could be used to combat some of the negative effects of a HFM (such as reduced endothelial function and reduced oxidative defence), there is little information regarding HIIE and its postprandial effects. At the start of this Ph.D. study there were no studies specifically focusing on the potential of HIIE to attenuate the deleterious effects of a HFM. However, a study by (Freese et al. 2011) which was published during this PhD period, demonstrated that HIIE can reduce postprandial TG measured for 3 hours after a single HFM. The study used a repeated-measures crossover design using 6 men and 6 women, who participated in three 2-day trials, a control trial, and 2 trials where HIIE was performed on the evening of the first trial day - with and without replacement of the energy deficit induced by the exercise. The exercise consisted of 4 repetitions of 30-sec maximal cycling and 4 mins rest. On day 2 of the trials, subjects consumed a HFM after a 13-hour overnight fast. The meal consisted of 1.2 g fat, 0.9 g carbohydrate, and 0.4...
g protein/kg and provided ~68 kJ/kg. The postprandial area AUC (mmol·l⁻¹·3 h⁻¹) TG response was significantly lower in the exercise trial with the energy deficit (21%, P<0.05) than in control. There was also a decreased (10%, P<0.05) postprandial AUC TG response in the second HIIE trial (with energy deficit replacement) compared to the control trial, despite these 2 trials (control and HIIE with energy replacement) being isocaloric (energy balance status was the same between trials). Overall this study demonstrated that a single bout of HIIE reduces postprandial TG even when energy deficit during the exercise is replaced. What remains to be determined is whether these effects remain over a longer time period, the underlying mechanisms and whether any other physiological benefits (e.g. in reducing oxidative stress) are observed. Thus the aim of this thesis is to determine whether a session of HIIE attenuates postprandial TG and to determine the longevity of any response. A second aim is to identify potential mechanisms responsible for HIIE’s increased disposal of plasma TG. This thesis has a final aim of studying in vitro. Skeletal muscle lipid metabolism, the rationale for which will be discussed in the subsequent sections.
1.21. Skeletal muscle metabolism and insulin sensitivity

As discussed in detail previously insulin sensitivity can play a key role in the control of postprandial lipid metabolism and it is also a modifiable risk factor for CVD (Berlin & Colditz 1990). Furthermore, exercise training or increased physical activity is well known to increase insulin sensitivity (Richter et al. 1984, Richter et al. 1982, Rodnick et al. 1990) (for review see (Sakamoto & Goodyear 2002). While the mechanism behind this has not been precisely elucidated, there is substantial evidence that alterations to skeletal muscle metabolism (such as increased substrate oxidation) are central to the exercise training induced increases in insulin sensitivity, as skeletal muscle is the main depot for the uptake of glucose in response to insulin (DeFronzo & Tripathy 2009, Yki-Jarvinen et al. 1987, Wackerhage 2014).

1.22. Insulin resistance

The main feature of insulin-resistant states, including type 2 diabetes, is a reduction of glucose uptake and storage (as glycogen) in skeletal muscle in response to insulin (Roden 2004, Baron et al. 1991). Individuals with insulin resistance also
exhibit impaired fasting and postprandial fatty-acid oxidation and an inability to switch between fatty acid to glucose oxidation during hyperinsulinemia (Simoneau & Kelley 1997, Kelley & Simoneau 1994). One hypothesised mechanism underlying insulin resistance is that skeletal muscle mitochondria are dysfunctional or are reduced in content. It has been suggested that both fewer and/or less functional mitochondria reduce the ability of the cell to oxidise glucose and other nutrients such as FA, which contributes to insulin resistance (Lowell & Shulman 2005). In support of this several studies have demonstrated reduced mitochondrial content or oxidative capacity of mitochondria in skeletal muscle of patients with insulin resistance (Sivitz & Yorek 2010, Kraegen, Cooney & Turner 2008, Erion & Shulman 2010). Interestingly, exercise training improves these factors as demonstrated by studies which show increased mitochondrial density (Morgan et al. 1971, Hoppeler et al. 1973), mitochondrial function (Phielix et al. 2010) and whole-body and muscular insulin sensitivity (Hawley & Lessard 2008, Meex et al. 2010, Ryder, Chibalìn & Zierath 2001) in response to endurance training. In particular, the ability to upregulate oxidation of glucose or FA postprandially is thought to be important to preventing insulin resistance. This was shown by Phielix et al. (2012) who assessed
9 young endurance athletes and 10 young untrained subjects who underwent a clamp with either infusion of glycerol or Intralipid. Trained subjects had a ~32% higher skeletal muscle mitochondria content and ~22% higher insulin sensitivity (P<0.05). Lipid infusion reduced insulin-stimulated glucose uptake by 63% in untrained subjects (P<0.05), whereas this was reduced by 29% in trained subjects. Trained subjects also had reduced phosphorylated glycogen synthase in skeletal muscle after lipid infusion, indicating that increased glycogen storage may be partly behind this effect. This would seem logical as trained subjects are known to have increased skeletal muscle glycogen storage compared to untrained individuals (Greewe et al. 1999). This evidence indicates that trained endurance athletes maintain the ability to uptake and store glucose even with elevated plasma lipid levels, and that this effect may be linked to the well documented increase in mitochondrial capacity after endurance training (Morgan et al. 1971, Hoppeler et al. 1973, Phielix et al. 2010, Ryder, Chibalin & Zierath 2001).

As mentioned previously, although adipose tissue and liver play a major role in insulin resistance, skeletal muscle accounts for the majority of the body’s glucose
disposal (DeFronzo & Tripathy 2009, Yki-Jarvinen et al. 1987), thus study of the pathology of diabetes regularly focuses on the mechanisms behind muscular insulin resistance and this is indeed the focus of this section of the current thesis. Obesity can often result in insulin resistance and obese individuals are at an elevated risk of developing type 2 diabetes (although genetic pre-disposition and other factors play a role) (Tuomilehto et al. 2001, Kirchner et al. 2013). Part of obesity’s role in the increased risk of insulin resistance may be explained by the increased levels of stored FA in subjects with increased obesity (Goodpaster et al. 2001). Supporting this, there is a correlation between skeletal muscle intramuscular TG (IMTG) and insulin resistance in subjects with obesity (Goodpaster et al. 2001). This may be due to excess accumulation of IMTG due to saturated adipocyte storage of FA as both insulin resistance (measured by glucose clamp technique, r=0.53 with IMTG) and IMTG (measured by nuclear magnetic resonance spectroscopy (NMR)) are also associated with increased subcutaneous adipose mass (r=0.61 with insulin resistance) and increased visceral fat mass (r=0.52 with insulin resistance, r=0.62 for IMTG), as measured by duel-energy x-ray absorptiometry (DEXA) (Goodpaster et al. 1997, Forouhi et al. 1999). These associations are likely due to the limited
capacity of adipocytes within adipose tissue to store FA and demonstrate the apparent harmful effects (i.e. causing insulin resistance) of ectopic FA accumulation.

1.23. Skeletal muscle lipotoxicity

More recent research has shown that it is not only global IMTG but particular FA that have an increased potential to cause insulin resistance when stored ectopically, in particular long chain saturated fatty acids (LCSFA). One of the most potent metabolically deleterious of all FA is palmitate (C16:0), which is a saturated FA that is common in the western diet (accounting for 52-57% of saturated FA which account for ~16% of total caloric consumption (Jonnalagadda et al. 1995)). Palmitate is often elevated in the plasma (Roden et al. 2004) and skeletal muscle (measured in the phospholipid fraction) (Pan et al. 1995) of obese subjects. Looking at the physiological effects of palmitate it has been shown that short-term palmitate incubation stimulates FA oxidation in vitro in skeletal muscle cells (Hirabara et al. 2003). On the other hand exposure to high concentrations of palmitate for a prolonged period of time results in the build-up of toxic intracellular FA
intermediates: ceramide, LCA-CoA and DAG (Szendroedi, Phielix & Roden 2011), a reduction in oxidative capacity (Hulver et al. 2003), reduced lipid oxidation (Pimenta et al. 2008), insulin resistance (Kelley et al. 1999) and increased activation of stress signalling pathways such as NF-κB (Coll et al. 2008), JNK, P38 (Senn 2006) and protein kinase Cδ (PKCδ) (Szendroedi, Phielix & Roden 2011) with cells ultimately undergoing apoptosis (Hulver et al. 2003) (Figure 8). This combination of occurrences resulting from increased LCFA and increased concentrations of ceramide, LCA-CoA and DAG is often known as 'lipotoxicity'. Indeed, the build-up of fatty-acid intermediates (DAG, ceramide, LCA-CoA), may mediate the activation of stress/inflammatory pathways followed by serine phosphorylation of insulin receptor 1 (IRS1), and ultimately impaired insulin signalling (Szendroedi, Phielix & Roden 2011, Roden et al. 1996, Holland et al. 2007) (Figure 8). These, and further studies (Dresner et al. 1999), demonstrated that activation of inflammatory pathways by DAG, acyl-CoAs and ceramides can activate atypical protein kinase C (PKC) isoforms which results in serine phosphorylation and deactivation of insulin receptor 1 (IRS1). These factors were measured in healthy subjects infused with TG emulsion (1.5ml/min; Liposyn II) and heparin (0.2 IU/kg/min) for 5 hours in order to
increase plasma FFA, activate LPL and increase FA tissue uptake, thus inducing skeletal muscle lipotoxicity (Dresner et al. 1999). Since IRS1 stimulates insulin’s actions, including glucose uptake, the impairment of IRS1 mediated signalling reduces insulin sensitivity and glucose uptake (Szendroedi, Phielix & Roden 2011, Roden et al. 1996, Dresner et al. 1999). The accumulation of intracellular skeletal muscle lipids also has other negative effects such as the degradation of mitochondria and inhibition of mitochondrial biogenesis. Lipid infusion for 48 hours in seven healthy subjects decreases the expression (~0.7 fold decrease P<0.05) of PGC1α (master regulator of mitochondrial biogenesis) and other nuclear encoded mitochondrial genes such as glycine amidinotransferase (GATM) (~0.6 fold decrease P<0.05) and isocitrate dehydrogenase [NAD+]–3β (IDH3β) (~0.6 fold decrease P<0.05) (Richardson et al. 2005). Additionally, cell culture (C2C12) studies demonstrate increased mitochondrial damage (determined by cytochrome-c release from mitochondria in cytosolic fractions) in addition to increased caspase 3 activity (indicative of cell apoptosis) after 18 hours infusion with 0.8mM palmitate solution (Henique et al. 2010). Furthermore, long-term exposure to increased intracellular concentration of skeletal muscle lipids, DAG, ceramides and acyl CoAs...
appears to reduce cross-talk between PGC1α and PPARγ [upregulated during- and promotes- normal mitochondrial biogenesis (Finck 2006)] and increase cross-talk between PGC1α and peroxisome proliferator-activated receptor-α (PPARα). This is demonstrated by an increase in PPARγ protein expression (22% P<0.05) in skeletal muscle protein of obese subjects compared to healthy lean individuals (Holloway, Bonen & Spriet 2008), despite the reduced PGC1α levels often reported in obese subjects (Richardson et al. 2005). However, in obese subjects there is a positive correlation between PGC1α and PPARα protein expression (r=0.72, P=0.03), while there is not in lean healthy subjects, suggesting a discord in mitochondrial biogenesis signalling compared to healthy subjects. Furthermore, PPARγ protein expression was up-regulated in response to the decreased lipid oxidation in obese individuals, again suggesting an altered signalling response to chronic metabolic conditions (r=0.64, P<0.05). It is not fully understood how these changes relate to mitochondrial biogenesis and palmitate oxidation, although it is likely that they play a major role in obese individuals as PPARγ and PGC1α cross-talk is central in normal mitochondrial biogenesis (Finck 2006) (Figure 8). Therefore, uncovering strategies to prevent palmitate induced skeletal muscle lipotoxicity are an important
area of study.

Figure 8 Role of mitochondria in insulin resistance

Figure from Szendroedi, Phielix & Roden (2011). In the insulin-resistant state, availability of FFA is increased, which raises triglyceride storage and intracellular concentrations of lipid metabolites (DAG, ceramides, LCA-CoA). DAG and ceramides induce impairment of the insulin signalling pathway via activation of inflammatory/stress messengers (for example, PKCδ), which results in inhibitory serine phosphorylation of IRS. Glucose transport and phosphorylation is reduced. Stimulation of PGC1α and PGC1β, the major regulators of mitochondrial biogenesis and fatty acid oxidation, is induced by insulin in skeletal muscle. FFA stimulate PPARγ and PPARδ. However, stimulation of oxidative capacity, mitochondrial biogenesis and mitochondrial lipid uptake is impaired in the insulin-resistant state. Therefore, whole-body lipid oxidation is impaired in humans with obesity and insulin resistance as a result of impaired mitochondrial plasticity. It is yet unknown if insulin has direct, rapidly acting effects on mitochondrial function. These defects may reflect dysregulation of the lipid-induced PPAR–PGC1 interaction subsequent to prolonged hyperlipidaemia, which might lead to decreased lipid uptake into mitochondria, in order to compensate for lower mitochondrial content and increased lipid availability; lipid-induced uncoupling of the respiratory chain; reduced oxidation of glycolytic substrates, which uncouples fatty acid oxidation rates from TCA cycle rates; and metabolic inflexibility. Abbreviations: CPT1 (carnitine O-palmitoyltransferase 1) DAG (diacylglycerol) IRS (insulin receptor
substrate), FAT (fatty acid translocase also known as CD36); fATP (ATP flux) FFA (free fatty acids), GLUT (glucose transporter) G6P (glucose-6-phosphate), LCA-CoA (long-chain acyl-CoA) PKCδ (protein kinase Cδ), PPAR (peroxisome proliferator-activated receptor), PGC (PPAR coactivator), ROS (reactive oxygen species), TCA (tricarboxylic acid) (Szendroedi, Pfielix & Roden 2011). Image rights obtained from Nature Publishing group 2015.

As detailed it is known that increased mitochondrial oxidative capacity prevents insulin resistance in skeletal muscle tissue (van Loon, Goodpaster 2006) and this appears to transfer to the in vitro environment. Supporting this, C2C12 cell lipid oxidation was increased by using a mutant form of carnitine palmitoyltransferase 1 (CPT1), which was active but not inhibited by malonyl-CoA and so had a high level of mitochondrial FA uptake. These cells displayed protection against palmitate induced apoptosis and insulin resistance (Henique et al. 2010), highlighting the beneficial effects of increased cellular capacity to oxidise FA during lipotoxic conditions. Other studies have also demonstrated the benefit of increased oxidative capacity in dealing with lipotoxicity (for review see (Galgani, Moro & Ravussin 2008). Additionally, the reduced ability to upregulate lipid oxidation in response to a HFM is thought to be a precursor of the development of insulin resistance in genetically susceptible individuals (Heilbronn et al. 2007). The evidence from these
studies supports the hypothesis that the ability to upregulate lipid or glucose oxidation in response to high postprandial concentrations of lipid or glucose is of the upmost importance for attenuating the negative effects of a western diet in skeletal muscle. While one way to achieve this is through chronic exercise which increases mitochondrial density and capacity, it is worth considering the mechanisms underlying mitochondrial fuel selection.

1.24. Randle cycle

Mammalian mitochondrial fuel selection has long been a topic of debate. In 1963 Sir Phillip Randle and others (Randle et al. 1963) described a “glucose-fatty acid cycle” that proposed a mechanism by which glucose and FA act as competitive substrates for their oxidation in muscle and adipose tissue in mammals [for review see Frayn (2003), Sugden & Holness (2003), Sugden (2007)]. The cycle controls mitochondrial fuel selection and adapts glucose and FA cellular uptake and oxidation in response to their respective availability (Hue & Taegtmeyer 2009).
1.25. Fatty acid metabolism

To explain the Randle cycle and its mechanism of action, FA metabolism will be addressed. In the fasted state, increased lipolysis increases plasma NEFA concentration and increases the supply of lipids to tissues. In most peripheral tissues lipids then become the preferred substrate for oxidation through an inhibition of glucose oxidation (Garland, Randle & Newsholme 1963). By inhibiting glucose oxidation FA spare glycogen which is beneficial during starvation as glycogen is broken down to glucose, the preferred fuel of the brain under normal conditions (Frayn 2010). Therefore, in the fasted state, the mammalian body (excluding the brain) is predisposed to prefer lipid as a substrate for oxidation [for review (Hue, Taegtmeyer 2009]. In addition, inhibition of glucose metabolism prior to the degradation of glycolytic pyruvate to acetyl-CoA inhibits further metabolism of pyruvate and lactate, both of which are hepatic gluconeogenic precursors that increase hepatic glycogen synthesis (Garland, Newsholme & Randle 1962). The inhibition of glucose oxidation by FA has been demonstrated in several tissues including heart, liver, pancreatic β-cells and muscle (Hue & Taegtmeyer 2009, Jensen 2002).

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The cycle is not exclusive to the fasted state, and is also observed in the fed state after a HFM or during exercise, where plasma concentrations of NEFA are often increased. During these conditions, some of the spared glucose is rerouted to glycogen synthesis, which is partly responsible for the rapid resynthesis of muscle glycogen after exercise (Depre et al. 1998). The inhibition of glucose metabolism, initially induced by FA metabolism, is a graded process which results in greater levels of inhibition of 6-phosphofructo-1-kinase (PFK-1) and PDH (Hue & Taegtmeyer 2009) (illustrated in Figure 8). This occurs in the fed state in order to replenish glycogen stores that may have been depleted in the prior fasted state.

PDH is part of an enzyme complex that catalyses the conversion of pyruvate into acetyl-CoA by pyruvate decarboxylation, whereas PFK-1 phosphorylates fructose 6-phosphate (Wegener & Krause 2002). Initially the first step of inhibition of PDH is an increase in the mitochondrial ratios of acetyl-CoA/CoA and NADH/NAD+, caused by FA oxidation, which both act to transiently allosterically inhibit PDH. PDH is regulated as a whole complex of enzymes including PDH phosphatase (PDP), PDH kinase (PDK) and PDH itself, as a whole, these enzymes are referred to as the pyruvate dehydrogenase complex (PDC). PDK phosphorylates and inactivates
PDH, and PDP mediates the reverse reaction thus activating PDH. PDP is allosterically stimulated by insulin, phosphoenolpyruvic acid (PEP) and AMP, while it is competitively and allosterically inhibited by ATP, NADH and acetyl-CoA (the latter two are products of PDH). Thus, through increased allosteric inhibition and a reduction of allosteric stimulation of PDP high ratios of acetyl-CoA/CoA and NADH/NAD+ can reduce the activity of PDP, increasing the amount of PDH phosphorylation and ultimately reducing the activity of PDH (Arjunan et al. 2002). Alongside the reduced activity of PDH, an accumulation of cytosolic citrate can also occur due to the high levels of acetyl-CoA and the high capacity of CS compared to other enzymes of the Krebs cycle (Hue & Taegtmeyer 2009) (Figure 9). The higher capacity of CS can result in increased product production (i.e. citrate), while Isocitrate dehydrogenase (downstream of CS in the Krebs cycle) has a lower maximal activity rate than CS and thus a lower rate of utilization of citrate than the rate of CS citrate production. This can lead to an accumulation of intracellular citrate. Citrate in turn inhibits PFK-1, which causes an upstream increase in the accumulation of glucose-6-phosphate, and subsequent inhibition of hexokinase resulting in reduced glucose uptake (Hue & Taegtmeyer 2009, Garland, Newsholme ...
& Randle 1962). Cytosolic citrate can inhibit PFK-1’s isomer PFK-2 which normally produces fructose-2,6-bisphosphate, a potent activator of PFK-1 (Depre, Veitch & Hue 1993). Thus cytosolic citrate inhibits PFK-1 via a double inhibition of PFK-1 and its isomer PFK-2. In summary, a high conversion of FA into acetyl-CoA and citrate can result in a high rate of citrate efflux into the cytosol and inhibition of PFK-1. Acetyl-CoA build up can also inhibit PDH.
Figure 9 Pathways of enzyme modulation by citrate and other mechanism involved in intracellular regulation of FA and glucose metabolism

Abbreviations are as follows in order as they appear on the diagram from top-bottom and left-right: Glucose-6-P (glucose-6-Phosphate), Fructose-6-P (fructose-6-Phosphate), Fructose 1,6-BP (Fructose 1,6-Bi-phosphate), PC (Pyruvate Carrier), PDH (pyruvate Dehydrogenase), MCD (malonyl CoA carboxylase), ACC (Acetyl CoA Carboxylase), CD36 (cluster of differentiation 36), ACL (ATP-Citrate Lyase), LCF (Long-chain Fatty), CPT1 (Carnitine palmitoyltransferase 1) β-Ox (β-Oxidation), CS (citrate synthase), ACN (Aconitase), ICD (Isocitrate-dehydrogenase), α-KD (α-Ketoglutarate Dehydrogenase), SDS (Succonyl CoA synthetase), SDH (Succinate Dehydrogenase) FMR (Fumarase), MDH (Malate Dehydrogenase).
1.26. Glucose metabolism

Glucose metabolism can also competitively inhibit lipid metabolism and FA oxidation under certain conditions such as when plasma glucose concentration is high. McGarry et al. (1977) discovered a potential mechanism for this, involving malonyl-CoA. This can be derived from both glucose and lipid oxidation through CS production of citrate and conversion of citrate into malonyl-CoA, (Figure 9). Malonyl-CoA is formed in the cytosol by carboxylating acetyl-CoA, a reaction catalysed by the enzyme acetyl-CoA carboxylase (ACC). One molecule of acetyl-CoA joins with a molecule of bicarbonate to form malonyl-CoA, a reaction utilising a molecule of ATP (Nelson & Cox 2008).

Malonyl-CoA inhibits FA oxidation by inhibiting CPT1, the only known transporter of long chain FA (LCFA) across the mitochondrial membrane. Consequently FAs are stopped from entering the mitochondria and being oxidised (Ruderman et al. 1999). Glucose metabolism also has some self-regulating mechanisms such as the inhibition of PDK by pyruvate. In this instance, an increase in fructose-2,6-bisphosphate (synthesised from PFK-2) stimulates glycolysis, resulting in pyruvate
production. Pyruvate then acts to inhibit PDK (an inhibitor of PDH), stimulating PDH activity, increasing pyruvate entry into the Krebs cycle via conversion to acetyl-CoA and thus stimulating glucose oxidation (Hue & Taegtmeyer 2009).

As discussed earlier, citrate can act to inhibit PFK-1 which is generally the role it plays during FA induced inhibition of glucose metabolism. However, it can also play an alternative and opposite role in the Randle cycle. An excess accumulation of citrate in the mitochondria can lead to citrate efflux via the citrate carrier (CiC), which carries citrate across the mitochondrial membrane and into the cytosol (Sun et al. 2010). The mechanism by which citrates fate (i.e. conversion to malonyl-CoA or to remain as citrate) is decided in the cytosol is not fully understood. If citrate is converted to malonyl-CoA, this process begins as cytosolic citrate is converted to acetyl-CoA by ATP citrate lyase (Sun et al. 2010), with the consumption of a molecule of ATP and CoA. Acetyl-CoA can then be converted to malonyl-CoA by ACC and inhibit LCFA mitochondrial entry and subsequent oxidation. Malonyl-CoA is also the first step in de novo lipogenesis in adipose and hepatic tissue (Frayn 2010). This first stage of lipogenesis is necessary to prevent the oxidation of newly
synthesized FA during periods of cellular substrate abundance, *e.g.*, postprandially (Ruderman *et al.* 1999); thus favouring FA esterification and storage over their oxidation. However, as skeletal muscle has little or no FA synthase (FAS), the enzyme that converts malonyl-CoA into fatty acyl-CoA, the build-up of malonyl-CoA appears to have a purely regulatory function in respect to controlling the rate of LCFA transport across the mitochondrial membrane (Rasmussen, Wolfe 1999). Thus, in skeletal muscle malonyl-CoA plays a central role in the inhibition of LCFA oxidation when glucose concentrations are elevated. Cytosolic citrate can be converted to malonyl-CoA and its metabolic role is determined firstly by its rate of efflux from the mitochondria via the CiC, and secondly via its rate of conversion to acetyl-CoA by ATP citrate lyase in the cytosol.

Another important regulator of glucose and lipid oxidation, and integrated metabolism is AMPK. AMPK is a protein kinase that plays a decisive role in intracellular and systemic metabolic regulation, and is activated when cytosolic concentrations of AMP rise and the ATP/AMP balance is shifted in favour of AMP (Hue, Rider 2007). AMPK is a highly conserved eukaryotic serine/threonine kinase.
containing one catalytic (α) and two (β and γ) regulatory subunits. Metabolic stress, a term defined in this case specifically by a decrease in substrate supply or an increase in energy demand, often leads to the activation of AMPK. Metabolic stress, for example, can be caused by glucose or oxygen deprivation or by muscular contraction during exercise, with both conditions increasing the AMP/ATP ratio (Hue, Rider 2007, Hayashi et al. 2000). During its activation, AMPK regulates metabolism by activating catabolic ATP synthesizing mechanisms such as lipid oxidation and glycolysis, while de-activating anabolic processes such as lipid and protein synthesis (Hardie 2007). The master regulatory capacity of AMPK is bestowed by the fact that key enzymes involved in the control of carbohydrate, lipid and protein metabolism are known to be AMPK substrates (Hue, Rider 2007). Specifically, AMPK can reduce the activity of acetyl CoA carboxylase 2 (ACC2) (Dyck et al. 1999), and increase the activity of Akt substrate 160 (Treebak et al. 2006) (responsible for GLUT4 recruitment to the plasma membrane). Thus AMPK provides an alternative and powerful control mechanism to intracellular metabolism in addition to the Randle cycle (Figure 10). Phosphorylation of AMPK at threonine 172 indicates lower levels of available intracellular ATP and is a useful tool in
molecular biochemistry to identify defects in cellular energy metabolism (Stein et al. 2000), which will be utilised in chapter 5.
Figure 10 Regulation of ACC by AMPK and citrate.

AMPK activation, such as occurs in many tissues during exercise or glucose deprivation, phosphorylates ACC and inhibits its activity. Conversely, a sustained excess of glucose, and possibly inactivity, decrease AMPK phosphorylation and activity and cause ACC activation. In skeletal muscle cells, glucose availability also determines the concentration of cytosolic citrate, due to changes in the ATP/AMP ratio. Citrate is an allosteric activator of ACC and a precursor of its substrate, cytosolic acetyl-CoA. Such changes in citrate occur rapidly (in minutes) and may be responsible for early changes in malonyl-CoA concentration and for sustained changes in malonyl-CoA under conditions in which assayable AMPK activity is not altered. ACC (acetyl-CoA carboxylase), AMPK (AMP kinase). Figure from (Ruderman, Prentki 2004), image rights obtained from Nature Publishing Group 2015.
1.27. Further complexities of intracellular metabolic regulation

As discussed above, LCFA oxidation is regulated by malonyl-CoA concentrations via CPT1 modulation, alongside other factors. The main determinants of malonyl-CoA concentration are the ratio of activity between ACC and malonyl-CoA decaboxylase (MCD), which have strictly controlled activities (Goodwin & Taegtmeyer 2000). ACC activity is regulated by reverse phosphorylation with dephosphorylation indicating an active form. AMPK phosphorylates and inactivates both forms of ACC, and there is also some evidence that AMPK plays a role in activating MCD, however these studies have not been confirmed (Pentyala & Benjamin 1995, Saha et al. 2000). MCD also appears to be activated by muscular contraction (Saha et al. 2000), which, along with the proposed AMPK activation would reduce levels of malonyl-CoA during exercise, and might be one mechanism by which fat oxidation is increased at sub-maximal exercise intensities (<70% \( \dot{V}O_{2\text{max}} \)) (Wall et al. 2013).

Thus, for the activation of FA oxidation, and the resulting inhibition of glucose utilization to occur, ACC activity must be low or MCD activity must be high in order
to break down malonyl-CoA. However, FA catabolism may also stimulate FA oxidation in a feed-forward loop with AMPK being (partially) activated by the AMP produced from acyl-CoA synthase facilitated activation of long-chain FA when broken down into CoA derivatives (Clark, Carling & Saggerson 2004). This has been reported in heart tissue; but whether this effect occurs in other tissues remains to be elucidated. Conversely, ACC must be active or MCD activity must be low in order to inhibit FA oxidation as a result of increased glucose oxidation. Furthermore AMPK must be inactive, and there is evidence that increased concentrations of glucose inactivate AMPK directly, increasing malonyl-CoA concentration, increasing glycolysis and potentially inhibiting FA oxidation (Itani et al. 2003). This study assessed the activity of the alpha2 isoform of AMPK and 2-deoxyglucose uptake in incubated rat extensor digitorum longus muscle after preincubation for 4 h in media containing 0, 3, 6, or 25 mmol/l glucose. Decreases in AMPK activity at higher glucose concentrations (25 mmol/l) correlated closely with decreases in glucose transport (2-deoxyglucose uptake), measured during a subsequent 20-min incubation at 6 mmol/l glucose ($r^2=0.93$, $P<0.001$). The decrease in AMPK activity at the higher glucose concentrations correlated with increases in the
rate of glycolysis, as estimated by lactate release (Itani et al. 2003).

Although malonyl-CoA is clearly important to the control of LCFA oxidation, mitochondrial events also appear to play a role in regulating fuel selection. For example, octanoate oxidation which is independent of CPT1 has confirmed that FA oxidation is preferred by the mitochondria (Eaton 2002). FA oxidation is known to further stimulate respiration in skeletal (Turner et al. 2007, Montgomery et al. 2013). This is accompanied by a suspected loss of efficiency in oxidative phosphorylation (Borst et al. 1962, Rigoulet et al. 1998). The reasons for this apparent decrease in efficiency when switching from glucose to FA oxidation are complex, but include a greater proportion of electrons transferring to complex 2 rather than complex 1 of the electron transport chain (ETC), resulting in intrinsic uncoupling that can be somewhat quantified by the reduced efficiency of the ATP/O ratio (Borst et al. 1962, Nobes, Hay & Brand 1990). Secondly, proton leak across the inner mitochondrial membrane reduces efficiency of respiration and is partly behind the stimulation of respiration by FA (Brand et al. 1994, Brand 1990). To keep sufficient supplies of cellular ATP in spite of reduced efficiency, mitochondria are
forced to increase respiration. The uncoupling effect has also been shown in skeletal muscle (Hirabara et al. 2006), particularly with medium-chain FA (Montgomery et al. 2013). However, the mechanisms are yet to be elucidated to the same extent as other tissue. This information is of importance when considering the complex regulation of regulation of FA and glucose metabolism.

Despite the complex regulation of intracellular FA and glucose metabolism, given the information presented in sections 1.27 and 1.28 it is clear that citrate plays a central role in the regulation of both glucose and FA via its direct inhibition of PFK-1 and the upstream effects of this on glucose metabolism. While citrate’s effects on fat metabolism are indirect and are mediated by its conversion to malonyl-CoA and the subsequent inhibition of CPT1. Thus, citrate is of central importance to the regulation lipid and glucose oxidation and its regulation will be discussed further.

1.28. Citrate and citrate synthase

As citrate is of central importance, it is prudent to discuss the enzymes that regulate its metabolism. Although ATP-citrate lyase controls the rate at which cytosolic citrate is converted to acetyl-CoA, the initial control of citrate’s metabolic fate is via
CS. CS is a key enzyme of the mitochondrial Krebs or TCA cycle, it catalyses the reaction between acetyl-coA and oxaloacetate to form citrate and coenzyme A (CoASH), shown in the reaction below (Equation 2).

\[ \text{Acetyl CoA + oxaloacetate + H}_2\text{O} \rightleftharpoons \text{Citrate + CoASH} \]

Equation 2 CS conversion of substrates to products

CS is located in the mitochondrial matrix in mammalian cells and is encoded by nuclear (rather than mitochondrial) DNA (Wiegand & Remington 1986). Although CS is often thought of as a rate limiting enzyme for the Krebs cycle (Wiegand & Remington 1986), it is unclear if this is always the case. Blomstrand et al. (1997) have shown that CS’s maximal activity far outstrips that of its neighbouring enzymes in the Krebs cycle, indicating that it is not the maximal activity that is rate-limiting. CS itself may not be a rate-limiting enzyme for the Krebs cycle, however its production of citrate and the potential of this molecule to enter the cytosol and modulate metabolic enzymes means that CS activity may play a role in upstream regulation of substrate metabolism. Thus, the regulation of CS activity levels warrants further investigation. Chronic endurance training alters skeletal muscle CS

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activity with trained young subjects displaying a \(~\)32\% increase and older subjects a \(~\)40\% increase in CS activity after a 12-week endurance training program (Murias et al. 2011). The opposite side of CS activity data in humans comes from obese and diabetic patients. Ritov et al. (2010) demonstrated that sedentary lean subjects have reduced total CS skeletal muscle activity (\(~\)46\%) compared to obese, insulin resistant individuals (Ritov et al. 2010). Therefore, in this study, CS activity is lower in sedentary lean subjects compared to sedentary obese subjects. Again linking CS to obesity recent evidence from mouse studies has demonstrated that the A/J mouse has a resistance to obesity coupled with a reduced activity of CS (Ratkevicius et al. 2010). This lower CS activity does not seem to alter the mouse’s health (aside from an apparently beneficial effect of obesity resistance) or activity levels which may be due to the fact that CS’s capacity to produce citrate exceeds the rate of substrate flux through the Krebs cycle as stated earlier. This is highlighted by maximal aerobic exercise performance of A/J mice being similar to B6, D2 and C3H (Lightfoot et al. 2001). The mechanisms behind the A/J mouse resistance to obesity is unknown but several studies have implicated the same region of chromosome 10 that the CS gene occupies in association with obesogenic
phenotypes (Burrage et al. 2010, Singer et al. 2004).

In summary, regulation of citrate is central in controlling mammalian intracellular fuel selection and there is an indication that in the absence of chronic exercise training, lower CS levels may be beneficial in terms of metabolism. However, the role of CS and citrate is still not fully understood in skeletal muscle under conditions such as during lipotoxicity or high acute concentrations of palmitate and glucose.
1.29. Aims

Therefore the aims of this thesis are to:

- Determine whether a session of HIIE attenuates postprandial TG and to determine the longevity of any response.

- Identify potential mechanisms responsible for HIIE’s increased disposal of plasma TG’s, by measuring plasma β-hydroxybutyrate concentrations and TRL-LPL lipolysis levels.

- Determine if a moderate reduction CS activity improves glucose and FA metabolism of cultured murine C2C12 cells during lipotoxicity and/or high acute concentrations of palmitate and glucose.
2. General Methods
2.1. Subjects

Human studies were conducted in accordance with current local guidelines and the Declaration of Helsinki following approval from the College of Life Sciences and Medicine Ethics Review Board. Information on the aims, risks and discomfort associated with this study were provided to all the subjects before giving written informed consent. All subjects engaged in regular physical activity (at least three 30 minutes sessions per week) but none were specifically trained (e.g. a competitive athlete). Subjects were excluded from the studies if they had a history of CVD, diabetes, obesity (BMI >30 kg/m²), hypertension (systolic/diastolic blood pressure >140/90 mmHg), smoking, or a musculoskeletal injury of any form.

2.2. Anthropometric measurements

Height was measured using a stadiometer to the nearest 1 cm (Holtain Ltd., Crymych, Dyfed, Wales, UK). Weighing scales were used to measure body mass to the nearest 0.1 Kg (Ohaus chamo 2, Ohaus UK Ltd., Leicester, England, UK). Skinfold thickness was measured to the nearest 0.1mm at 4 sites (bicep, triceps, sub scapula and supra iliac) with callipers (Idass, Hapenden skinfold callipers,
England, UK) on the right side of the body. Body density was calculated (Siri 1993) and then using the Siri equation an estimation of body fat percentage was calculated (Siri 1993).

2.3. Estimation of energy expenditure

EE was estimated during the walking trials from the measurement of $\dot{V}O_2$ and the expiratory exchange ratio (Weir 1949). To estimate EE during the high-intensity trials the mean power output during the 30 s sprint and an estimate of mechanical efficiency of 18.5% were used (Smith & Hill 1991).

2.4. Blood handling & biochemical measurements

Blood samples were collected into 6ml K+EDTA non-ridged vacutainers (Vacuette, greiner bio-one, Kremsmunster, Austria) (at all-time points). The blood samples were centrifuged (Eppendorf Centrifuge 5702/R, UK) for 10 minutes at 1000G (4°C). 200µL aliquots of plasma were collected and frozen at -20°C until later analysis. 4µL of 1x butylated hydroxytoluene (BHT), an antioxidant, was added to two plasma samples to prevent further lipid peroxidation, before storage for later analysis of lipid peroxidation (time points; fasting, 2, 5, and 7 hours only).
2.5. Glucose assay

The glucose assay was performed using a glucose oxidase enzymatic method (Bergmeyer, Gawehn 1974). The principle of determining glucose concentrations in the plasma was by the formation of a red quinoneimine dye measured using a colorimetric method (Equation 3). The buffer solution and enzyme reagent come pre-mixed by the manufacturer; the compositions can be seen in Table 8.

\[
\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O_2
\]

\[
2H_2O_2 + 4 - \text{aminophenazone} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O_2
\]

Equation 3 Principle of glucose determination

Table 8 Glucose reagents composition

<table>
<thead>
<tr>
<th>Contents</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Reagent</td>
<td></td>
</tr>
<tr>
<td>4-aminophenazone</td>
<td>0.77 mmol/l</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>≥ 1.5 U/ml</td>
</tr>
<tr>
<td>MOPS Buffer</td>
<td>50 mmol/l, pH 7.0</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>≥ 1.5 U/ml</td>
</tr>
<tr>
<td>Phenol</td>
<td>11 mmol/l</td>
</tr>
<tr>
<td>Phosphate Buffer</td>
<td>50 mmol/l, pH 7.0</td>
</tr>
</tbody>
</table>

Product information taken from manufacturer's manual.

1000µL of the enzyme reagent was added to the cuvettes. 10µL of unknown samples or standard (5.43 mmol/l) was then added, vortexed, and incubated for 25 minutes at room temperature. All testing was performed in duplicate (CV shown in
Table 10). A dual-beam spectrophotometer (Camspec M330b, Leeds, UK) was set at a wavelength of 500nm and zeroed against a blank (1000µL of enzyme reagent only). The absorbance of unknown samples and standards was then measured against the blanks. Subsequently, mean absorbance’s and glucose concentrations in the plasma were calculated.

2.6. Triglyceride assay

The principle for determining the concentration of TG in plasma was hydrolysis with lipases (Bucolo, David 1973) (Equation 4), and by the formation of a red quinoneimine dye measured using a colorimetric method. In preparation, the buffer solution (R1a) was reconstituted with the enzyme reagent (R1b); the compositions can be seen in Table 9.
Triglycerides $+ \text{H}_2\text{O} \xrightarrow{\text{lipases}} \text{Glycerol} + \text{FA}$

Glycerol + ATP $\xrightarrow{\text{Glycerol kinase}} \text{Glycerol} - 3-\text{phosphate} + \text{ADP}$

Glycerol – 3 – phosphate $+ \text{O}_2 \xrightarrow{\text{G-3-P Oxidase}} \text{Dihydroxyacetone} + \text{phosphate} + \text{H}_2\text{O}_2$

$2\text{H}_2\text{O}_2 + 4 – \text{aminophenazone} + 4 \text{chlorophenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + \text{HCl} + 4\text{H}_2\text{O}$

Equation 4 Principle of TG determination

Table 9 TG reagent composition

<table>
<thead>
<tr>
<th>Contents</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R1a. Buffer</strong></td>
<td></td>
</tr>
<tr>
<td>4-chloro-phenol</td>
<td>5.5 mmol/l</td>
</tr>
<tr>
<td>Magnesium-ions</td>
<td>17.5 mmol/l</td>
</tr>
<tr>
<td>Pipes Buffer</td>
<td>40 mmol/l, pH 7.6</td>
</tr>
<tr>
<td><strong>R1b. Enzyme Reagent</strong></td>
<td></td>
</tr>
<tr>
<td>4-aminophenazone</td>
<td>0.5 mmol/l</td>
</tr>
<tr>
<td>ATP</td>
<td>1.0 mmol/l</td>
</tr>
<tr>
<td>Glycerol-3-phosphate oxidase</td>
<td>$\geq 1.5$ U/ml</td>
</tr>
<tr>
<td>Glycerol-kinase</td>
<td>$\geq 0.4$ U/ml</td>
</tr>
<tr>
<td>Lipases</td>
<td>$\geq 150$ U/ml</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>$\geq 0.5$ U/ml</td>
</tr>
</tbody>
</table>

Product information taken from manufacturer’s manual.

1000µL of the enzyme reagent (Table 9) was added to the cuvettes. 10µL of unknown samples or standard (2.21mmol/l) was then added, vortexed, and incubated for 10 minutes at room temperature. All testing was performed in duplicate. A dual-beam spectrophotometer (Camspec M330b, Leeds, UK) was set at a wavelength of 500nm and zeroed against reagent blanks (1000µL of enzyme reagent only). The absorbance of unknown samples and standards was then
measured against the blanks. Subsequently, mean absorbance and TG concentration in the plasma were calculated. The inter assay Coefficient of variation for chapters 3 and 4 is shown in table 10.

Table 10 Inter-assay coefficient of variations for TG and glucose for chapters 3 and 4.

<table>
<thead>
<tr>
<th></th>
<th>Chapter 3</th>
<th>Chapter 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose</strong></td>
<td>4.3%</td>
<td>2.22%</td>
</tr>
<tr>
<td><strong>TG</strong></td>
<td>2.02%</td>
<td>0.81%</td>
</tr>
</tbody>
</table>

2.7. Insulin

Insulin was measured by ELISA (Mercodia Insulin ELISA). A 96-well plate coated with murine monoclonal anti-insulin antibodies was used. 25 μL each of standards (recombinant human insulin) and samples were pipetted into appropriate wells. 100 μL of enzyme conjugate solution (peroxidase conjugated mouse monoclonal anti-insulin) was added to each well. The plate was then incubated on a plate shaker (700-900 rpm) for 1 hour at room temperature (18–25°C). The plate was washed 6 times with 700 μL wash buffer solution per well and was inverted and tapped firmly after final wash. 200 μL of substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was added into each well. The plate was incubated for 15 minutes at room temperature.
(18–25°C). 50 μL 10% sulphuric acid was added to each well. The plate was read at 450 nm within 30 minutes using a spectrophotometric plate reader (Synerg HT Multi-mode microplate reader; BioTek, Bedfordshire, UK) at 450nm. The inter-assay coefficient of variation was 7.2%.

2.8. Intracellular-adhesion molecule-1 and vascular-cellular adhesion molecule -1

sICAM-1 and sVCAM-1 concentrations were determined using ELISA kits (R&D Systems) and absorbance was measured using a spectrophotometric plate reader (Synerg HT Multi- mode microplate reader; BioTek, Bedfordshire, UK).

2.8.1. Intracellular-adhesion molecule-1

A 96-well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against human sICAM-1 was used. 100 μL of sICAM-1 conjugate was added to each well. 100 μL of standard, control, or sample were added per well. The plate was incubated for 1.5 hours at room temperature on a horizontal orbital microplate shaker set at 50 rpm. Samples were aspirated and washed with 400 μL wash buffer 4x. 200 μL of substrate solution (H₂O₂ and
tetramethylbenzidine) was added to each well and the plate was incubated for 30 mins protected from the light. 50 µL of 2 N sulphuric acid was added to the wells to stop the reaction. Absorbance was measured at 540 nm (with wavelength correction at 450 nm) within 30 mins.

2.8.2. Vascular-cellular adhesion molecule-1

A 96 well polystyrene microplate coated with a monoclonal antibody against human sVCAM-1 was used. 100 µL of sVCAM-1 conjugate was added to each well. 100 µL of standard, control, or sample were added per well. The plate was incubated and washed using the same procedure as sICAM-1. 100 µL of substrate solution (H₂O₂ and tetramethylbenzidine) was added to each well and incubated for 20 mins protected from light. Stop solution was added and the plate was read using the same procedure as for sICAM-1.

Concentrations from both ELISA’s were calculated through the interpolation of sample absorbance values compared with generated standard curves. The inter-assay coefficient of variations were ICAM-1 = 3.38% and sVCAM-1 = 6.3%.

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2.9. Lipid peroxidation-thiobarbituric acid reactive substances

Lipid peroxidation was estimated by measurements of malondialdehyde (MDA) content using a thiobarbituric acid reactive substances (TBARS) assay to produce a pink chromogen in a heated, acidic environment. An 8 point MDA standard curve was created, in duplicate; with a concentration range of 0.98µM to 125µM. 100µL of unknown samples or MDA standards and 100µL of SDS lysis solution was added to microcentrifuge tubes and samples incubated for 5 minutes at room temperature. 250µL of the TBA reagent was added to all the tubes and incubated for 60 minutes at 95°C on a heat block. Subsequently, tubes were cooled to room temperature and placed in a centrifuge for 15 minutes at 1,000G (4°C) (1-15k, Sciquip, Sigma, Shrewsbury, UK). Using a new microcentrifuge tube, 300µL of the supernatant was transferred across and 300 µL of n-Butanol was added before being vortexed for 1 minute and then centrifuge for 5 minutes at 10,000G (4°C). All standards were performed in duplicates, with a blank control (0µM MDA). Using a 96-well microplate, 200µL of the unknown samples and MDA standards were added, and absorbencies were recorded using a spectrophotometric microplate reader (Synerg HT Multi-mode microplate reader, BioTek, Bedfordshire, UK) set at a wavelength of
532nm. Subsequent quantification of MDA concentration in unknown samples was calculated using the standard curve. The inter-assay coefficient of variation (CV) was 6.67%

2.10. Protein carbonyls

To estimate protein oxidation, a fluorometric assay kit for protein carbonyls was used (Cayman Chemical, MI, USA). The assay used the 1:1 binding of fluorophore to protein carbonyl. Protein concentration was determined for each sample via Bradford assay (Noble 2014). The Bradford assay was performed by adding 4-8µL of sample lysate to cuvettes with Bradford reagent (Bio-rad, Hertfordshire, UK), taking the total volume to 1ml. Absorbance was measured at 595nm on a spectrophotometer (GENESYS 10 Bio UV-Vis Spectrophotometer, Thermo Fisher Scientific, Waltham, MA). The protein concentration was adjusted to 5-10mg/ml with HEPES buffered saline solution (HBS). 50 µL of fluorophore was added to each sample, the tubes were incubated overnight protected from light. 400 µL of 20% trichloroacetic acid solution (TCA) was added to each tube, and then tubes were vortexed and incubated on ice for 10 mins. The supernatant was discarded.
1ml of acetone was added to each tube and vortexed vigorously. Tubes were centrifuged at 10,000 G for 10 mins at 4°C. The acetone wash was repeated twice more. The supernatant was decanted and tubes were left to dry for one hour with the lids open. 50 µL of 6 M guanidine hydrochloride was added to each tube and incubated for 10 mins. 450 µL of 1 M potassium phosphate was added to each tube and samples were vortexed then centrifuged at 10,000 G for 10 mins at 4°C. A standard curve was prepared, using diluted fluorophore. 50 µL of supernatant was transferred from the sample or standard to a 96-well plate and fluorescence was measured to determine protein carbonyl concentration in a spectrophotometric microplate reader (Synerg HT Multi-mode microplate reader, BioTek, Bedfordshire, UK) set at a wavelength of 490 nm and an emission wavelength of 530 nm. Data was expressed per mg of protein. The inter-assay CV was 3.8%.

2.11. β-Hydroxybutyrate

To estimate hepatic lipid oxidation (Fritz 1961), β-hydroxybutyrate concentration was assessed using an enzyme assay kit (Cayman Chemical, MI, USA). A standard curve was made using supplied standards. 50 µL of sample was added in duplicate
to the wells. The reaction was initiated by adding 50 µL of developer solution (lyophilized enzyme mixture and WST-1 solution). The plate was incubated at room temperature for 30 mins protected from light. Absorbance was measured at 450 nm using a spectrophotometric plate reader (Synerg HT Multi-mode microplate reader; BioTek, Bedfordshire, UK). Concentrations from the assays were calculated through the interpolation of sample absorbance values compared with generated standard curves. The inter-assay CV was 4.5%.

2.12. Measurements of immune activation

Two monoclonal antibodies for CD11b and CD36 were used as markers of leukocyte activation. The expression of CD11b on leukocytes was determined by Alexa Fluor-488 anti-human CD11b generated in mouse. The determination of leukocytes CD36 expression was by APC mouse anti-human CD36. As negative controls Alexa Fluor-488 mouse IgG1, k isotype and APC mouse IgM, k isotype controls were used, respectively.

Measurements of leukocyte activation were performed on fasting, 2, 5 and 7 hours blood samples from day 1 and 2 of the OFTT. 5µL of Alexa Fluor-488 and 20µL of
APC was added to a FACS tube, prior to adding 100µL of whole blood. Samples were then incubated for 30 minutes in a darkroom (room temperature). Red blood cells were lysed by adding 2mls of 1x pharmlyse solution (in distilled water) and further incubated for 15 minutes. Samples were centrifuged for 5 minutes (250 G, 4°C), washed in phosphate buffered saline (PBS) (0.2% BSA) and centrifuged again before being fixed in 0.5ml of 1% paraformaldehyde (in PBS) and stored until later analysis at 4°C. For the negative controls 100µL of whole blood was added along with each isotype control (CD11b and CD36), and 100µL of whole blood was added separately as an unstained control.

Samples were analysed using a FACS Calibur (BD, Oxford, UK) and the data processed using FlowJo software. Granulocytes, monocytes, and lymphocytes were identified using forward and side scatter plots (Figure 11). Percentage surface expression of CD11b and CD36 was recorded as the percentage positive cells on a green fluorescence histogram and an orange fluorescence histogram, respectively (for representative examples of CD36 see Figure 12 and Figure 13 for CD11b). MFI was also measured to indicate the surface expression quantity per leukocyte, given
in arbitrary units. 50,000 cells were analysed for each sample.

Figure 11  A representative example of the gating used for granulocytes

Lymphocytes and monocytes identified the forward (X-axis) and side scatter (Y-Axis) in the FlowJo software for CD11b and CD36 expression. Forward scatter is the light scattered forward, indicating the size of the cell. Side scatter is the light scattered side-ways indicating the granularity of the cell. Gating was initially set using the whole blood sample (no antibodies applied). The values correspond to the percentage of cells gated from the overall count – 50,000 events were recorded for each sample.
Figure 12 Representative histograms charting the degree of CD36 expression

Determined by APC in lymphocytes (A) and monocytes (B). Red lines represent the isotype control against a typical sample (blue lines). X-axis denotes fluorescence intensity, while Y-axis denotes number of cells as a % of the total. Gating was set at 99% of the population of isotype control cells.
Figure 13 Representative histograms charting the degree of CD11b expression

_Determined by Alexa fluor-488 in granulocytes (A), and monocytes (B). Red lines represent the isotype control against a typical sample (blue lines). X-axis denotes fluorescence intensity, while Y-axis denotes number of cells as a % of the total. Gating was set at 99% of the population of isotype control cells._

### 2.13. Measurements of leukocyte oxidative burst

Reagents from a commercially available kit (ORPEGEN Pharma, Germany): Wash buffer: instamed-salts were reconstituted in 1000mL of double-distilled water (ddH₂O). 1x unlabelled opsonixed E.coli bacteria. Phorbol 12-myristate 12-acetate (PMA): 5µL in 1mL wash buffer. N-formyl-Methionine-Leucine-Phenylalanine (fMLP): 5µL in 1mL wash buffer. Dihydrorhodamine (DHR) 123 substrate: 1mL of wash buffer was added and left to stand at room temperature, prior to use. 1 x Lyse
fix solution in ddH$_2$O.

The oxidative burst capacity of granulocytes and monocytes upon stimulation, with E-Coli, was determined at fasting, 2, 5 and 7 hours in days 1 and 2 of the OFTT. 100 µL of whole blood was added to a FACS tube and placed on ice for 10 minutes. Unknown samples were then stimulated by pipetting 20µL of unlabelled E.coli. For the controls, 20µL of PMA (high stimulus), fMLP (low stimulus), and wash buffer (unstimulated) were added to the whole blood, and a blank containing 100µL of whole blood only. All samples were then incubated for 10 minutes at 37°C in a water bath. 20µL of DHR 123 substrate was then added to all the samples, which were vortexed and incubated for a further 10 minutes at 37°C. Subsequently, 2mL of lyse fix solution was added, samples vortexed, and incubated in a dark room at room temperature for 20 minutes. The samples were then placed in a centrifuge for 5 minutes at 250G (4°C). The supernatant was discarded and 3mL of wash buffer was added before centrifuging again and removing the supernatant. 200µL of DNA stain was added to all samples, except the blank where 200µL of wash buffer was added.
Samples were analysed using a FACS Calibur (BD, Oxford, UK) and the data processed using FlowJo software. A gate was set in the red fluorescence histogram to identify cells with DNA content, using phycoerythrin (PE), and granulocytes and monocytes were identified using forward and side scatter plots (Figure 14). Detected by fluorescein isothiocyanate (FITC), percentage oxidative burst was recorded as the percentage positive cells on the green fluorescence histogram with mean fluorescence intensity (MFI) also measured to indicate the oxidation quantity per leukocyte, given in arbitrary units (for representative examples see Figure 15). 50,000 cells were analysed for each sample.
Figure 14 Representative histogram charting the gating applied to samples collected for oxidative burst capacity in leukocytes

Gating was initially set using the wash buffer (unstimulated) sample and identified using PE. Value represents the percentage of cells gates from the overall count collected. 50,000 events were recorded for each sample. (B) Representative example of the gating used for granulocytes and monocytes identified by forward and side scatter in the FlowJo software, for oxidative burst capacity. Forward scatter is the light scattered forward, indicating the size of the cell. Side scatter is the light scattered side-ways indicating the granularity of the cell. The values correspond to the percentage of cells gated from the overall percentage gated in A.
Figure 15 Representative histograms charting the degree of oxidative burst

Granulocytes (A) and monocytes (B). Red lines represent the wash buffer (unstimulated sample) against a typical sample (blue lines). X-axis denotes fluorescence intensity, while Y-axis denotes number of cells as a % of the total. Gating was set at 99% of the population of unstimulated cells.

2.14. Determination of lipoprotein lipase dependant triglyceride rich lipoprotein- triglyceride hydrolysis

A sample group of 5 subjects was used for this assay, where we had sufficient volume of frozen plasma to carry out this analysis. No initial sample size calculations were carried out, but using the mean and SD of the collected data (HIIE: 204.3±48.7 nmol NEFA/ml.h, control: 142.6±28.6 nmol NEFA/ml.h), the retrospective power of 5 subjects was 98% at an alpha level of 0.05. Analysis was carried out in a batch at the end of the study. Samples were taken and stored by Brendan Gabriel
myself, but all further LTTH analysis was carried out by Valerie Pruneta-Deloche and Phillipe Moulin at the INSA de Lyon (Lyon, France). Spontaneous lipolytic activity in TRLs resulting from LPL bound on their surface was measured using the LTTH assay as previously described (Pruneta-Deloche et al. 2004). TRLs were isolated by fast-protein liquid chromatography (FPLC) using a Superose 6 HR 10/30 column (Pharmacia) at 4°C to separate lipoproteins in TSE buffer containing 10IU/ml heparin for stabilisation of LPL during the procedure. 1 ml of filtered plasma was applied to the column and chromatographed at a flow rate of 0.3 ml/min under a pressure of 150 psi. Fractions corresponding to total TRLs, including both chylomicrons and VLDL (fractions 9 to 18), were pooled and immediately assayed for LPL activity. Aliquots of the pooled TRLs corresponding to 0.3 μmol of TG were incubated for one hour at 37°C of buffer and lipolysis monitored over time. Blanks were obtained by incubations of samples in the presence of 2 mmol/L Paraoxon (Sigma), which totally blocks LPL activity. The resulting amounts of NEFA released by LPL bound to TG rich lipoprotein were then measured in triplicate, and after correction for plasma TG concentrations, LTTH was finally expressed as the amount of NEFA released per ml of plasma per hour. This assay has been
validated as a measure of LPL activity in response to endogenous TRL’s, as LTTH values correlate with post-heparin LPL activity values in healthy subjects, and plasma TG concentration exhibits an inverse relationship with LTTH (Pruneta-Deloche et al. 2004, Pruneta et al. 2001). The coefficient of variation, for duplicate samples, for the LTTH assay was 7.3%.

2.15. C2C12 cell culture

Mouse C2C12 muscle cells were cultured in 10 ml growth medium (GM) containing 88% high-glucose (20 mM glucose) Dulbecco’s Modified Eagles’ medium (DMEM) (Sigma-Aldrich, St. Louis, USA), 10% foetal calf serum (FCS) and 2% glutamine (Thermo Fisher, Waltham, MA, USA) in T75 cm² flasks at 37°C and 5% CO₂.

For all experiments unless stated otherwise, 2x10⁶ cells were seeded in plastic 6-well plates coated with ECM gel (E6909, Sigma-Aldrich, MO, USA), to avoid detachment, and containing 2 ml GM. When 100% confluence was reached myoblasts were induced to fuse and form myotubes by switching the media to 2 ml differentiation medium (DM) containing 96% high-glucose DMEM, 2% horse serum (HS) and 2% glutamine. DM was changed every 24 h for at least 5 days, at which point...
point cells were used for experiments.

2.16. Short-hairpin ribonucleic acid targeting citrate synthase messenger-ribonucleic acid

C2C12 mouse muscle cells were infected with shRNA1 [CS-KD(citrate synthase knockdown)] and shRNA2 (CS-KD 2) constructs targeting Cs mRNA as well as empty construct (EV) using standard methods for lentiviral constructs (MISSION shRNA Lentiviral transduction particles, Sigma-Aldrich, U.K.) as described previously (Martin-Granados et al. 2008, Voss et al. 2011). Viruses were produced following manufacturer's instructions by co-transfecting HEK293 cells with a plasmid carrying a phosphatase targeting shRNA construct and the Mission lentiviral packaging mix (Sigma–Aldrich) containing the two plasmids expressing the key structural viral packaging genes and a heterologous viral envelope gene. Control lines (EV) were made using the empty plasmid vector (pLKO.1-puro) containing the puromycin resistance marker (P) and plasmid vector containing a scrambled shRNA sequence (non-target, NT) instead of the vector carrying the protein phosphatase shRNA. Virus was harvested at 72 h post-transfection and infections of C2C12 cells at 20% confluence were carried out in the presence of 10 μg/ml of polybrene.
Following transduction, cells were selected with 3 µg/ml of puromycin. After 3–4 days of puromycin selection, cells were incubated for 1–2 days in non-selective medium unless otherwise stated.

The target sequence of shRNA 1 was:

CCGGGCACCCAAACATTGGTTATTCTCGAGAATAACTCAAATGTTGGGTGCTTTTTG

In the 3’ untranslated region (3’UTR) region.

The target sequence of shRNA 2 was:

CCGGGACGTGTCAGATGAGAAGTTACTCGAGTAACTTCTCATCTGACAGCTTTTTG

In the coding DNA sequence (CDS) region.

The extent of the knockdown was verified by measuring specific CS activity.

2.17. Citrate synthase activity
A spectrophotometer (GENESYS 10 Bio UV-Vis Spectrophotometer, Thermo Fisher Scientific, Waltham, MA) was used for assessment of CS. The molar extinction coefficient used was 13,600 l·mol⁻¹·cm⁻¹ for coenzyme A-DTNB at 412 nm. The CS reaction reagent consisted of 100 mM triethanolamine-HCl, DTNB (100 µM), Triton-X (0.25% vol/vol), oxaloacetate (0.5 mM), acetyl CoA (0.31 mM) with pH adjusted to 8.0. 1000 µl of reaction reagent included also 10 µl of muscle homogenate that was added to start the reaction. The absorbance changes were measured every 15 s over 3 min for determination of maximum CS activity (Vmax). Initial velocity (V0) of CS was also determined at varied substrate concentrations by measuring absorbance changes every 5 s. All assays were carried out at room temperature of 21°C. CS from porcine heart was used as a standard (C3260-200UN, Sigma-Aldridge, UK) for assay calibration.

2.18. Cell impedance assay

A cell impedance assay was carried out on myoblasts treated with EV, CS-KD 1 or CS-KD 2 to assess proliferation rate, and growth rate once cells had reached differentiation stage. Myoblasts were seeded at ~40% confluency in a specific well
plate (E-Plate 16, ACEA Biosciences, CA, US), which was inserted in a cell impedance analyser (RTCA DP Analyzer, ACEA Biosciences, CA, US). Cells were kept in GM for 2 days before being changed to DM when ~90% confluent. Readings of cell impedance were taken every 5 mins from 4 different wells for each treatment. Values are expressed as arbitrary Cell Index units. The Cell Index at each time point is defined as \((R_n-R_b)/15\); where \(R_n\) is the cell-electrode impedance of the well when it contains cells and \(R_b\) is the background impedance of the well with the media alone. Photos were taken (EVOS f1, Life Technologies, Paisely, UK) directly before every media change at 11:00 hours.

2.19. Palmitate incubation

Myotubes were incubated for 24 hours with 0.8 mM palmitate (dissolved in ethanol and conjugated to 2% FA -free BSA for 24 hr) and 1.2 mM L-carnitine. Following treatment, substrate uptake and oxidation were assessed as described below, or alternatively cells were lysed and assessed by other means as described below.

2.20. Crystal violet cell proliferation assay

Cells were cultured as previously described in C2C12 Cell Culture. A crystal violet
proliferation assay was performed according to previously described methods (Vega-Avila, Pugsley 2011). Both EV and CS-KD cells were seeded in 12 6-well plates (6 for EV and 6 for CS-KD) at 5,000 cells per-well and allowed to proliferate in proliferation media. Both EV and CS-KD had one plate removed from culturing at 24, 48 and 72 hours after seeding and had media removed before being washed with PBS and being incubated with crystal violet solution (HT90132, Sigma-Aldrich, UK) for 10 minutes at room temperature. Cell counts were performed at 20x magnification by 2 blinded individuals on 10 fields for each well, a mean was taken of these readings. Cells were fixed in separate plates at 24, 48 and 72 hours.

2.21. Immunoblotting

Antibodies were from Cell Signalling Technology (Danvers, USA); B-actin AMPK P-AMPK and Anti-rabbit HRP-linked secondary antibody ( # 4967, 2532, 2531, 7074 respectively) except CS (Alpha Diagnostic, San Antonio, USA # CISY11-A). The cell and tissue samples were homogenised in ice cold lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1% (vol/vol) Triton X-100, 0.1% (vol/vol) 2-mercaptoethanol, pH was adjusted to 7.5) supplemented with protease inhibitor

Brendan Gabriel
cocktail, 10 mM β-glycerophosphate, 50 mM NaF and 0.5 mM Na3VO4. Protein concentration was determined using the Bradford Assay (Noble 2014) (Bio-rad, Hertfordshire, UK) as previously described (2.10). Gels were cast using the ingredients in Table 11. Firstly, running mix was pipetted into a gel cast (Bio-Rad, CA, USA) and left to polymerise for ~1hr. Secondly, stacking buffer was pipetted into the cast and a comb was added and a lane dividing comb was inserted (Bio-Rad, CA, USA).

Table 11 Ingredients of pre-cast gels used for western-blot

<table>
<thead>
<tr>
<th></th>
<th>Stacking Mix</th>
<th>Running Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% AMPS (Sigma-Aldrich, St Louis, USA)</td>
<td>33µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>30% Acrylamide (Sigma-Aldrich, St Louis, USA)</td>
<td>0.66ml</td>
<td>1.67ml</td>
</tr>
<tr>
<td>4x Tris/SDS, pH6.8</td>
<td>1ml</td>
<td></td>
</tr>
<tr>
<td>4x Tris/SDS, pH8.8</td>
<td>1.25ml</td>
<td></td>
</tr>
<tr>
<td>Double distilled H2O</td>
<td>2.35ml</td>
<td>2.03ml</td>
</tr>
<tr>
<td>Temed (Sigma-Aldrich, St Louis, USA)</td>
<td>5µL</td>
<td>4µL</td>
</tr>
</tbody>
</table>

For immunoblotting, the samples were loaded on the 10% polyacrylamide gel, separated using SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membrane. Then membranes were washed with Tris buffered saline (TBS) containing 0.1% (vol/vol) Tween-20 (TBS-T buffer) before being blocked with 5% Brendan Gabriel
(wt/vol) non-fat milk in TBS-T buffer. Afterwards, membranes were incubated for >3 hours at 4°C with the primary antibody followed by 2-h exposure to HRP-conjugated secondary antibodies (1:2000 dilution, #7071, Cell Signalling, Beverly, MA, USA), and detected using ECL detection reagent (Amersham Biosciences, Buckinghamshire, UK) and Biorad Imager for immunobloting (Bio-rad, Hertfordshire, UK). All preparation and quantification was carried out by myself. Immunoblots were performed by myself, Aivaras Ratkevicius and Holly Cribes.

2.22. **Cytochrome c oxidase activity assay**

Cytochrome c oxidase (COX) activity was determined in order to assess mitochondrial density in cellular samples (Larsen et al. 2012). Cells were cultured as previously described in ‘C2C12 Cell Culture’. EV and CS-KD Cells were then changed to either 5mM glucose HBS medium for 1 hour or 5mM glucose and 0.8mM palmitate DMEM for 24 hours. Cells were then lysed in ice-cold lysis buffer [tris-HCL 50mM, EDTA 1mM, EGTA 1mM, Triton X-100 1%, protease inhibitor cocktail 2% (P8340, Sigma-Aldrich, St Louis, USA), β-glycerophosphate 10mM, NaF 50mM, Sodium orthovanadate 1mM] and centrifuged at 13,000 G for 20 mins
in 0.5 ml Ultra spin filters (Amicon Ultra, Millipore, MA, USA) in order to isolate protein. Protein pellets were re-constituted with 300ul enzyme dilution buffer (CYTOCOX1-1KT, Sigma-Aldrich, MO, USA- 10 mM Tris-HCl, pH 7.0, containing 250 mM sucrose and diluted 2-fold). COX activity was then assessed by kit assay (CYTOCOX1-1KT, Sigma-Aldrich, MO, USA). A positive control (COX 5mg/ml) was used to verify the assay. 0.95ml of assay buffer (10 mM Tris-HCl, pH 7.0, containing 120 mM KCl, diluted 5-fold with water) was added to cuvettes to zero the spectrophotometer. 30µl of sample was added to the cuvettes along with 70µl of enzyme dilution buffer and 50µl of 0.5mM ferrocytochrome c substrate solution. Cytochrome c's change in absorbance was measured over 1 minute at 550nm (GENESYS 10S UV-Vis, Thermo Fisher Scientific, MA, USA) as it was reoxidised by COX.
2.23. Glucose oxidation and incorporation

EV and CS-KD cells were differentiated to myotubes as previously described in ‘C2C12 Cell Culture’. Myotubes were then washed with Hepes buffered saline solution (HBS) and subsequently incubated with HBS media containing 5mM glucose [14C]glucose at 2 μCi/ml (PerkinElmer - NEC042X050UC) for 1 h. Following this incubation period, 1 ml of the culture medium was carefully transferred to a sealable tube, the cap of which housed a filter paper disc (GF/B – Whatman, GE, CT, USA) that had been pre-soaked with 200μL of 1 M KOH. 14CO2 trapped in the media was then released by acidification of media using 60% (vol/vol) perchloric acid and gentle agitation of the tubes at 37°C for 2 h. Radioactivity that had become adsorbed onto the filter discs was quantified by liquid scintillation counting (Wallac 1409 Liquid Scintillation counter, PerkinElmer, Waltham, MA, USA).

Cells were subsequently washed with PBS and lysed in 1M NaOH. Intracellular [14C]glucose was then quantified using a Wallac 1409 Liquid Scintillation counter (PerkinElmer, Waltham, MA, USA). Protein content was determined in each sample.
via the Bradford assay (Noble 2014) (B6916- Sigma-Aldrich, St Louis, USA) as previously described (2.10) by a GENESYS 10 Bio UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Values are expressed as pmol relative to protein content.

2.24. Palmitate incorporation and oxidation

Myotubes were cultured as previously described in ‘C2C12 Cell Culture’. Myotubes were then washed with HBS and subsequently incubated with HBS media containing 0.8 mM palmitate (dissolved in ethanol and conjugated to 2% FA -free BSA)-[14C]palmitate at 2 μCi/ml (PerkinElmer - NEC534050UC) for 2 h. Following this incubation period, Incorporation, oxidation and protein concentration were measured as previously described for glucose.

2.25. Citrate assay

Cells were cultured as previously described in ‘C2C12 Cell Culture’. Cells were lysed in ice-cold lysis buffer [tris-HCL 50mM, EDTA 1mM, EGTA 1mM, Triton X-100 1%, protease inhibitor cocktail 2% (P8340, Sigma-Aldrich, St Louis, USA), β-glycerophosphate 10mM, NaF 50mM, Sodium orthovanadate 1mM], centrifuged
and protein was determined as described in ‘Cytochrome c oxidase activity assay’.

Intra-cellular citrate concentration was determined via a colorimetric-enzyme assay kit (MAK057, Sigma-Aldrich-Aldrich, MO, USA). A standard curve was prepared in a 96-well plate. A reaction mix containing 44µL assay buffer (MAK057A, Sigma-Aldrich-Aldrich, MO, USA), 2µL enzyme mix (MAK057D, Sigma-Aldrich-Aldrich, MO, USA), 2µL citrate developer (MAK057E, Sigma-Aldrich-Aldrich, MO, USA) and 2µL enzyme probe in DMSO (MAK057B, Sigma-Aldrich-Aldrich, MO, USA) was added to the sample and standard wells. Samples were measured in duplicate. A blank was also created for each sample using the reaction mix without the enzyme mix. The plate was left mixed and left at room temperature for 30 mins. Absorbance was measure by a plate reader (Synergy HT, Biotek, Bedfordshire, UK). The blank value was deducted from each sample and values were calculated using the standard curve.

2.26. Acetyl CoA assay

Intra-cellular Acetyl CoA concentration was determined via a fluorescent assay kit (ab87546, Abcam, UK). Cells were lysed in ice-cold lysis buffer [tris-HCL 50mM,
EDTA 1mM, EGTA 1mM, Triton X-100 1%, protease inhibitor cocktail 2% (P8340, Sigma-Aldrich, St Louis, USA), β-glycerophosphate 10mM, NaF 50mM, Sodium orthovanadate 1mM] then centrifugated at 14,000 G for 10-20 minutes (depending on lysate vol.) in centrifugal filter devices (UFC501096, Amicon, Millipore, MA, USA) to remove protein. Protein concentration was determined by Bradford assay (Noble 2014) as previously described (2.10). Free CoA was quenched and Acetyl CoA was converted to CoA. This was then reacted to form NADH which generated fluorescence when a probe was added. A standard curve was prepared in the 0-100pmol range. Samples had 10µL of Coenzyme A quencher, and were incubated for 5 mins at room temperature. 2µL of quench remover was then added. Samples were mixed and incubated for 5 mins. A reaction mix consisting of 40µL buffer, 2µL substrate mix, 1µL conversion enzyme, 5µL enzyme mix and 2µL picoprobe was added to each sample and standard well. Samples were measured in duplicate. A blank mix was also made for each sample with the conversion enzyme removed. Fluorescence was measured at EX/EM=535/589 by a plate reader (Synergy HT, Biotek, Bedfordshire, UK). The blank value was deducted from each sample and values were calculated using the standard curve.
2.27. **Statistical analysis**

Prism version 5 software was used to analyse all data. Data were compared via independent t-tests, one-way analysis of variance (ANOVA) or two-way ANOVA where appropriate as described in each results chapter. P<0.05 was considered to represent statistical significance.
3. High intensity exercise attenuates postprandial lipaemia and markers of oxidative stress


3.1. Introduction

CVD is a major cause of mortality and is becoming more prevalent (Bjãrn 2010).

The most common form of CVD is CHD, a condition with atherosclerosis at the
centre of the pathology (Madamanchi, Vendrov & Runge 2005). Atherosclerosis is
often linked to high fasting serum levels of lipids and low density lipoproteins.
However, fasting levels of TG are not a good predictor of atherosclerosis (Eberly,
Stamler & Neaton 2003) and as people spend the majority of the day in a
postprandial state, it has been suggested that atherogenesis is a postprandial
phenomenon (Zilversmit 1979). This contention has recently been supported by
several studies demonstrating that postprandial TG concentration is a strong
independent risk factor for cardiovascular disease (Nordestgaard et al. 2007,
Bansal et al. 2007).

A single HFM can induce endothelial dysfunction, likely due to oxidative stress and
activation of leukocytes, one of the early stages in the development of
2008). The importance of leukocyte activation is indicated by early research
demonstrating that leukocyte counts are a predictor of future MI (Friedman, Klatsky & Siegelaub 1974, Karabinos et al. 2009). However, it should be noted that whilst current data suggest that leukocytes are involved in the process of endothelial dysfunction their precise role has yet to be established. Recent research from Azekoshi et al. (2010) has provided some evidence of a definite role for leukocytes in endothelial dysfunction via activation of the rennin angiotensin system (Azekoshi et al. 2010). Moreover, the immune reaction appears to couple dyslipidaemia to atherosclerotic plaque formation partially through the induction of the endothelium to express adhesion molecules, such as ICAM-1 and VCAM-1 on their surface (Nakashima et al. 1998). This induction of ICAM-1 and VCAM-1 expression can be induced by oxidative stress (Wang et al. 2008).

In support of a role for oxidative stress in endothelial dysfunction it has been demonstrated that the consumption of antioxidants can attenuate the deleterious effects of a HFM (Anderson et al. 2006). Exercise is also proposed to be a mechanism to reduce postprandial TG and adults are advised to accumulate thirty minutes of moderate intensity exercise on five days a week (Haskell et al. 2007).
Several studies have shown that such moderate intensity exercise attenuates postprandial TG levels after a HFM (Miyashita, Tokuyama 2008, Gill et al. 2004). However, the current numbers of people meeting these recommendations remains low (Scottish Government 2006) with lack of time frequently cited as the greatest barrier to performing exercise (Trost et al. 2002). HIIT has, therefore, been proposed to be a time efficient method of improving cardiovascular health (Rakobowchuk et al. 2008, Freese et al. 2011).

Previous studies have shown that a time efficient exercise protocol, involving 4-6 30s maximal sprits per session, can lead to improvements in endurance performance and muscle oxidative capacity (Burgomaster et al. 2008). Further work by this group has also demonstrated that this exercise protocol can reduce hyperglycaemia and improve mitochondrial capacity in patients with type 2 diabetes (Little et al. 2011a), and also improve peripheral arterial stiffness and flow mediated dilation in healthy untrained individuals (Rakobowchuk et al. 2008). Similar improvements in flow mediated dilation have also been shown after a HFM preceded by aerobic HIIT by Tyldum et al. (2009). Furthermore in a recent study it
has been shown that HIIE can reduce postprandial TG for 3 hours after a high fat
breakfast (Freese et al. 2011). What remains to be determined is if these effects
remain over a longer time period (i.e. 7-8 hours), which more closely reflects daily
food intake (i.e. breakfast and lunch meals) where elevated TG and endothelial
dysfunction can still be observed [e.g. (Anderson et al. 2001) ] and whether the
beneficial effects extend to improvements in markers of oxidative stress and/or
adhesion molecules.
3.2. Aims

The primary aim of the present study was to determine the effect (in healthy young men) of HIIE on postprandial plasma levels of TG, soluble adhesion molecules and markers of oxidative stress.

A second aim was to investigate whether changes in postprandial TG could be associated with a decrease in hepatic VLDL secretion, indicated by measures of β-hydroxybutyrate.
3.3. Methods

Nine healthy male volunteers took part in this study (age, 24±3 years; body fat, 14.9 ± 4.1%; weight, 81.5 ± 8.5 kg; height, 1.81 ± 0.10 m; BMI, 24.9±2.0 kg/m²). Ethics and exclusion criteria were approved and defined as described in chapter 2.1. Healthy young males were chosen as an accessible and metabolically stable population in which the null hypothesis that HIIE does not alter postprandial TG levels could be confirmed or denied with less confounding factors than other populations. A sample size calculation was carried out using the change in area under the curve for triglycerides after 30 min walking, assuming a similar or larger change with HIIE, from the study of Miyashita, Burns & Stensel (2008). This study compared a group of participants over 3 trials: control, continuous walking and intermittent walking in fifteen young healthy males. Using this data a total of 10 subjects would be needed to give this study 80% power, with an alpha level of 0.05. Although 10 subjects were recruited one subject dropped out through choice.
3.3.1. Experimental protocol

A randomised cross-over study design was implemented in a randomised control trial. All subjects completed three 2 day trials; a HIIE trial, walking trial and a control trial. The order of these trials was randomised with a minimum of 7 days interval between the trials (Figure 16). Two days prior to each trial subjects were asked to refrain from exercising, and the consumption of caffeine and alcohol. Subjects recorded their dietary intake for 24 hours prior to the start of the trial and throughout the 2 days of the trial itself (for the subject’s personal use). Each subject was instructed to then replicate diet for these days in the subsequent trials.
3.3.2. Day 1 trials

3.3.2.1. Walking

The walking trial took place on a treadmill (Cybex International). Subjects were started at a speed of 7 km/h and asked to walk at an intensity similar to that of the current recommendations for 30 min (Haskell et al. 2007), i.e. a brisk walk (out of breath, but still able to talk). Pulmonary gas exchange $\dot{V}O_2$ (oxygen consumption)
and $\dot{V}_{\text{CO}_2}$ (carbon dioxide output) were monitored breath-by-breath during several time intervals, *i.e.* 8–10, 18–20 and 28–30 min respectively using an online gas analysis system (CPX Ultima; Medgraphics).

### 3.3.2.2. High intensity interval exercise

Subjects performed a 4 minute warm up (no resistance) on a cycle ergometer (Monark 894, Wingate bike, UK). Subjects then performed 5 x 30 second maximal sprints, with 4 minutes rest intervals, and a resistance of 7.5% body mass. Peak power (Watts), average power (Watts) and peak pedal rate (RPM) during each sprint were recorded.

### 3.3.2.3. Control

During the control trial participants sat and rested for 30 min.

### 3.3.3. Day 2

Subjects arrived at 08.45 hours after an overnight fast from 22:00 hours and rested for 15 min before a cannula (20 gauge) was inserted into a vein in the antecubital fossa, and a baseline blood sample was collected. The cannula was flushed regularly with saline throughout the day. A standardised HFM was then consumed.
for breakfast. The mean energy content of the meal was 812±95 kcal. This consisted of white bread, mayonnaise, butter, whole milk, cheddar cheese and potato crisps. The meal contained 0.7 g of fat, 1 g of carbohydrate, 0.3 g of protein and 11 kcal per kg of body weight. The mean macronutrient content of the meal was 56.8±6.1 g of fat, 75.7±8.2 g of carbohydrate and 25.8±2.8 g of protein (calculated from nutritional information provided on food packaging). The mean time taken to consume the meal was 13:14±4:30 min:secs for breakfast and 13:42±3:56 min:secs for lunch.

Further blood samples were collected 0.5, 1, 2 and 3 h after the breakfast meal. A second identical meal was then consumed for lunch with subsequent blood samples taken 3.5, 4, 6 and 7 h after the consumption of the first meal. Water was provided ad libitum throughout the day of the first trial and this volume of water was consumed during subsequent trials.

3.3.4. Analysis

Blood plasma samples were analysed for TG, glucose, insulin, protein carbonyls, TBARS, ICAM-1 and VCAM-1 as described in chapters 2.5-2.10. Power output and
EE of HIIE and EE of walking were determined as described in chapter 2.3.

### 3.3.5. Statistical analysis

All data is presented as mean ± SD. Prism 5 statistical software (GraphPad Software, La Jolla, CA) was used. A test for normality on data sets was performed before statistical analysis. A 2-way (trial x time) ANOVA with repeated measures was employed to determine differences between groups and time. Post-hoc Tukeys were performed where a significant effect was shown to identify the location of any differences. Where a time effect was found, a paired t-test with Bonferroni correction was performed (significance level was taken at $P<0.016$). The trapezoid rule was used to calculate total AUC and incremental AUC (iAUC), derived from numerical integration (Dalquist & Bjorck, 2008). Significance was taken at $P<0.05$.

In a small number of cases where single data points were missing, the point between the prior time point and the following time point was used (interpolation). Where the data were missing at the start or end of a data set, the difference between the prior/post time points was extrapolated to the missing value using forward and backwards forecasting. There was 1 missing timepoint for glucose,
insulin and TG and 3 for the power measurements of the Wingate test.
3.4. Results

3.4.1. Exercise

Volunteers did not perform any exercise in the control trial. Subjects walked at an average speed of 6.7±0.2 km/h and an average $\dot{V}O_2$ of 20± 2.3 ml/kg/min. Estimated EE was 240.9±35.2 kcal during the walking trial. The maximum power output during HIIE was 869.1±198.4 Watts and mean power output was 632.6±102.2 Watts. This corresponded to an average EE of 103.2±5.1 kcal during the HIIE trial, which was lower (P<0.001) than during the walking trial.

3.4.2. Insulin and glucose

There were no differences between the 3 trials in either insulin or glucose concentration in the plasma samples collected. The ANOVA did reveal a significant effect of time for both insulin and glucose in response to the meals (P<0.0001) (Figure 17).
3.4.3. **Triglyceride**

The initial ANOVA revealed no differences between the 3 trials in plasma TG concentration. There was a significant effect of time in response to the meal (P<0.0001) (Figure 17).

The data were analysed in the form of AUC in order to assess the TG data as a
whole day for each trial. This showed that there was a lower (P<0.05) iAUC in HIIE trial compared to the control trial (Figure 18 A and Table 12) (6.42±2.24 mmol/l/7h in HIIE vs. 9.68±4.77 mmol/l/7h in control). There was also a trend (P=0.056) for a lower total AUC in HIIE compared to the control trial (Figure 18 B and Table 12) (14.13±2.83 mmol/l/7h in HIIE vs. 17.18±3.92 mmol/l/7h in control). There were no differences between the control and walking trials or fasting levels in any trial (Table 12).

Figure 18 TG incremental (Figure 18A) and total (Figure 18B) AUC over the 7 hour experimental period during prior walking, HIIE and control trials. Values are Mean±SD. †denotes a trend (P=0.056) between HIIE and control trials, *denotes a significant difference (P<0.05) between HIIE and control trials.
Table 12 Fasting, AUC total triglyceride and incremental triglyceride on the control, walking and high intensity intervals trials.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Fasting Triglyceride</th>
<th>Total AUC Triglyceride</th>
<th>Incremental AUC Triglyceride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.75±0.20</td>
<td>17.18±3.92</td>
<td>9.68±4.77</td>
</tr>
<tr>
<td>Walking</td>
<td>0.73±0.28</td>
<td>16.66±3.60</td>
<td>8.98±2.84</td>
</tr>
<tr>
<td>HIIE</td>
<td>0.77±0.25</td>
<td>14.13±2.83†</td>
<td>6.42±2.24*</td>
</tr>
</tbody>
</table>

*Area under the curve (AUC). †denotes a trend (P=0.056) between HIIE and control trials, * denotes a significant (P<0.05) difference compared to the control trial, data are presented as mmol/7h.L⁻¹ for AUC or mmol/L for fasting.*
3.4.4. **Soluble adhesion molecules**

There were no differences between the 3 trials in soluble adhesion molecule concentrations (Figure 19). However, sICAM-1 did show a significant effect of time with values rising throughout the day (P<0.0001).

![Plasma sVCAM-1 and sICAM-1 concentrations](image)

Figure 19 Plasma sVCAM-1 (Figure 19A) and sICAM-1 (Figure 19B) concentrations in response to prior walking, HIIE and control trials. Values are Mean±SD.
3.4.5. Markers of oxidative stress

Protein carbonyl levels (Figure 20 A) increased (P<0.05) at 2 and 5 hour above baseline in both walking and control trials. However, HIIE completely prevented this effect, with no change in protein carbonyl levels after HIIE. TBARS (Figure 20 B) were raised above baseline in all 3 trials, however, HIIE reduced the magnitude of this effect. There were no differences in either protein carbonyls or TBARS between walking and control trials.

Figure 20 Plasma markers of oxidative stress

Plasma protein carbonyls (Figure 20A) and TBARS (Figure 20B) concentrations in response to prior walking, HIIE and control trials. Values are Mean±SD. *denotes a significant difference (P<0.01) between HIIE and the control/walking trials. †denotes a significant difference (P<0.01) between HIIE and walking trials. ‡ denotes a significant difference (P<0.05) between HIIE and control trials.
3.4.6. β-hydroxybutyrate

β-hydroxybutyrate (Figure 21) increased between baseline and 5 hours in all 3 trials (P<0.0001), with no difference between the 3 trials.

Figure 21 Plasma β-hydroxybutyrate

*Plasma β-hydroxybutyrate concentration in response to prior walking, HIIE and control trials. Values are Mean±SD.*
3.5. Discussion

The main finding of the current study was that a prior bout of HIIE attenuated the postprandial rise in TG, compared to a control trial, with no differences in glucose or insulin between trials. Thirty minutes of brisk walking, which is equivalent to the current physical activity recommendations, had no effect on postprandial TG concentrations. The first of these findings agree with the recent work of Freese et al. (2011) who employed a similar exercise protocol, although in their work TG concentrations were only monitored for 3 hours postprandially. The current study has extended these findings by also demonstrating that these effects are present for a 7 hour period (after breakfast and lunch) and that HIIE also results in a decrease in markers of oxidative stress, with no change in soluble adhesion molecules. These findings have important clinical implications as HIIE may be of a greater benefit than the currently recommended forms of physical activity. The importance of a reduction in exercise time is highlighted by the consistent findings that a lack of time is frequently cited as the major barrier to participation in exercise (Trost et al. 2002). However, while the total exercise time in the current study was only 2.5 min the session did last ~25 min, which is slightly shorter than the current
recommendations, it remains to be investigated whether this would increase exercise participation.

The HIIE protocol employed also has an approximately 57% lower EE. Whilst this may be seen as a negative observation if one was aiming to reduce body fat, HIIE has, in fact, been found to be more effective than traditional steady state endurance exercise in reducing body fat (Trapp et al. 2008). However this study used a different protocol to the current study, i.e. subjects performed 15 weeks of HIIE consisting of 8 secs of maximal cycling on an ergometer followed by 12 secs passive recovery for a maximum of 60 repetitions each session, sessions were between 5-20 minutes long. Nevertheless, the greater TG reducing effect of HIIE may be surprising as EE is thought to be a crucial determinant of this response in moderate intensity exercise (Gill, Hardman 2000). Both these observations may be due to an elevation in resting metabolic rate and/or a more prolonged/greater elevation in post-exercise oxygen consumption after HIIE (Laforgia et al. 1997, Brockman, Berg & Latin 1993). Further work is needed to clarify these assertions.
It is well established that, in healthy individuals, exercise can reduce postprandial TG concentrations. Early work demonstrated that a 90 min brisk walk can reduce postprandial TG concentrations by approximately 20% (Gill, Hardman 2000) and these findings have been supported by several further studies (for review, see (Gill, Hardman 2003b). This duration of exercise is, however, three fold higher than the current recommendations and is unlikely to be achieved by the general population. Recent work has demonstrated that 30 min of brisk walking results can also reduce postprandial TG concentrations, by approximately 15%, in healthy young men (Miyashita, Tokuyama 2008). A similar finding was not observed in the current study with 30 min of brisk walking, with no clear reason for the differences in findings. It may be that the current study did not have sufficient participant numbers to detect differences between walking and control trials. However, the main aim of the study was to determine the effect of HIIE, which had a clearly positive effect in reducing plasma TG.

The mechanisms responsible for an exercise induced decrease in plasma TG concentrations remain to be elucidated (for review see (Peddie, Rehrer & Perry
The proposed mechanisms are that either, or a combination of both, the uptake of TG in peripheral tissues is elevated or the production and release of TG, packaged in VLDL, from the liver is altered. With regards to augmented TG clearance after prior intense exercise it appears likely that this is mediated by an increase in LPL activity (Peddie, Rehrer & Perry 2012). However, after prior moderate intensity exercise there also appears to be a role for decreased secretion of VLDL from the liver. Indeed, Gill et al. (2007) showed increased circulating levels of β-hydroxybutyrate alongside reduced postprandial TG concentrations following prolonged exercise (Gill et al. 2007). This increase in β-hydroxybutyrate is suggested to be indicative of an increase in hepatic FA oxidation which would shift hepatic FA portioning away from VLDL synthesis. Early evidence has shown that there is an inverse relationship between ketogenesis and VLDL production (Mayes, Felts 1967), supporting the contention that increases in β-hydroxybutyrate would be reflected in a decrease in VLDL production. However further work has demonstrated that this relationship doesn’t hold under all situations (Laker, Mayes 1982) and so caution should be employed when interpreting these results. The current investigation found no difference between exercise trials in β-
hydroxybutyrate levels, with all 3 trials showing a rise between 2 and 5 hours after the first meal. It is therefore likely, that the attenuation of postprandial TG seen after HIIE comes about solely as a result of increased LPL activity, although further work is required to elucidate the effects of HIIE on hepatic VLDL secretion.

The magnitude of postprandial TG concentration has previously been shown to correlate with the magnitude of endothelial dysfunction and intima-media thickness of the carotid artery (Gaenzer et al. 2001). Furthermore both total and incremental TG AUC measures also correlate with intima-media thickness (Boquist et al. 1999), highlighting the physiological and clinical relevance of these measures of the TG response during the 7 hour test period. Whilst the current study did not make any measure of endothelial function, previous work (Tyldum et al. 2009) has demonstrated that aerobic HIIE completely abolished the reduction in brachial artery flow mediated dilation normally observed after a HFM. Although this has not been investigated using pure HIIE. These authors also found that this effect was associated with an increase in total antioxidant status in the blood, with no reductions in plasma TG. While this supports the assertion that the deleterious
effects of a HFM are associated with an increase in ROS production \( e.g. \) \cite{vanOostrom2006}, a single measure of total antioxidant status gives a limited view of redox status \cite{Bailey2007}. The current study has shown that whilst oxidative stress was increased for up to 5 hours in the control trial, high intensity intermittent exercise attenuated the rise in oxidative stress, as measured by plasma protein carbonyls and TBARS. During the walking trial there was no significant reduction in markers of oxidative stress when compared to the control trial.

Under normal conditions the state of oxidative stress will reduce the amount of bioactive NO, via chemical inactivation to form peroxynitrite, and may also make eNOS dysfunctional, producing \( O_2^- \) rather than NO \cite{Pritchard1995}. These conditions will ultimately result in endothelial dysfunction \cite{Forsterrmann2010} which is an independent predictor of the progression of atherosclerosis and CVD \cite{Schachinger2000}. The generation of a state of oxidative stress has many other effects in processes like intracellular signalling and also the induction of adhesion molecule expression \cite{Nakashima1998}. Previous work in people with type 2 diabetes has demonstrated that a HFM
can result in an increase in circulating levels of the soluble adhesion molecules sICAM-1 and sVCAM-1 and that this increase can be attenuated by the consumption of vitamin E and ascorbic acid (Nappo et al. 2002). However, others have found no rise in both sICAM-1 and sVCAM-1 after a HFM in healthy subjects (Tsai et al. 2004). The current study has demonstrated that whilst sICAM-1 increased throughout the day sVCAM-1 remained constant and that prior exercise has no effect on postprandial sICAM-1 and sVCAM-1 levels. Similar results have been found previously in overweight adolescent boys (MacEneaney et al. 2009), but not to our knowledge in healthy adults.

One limitation of this study is a relatively low number of participants compared to other similar studies. This may explain the differences in postprandial TG results after 30 mins brisk walking between Miyashita, Burns & Stensel (2008) and the current study. Nevertheless, the results from the current study still show a beneficial effect from HIIE and this would suggest that HIIE is more efficacious compared to 30 mins walking in attenuating postprandial TG. Secondly, the current study only used healthy males and it remains to be elucidated whether similar adaptations will
be seen in patients and in other populations. Additionally, this study was powered to
detect differences in TG concentration, this must be taken into account for variables
that the power calculation was not based on, and results (particularly those which
confirm the null hypothesis) must be viewed in this light. Lastly, the blood sampling
technique used in this chapter may affect the inflammatory marker results, as
cannulas may increase the local inflammatory response to a greater degree than a
single-sampling method of venepuncture (Seiler, Muller & Hiemke 1994).

In conclusion, the current study has demonstrated that prior high intensity
intermittent exercise reduces postprandial TG, with no effect of 30 min brisk
walking. This decrease in TG was not associated with an increase in β-
hydroxybutyrate, indicating that this effect is not due to a reduction in hepatic VLDL
secretion. The reduction in postprandial TG was associated with an almost
complete abolition of the postprandial increase in markers of oxidative stress. High
intensity intermittent exercise may therefore be a useful tool in the prevention of
atherosclerosis and reduction in the development of CVD, although further work is
required to confirm this and also the longevity of these responses.
4. The effect of high intensity interval exercise on postprandial triglyceride and leukocyte activation – monitored for 48h post exercise


ACERO Main Meeting, Aberdeen, UK (2013) in an oral presentation entitled “The effect of high intensity interval exercise on postprandial Triglyceride and leukocyte activation” Brendan M. Gabriel
4.1. Introduction

In chapter 3, it was demonstrated that a single bout of HIIE attenuated the rise in postprandial TG levels. What remains to be established is whether this effect persists on the second day after HIIE, information which will have clear implications for the required frequency of such exercise. Furthermore, the mechanisms underlying the reductions in TG after HIIE have yet to be investigated. Potential candidates are either a reduction in hepatic VLDL secretion or an increase in LPL activity. In chapter 3 we have shown that HIIE does not affect plasma β-hydroxybutyrate, a marker of hepatic FA oxidation changes which would shift hepatic FA partitioning and alter VLDL synthesis, making an increase in LPL activity the likely candidate.

Additionally, several studies have shown that TRL activate monocytes (monitored via increased CD11b expression) and to a lesser extent neutrophils (monitored via increased CD11b and CD66b expression) via uptake of TG (Alipour et al. 2008). These findings have recently been extended by Gower et al. (2011) who found that after a HFM monocytes internalise lipid, upregulate CD11c and increase adhesions.
to VCAM-1. This leukocyte activation has several consequences including an increase in pro-inflammatory cytokine production, oxidative stress, adhesion, activation of endothelial cells and ultimately an increase in migration of leukocytes and lipoproteins to the sub endothelium (for review see (Klop et al. 2012). Leukocyte activation can also lead to the uptake of oxLDL by monocyte derived macrophages in the vascular wall, a well-established step in the development of atherosclerosis (Park 2014). The scavenger receptor CD36 has been shown to be central in facilitating monocyte/macrophage uptake of oxLDL (Silverstein, Febbraio 2000, Park 2014), and appears to be linked to the pathology of atherosclerosis (Silverstein, Febbraio 2000, Park 2014). The effect of a HFM on leukocyte CD36 expression, however, remains to be established.
4.2. Aims

The aims of this study were to:

- Determine whether the attenuation in postprandial TG remained 2 days after a bout HIIE
- Determine HIIE’s effects on leukocyte activation
- Determine the mechanisms underlying the reduction in postprandial TG observed after HIIE by measuring LTTH.
4.3. Methods

4.3.1. Subjects and measurements

Eight healthy male participants took part in this study (age; 25±4 years, body fat; 11.7±4.1%, weight; 72.9±15.3 kg, height; 1.77±0.13 m, BMI; 23.4±3.0 kg/m²). All participants were regularly physically active but none were specifically trained. Subjects were recruited and EE and anthropometric measurements were made in the same way as described in chapters 2.1-2.2. Healthy young males were chosen as an accessible and metabolically stable population in which the null hypothesis that HIIE does not alter postprandial TG on day 3 of the trial levels could be confirmed or denied with less confounding factors than other populations. The power calculation for this study was based on the results from chapter 3 with the change in TG AUC after HIIE and showed that 10 subjects would give this study 80% power, with an alpha level of 0.05. Two subjects were discarded from the study due to admissions of changes of diet in the final trial.

4.3.2. Experimental protocol

A randomised cross-over study design was implemented in a randomised control
trial. All subjects completed two 3 day trials; a HIIE trial and a control trial. The order of these trials was randomised with a minimum of 7 days interval between the trials (Figure 22). Two days prior to each trial subjects were asked to refrain from exercising, and the consumption of caffeine and alcohol. Subjects recorded their dietary intake for 24 hours prior to the start of the trial and throughout the 3 days of the trial itself (for the subject’s personal use). Each subject was instructed to then replicate diet for these days in the subsequent trials.
4.3.3. Day one

Subjects arrived at the laboratory at 15.00 hours. Subjects either performed HIIE or control trials in the same manner described in chapter 3.3.2. At approximately 15.30 hours the subjects left the laboratory and for the remainder of the evening they were instructed to rest. From 22.00 hours subjects fasted.

4.3.4. Days two & three

Subjects arrived at the laboratory at 08.45 hours, and rested in a supine position for 15 minutes. A cannula (20G) was inserted in the same manner as chapter 3.3. At
this point the trial started (0 hours, approximately 09.00 hours) and subjects consumed the high-fat breakfast. The time taken to consume the meal was recorded. Further blood samples measurements and meals were taken at the same time points as chapter 3. Procedures considering water and evening meals were also the same as chapter 3. On day 3 subjects entered the laboratory at 08.45 hours after an overnight fast and repeated the procedures for day 1.

The HFM was prepared in the same manner described in chapter 3.3. Assessed together, both breakfast and lunch provided mean ± standard deviation (SD) energy content of 831±164 kcal. The mean macronutrient content of the meal was 51.2±10.8g fat, 68.4±14.4g CHO and 23.3±4.9 g protein. The mean time taken to consume the meal was 10:47±3:19 min:sec for breakfast and 11:12±3:30 min:sec for lunch.

4.3.5. Analytical methods

Blood samples were collected in EDTA vacutainers in the same method as chapter 2.4, blood was additionally collected in 3mL lithium heparin vacutainers (at time points fasting, 2, 5, and 7 hour). The latter were placed on a rotating platform at
4.3.6. *Plasma triglyceride & glucose assays*

All blood sampling and assay protocols for plasma TG and glucose were the same as described in chapter 2.5 and 2.6.

4.3.7. *Statistical analysis*

Statistical analysis and missing data were performed and dealt with in the same manner described in chapter 3.3.5. All data are presented as means±SEM. One subject was discarded from all ex-vivo immune activation results due to technical errors in data collection. There were also 4 missing timepoints for CD36 expression on monocytes and 5 missing timepoints for CD11b expression on granulocytes.

4.4. *Results*

4.4.1. *Power output and energy expenditure*

The average peak power output during HIIE was $870.3 \pm 140.4$W and the average mean power output was $464.3 \pm 119.7$W. This corresponded to an average EE of $93.1 \pm 20.1$ kcal during HIIE.

Brendan Gabriel
4.4.2. Plasma glucose and triglyceride

There were no differences between the trials in plasma glucose or TG concentrations (Figure 23). The data was analysed as AUC for the reasons outlined previously and in order for comparison with chapter 3 results. When comparing TG total AUC using a one-way ANOVA this was lower (P<0.05) on day 2 of HIIE (7.46±0.54mmol/l/7h) compared to the control trial (9.47 ±1.07mmol/l/7h) (Figure 24 and Table 13). There were no differences in total AUC (9.05±0.9226vs 9.36±1.07mmol/l/7h) between HIIE and control groups during day 3 of the trial (Figure 24 and Table 13). Analysis of variation revealed a significant (P<0.05) treatment effect for TG iAUC, however Bonferroni post hoc tests found no differences between HIIE or control trials on either day 2 (2.64±0.15vs 3.87±0.68mmol/l/7h) or day 3 (4.98±0.75vs 4.01±0.67 mmol/l/7h) (Figure 24B and Table 13). There were no differences in fasting values between trials (Table 13).
Figure 23  Plasma TG (A) and glucose (B) concentrations on day 2 and 3 of the HIIE and control trials.
Figure 24 TG total (A) and incremental (B) AUC over the 7 h experimental period of day 2 and 3 of the HIIE and control trials.

(A) * denotes a significant difference ($P < 0.05$) between HIIE and control trials on Day 2. (B) * denotes a significant ($P < 0.05$) treatment/column effect, however Bonferroni post hoc tests found no differences between HIIE or control.
Table 13 TG total and incremental AUC over the 7 h experimental period of day 2 and 3 of the HIIE and control trials.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th></th>
<th>Control</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
<td>Day 3</td>
<td>Day 2</td>
<td>Day 3</td>
</tr>
<tr>
<td><strong>Fasting TG (mmol/L)</strong></td>
<td>0.81±0.08</td>
<td>0.72±0.09</td>
<td>0.69±0.08</td>
<td>0.63±0.06</td>
</tr>
<tr>
<td><strong>Total TG AUC (mmol/l/7h)</strong></td>
<td>9.47±1.07</td>
<td>9.36±1.07</td>
<td>7.46±0.54*</td>
<td>9.05±0.92</td>
</tr>
<tr>
<td><strong>Incremental TG AUC (mmol/l/7h)</strong></td>
<td>3.87±0.68</td>
<td>4.01±0.67</td>
<td>2.64±0.15</td>
<td>4.98±0.75</td>
</tr>
</tbody>
</table>

* denotes a significant difference (P <0.05) from day 2 of the control trial.

4.4.3. *Ex. vivo immune activation*

Analysis of variance revealed CD11b expression increased with time over day 2 and 3 of the study in lymphocyte (P<0.0001), monocyte (P<0.0001) and granulocyte (P<0.0001) populations (Figure 25A). Lymphocyte and monocyte CD36 expression decreased with time over day 2 and 3 (P<0.05) (Figure 25B). There were no differences between trials in the expression of CD36 or CD11b in lymphocyte, monocyte or granulocytes.
Figure 25 CD11b (A) and CD36 (B) mean fluorescence intensity on gated cell populations on day 2 and 3 of the control trials.

(A) *** denotes an effect of time for granulocytes and monocytes ($P<0.001$)
(B) * denotes an effect of time for monocytes ($P<0.005$) and lymphocytes ($P<0.05$).

■ denotes laboratory meal times.
4.4.4. **Oxidative burst**

There was no difference between trials in ROS production in any of the monocyte or granulocyte populations. There were also no time effects (Figure 26).

![Figure 26 Granulocyte (A) and monocyte (B) oxidative burst mean fluorescence intensity on gated cell populations on day 2 and 3 of the control trials. ■ denotes laboratory meal times.](image)

**Figure 26** Granulocyte (A) and monocyte (B) oxidative burst mean fluorescence intensity on gated cell populations on day 2 and 3 of the control trials. ■ denotes laboratory meal times.
4.4.5. Lipoprotein lipase dependant triglyceride rich lipoprotein- triglyceride hydrolysis assay

Analysis of variance revealed interaction and time (P<0.05) effects in LTTH activity, with no group effect (P=0.09) observed. Post hoc tests showed that LTTH activity was higher (P<0.05) at 2 hours on day 2 of HIIE (204.3±31 nmol NEFA/ml.h) compared to control trial (142.7±17.9 nmol NEFA/ml.h) (Figure 27).

Figure 27 LTTH activity on day 2 of the HIIE and control trials.

* denotes a significant (P<0.05) difference between HIIE and control trials. (N=5)
4.5. Discussion

The main finding of the present study was that HIIE attenuated postprandial TG on day 2 of the study compared with a control trial, with this effect abolished by day 3.

The attenuation of postprandial TG the day following HIIE is supported by the recent study by (Freese et al. 2011) and in chapter 3, and the current study extends this finding by demonstrating that this reduction in plasma TG is associated with an increase in LTTH. Prior to the current study the longevity of, or mechanisms underlying, these effects had not been investigated.

The novel finding of the current study, that the beneficial effect of HIIE on postprandial TG is abolished by day 3, suggests that one must engage in HIIE every 1-2 days to attenuate the rise in postprandial TG which is a chronic risk factor for CHD (Nordestgaard et al. 2007). Previous studies have proposed that the effect of endurance exercise on postprandial TG is acute (Herd et al. 1998, Herd et al. 2000, Hardman, Lawrence & Herd 1998), with the current study indicating that HIIE induces an acute effect, in young healthy males. On top of this benefit of HIIE, in reducing postprandial TG, training studies ranging from two to six weeks in duration
have shown that HIIE can improve endurance performance, muscle oxidative capacity (Gibala et al. 2006), insulin action (Babraj et al. 2009) and endothelial function (Rakobowchuk et al. 2008). It is also possible that more chronic improvements in insulin sensitivity, observed after HIIE, would also result in long term reductions in TG levels; however this remains to be investigated. Whilst similar benefits have been found after more prolonged endurance training HIIE may also be beneficial as a public health tool.

The mechanisms responsible for the reduction in postprandial TG concentrations after exercise remain to be elucidated. Previous studies using moderate intensity, continuous exercise have shown an upregulation in LPL activity for up to 18 hours, an observation associated with a fall in serum TG after exercise bouts lasting several hours (Kantor et al. 1987, Sady et al. 1986). Some studies have suggested that there is a threshold EE above which LPL activity is increased following moderate intensity exercise. A study comparing 4 separate bouts of moderate intensity exercise with EEs of 800, 1,100, 1,300 or 1,500 kcal, found that the 800 kcal exercise induced a reduction of postprandial TG, but there was no increase in
LPL activity (Ferguson et al. 1998). On the other hand the 1,100, 1,300 and 1,500 kcal exercise bouts showed a significant increase in LPL activity 24 hours after the bout of exercise, again with a reduction of postprandial TG. Further studies have shown that moderate intensity, continuous exercise (215-1075 kcal EE) reduces postprandial TG but does not induce a statistically significant increase in LPL activity (Miyashita, Tokuyama 2008, Herd et al. 2001, Gill et al. 2003a). However, these studies use a variety of methods for LPL assessment and there is also conflicting evidence regarding this subject (see chapter 1.17-1.18). It has also been proposed that altered hepatic VLDL kinetics also play a role in reducing postprandial TG after moderate intensity exercise (for review see (Peddie, Rehrer & Perry 2012). Previous studies have shown indirect evidence of this effect on hepatic VLDL secretion after moderate intensity exercise (Gill et al. 2001b). Further support of this comes from the same group, where moderate intensity exercise had no effect on TG clearance, monitored after Intralipid infusion (Gill et al. 2001a). On the other hand further study has shown that there was no effect of moderate intensity exercise on hepatic VLDL-TG secretion rate (Magkos et al. 2006, Tsekouras et al. 2007). It seems then, that moderate intensity exercise may attenuate postprandial
TG via increased LPL hydrolysis of TRL, with conflicting evidence as to whether decreased hepatic VLDL-TG kinetics play a major role. However, the mechanisms behind the reductions in postprandial TG after HIIE have not been studied. It is worth noting, at this point, that the current study measures LTTH in pre-heparin LPL which is more likely to reflect physiological processes due to the ability to measure a succession of timepoints rather than studies employing heparin infusion where the majority of the LPL is released after one sample.

There is some evidence in rats that muscle contractile activity is the main regulator of LPL activity (Hamilton et al. 1998). This study also indicated that the intensity of physical activity required to facilitate upregulation of LPL activity may be fibre type specific with LPL activity, following HIIE, increasing only in fast fibres. Additionally, increases in LPL activity seemed to be due to local contractile activity rather than hormonal changes (Bey, Hamilton 2003), although these findings cannot be directly applied to humans. In chapter 3, we found that β-hydroxybutyrate was not changed after HIIE. This would suggest that a change in VLDL secretion rate was not responsible for the reduction in postprandial TG observed after HIIE, although LPL
activity was not measured in the previous study. In the current study, lipolysis in TRLs resulting from LPL bound on their surface was measured using the LTTH assay as previously described (Pruneta-Deloche et al. 2004). LTTH was greater after HIIE at 2 hours of day 2 of the trial, compared with the control group, indicating that the main mechanism through which HIIE reduces postprandial TG is likely to be an increase in TRL bound TG hydrolysis by LPL and subsequent peripheral tissue, most likely skeletal muscle, uptake. This increase in LPL dependent TG hydrolysis could be due to an increase in LPL activity and/or an increased affinity of TRL for LPL. Indeed recent work has indicated that after moderate intensity exercise there may be compositional changes to VLDL1 particles. Additionally, increases in particle size may increase TRL affinity to LPL for clearance from the circulation (Al-Shayji, Caslake & Gill, 2012). Whether such a change occurs after HIIE merits further examination.

If it is the case that LPL activity is increased by HIIE then this is would be somewhat surprising if HIIE is viewed in light of the EE which often elicits increased LPL activation after moderate intensity exercise (>1,100 kcal). The estimated EE of HIIE
in the current study was only 93.1±20.1 kcal. Even allowing for a large error in
estimation or post-exercise oxygen consumption this is well below normally
reported EE for moderate intensity exercise which elicits similar changes. It is,
therefore, possible that LPL activation is fibre type specific in humans, as well as
rats, and that if exercise is of sufficient intensity/duration to recruit fast fibres then
LPL activity will be increased. Further work is clearly required to firstly confirm our
hypothesis that there is an increase in LPL activity after HIIE and secondly to
investigate the potential for this to be a fibre type specific effect.

As many of the deleterious effects of elevated postprandial TG are thought to be
due to leukocyte activation we investigated whether HIIE altered expression of cell
surface markers of leukocyte activation. We found that there was no difference in
any of the markers of leukocyte activation used in the current study between trials,
although effects of time were noted. The rise in CD11b during day 2 and 3 of the
study in monocyte, lymphocyte and granulocyte populations is likely due to the rise
in postprandial TG, as increased TG levels after a HFM have previously been
shown to activate leukocytes and increase CD11b expression (Gower et al. 2011).
This may be caused by endothelial inflammation which is enhanced in response to lipid exposure. The majority of this increase was seen in day 3 and it may be that this more prolonged, compared to a single HFM (i.e. early on day 2), level of exposure to lipids is required for this immune activation to occur. Further work designed to specifically investigate this is needed. To the best of our knowledge, the current study is the first to assess CD36 expression in human leukocytes after a HFM. The current study showed that CD36 expression was decreased in lymphocyte and monocyte populations over day 2 and 3 of the study. This decrease in CD36 surface expression might indicate increased CD36 internalization as Zamora et al. (2012) have shown that CD36 can become internalized in response to a pro-inflammatory environment. Furthermore, it is believed that decreased expression of CD36 in macrophages, induced by various cytokines, is linked to the development of atherosclerosis (Han et al. 2000, Han et al. 2009), possibly due to toll-like receptor pathway activation arising from the increase in cytokines, such as TNF-α. This can occur during atherogenic conditions, often seen after a HFM (Zamora et al. 2012), where CD36 takes up and internalises oxLDL and may eventually lead to foam cell formation in macrophages (Han et al. 2000).
current study found no effect of HIIE on markers of leukocyte activation only a limited number of markers, from a wide number available, were monitored and clearly further work is needed in this area. Indeed a recent study found that moderate intensity exercise blunted the postprandial rise in the markers of leukocyte activation CD11a and CD18 (Strohacker et al. 2012), however this has not been investigated after HIIE. Additionally, this study was powered to detect differences in TG concentration, this must be taken into account for variables that the power calculation was not based on, and results (particularly those which confirm the null hypothesis) must be viewed in this light due to the possibility of a type II error. Lastly, the blood sampling technique used in this chapter may affect the inflammatory marker results, as cannulas may increase the local inflammatory response to a greater degree than a single-sampling method of venepuncture (Seiler, Muller & Hiemke 1994).

In conclusion, the beneficial effects of HIIE on postprandial TG are abolished 2 days after exercise. This suggests that although HIIE may be useful in the prevention of CVD, it will have to be performed 3-4 times a week to be effective. Furthermore,
whilst the current study demonstrates a reduction in postprandial TG after HIIE, this is in young healthy participants. Whilst this exercise may be of benefit in this group in the prevention of future disease there is a need to carry out such studies in populations who are at risk of CVD, such as in obesity or type 2 diabetes. This reduction in postprandial TG after HIIE was associated with an increase in LTTH activity, with no difference in markers of leukocyte activation between trials.
5. Citrate synthase manipulation and skeletal muscle metabolism


NHS Endowment Symposium, Aberdeen, UK (2013) in a poster entitled “Inhibition of citrate synthase impairs fatty acid oxidation in muscle cells” Aivaras Ratkevicius, Brendan M. Gabriel, Stuart R. Gray

5.1. Introduction

There is an increasing amount of evidence suggesting that mitochondria play a key role in metabolic health (Powers et al. 2012). Activity of mitochondrial CS has been used as a biomarker of mitochondrial content and function (Larsen et al. 2012). However, we have recently observed that low CS activity in the skeletal muscles of A/J mice was due neither to reduced mitochondrial content nor to low CS protein content (Ratkevicius et al. 2010, Kilikevicius et al. 2012). We attributed this phenomenon to the missense mutation, in exon 3 of Cs gene of A/J mice, i.e. H55N substitution (A for C, rs29358506). This substitution is unique to A/J mice compared to other mice and animal species, and is predicted to induce a change in amino acid that might affect protein function (Ratkevicius et al. 2010). A study by Johnson et al. (2012) has linked this H55N polymorphism to the accelerated age-related hearing loss in the congenic B6.A-(rs3676616-D10Utsw1)/KjnB6 (B6.A) mice which carry the A/J allele in the telomeric region of chromosome 10 on B6 mouse background (Johnson et al. 2012). These findings suggest that genetic factors regulating CS activity might be of particular importance for functioning of various tissues, including skeletal muscle, and thus warrant research into the associated mechanisms.
In muscle cells, CS is a key enzyme of the mitochondrial Krebs or TCA cycle, it catalyses the reaction between acetyl CoA and oxaloacetate to form citrate (Described in detail in Chapter 1.22-1.29). When mitochondrial substrate flux is near maximal, citrate can be exported out of the mitochondria by the CiC, which mediates its efflux across the mitochondrial membrane in exchange for malate (Sun et al. 2010). In the cytoplasm of adipose and hepatic tissue (tissues with a high degree of lipid synthesis), citrate supports lipid synthesis via conversion to malonyl CoA (the first committed step of lipid synthesis), subsequent inhibition of CPT1 and a reduction in lipid oxidation (for review see (Owen, Kalhan & Hanson 2002). It also blunts glycolysis by inhibition of PFK1 (Blomstrand, Radegran & Saltin 1997; Newsholme, Sugden & Williams 1977) which can indirectly lead to hexokinase inhibition and reduced glucose uptake (Garland, Randle & Newsholme 1963). Therefore, citrate can allosterically suppress both lipid oxidation and glucose oxidation/uptake, but it is still not fully understood which role citrate plays in skeletal muscle under conditions such as during lipotoxicity (described in Chapter 1.24-1.29) or acute high concentrations of palmitate and glucose.
There is some evidence in mice that a decrease in CS activity may decrease the hepatic synthesis of lipids, possibly due to decreased cytosolic citrate and decreased conversion to malonyl CoA (Crumbley et al. 2012). Additionally, there is also evidence in humans that sedentary lean subjects have reduced total CS skeletal muscle activity (~46%) compared to obese, insulin resistant individuals (Ritov et al. 2010), potentially supporting the hypothesis that a reduction of skeletal muscle CS activity could promote FA oxidation and improve resistance to obesity and reduce skeletal muscle insulin resistance, although this is yet to be tested.
5.2. Aims

The aims of this study, therefore, were to assess whether a moderate reduction in CS activity:

- Alters skeletal muscle cell viability or metabolic stress
- Affects glucose and palmitate metabolism in skeletal muscle
- Alters citrate and acetyl CoA metabolism in cells (precursor and product of CS, respectively)
5.3. Methods

Details of methods are presented in the General Methods section (2.15-2.27).

5.3.1. Study design

Firstly, CS activity and protein values were stably decreased in C2C12 cells using shRNA, this was verified by western blot and enzyme activity assays (described in Chapter 2.17 & 2.21). Samples of these cells were frozen and stored at -80°C for future use. The growth rate, viability of these cells and the metabolic stress levels (AMPK/AMPK-P ratio) were also assessed because oxidative phosphorylation is important in maintaining eukaryotic cell growth and viability (Kovácová, Irmlerová & Kovác 1968). These were assessed using a crystal violet assay, an ‘Xcelligence’ cell impedance assay and western blotting, respectively (General Methods). CS-KD2 cells were discontinued as the cell viability was too low to maintain a culture, there was sufficient protein for enzyme activity assays but not for immunoblot in CS-KD2 cells. The protein content of CS-KD and EV in response to lipotoxicity (high palmitate and glucose- 0.8mM and 5mM, respectively- for 24 hours) was measured to assess cell apoptosis in response to lipotoxicity (Henique et al. 2010) (General
Methods). COX activity was measured to assess basal mitochondrial density and during lipotoxic conditions as CS is a marker of mitochondrial density (Larsen et al. 2012) (thus a decrease in CS activity may affect mitochondrial density) and lipotoxic conditions decrease mitochondrial density (Henique et al. 2010). The glucose and palmitate oxidation rate of these cells was then assessed under basal (5mM glucose), acutely high palmitate and glucose (0.8mM and 5mM, respectively) and under high palmitate and glucose (0.8mM and 5mM, respectively) with prolonged incubation (24 hours). These experiments were designed to test cells metabolic responses to conditions traditionally associated with skeletal muscle insulin resistance (Roden 2004, Galgani, Moro & Ravussin 2008). Finally, the intra-cellular concentrations of the main substrate and product of CS were measured to verify that reduced CS activity reduced intracellular concentrations of citrate and whether reduced CS activity reduced acetyl CoA conversion to citrate (detail on experiments in General Methods). A flowchart of the experimental design is presented in Figure 28.
Figure 28 Illustration of timeline of chapter 5 experimental work

*Illustration of experimental work order. Citrate synthase (CS), AMP kinase (AMPK), Phosphorylated AMPK (AMPK-P).*

### 5.3.2. Statistical analysis

All data were expressed as means±SD. Prism version 5 software was used to analyse all data. Data were compared between control and CS-KD treated cells via independent t-tests where there were 2 groups or one-way ANOVA where there were more than 2 groups with post-hoc t-tests. P<0.05 was considered as a statistical significant difference. Three to five independent experiments per condition
were carried out for each outcome.

5.4. Results

5.4.1. Citrate synthase activity and protein expression of short-hairpin ribonucleic transduced cells

Data on CS activity in C2C12 muscle cells transduced with EV, shRNA1 and shRNA2 are presented in Figure 30. CS activity was reduced (P<0.001) by ~48% and ~73% in the cells treated with CS-KD and CS-KD 2, respectively, compared to cells treated with EV. An immunoblot of CS protein was quantified (Figure 31 A) and CS-KD had ~55% lower (P<0.005) band intensity than EV (for illustration of techniques and enzyme pathway see Figure 29 and General Methods 2.16 & 2.21). Stable shRNA transduction was used to reduce activity and protein concentration of citrate synthase in CS-KD cells. Control cells had empty vector shRNA added (Method described in section 2.16).
Red dotted circle illustrates the use of stable shRNA transduction to reduce activity and protein concentration of citrate synthase in CS-KD cells. Control cells had empty vector shRNA added. Abbreviations are as follows in order as they appear on the diagram from top-bottom and left-right: Glucose-6-P (glucose-6-Phosphate), Fructose-6-P (fructose-6-Phosphate), Fructose 1,6-BP (Fructose 1,6-Bi-phosphate), PC (Pyruvate Carrier), PDH (pyruvate Dehydrogenase), MCD (malonyl CoA carboxylase), ACC (Acetyl CoA Carboxylase), CD36 (cluster of differentiation 36), ACL (ATP-Citrate Lyase), LCF (Long-chain Fatty), CPT1 (Carnitine palmitoyltransferase 1) β-Ox (β-Oxidation), CS (citrate synthase), ACN (Aconitase), ICD (Isocitrate-dehydrogenase), α-KD (α-Ketoglutarate Dehydrogenase), SDS (Succinyl CoA synthetase), SDH (Succinate Dehydrogenase) FMR (Fumarase), MDH (Malate Dehydrogenase).
Figure 30 CS activity in cells transduced with EV, shRNA1 and shRNA2.

* denotes difference (P<0.05) between CS-KD and CS-KD 2. *** (P<0.001) EV compared to CS-KD and CS-KD 2. Values are units/g protein. N=3 for each group in duplicate.
Figure 31: CS protein expression in cells transduced with EV and shRNA1

(A) Quantified immunoblot of CS protein, and (B) representative image of immunoblot. (A) ** denotes difference $P<0.005$ CS-KD compared to EV. Values are arbitrary units measuring band intensity. $N=3$ for each group in duplicate. CS-KD2 cells did not have sufficient protein levels to assess CS protein via immunoblot.
Many CS-KD 2 batches were extremely slow growing (as demonstrated by Figure 32) or did not survive at all. Therefore, experiments on CS-KD 2 were discontinued.

Figure 33 demonstrates consistent differentiation of EV and CS-KD into myotubes.

Figure 32 EV, CS-KD and CS-KD 2 (respectively) after 3 days PM (4x)
Images of shRNA transduced cells after 3 days of PM. Images are 4x magnified.

Figure 33: EV and CS-KD, respectively after 6 days DM (4x)
Images of shRNA transduced cells after 6 days of DM. Images are 4x magnified.
5.4.2. Cell impedance assay

A two-way ANOVA revealed CS-KD had a growth curve with a greater magnitude (P<0.001) than EV (Figure 34). This indicated a faster proliferation rate. The faster proliferation rate of CS-KD compared to EV was separately confirmed by crystal violet assay (Figure 35).

![Cell Impedance Assay](image)

**Figure 34** Cell impedance assay

*Values are plotted as means of N=4. *** denotes difference between CS-KD and EV (P<0.001) from each other. Error bars are not shown for clarity of graph. Medium was changed at 11:00 hr each day, indicated by the day markers.*

Although there were differences in growth rates of shRNA treated cells, when the data were split into a growth phase and a differentiated phase (after day 4) there was no difference between CS-KD and EV in a 1-way ANOVA of the raw data (Figure
34). The data were split in this way in order to assess differentiated myotubes rather than myoblasts. There were also no apparent visual differences between EV and CS-KD, as demonstrated by Figure 33. Therefore, for experimental purposes when comparing EV and CS-KD, cells were used that had been treated with DM for 5 days.
5.4.3. *Crystal violet cell proliferation assay*

CS-KD had an increased (P<0.001) number of cells at 48 hours compared to EV, indicating faster proliferation at this timepoint. There was no difference at either 24 hours or 72 hours between groups (Figure 35).

![Crystal violet proliferation assay](image)

**Figure 35 Crystal violet proliferation assay**

*Values are plotted as means±SD, N=6 (with 10 reps of counting) for each group. Each field was 20x magnified, 10 fields were counted per well. CS-KD had increased *** proliferation at 48 hours (P<0.001) compared to EV. Values are numbers of cells field.*
5.4.4. Markers of energy stress

In spite of this significant impairment in CS activity, phosphorylation of AMPK at threonine 172 (phospho-AMPK) did not change, indicating that CS-KD cells were not experiencing energy stress at basal (5mM glucose) conditions. There was also no difference between the groups of total AMPK protein levels (Figure 36). Statistical power to detect a difference in the ratio of PAMPK/AMPK between EV and CS-KD was retrospectively calculated as 91.5% at an alpha level of 0.05.
Figure 36: P AMPK and AMPK protein expression in cells transduced with EV and CS-KD

(A) Quantified immunoblot of the ratio of P AMPK/AMPK protein, and (B) representative image of immunoblot for P AMPK and total AMPK with β-actin as a loading control. Values are arbitrary units measuring band intensity. N=3 for each group in duplicate. Statistical power was retrospectively calculated as 91.5% at an alpha level of 0.05.
5.4.5. *Protein concentration in response to lipotoxicity*

EV cells incubated in 5mM glucose had higher (P<0.001) protein concentration than EV and CS-KD cells after 24 hours incubation with 0.8mM palmitate and 5mM glucose (Figure 38), indicating protein degradation in response to lipotoxicity (Figure 37). Bradford assay (Noble 2014) was used to determine protein content as previously described (2.10). Protein and mitochondrial degradation in response to prolonged lipid and glucose cellular overload is a known phenomenon, as previously described (see section 1.22 & 1.23).
Figure 37 Metabolic pathway diagram – illustration of experimental design – lipotoxicity

Illustration of protein and mitochondrial degradation in response to prolonged lipid and glucose cellular overload. Abbreviations are as follows in the order that they appear on the diagram from top-bottom and left-right: Glucose-6-P (glucose-6-Phosphate), Fructose-6-P (fructose-6-Phosphate), Fructose 1,6-BP (Fructose 1,6-Bi-phosphate), PC (Pyruvate Carrier), PDH (pyruvate Dehydrogenase), MCD (malonyl CoA carboxylase), ACC (Acetyl CoA Carboxylase), CD36 (cluster of differentiation 36), ACL (ATP-Citrate Lyase), LCF (Long-chain Fatty), CPT1 (Carnitine palmitoyltransferase 1) β-Ox (β-Oxidation), CS (citrate synthase), ACN (Aconitase), ICD (Isocitrate dehydrogenase), α-KD (α-Ketoglutarate Dehydrogenase), SDS (Sucononyl CoA synthetase), SDH (Succinate Dehydrogenase) FMR (Fumarase), MDH (Malate Dehydrogenase).
5.4.6. Cytochrome C oxidase activity

COX activity was measured in EV and CS-KD cells, in basal glucose conditions and also in media with 5mM glucose with 0.8mM palmitate, as a marker of mitochondrial density (Larsen et al. 2012) (Figure 39). Activity was the same in both EV and CS-KD cells within both conditions. Treatment with 0.8mM palmitate and 5mM glucose for 24 hours reduced activity in both cell types (P<0.05), indicating reduced mitochondrial content under lipotoxic conditions (illustrated by Figure 37 and described in 1.22-1.24).
Figure 39 COX activity in cells treated with EV, CS-KD and also in response to 24 hour 0.8mM palmitate incubation.

*denotes (P<0.05) difference between EV incubated with 5mM glucose for 1 hour and EV incubated with 5mM and 0.8mM palmitate for 24 hours and CS-KD incubated with 5mM glucose for 1 hour and CS-KD incubated with 5mM and 0.8mM palmitate for 24 hours. Values are μmol/min/mg protein. N=3 for each group in duplicate.
5.4.7. Glucose oxidation and incorporation

EV and CS-KD treated cells were used in a glucose oxidation assay (Figure 40). Cells were also incubated with 5mM glucose and 0.8mM palmitate (mixed substrate media) during the assay to assess glucose oxidation under these conditions. There was a trend (P=0.0575) for CS-KD to have increased glucose oxidation after 24 hours incubation with mixed substrate media compared to EV. There was no difference between CS-KD and EV glucose incorporation under 5mM glucose conditions. CS-KD had increased (P<0.05) glucose incorporation over EV when both were incubated with 5mM glucose and 0.8mM palmitate (Figure 41) and under the same conditions with 24 hours incubation (P<0.05). CS-KD had increased (P<0.05) glucose incorporation over EV when both were incubated with 5mM glucose and 0.8mM palmitate (Figure 41) and under the same conditions with 24 hours incubation (P<0.05). Overall, C0₂ production from glucose-fed Krebs cycle and glucose incorporation into the cell was measured (described in section 2.23 and illustrated in Figure 40).
Figure 40 Metabolic pathway diagram – illustration of experimental design – glucose oxidation

Illustration of pathways being assessed, red dotted circle covers: C0₂ production from glucose-fed Krebs cycle and glucose incorporation into the cell. Abbreviations are as follows in order as they appear on the diagram from top-bottom and left-right: Glucose-6-P (glucose-6-Phosphate), Fructose-6-P (fructose-6-Phosphate), Fructose 1,6-BP (Fructose 1,6-Bi-phosphate), PC (Pyruvate Carrier), PDH (pyruvate Dehydrogenase), MCD (malonyl CoA carboxylase), ACC (Acetyl CoA Carboxylase), CD36 (cluster of differentiation 36), ACL (ATP-Citrate Lyase), LCF (Long-chain Fatty), CPT1 (Carnitine palmitoyltransferase 1) β-Ox (β-Oxidation), CS (citrate synthase), ACN (Aconitase), ICD (Isocitrate-dehydrogenase), α-KD (α-Ketoglutarate Dehydrogenase), SDS (Succonyl CoA synthetase), SDH (Succinate Dehydrogenase) FMR (Fumarase), MDH (Malate Dehydrogenase).
Figure 41 Oxidation and incorporation assay of [14C]glucose radio-labelled substrate.

Values are % fold change from baseline (EV 5mM glucose). N=3 for each group in duplicate. There was a trend P=0.0575 for CS-KD to have increased glucose oxidation over EV when incubated with 5mM glucose and 0.8mM palmitate for 24 hours. * denotes P<0.05 difference between EV and CS-KD.
5.4.8. Palmitate oxidation and incorporation

EV and CS-KD treated cells were used in a palmitate incorporation and oxidation assay (Figure 43) with 0.8mM palmitate assay buffer (Figure 42). In a second experiment, cells were also incubated with 5mM glucose in addition to the 0.8mM palmitate (mixed substrate media) during the assay to assess palmitate incorporation and oxidation under these conditions. EV had increased (P<0.05) palmitate oxidation over CS-KD in mixed substrate media, when incubated for 2 hours. Cells were also incubated with 0.8mM palmitate and 5mM glucose for 24 hours and assessed with a mixed substrate assay buffer. EV cells had increased (P<0.05) oxidation over CS-KD cells after 24 hours incubation in mixed substrate media (oxidation was assessed over a 2 hour period). There were no differences in incorporation. Overall, CO₂ production from palmitate-fed Krebs cycle and glucose incorporation into the cell was measured (described in section 2.24 and illustrated in Figure 42).
Figure 42 Metabolic pathway diagram – illustration of experimental design – palmitate oxidation

Illustration of pathways being assessed, red dotted circle covers: CO₂ production from palmitate-fed Krebs cycle and palmitate incorporation into the cell. Abbreviations are as follows in order as they appear on the diagram from top-bottom and left-right: Glucose-6-P (glucose-6-Phosphate), Fructose-6-P (fructose-6-Phosphate), Fructose 1,6-BP (Fructose 1,6-Bi-phosphate), PC (Pyruvate Carrier), PDH (pyruvate Dehydrogenase), MCD (malonyl CoA carboxylase), ACC (Acetyl CoA Carboxylase), CD36 (cluster of differentiation 36), ACL (ATP-Citrate Lyase), LCF (Long-chain Fatty), CPT1 (Carnitine palmitoyltransferase 1) β-Ox (β-Oxidation), CS (citrate synthase), ACN (Aconitase), ICD (Isocitrate-dehydrogenase), α-KD (α-Ketoglutarate Dehydrogenase), SDS (Succonyl CoA synthetase), SDH (Succinate Dehydrogenase) FMR (Fumarase), MDH (Malate Dehydrogenase).
Figure 43 Oxidation and incorporation assay of [14C]palmitate radio-labelled substrate.

Values are pmol/mg/min. N=3 for each group in duplicate. ** denotes \( P<0.005 \) between CS-KD and EV incubated with 5mM glucose and 0.8mM palmitate for 24 hours. *** denotes \( P<0.001 \) difference between CS-KD and EV incubated with 5mM glucose and 0.8mM palmitate.
5.4.9. *Intracellular citrate concentration*

Citrate concentration was measured in cells treated with EV and shRNA1 under 4 conditions: 5mM glucose, 5mM glucose and 0.8mM palmitate, 0.8mM palmitate and a 24 hour incubation with 5mM glucose and 0.8mM palmitate. 5mM glucose was taken to represent basal conditions, whereas palmitate acutely increases mitochondrial oxygen consumption rate (Hirabara *et al.* 2006), resulting in increased mitochondrial flux and increased CS activity. Results of these measurements are presented in Figure 45. There was no difference between CS-KD and EV during basal conditions. There was a trend (P=0.06) for an increased citrate concentration when cells were treated with glucose and palmitate acutely (1hr). When EV cells were acutely (2hr) treated with palmitate they had increased (P<0.005) citrate concentration over palmitate treated CS-KD cells, this was also the case when cells were treated with palmitate and glucose over 24 hours. Citrate was tested as it is the product of CS (illustrated in Figure 44, described in section 1.29).
**Figure 44** Metabolic pathway diagram – illustration of experimental design

*Red circles illustrates citrate being measured, in whole cell lysates. Abbreviations are as follows in order as they appear on the diagram from top-bottom and left-right: Glucose-6-P (glucose-6-Phosphate), Fructose-6-P (fructose-6-Phosphate), Fructose 1,6-BP (Fructose 1,6-Bi-phosphate), PC (Pyruvate Carrier), PDH (pyruvate Dehydrogenase), MCD (malonyl CoA carboxylase), ACC (Acetyl CoA Carboxylase), CD36 (cluster of differentiation 36), ACL (ATP-Citrate Lyase), LCF (Long-chain Fatty), CPT1 (Carnitine palmitoyltransferase 1) β-Ox (β-Oxidation), CS (citrate synthase), ACN (Aconitase), ICD (Isocitrate-dehydrogenase), α-KD (α-Ketoglutarate Dehydrogenase), SDS (Succonyl CoA synthetase), SDH (Succinate Dehydrogenase) FMR (Fumarase), MDH (Malate Dehydrogenase).*
Figure 45 Citrate concentrations in cells transduced with EV, CS-KD and also in response to 0.8mM palmitate incubation.

* denotes (P<0.05) difference between CS-KD + EV incubated for 24 hour in 0.8mM palmitate and glucose and between CS-KD and EV when incubated with 0.8mM palmitate for 2 hours. Values are ng/mg. N=3 for each group in duplicate.

5.4.10. Intracellular acetyl CoA concentration

Cells were treated with EV or CS-KD and intra-cellular acetyl CoA was assessed after basal conditions (5mM glucose) or 24 hour palmitate 0.8mM and 5mM glucose incubation (Figure 47). These conditions were chosen as they represent basal levels of citrate and maximal stimulation of citrate production as demonstrated in
Figure 45. High levels of citrate would likely indicate conditions which induced near maximal CS activity and therefore acetyl CoA levels may be altered as it is a substrate of CS (illustrated in Figure 46 and described in section 1.29). CS-KD with glucose and palmitate 24 hour incubation had higher (P<0.001) acetyl CoA than CS-KD basal. The fold change of acetyl CoA from acute (2 hr) glucose to 24 hour palmitate and glucose conditions was higher (P<0.05) in CS-KD compared to EV cells.
Figure 46 Metabolic pathway diagram – illustration of experimental design – acetyl CoA

Red circles illustrates acetyl CoA being measured, in whole cell lysates. Abbreviations are as follows in order as they appear on the diagram from top-bottom and left-right: Glucose-6-P (glucose-6-Phosphate), Fructose-6-P (fructose-6-Phosphate), Fructose 1,6-BP (Fructose 1,6-Bi-phosphate), PC (Pyruvate Carrier), PDH (pyruvate Dehydrogenase), MCD (malonyl CoA carboxylase), ACC (Acetyl CoA Carboxylase), CD36 (cluster of differentiation 36), ACL (ATP-Citrate Lyase), LCF (Long-chain Fatty), CPT1 (Carnitine palmitoyltransferase 1) β-Ox (β-Oxidation), CS (citrate synthase), ACN (Aconitase), ICD (Isocitrate-dehydrogenase), α-KD (α-Ketoglutarate Dehydrogenase), SDS (Succonyl CoA synthetase), SDH (Succinate Dehydrogenase) FMR (Fumarase), MDH (Malate Dehydrogenase).
Figure 47 Intra-cellular acetyl CoA concentrations in cells treated with EV, CS-KD and also in response to 24 hour 0.8mM palmitate incubation.

*** denotes (P<0.001) difference between CS-KD basal and CS-KD incubated with 0.8mM palmitate and 5mM glucose for 24 hours. * denotes an increase of CS-KD % fold change from basal (5mM glucose conditions) over EV (P<0.05). Values are pmol/µg. N=3 for each group in duplicate.
5.5. Discussion

The novel findings from this study were that a moderate knockdown of CS activity reduced FA oxidation compared to control cells when cells were exposed to high levels of glucose and high levels of palmitate. The knockdown of CS did not affect energy stress of C2C12 cells measured by the ratio of phospho-AMPK to total AMPK protein, or mitochondrial density. Additionally, the data indicated that the reduced palmitate oxidation in CS-KD cells corresponded with decreased intracellular citrate concentrations and possibly decreased clearance of acetyl CoA compared to EV cells.

Defective skeletal muscle cell metabolism is seen as a major contributor to the pathology of obesity and insulin resistance, particularly the inability to upregulate lipid oxidation in response to a high glucose and palmitate substrate load (Galgani, Moro & Ravussin 2008). It was not known what affect reducing CS activity would have on the metabolism of C2C12 myotubes. We hypothesised that a reduction of CS activity would reduce citrate efflux from the mitochondria, thereby reducing its conversion to acetyl CoA and subsequently to malonyl CoA. As malonyl CoA is a
potent inhibitor of CPT1 (Hue & Taegtmeyer 2009), which is responsible for long-chain FA transport into the mitochondria, we hypothesised that a moderate reduction of CS would promote FA oxidation.

Firstly, however, as oxidative phosphorylation is important in maintaining eukaryotic cell growth and viability (Kovácová, Irmlerová & Kovác 1968) the effect of a moderate shRNA induced CS knockdown on cell viability and metabolism was assessed. Two constructs were used, CS-KD and CS-KD 2. The CS enzyme activity assay showed that both constructs significantly reduced CS activity compared to EV (CS-KD ~45%, CS-KD 2 ~ 72% reduction). CS-KD shRNA induced CS activity reduction of ~45% was similar to the ~46% reduction seen in sedentary, lean humans when compared to obese, insulin resistant individuals (Ritov et al. 2010). CS-KD transduced cells were always viable (Figure 32) and displayed no metabolic stress under basal (5mM glucose) conditions (Figure 41). CS-KD2 transductions were discontinued due to the difficulty in producing viable cells, and the fact that CS-KD served as a better model to study the CS knockdown. It may be that the greater CS knockdown in these experiments put cells near the threshold of
the required CS activity for survival. Cells were assessed at the myotube phase because the cell index assay revealed no differences between any of the cell lines at 5-days differentiation (Figure 34), therefore cells could be compared for markers of metabolism without differential growth rates affecting the results. CS-KD cells did have a faster proliferation rate then EV. The reason for this is unclear but silenced CS has previously been directly linked with increased glycolytic activity, proliferation rate and tumour growth of cancer cells (Lin et al. 2012).

Normally, muscle cells switch substrate according to fuel availability; in particular an inability to upregulate lipid oxidation in the face of high lipid and glucose conditions is thought to be key in the pathogenesis of insulin resistance (Galgani, Moro & Ravussin 2008). CS-KD cells had 42.1% reduced palmitate oxidation compared to EV cells while exposed to 5mM glucose and 0.8mM palmitate conditions. This difference between CS-KD and EV was also present after 24 hour incubation in these conditions. Both EV and CS-KD cells showed reduced ability to oxidise palmitate when incubated for 24 hours with glucose and palmitate compared to cells acutely (2 hr) incubated in the same conditions. Along with protein degradation
these results are common in lipotoxic conditions with similar findings in previous work (Pimenta et al. 2008). Despite the difference in palmitate oxidation when glucose and palmitate were present, CS-KD cells still had normal capacity to oxidise palmitate when glucose was absent from the media (Figure 43) compared to EV, indicating that mitochondrial capacity was not reduced under these conditions. This suggests that any differences in oxidation between the cell types were due to changes in Randle cycle regulation in response to mixed substrate conditions rather than a decrease in intrinsic mitochondrial capacity in CS-KD cells. Additionally, CS-KD cells had normal glucose oxidation when presented with 0.8mM palmitate compared to EV and COX activity was similar between EV and CS-KD, suggesting that overall mitochondrial density was unchanged by the CS-KD phenotype. Although COX is a good measure of mitochondrial density, cardiolipin is thought to be a better indicator (Larsen et al. 2012). Mitochondrial degradation in lipotoxicity was not tested directly, although COX activity was similarly decreased in both CS-KD and EV (Figure 39), additionally protein concentration was reduced to the same extent in both cell types after incubation with palmitate and glucose for 24 hours (P<0.001) (Figure 38). This indicates that increased FA oxidation did not protect the
EV cells from mitochondrial degradation or cell apoptosis compared to CS-KD cells, as has been shown in other studies (Henique et al. 2010) the reason for this is unknown.

Considering the lower palmitate oxidation displayed by CS-KD under mixed substrate conditions, and the fact that this often precedes insulin resistance (Galgani, Moro & Ravussin 2008), CS-KD appear to display a pathogenic phenotype. Although it is unclear how this negative effect of lowering CS activity relates to humans, there is evidence that type 2 diabetic patients have an intrinsic defect in CS (Ortenblad et al. 2005). However, in this study reduced CS did not affect the maximal oxidative ability of the myotubes from patients with type 2 diabetes (Ortenblad et al. 2005). Both of these findings are similar to the current study, providing possible evidence that our cell model is somewhat similar to a diabetic pathogenic phenotype.

Therefore it appears that under conditions which induce a change in mitochondrial flux either through high concentrations of glucose and palmitate or under lipotoxic conditions, a reduction of ~45% CS gives C2C12 muscle cells a reduced ability to
oxidise palmitate when glucose is also present. In both conditions (24 hour glucose and palmitate and 2hr glucose and palmitate) in which EV palmitate oxidation was higher than CS-KD, intracellular citrate was also increased or had a trend to be increased (P=0.06) (respectively) in EV compared to CS-KD. We hypothesised that lower levels of cytosolic citrate may increase FA oxidation via reduced inhibition of CPT1, however this does not appear to be the case in this situation. It must be noted that our values of intracellular citrate do not necessarily equate to cytosolic citrate. Also, CS is the initial step in regulating the mitochondrial efflux of citrate. It may be that in EV cells citrate metabolism is regulated at other steps, such as the ratio of the activities of ACC and MCD, which are central in controlling the rate conversion of acetyl CoA to malonyl CoA (ACC), and the reverse decarboxylation of malonyl CoA to acetyl CoA (MCD) thus controlling the amount of CPT1 inhibition (Hue & Taegtmeyer 2009). The activity of these enzymes is strictly controlled (Goodwin & Taegtmeyer 2000), and may explain why a decrease in intracellular citrate did not apparently decrease CPT1 inhibition and promote FA oxidation in CS-KD cells (Figure 48). However, since EV cells have higher intracellular citrate levels (compared to CS-KD cells), and citrate wasn’t apparently being routed to
malonyl CoA conversion (since there was no decrease in palmitate oxidation), it might be expected that EV cells would have lower levels of glucose oxidation compared to CS-KD due to citrates allosteric inhibition of PFK1 (Hue & Taegtmeyer 2009). However, this was not the case, except after 24 hours incubation with palmitate and glucose where CS-KD cells had a trend for higher glucose oxidation compared to EV (P=0.0575), glucose oxidation levels were similar between the two cell types. It is possible that CS-KD cells have increased accumulation of mitochondrial acetyl CoA due to the decreased activity of CS, as CS converts acetyl CoA and oxaloacetate into citrate (Wiegand, Remington 1986). Acetyl CoA inhibits PDH (Hue & Taegtmeyer 2009) and would potentially reduce the amount of glucose oxidation in CS-KD cells. Therefore, although there might be less allosteric modulation of PFK1 by likely reduced cytosolic citrate levels, glucose oxidation levels do not appear to be increased in CS-KD cells which may be potentially due to increased mitochondrial acetyl CoA concentrations (Figure 48). Although, we did not test mitochondrial concentrations of acetyl CoA directly, intracellular concentrations of acetyl CoA appear to accumulate faster in CS-KD cells compared to EV. This finding provides support for this hypothesis, however further work is
needed to confirm this. It is unclear why FA oxidation levels were reduced in CS-KD cells when incubated with glucose and palmitate. This may also be due to increased mitochondrial acetyl CoA levels. The conversion of ketoacyl CoA to acyl CoA catalysed by the enzyme medium-chain ketoacyl-CoA thiolase (MCKAT) is an essential step in β-oxidation (demonstrated in Equation 5). MCKAT activity is inhibited by an increased acetyl CoA:CoASH ratio via feedback inhibition (Schulz 1994), thus an increased accumulation of acetyl CoA in CS-KD in glucose and palmitate media cells may be partly behind the reduction of FA oxidation in the same media (Figure 48).

\[
\text{Ketoacyl CoA} + \text{CoASH} \rightleftharpoons \text{Acetyl CoA} + \text{Acyl CoA}
\]

Equation 5 Reaction of β-oxidation catalysed by MCKAT

In summary, a moderate knockdown of CS activity reduced FA oxidation compared to control cells when cells were exposed to glucose and palmitate. The knockdown of CS did not affect energy stress of C2C12 cells measured by the ratio of P-AMPK to total AMPK protein or appear to affect intrinsic mitochondrial density. Additionally, there is some evidence that the decreased palmitate oxidation with no
corresponding increase in glucose oxidation in CS-KD cells is mediated by reduced clearance of acetyl CoA compared to EV cells. These findings suggest a pathological mechanism by which a decrease in CS activity may be partly behind the development of insulin resistance in skeletal muscle.
Figure 48 A depiction of potential allosteric sites of modulation of enzymes involved in cellular metabolism.

Abbreviations are as follows in order as they appear on the diagram from top-bottom and left-right: Glucose-6-P (glucose-6-Phosphate), Fructose-6-P (fructose-6-Phosphate), Fructose 1,6-BP (Fructose 1,6-Bi-phosphate), PC (Pyruvate Carrier), PDH (pyruvate Dehydrogenase), MCD (malonyl CoA carboxylase), ACC (Acetyl CoA Carboxylase), ACL (ATP-Citrate Lyase), CD36 (cluster of differentiation 36), PDH (Pyruvate Carrier), β-Ox (β-Oxidation), CS (citrate synthase), ACN (Aconitase), ICD (Isocitrate-dehydrogenase), α-KD (α-Ketoglutarate Dehydrogenase), SDS (Succonyl CoA synthetase), SDH (Succinate Dehydrogenase) FMR (Fumarase), MDH (Malate Dehydrogenase).
6. General Discussion
This thesis has demonstrated that postprandial TG is attenuated after a bout of HIIE for up to 18 hours, with this effect abolished on the 2nd day after HIIE. This finding should be taken into account when recommending exercise regimes with it being necessary for HIIE to be performed 3-4 times a week in order to maintain the beneficial effect on postprandial TG. The mechanism of this reduction in postprandial TG appears to be an increased LPL activity or/and an increase in lipoprotein affinity for LPL, rather than reduced hepatic VLDL secretion. Postprandial TG levels are an independent risk factor for CVD (Nordestgaard et al. 2007, Bansal et al. 2007, Eberly, Stamler & Neaton 2003) and the subsequent risk for CVD may be reduced with HIIE. However, further long term work is needed to assess whether regular HIIE reduces the risk of CVD. The current thesis also demonstrated that a bout of HIIE reduces the rise in oxidative stress seen after HFMs, however it does not affect markers of leukocyte activation or soluble adhesion molecules.

In a separate research direction we assessed the effects of reducing citrate synthase activity in skeletal muscle on lipid oxidation. The results of this thesis demonstrated that a moderate (~45%) reduction in CS reduces fatty acid oxidation in C2C12 cells
when incubated with high concentrations of glucose and palmitate (5mM and 0.8mM, respectively). This may be mediated by reduced citrate efflux from the mitochondria and potential accumulation of mitochondrial acetyl CoA. Also a reduction in CS of ~45% did not appear to affect mitochondrial density or the ability to oxidise palmitate when glucose was absent from the media. Therefore, the effect appears to be a consequence of altered substrate preference rather than reduced oxidative capacity. We hypothesised that a moderate reduction of CS would increase lipid oxidation, however this was not the case, and these cells actually had similarities to ex vivo skeletal muscle cells from type 2 diabetic patients such as decreased CS activity and unaltered maximal oxidative capacity (Ortenblad et al. 2005).

The first two aims of this thesis were to determine whether a session of HIIE attenuates postprandial TG and to determine the longevity of any response, and to identify potential mechanisms responsible for HIIE’s increased disposal of plasma TG, by measuring plasma β-hydroxybutyrate concentrations and TRL-LPL lipolysis levels. HIIE’s effect on postprandial lipaemia has now been verified by several studies in this area which are summarised in Table 5. Including 56 subjects over 6
separate effect outcomes, this meta-analysis demonstrated that prior HIIE consistently attenuates postprandial lipaemia, both measured by iAUC and AUC (Freese, Gist & Cureton 2013). HIIE may even be more effective than moderate intensity exercise at attenuating postprandial lipaemia as Freese et al. 2013 found that HIIE’s effect on iAUC was greater (P<0.05) than moderate intensity exercise. The mechanism of HIIE’s attenuation of postprandial TG has not been furthered studied since the completion of chapter 2 and 3, although the mechanism may prove to be different to moderate intensity exercise, as the EE of HIIE is far lower despite the similar if not greater effect size (Freese, Gist & Cureton 2013).

More long-term studies need to be carried out before HIIT is recommended to all populations, although there is now substantial evidence of its acute and short-term training benefits in healthy, young populations (Freese, Gist & Cureton 2013, Gibala et al. 2012). The ACSM recommends that a base-level of fitness is achieved before embarking on an exercise regime which includes HIIT (Kravitz 2014), however the long-term benefits/risks of HIIT have not been studied. Thus future work, could involve 6-12 month training studies in a variety of populations and assess various...
health criteria such as insulin sensitivity, postprandial lipaemia, adipose tissue mass, 
\( \dot{V}O_{2\text{max}} \), LDL-C, total cholesterol, HDL-C et al., additionally, epidemiological studies 
could assess the disease and mortality outcomes of cohorts that engage in regular 
HIIT. Although previous studies had only assessed HIIT in healthy, young 
populations, recent studies have demonstrated that HIIT may be an effective 
exercise protocol for elderly and middle-aged populations (Adamson et al. 2014b, 
Adamson et al. 2014a). The former of these studies recruited middle-aged (43±8 
years), untrained subjects to either a control or a HIIT 8-week training protocol (10 x 
6-second sprints interspersed with a one-minute recovery – twice a week). The 
results of training were that HIIT improved aerobic capacity (P<0.001), physical 
function (P<0.05, demonstrated by 3 functional walking or sit to stand tests) and 
reduced blood glucose AUC (P<0.05) (Adamson et al. 2014a). The latter of these 
studies recruited elderly adults, who were randomly assigned to either a HIIT trial (6- 
10 6-second sprints, 1 min recovery) or a control trial (HIIT: N=7, 64±2 years old, 
Control: N=6, 65±4 years old). The HIIT training reduced systolic BP (9%, P<0.05), 
increased \( \dot{V}O_{2\text{max}} \) (8%, P<0.05) and the functional walking test time (11%, P<0.05) 
(Adamson et al. 2014b). Thus, HIIT may be a suitable and effective method of 

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training for populations other than healthy, young individuals; however, further studies are needed to confirm this. Therefore, at present the current exercise guidelines recommendations are correct in their recommendation of engaging in regular moderate intensity exercise and higher intensity exercise [while the recommendations of ‘high intensity exercise’ are generally classed as 6-13.5 METs for healthy individuals (O’Donovan et al. 2010), the HIIT protocol used in this study would likely be >13.5 (Cochran et al. 2014)], while HIIT has become one of a suite of options for allowing healthy individuals to become more active.

The third aim of this thesis was to determine if a moderate reduction of CS activity improves glucose and FA metabolism of cultured murine C2C12 cells. As discussed previously, a reduction of CS appeared to reduce metabolic flexibility of cells, which is a hallmark of insulin resistance (Galgani, Moro & Ravussin 2008). It seems that citrate synthase acts as somewhat of a rate-limiting enzyme in mitochondrial oxidation in skeletal muscle. This is supported by a recent meta-analysis (Vigelso, Andersen & Dela 2014) which analysed 70 publications with 97 intervention groups looking at training induced changes in skeletal muscle CS activity and \( \dot{V}O_{2\text{max}} \), in

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human subjects. The study found that there was a positive correlation ($r=0.45$, $P<0.001$) between the relative (training induced) change in $\dot{V}O_2\text{max}$ and CS activity. Thus, CS activity in skeletal muscle appears to be key to exercise induced increase in oxidative capacity (Vigelso, Andersen & Dela 2014). This meta-analysis (Vigelso, Andersen & Dela 2014) also included studies using diabetic subjects which also demonstrated that CS was increased to the same extent (~80%, $p<0.001$) in both a group with type 2 diabetes and a control group (n=7 and 6, respectively) in response to 8 weeks aerobic training and the training-induced increase in $\dot{V}O_2\text{max}$ (~20%, $p<0.01$) was similar in both groups (Bruce et al. 2004). This is supported by other studies included in the meta-analysis (Vigelso, Andersen & Dela 2014), demonstrating that diabetics (Harmer et al. 2008, Daugaard et al. 2000, Allenberg, Johansen & Saltin 1988, Mogensen et al. 2009) and the offspring of diabetics (Irving et al. 2011) also show similar improvements in CS and $\dot{V}O_2\text{max}$ in response to training programs.

Additionally, there is evidence that subjects with type 2 diabetes may have decreased CS compared to other mitochondrial enzymes in skeletal muscle
(Ortenblad et al. 2005) (discussed in chapter 5.5). It is unclear whether this is a
 genetic, epigenetic or environment mediated decrease. As discussed previously,
 exercise training appears to improve CS activity in subjects with type 2 diabetes. It
 may be that a decrease in PGC1-α promoter methylation is behind this effect. This is
demonstrated by human subjects with type 2 diabetes that have alterations in
cytosine methylation of promoters of genes, such as PGC-1α which inversely
 correlate with PGC-1α mRNA, CS mRNA and mitochondrial DNA (mtDNA) (Barres
 et al. 2009). Furthermore, palmitate incubation of primary skeletal muscle cells from
 the same subjects induced similar altered methylation of PGC-1α and reduced
 mtDNA/DNA along with PGC-1α and CS gene expression. However, specific
 inhibition of DNA methyltransferase 3B (DNMT3B) attenuated palmitate induced
 PGC-1α methylation and also attenuated palmitate induced downregulation of PGC-
 1α expression, mtDNA, CS and several other mitochondrial genes (Barres et al.
 2009). Taken together this evidence strongly suggests that DNA hypermethylation of
 the PGC-1α promoter plays an important role in the downregulation of mitochondrial
density and CS in subjects with T2D. Thus future work in this area could assess the
role of PGC-1α promoter DNA methylation in the control of CS activity in diabetic
subjects, and potentially, the role of exercise training in this mechanism, as chronic exercise is known to induce hypomethylation of the PGC-1α promoter in type 2 diabetics (Barres et al. 2012).

A limitation of this thesis was that although the HIIE protocol used in this thesis takes less time than traditionally recommended moderate intensity exercise, it is still a relatively long duration compared to other HIIE protocols, which take ~4 mins in total and elicit impressive training responses (Tabata et al. 1996). Therefore future work could involve shorter protocols and assess whether such protocols can still attenuate postprandial TG. Indeed, recent research from this lab has indicated no effect on postprandial TG of twenty 6 sec maximal sprints, interspersed with 24 sec recovery (total exercise time – 14 mins) when performed the day before a HFM (Allen et al. 2014). On the other hand, a single maximal sprint (~198 sec) matched for work with a Wingate test showed an improvement in insulin sensitivity the following day compared to a Wingate test (Whyte et al. 2013), suggesting that such shorter protocols can still be of benefit to health. However, the longer duration (~25 mins overall exercise time) Wingate test induced greater fat and carbohydrate oxidation
the following day compared to the extended sprint (Whyte et al. 2013), which may be
of additional benefit to health. Thus further work needs to be done in order to clarify
the optimal exercise regime for health that is also time-efficient. Additionally,
longitudinal and potentially epidemiological studies could be performed to determine
if the shorter time protocol of HIIT (i.e. protocols with a time significantly shorter than
30 mins), would increase exercise participation as this is yet to be determined.

An additional finding of this thesis was that HIIE does not alter markers of leukocyte
activation, although there were limitations of these testing methods. The numbers of
subjects were low for a test with such large inter-subject variation, giving this aspect
of chapter 4 low statistical power. Furthermore, cannulas have been found to
increase local inflammation to a greater extent than a single-sampling method of
venepuncture (Seiler, Muller & Hiemke 1994). More work, therefore, needs to be
carried out before these findings can be confirmed. Indeed a recent study found that
moderate intensity exercise blunted the postprandial rise in the markers of
leukocyte activation CD11a and CD18. However, the novel findings of a decrease in
CD36 expression on monocyte and lymphocyte in response to a HFM are
supported by *in vitro* studies (Zamora *et al.* 2012). This finding may confirm previous hypotheses that monocyte internalisation of CD36 is an important mechanism of monocyte activation postprandially and thus may be a key process in the pathology of atherosclerosis (Han *et al.* 2000, Han *et al.* 2009). There is also potential for further work in this area to link these observations to citrate metabolism in immune cells as the CiC transporter is critical in Lipopolysaccharides (LPS) signalling (O'Neill 2011). LPS are detected by toll-like receptor 4 (TLR4) (O'Neill 2011) which, under different circumstances, also directly induces CD36 signalling and FA uptake (Zamora *et al.* 2012, O'Neill 2011) demonstrated that the CiC transporter is central to inflammation in immune cells, and CiC gene expression is activated by NF-κB (a hallmark of inflammation (Sharif *et al.* 2007) which could be also be studied postprandially to determine what role citrate efflux plays in immune cell activation in the postprandial environment.

Another limitation of this thesis was the manner of testing used to determine intracellular acetyl CoA and citrate concentrations. Specifically, we were unable to determine if the location of the metabolites were mitochondrial or cytoplasmic.
Identifying the locations and concentrations of metabolites in differing situations would provide greater insight into the metabolic processes behind the cellular physiological effects seen in this study. Future work should isolate mitochondrial and cytoplasmic fractions in a method that preserves metabolite status to the best possible standard.

Future work in this area could also assess whether there is an individual response to exercise in terms of HIIE’s attenuation of postprandial lipaemia. In this thesis 17 subjects underwent an oral fat tolerance test the day after performing HIIE or resting in a control trial. Out of 17, there were 3 subjects who showed a negative response to HIIE in terms of postprandial TG, there were 8 who displayed a difference in postprandial AUC TG of 0.1-2 mmol/l/7h between control and HIIE trials. There were also 6 who displayed a 2-9.2 mmol/l/7h response, indicating variation in responses between subjects. Ferreira et al. (2013) used moderate intensity exercise and aerobic HIIE to show a large individual variation in the response to exercise in terms of postprandial lipaemia. Furthermore, subjects in this trial were categorised into 3 groups: subjects displaying the ε2 allele of the APOE
gene who had a large reduction of postprandial TG; Subjects who displayed the ε3 allele who showed a moderate reduction of postprandial TG; and carriers of the ε4 allele who showed no response to either exercise protocol. Carriers of the ε4 allele also show a 42% increased risk for CHD compared to the ε3 group (measured by a meta-analysis using 48 relevant studies including 15,492 case-patients and 32,965 controls (Song, Stampfer & Liu 2004), the response to exercise may be one mechanism by which this increased risk is mediated. Further work could study the response to HIIE of subjects with the different APOE alleles to identify if the responses are similar to the (Ferreira et al. 2013) study, as HIIE appears to modulate plasma TG by a different mechanism to moderate endurance exercise.

In summary, this thesis has demonstrated the novel findings that as single bout of HIIE is a low energy-expenditure and time-efficient method of attenuating postprandial TG after a HFM for <36 hours. This is clinically relevant as postprandial TG is an independent risk factor for CVD (Nordestgaard et al. 2007). These findings support other recent work which have used a 4 times a week training protocol of HIIE to elicit other health benefits, such as increased $\dot{V}O_2$max.
increased exercise stroke volume and increased cardiac output (Esfandiari, Sasson & Goodman 2013). Furthermore HIIE appears to attenuate the rise in oxidative stress after a HFM and the method of plasma TG disposal after a bout of HIIE appears to be LPL activity mediated, rather than due to altered hepatic metabolism. Additionally, substrate metabolism was studied in vitro in C2C12 muscle cells. In these cells a reduction in CS activity does not increase FA oxidation and actually reduced the ability to oxidise FAs in mixed substrate and likely lipotoxic conditions in C2C12 muscle cells. These findings highlight the importance of citrate’s role as an intracellular metabolic modulator in muscle. The findings of the thesis highlight that further research is warranted into how muscle citrate metabolism and muscular contractions may be manipulated to improve substrate and fat metabolism in diseases such as obesity, diabetes and CVD. Additionally, this thesis has added to the growing body of evidence that HIIE produces acute and chronic health benefits in healthy, young populations. However further work needs to be done in order to establish HIIE’s place amongst the range of training modalities available.
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8. Appendix

8.1. Appendix 1

The findings from chapter 3 are published in the journal below:
High-intensity exercise attenuates postprandial lipaemia and markers of oxidative stress

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ABSTRACT

Regular exercise can reduce the risk of CVD (cardiovascular disease). Although moderate-intensity exercise can attenuate postprandial TAG (triglycerides), high-intensity intermittent exercise might be a more effective method to improve health. We compared the effects of high-intensity intermittent exercise and 30 min of brisk walking on postprandial TAG, soluble adhesion molecules and markers of oxidative stress. Nine men each completed three 2-day trials. On day 1, subjects rested (control), walked briskly for 30 min (walking) or performed 5 × 30 min maximal sprints (high-intensity). On day 2, subjects consumed a high-fat meal for breakfast and 3 h later for lunch. Blood samples were taken at various times and analyzed for TAG, glucose, insulin, ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular adhesion molecule-1), TBA-R (thiobarbituric acid-reactive substances), protein carbonyls and 8-hydroxydeoxyguanosine. On day 2, the high-intensity trial there was a lower [P < 0.05] incremental TAG AUC (area under the curve, 6.42 ± 2.24 mmol/l per 7 h) compared with the control trial (8.68 ± 4.47 mmol/l per 7 h) with no differences during day 2 of the walking trial (8.95 ± 2.84 mmol/l per 7 h). A trend (P = 0.156) for a reduced total TAG AUC was also seen during the high-intensity trial (14.13 ± 2.83 mmol/l per 7 h) compared with control (17.10 ± 3.92 mmol/l per 7 h), walking showed no difference (16.23 ± 3.51 mmol/l per 7 h). On day 2, of the high-intensity trial plasma TBA-R and protein carbonyls were also reduced (P < 0.05) when compared with the control and walking trials. In conclusion, high-intensity intermittent exercise attenuates postprandial TAG and markers of oxidative stress after the consumption of a high-fat meal.

INTRODUCTION

CVD (cardiovascular disease) is a major cause of mortality and is becoming more prevalent [1]. The most common CVD is CAD (coronary artery disease), a condition with atherosclerosis at the centre of the pathalogy [2]. Atherosclerosis is often linked to high fasting serum levels of lipids and HDLs (high-density lipoproteins). However, fasting levels of TAG (triglycerides) are not a good predictor of atherogenesis [3] and, as people spend the majority of the day in a postprandial state, it has been suggested that atherogenesis is a postprandial phenomenon [4]. This conclusion has recently been supported by several studies demonstrating that postprandial TAG concentrations is a strong independent risk factor for CVD [5, 6].

A single high-fat meal can induce endothelial dysfunction, thought to be due to oxidative stress and...
activation of leucocytes, one of the early stages in the development of atherosclerosis [7-10]. The importance of leucocyte activation is highlighted by the early research demonstrating that leucocyte counts are a predictor of future myocardial infarction [11]. However, it should be noted that, although current data suggest that leucocytes are involved in the process of endothelial dysfunction, their precise role has yet to be established.

A recent study by Azzali et al. [12] has provided some evidence of a definite role for leucocytes in endothelial dysfunction via activation of the RAS (renin-angiotensin system). Moreover, the immune reaction appears to couple dyslipidaemia to atherosclerotic plaque formation partially through the induction of the expression of pro-adhesive molecules, such as ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular adhesion molecule-1) on their surfaces [13]. This induction of ICAM-1 and VCAM-1 expression can be induced by an increase in oxidative stress [14].

In support of a role for oxidative stress in endothelial dysfunction, it has been demonstrated that the consumption of antioxidants can attenuate the deleterious effects of a high-fat meal [15]. Exercise is also proposed to be a mechanism to reduce postprandial TAG, and adults are advised to accumulate 30 min of moderate-intensity exercise on 5 days a week [16]. Some studies have shown that such moderate-intensity exercise attenuates postprandial TAG levels after a high-fat meal [17,18]. However, the current number of people meeting these recommendations remains low [19], with lack of time frequently cited as the greatest barrier to performing exercise [20]. Short-duration high-intensity exercises has therefore been proposed as a time-efficient method of improving cardiovascular health [21,22].

Previous studies have shown that a time-efficient exercise protocol, involving four to six 30 s maximal sprint per session, can lead to improvements in endurance performance and muscle oxidative capacity [23]. Further work by this group has also demonstrated that this exercise protocol can reduce hyperglycaemia and improve insulin sensitivity in Type 2 diabetics [24], and improve peripheral arterial stiffness and flow-mediated dilation in healthy untreated individuals [25]. Similar improvements in flow-mediated dilation have also been shown postprandially by Taskinen et al. [25]. Furthermore, in a recent study, it has been shown that high-intensity intermittent exercise can reduce postprandial TAG for 3 h after a high-fat breakfast [26].

What remains to be determined is whether these effects remain over a longer time period (i.e. 7-8 h), which more closely reflects daily food intake (i.e. breakfast and lunch meals) where endothelial dysfunction can still be observed (e.g. [26]) and whether the beneficial effects extend to improvements in markers of oxidative stress and/or adhesion molecules.

The primary aim of the present study, therefore, was to determine the effect of high-intensity intermittent exercise on postprandial plasma levels of TAG, soluble adhesion molecules and markers of oxidative stress in healthy young men. The second aim was to investigate whether changes in postprandial TAG could be associated with a decrease in hepatic VLDL (very low density lipoprotein) secretion, indicated by measures of β-hydroxybutyrate.

MATERIALS AND METHODS

Subjects

The study conformed to current local guidelines, the Declaration of Helsinki and was approved by the local ethics committee. Nine healthy male volunteers took part in this study (age, 24 ± 3 years; body fat, 14.9 ± 4.1%; weight, 81.3 ± 8.5 kg; height, 1.86 ± 0.10 m). All participants were regularly physically active but none were specifically trained. Exclusion criteria for volunteers included a history of CVD, smokers, hypertension (systolic/diastolic blood pressure >140/90 mmHg), diabetes, obesity (body mass index >30 kg/m²) or subjects with any form of musculoskeletal injury. All participants were fully informed of the aims, risks and discomforts associated with the investigation before providing written informed consent.

Anthropometric measurements

Height was measured to the nearest 0.5 cm using a stadiometer (Holtain Ltd). Weight was measured to the nearest 0.1 kg using a weighing scale (Ohaus Classic 2). Skinfold thickness was measured on the right side of the body with calipers (Holtain) at four sites (biceps, triceps, subscapular and suprailiac) to the nearest 0.1 mm. The percentage body fat was calculated using standard methods [27].

Experimental protocol

Subjects completed three 2-day trials in a randomized order. On day 1 (14:00 hours) the subjects rested for 30 min (control), walked slowly for 30 min (walking) or performed 5 × 30 s maximal sprints with 4 min recovery between each sprint (high intensity). On day 2 (beginning at 08:45 hours) subjects arrived after an overnight fast and consumed a high fat meal for breakfast and 3 h later for lunch. Each trial was separated by at least 7 days. Subjects were instructed not to ingest alcohol or caffeine in the 24 h period prior to day 1 up until the end of day 2. During this time they were also asked to refrain from exercise or strenuous physical activity other than that of the trials. Subjects were also asked to record their diet on day 1 of the trial and replicate this on day 1 of the two subsequent visits.

Day 1 trials

Walking

The walking trial took place on a treadmill (Cybex International). Subjects were started at a speed of 7 km/h and asked to walk at an intensity similar to that of the current recommendations for 30 min [16], i.e., a brisk walk (out of breath, but still able to talk).

Pulmonary gas exchange $V_O_2$ (oxygen consumption) and $V_CO_2$ (carbon dioxide output) were monitored breath-by-breath during several time intervals, i.e., 8–10, 18–22 and 28–30 min, respectively, using an online gas analysis system (CPX Ultima; Medgraphics).

High-intensity

The high-intensity intermittent exercise was performed on a cycle ergometer (Monark 818E Wingate cycle ergomter). Subjects performed a 6 min warm-up with no load and then performed a 30 s maximal sprint against a load of 7.5% body weight, followed by unloaded cycling for 4 min. This sprint exercise was repeated a further four times, with 4 min rest in between each sprint. During each 30 s sprint average power (in W), peak power (in W) and peak pedal rate (rev/min) were recorded.

Control

During the control trial participants sat and rested for 30 min.

Day 2

Subjects arrived at 08:45 hours and rested for 15 min before a cannula (20 gauge) was inserted into a vein in the antecubital fossa, and a baseline blood sample was collected. The cannula was flushed regularly with saline throughout the day. A standardized high-fat meal was then consumed for breakfast. This consisted of white bread, mayonnaise, butter, whole milk, cheddar cheese and potato chips. This meal provided approximately 3.4 ± 0.4 MJ of energy with 56% of energy from fat, 35% from carbohydrate and 11% from protein. The meal consisted of 0.2 g fat, 1.1 g carbohydrate, 0.3 g of protein and 11 kcal/kg of body weight (1 kcal = 4.184 kJ). The mean macronutrient content of the meal was 50.8 ± 6.1 g of fat, 75.7 ± 8.2 g of carbohydrate and 25.8 ± 2.8 g of protein. The mean time taken to consume the meal was 13:28 ± 4.43 min.

Further blood samples were collected at 0.5, 1, 2 and 3 h after the breakfast meal. A second identical meal was then consumed for lunch with subsequent blood samples taken at 3.5, 4, 6 and 8 h after the consumption of the first meal. Water was provided to subjects throughout the day of the first trial and this volume of water was consumed during subsequent trials.

Measurements

Blood handling and analysis

Blood samples were collected with sterile 6 ml K$_3$ EDTA non-anticoagulated vacutainers (Vacutainers; Greiner Bio-one) and were centrifuged (Eppendorf Centrifuge 5702/R) at 1522 g at 4°C for 10 min. Then plasma was removed and frozen at −22°C until analysis. Blood TAG and glucose concentrations were assessed using manual enzymatic colorimetric assay kits (Random; Cramton) using a spectrophotometer (Camag M30CC); insulin was measured by ELISA (Mercodia; method ELISA) using a spectrophotometric plate reader (Synergy HT Multi-mode microplate reader; BioTek); sICAM-1 (soluble ICAM-1) and sVCAM-1 (soluble VCAM-1) concentrations were determined using ELISA kits (R&D Systems) and absorbance was measured using a spectrophotometric plate reader (Synergy HT Multi-mode microplate reader; BioTek). Concentrations from the ELISA were calculated through the interpolation of standard absorbance values compared with generated standard curves.

In order to investigate the effect of the exercise interventions on postprandial endocrine stress, we measured protein carbonyls and TBA RS (thiobarbituric acid-reactive substances) in blood samples collected at baseline, 2, 5 and 7 h.

To assess proteolysis, a fluorometric assay kit for protein carbonyls was used (Cayman Chemical). Protein content was also measured in each sample to express data per mg of protein. To assess lipid peroxidation, a fluorometric TBA RS assay kit was used (Cayman Chemical). In both assays, fluorescence was measured using a plate reader (Synergy HT Multi-mode microplate reader; BioTek).

To estimate hepatic lipid oxidation [20], $\beta$-hydroxybutyrate concentration was measured using an enzymatic assay kit (Cayman Chemical). Absorbance was measured using a spectrophotometric plate reader (Synergy HT Multi-mode microplate reader; BioTek).

Concentrations from the assays were calculated through the interpolation of sample absorbance values compared with generated standard curves. The coefficient of variation for each assay was: TAG, 2.02%; glucose, 4.3%; insulin, 1.2%; sICAM-1, 1.38%; sVCAM-1, 6.3%; $\beta$-hydroxybutyrate, 4.5%; TBA RS, 6.67%; protein carbonyls, 3.8%.

Estimation of energy expenditure

Energy expenditure was estimated during the walking trials from the measurement of $V_O_2$ and the respiratory exchange ratio [20]. To estimate energy expenditure during the high-intensity trials using the mean power output during the 30 s sprints and an estimate of mechanical efficiency of 18.3% [30].
Figure 1 Plasma glucose (A), insulin (B) and TAG (C) concentrations in response to the prior walking, high-intensity exercise and control trials. Values are mean ± SE.

Statistical analysis
Data were analyzed using the GraphPad Prism 5 software. Both total and incremental (taking into account changes in baseline concentrations) AUC (area under the curve) values for plasma TAG concentration were calculated using the trapezoidal rule. The AUC values were calculated to provide a summary of the TAG response during the 7 h test period. Fasting plasma concentrations and calculated incremental and total AUC values were compared between trials using a one-way ANOVA. To compare differences between the three trials over time, a two-way ANOVA with repeated measures was performed. Where a significant effect was observed post-hoc Tukey’s tests were performed to locate differences. Significance was taken at P < 0.05. Results are presented as mean ± SE.

RESULTS
Exercise
Volunteers did not perform any exercise in the control experiment. Subjects walked at an average of 6.7 ± 3.2 km/h and the average \( \dot{V}_O_2 \) was 22.03 ± 2.3 ml/kg of body weight per min an estimated energy expenditure of 240.9 ± 55.2 kcal (where 1 kcal = 4.184 kJ) during the walking exercise. The maximum power output during the high-intensity trial was 889.1 ± 198.4 W and mean power output was 632.6 ± 102.2 W. This corresponded to an average energy expenditure of 101.2 ± 5.1 kcal during the high-intensity trial, which was lower \( P < 0.001 \) than energy expended during the walking trial.

Insulin and glucose
There was no difference between the three trials in either insulin or glucose concentration in the plasma samples collected. ANOVA did reveal a significant effect of time for both insulin and glucose in response to the meals \( P < 0.001 \) (Figure 1).

TAG
The initial ANOVA revealed no differences between the three trials in plasma TAG concentration, although there was a trend. However, there was a significant effect of time in response to the meal \( P < 0.001 \) (Figure 2). The data were analyzed in the AUC form. This showed that there was a lower \( P < 0.05 \) incremental AUC in high-intensity exercise compared with the control trial (Figure 2). There was also a trend \( P = 0.036 \) for a lower total AUC in high-intensity exercise compared with the control trial (Figure 2). There were no differences between the control and walking trials.

Soluble adhesion molecules
There was no difference between the three trials in soluble adhesion molecule concentration (Figure 3). However, sICAM-1 did show a significant effect of time with values increasing throughout the day \( P < 0.001 \).
Figure 2. TAG total (A) and incremental (B) AUC over the 7 h experimental period in response to the prior walking, high-intensity exercise and control trials. Values are means ± SD.  *P < 0.05 compared with high-intensity exercise and control trials, †P < 0.05 between the high-intensity exercise and control trials.

Figure 3. Plasma sVCAM-1 (A) and sICAM-1 (B) concentrations in response to the prior walking, high-intensity exercise and control trials. Values are means ± SD.

Figure 4. Plasma protein carbonyls (A) and TBA-RS (B) concentrations in response to the prior walking, high-intensity exercise and control trials. Values are means ± SD. *P < 0.01 between the high-intensity and control walking trials. †P < 0.05 and ‡P < 0.01 between the high-intensity and walking trials.

Markers of oxidative stress
Protein carbonyl levels (Figure 4) increased (P < 0.05) at 2 and 5 h above baseline in both walking and control trials. However, high-intensity completely prevented this effect, with no change in protein carbonyl levels. TBA-RS (Figure 4) were raised compared with baseline in all three trials; however, high-intensity reduced the magnitude of this effect. There were no differences in either protein carbonyl or TBA-RS between walking and control trials.

β-Hydroxybutyrate
β-Hydroxybutyrate levels (Figure 5) increased between baseline and 5 h in all three trials (P < 0.0001), with no difference between the three trials.

Discussion
The main finding of the present study was that a prior bout of high-intensity intermittent exercise
attenuated the postprandial rise in TAG, compared with a control trial, with no differences in glucose or insulin between trials. A total of 30 min of brisk walking, which is equivalent to the current physical activity recommendations, had no effect on postprandial TAG concentrations. These findings agree with the recent study by Frontera et al. [22], who employed a similar exercise protocol, although in their work TAG concentrations were only monitored for 3 h postprandially. The present study has extended these findings by also demonstrating that these effects are present for a 7-h period (after breakfast and lunch) and that high-intensity exercise also results in a decrease in markers of oxidative stress, with no change in salivary adhesion molecules. These findings have important clinical implications as high-intensity exercise may be of greater benefit than the currently recommended forms of physical activity. The importance of a reduction in exercise time is highlighted by the consistent findings that a lack of time is frequently cited as the major barrier to participation in exercise [20].

Further to a reduced exercise time, the high-intensity exercise protocol employed also has an approximately 57% lower energy expenditure. Although this may be seen as a negative observation if one was aiming to reduce body fat, high-intensity intermittent exercise has, in fact, been found to be more effective than traditional steady-state endurance exercise in reducing body fat [31]. The greater TAG-reducing effect of high-intensity exercise may also be surprising, as energy expenditure is known to be a crucial determinant of this response [32]. Both these observations may be due to an elevation in resting metabolic rate and/or a more prolonged/greater elevation in post-exercise VO2 after high-intensity exercise [33,34]. Further work is needed to clarify these assertions. It is often assumed that high-intensity intermittent exercise would not be suitable or enjoyable for patient populations, yet, in patients with CAD, high-intensity intermittent exercise was noted as their preferred mode of exercise, when compared with constant-load endurance exercise [35].

It is well established that, in healthy individuals, exercise can reduce postprandial TAG concentrations. Early work demonstrated that a 90-min brisk walk can reduce postprandial TAG concentrations by approximately 20% [32] and three findings have been supported by several further studies (for a review, see [36]). The duration of exercise is, however, higher than the current recommendations and is unlikely to be achieved by the general population. Recent work has demonstrated that 30 min of brisk walking results can also reduce postprandial TAG concentrations, by approximately 15% in healthy young men [37]. A similar finding was noted in the present study with 30 min of brisk walking, with no clear reason for the differences in findings. It may be that the present study did not have sufficient number of participants to detect differences between walking and control trials. However, the main aim of the study was to determine the effect of high-intensity intermittent exercise, which had a clear positive effect in reducing plasma TAG.

The mechanisms responsible for an exercise-induced decrease in plasma TAG concentrations remain to be elucidated (for review, see [36]). The proposed mechanisms are that either, or a combination of both, the uptake of TAG in peripheral tissues is elevated or the production and release of TAG, packaged in VLDL, from the liver is decreased. With regard to augmented TAG clearance after prior intense exercise it appears likely that this is mediated by an increase in LPL (lipoprotein lipase) activity [37]. However, after prior moderate exercise there also appears a role for decreased secretion of VLDL from the liver. Indeed, Gill et al. showed increased circulating levels of β-hydroxybutyrate alongside reduced postprandial TAG concentrations following prolonged exercise [38]. This increase in β-hydroxybutyrate is suggested to be indicative of an increase in hepatic fatty-acid oxidation which would shift hepatic fatty-acid partitioning away from VLDL synthesis. Early evidence has shown that there is an inverse relationship between ketogenesis and VLDL production [39], supporting the contention that increases in β-hydroxybutyrate would be reflected in a decrease in VLDL production. However, further work has demonstrated that this relationship does not hold under all situations [40] and caution should be employed when interpreting these results. The present investigation found no difference between exercise trials in β-hydroxybutyrate levels, with all three trials showing a rise between 2 and 5 h after the first meal. It is therefore likely that the attenuation of postprandial TAG seen after high-intensity intermittent exercise comes about solely as a result of an increased LPL activity, although further work is required to elucidate the effects of
high-intensity intermittent exercise on hepatic VLDL secretion.

The magnitude of postprandial TAG concentration has previously been shown to correlate with the magnitude of endothelial dysfunction and intima-media thickness of the carotid artery [41]. Furthermore, both total and intercellular TAG AUC measures also correlating with intima-media thickness [42], highlighting the physiological and clinical relevance of these measures of the TAG response during the 7-hour period. Although the present study did not make any measure of endothelial function, previous work [43] has demonstrated that high-intensity intermittent exercise, albeit of a longer duration than in the present study, completely abolished the reduction in brachial artery flow-mediated dilation normally observed after a high fat meal. These authors found that this effect was associated with an increase in total antioxidant status in the blood, with no reductions in plasma TAG.

Although this supports the assertion that the deleterious effects of a high fat meal are associated with an increase in ROS (reactive oxygen species) production (e.g. [9]), a single measure of total antioxidant status gives a limited view of redox status [45]. The present study has shown that while oxidative stress was increased for up to 5 h in the control trial, high-intensity intermittent exercise ameliorated the rise in oxidative stress, as measured by plasma protein carbonyls and TBARS. During the walking trial, there was no significant reduction in markers of oxidative stress when compared with the control trial. Under normal conditions, the state of oxidative stress will reduce the amount of bioactive NO (nitric oxide), via chemical inactivation to form peroxynitrite, and may also make eNOS (endothelial nitric oxide synthase) dysfunctional, producing O₂⁻ rather than NO [46]. These conditions will ultimately result in endothelial dysfunction (for a review, see [45]), which is an independent predictor of the progression of atherosclerosis and CVD [46]. The generation of a state of oxidative stress has many other effects in processes such as intercellular signalling, and also the induction of adhesion molecule expression [47]. Previous work in Type 2 diabetes has demonstrated that a high-fat meal can result in an increase in circulating levels of the soluble adhesion molecules sICAM-1 and sVCAM-1, and that this increase can be attenuated by the consumption of vitamin E and ascorbic acid [48]. However, others have found no rise in both sICAM-1 and sVCAM-1 after a high-fat meal in healthy subjects [49]. The present study has demonstrated that while sICAM-1 increased throughout the day sVCAM-1 remained constant and that prior exercise had no effect on postprandial sICAM-1 and sVCAM-1 levels. Similar results have been found previously in overweight adolescent boys [49], but not to our knowledge in healthy adults.

One limitation of this study is a relatively low number of participants compared with other similar studies. This may explain the differences in postprandial TAG results after 30 min brisk walking between Miyashita et al. [17] and the present study. Nevertheless, the results from the present study still show a beneficial effect from a single bout of high-intensity intermittent exercise and this would suggest that high-intensity intermittent exercise is more efficacious compared with 30 min walking in ameliorating postprandial TAG. Secondly, the present study only used healthy males and it remains to be elucidated whether similar adaptations will be seen in patients with CVD.

In conclusion, the results of the present study demonstrate that prior high-intensity intermittent exercise reduces postprandial TAG, with no effect of 30 min brisk walking. This decrease in TAG was not associated with an increase in p-hydroxybenzoate, indicating that this effect is not due to a reduction in hepatic VLDL secretion. The reduction in postprandial TAG was associated with an almost-complete abolition of the postprandial increase in markers of oxidative stress. High-intensity intermittent exercise may therefore be a useful tool in the prevention of atherosclerosis and reduction in the development of CVD, although further work is required to confirm this.

AUTHOR CONTRIBUTION

Stuart Gray and Michael Pineau designed the research; Stuart Gray, Avinash Badwe, Brendan Gabriel and Patrick Gray conducted the research; Brendan Gabriel and Stuart Gray analysed the data and wrote the paper; Stuart Gray had primary responsibility for final content. All authors read and approved the final manuscript.

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8.2. Appendix 2

The findings from chapter 4 are published in the journal below:

The Effect of High Intensity Interval Exercise on Postprandial Triacylglycerol and Leukocyte Activation – Monitored for 48h Post Exercise

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Abstract

Postprandial phenomena are thought to contribute to atherosclerosis alongside activation of the immune system. A single bout of high intensity interval exercise attenuates postprandial triacylglycerol (TG), although the longevity and mechanisms underlying this observation are unknown. The aims of this study were to determine whether this attenuation in postprandial TG remained 2 days after high intensity interval exercise, to monitor markers of leukocyte activation and investigate the underlying mechanisms. Eight young men each completed two three-day trials. On day 1, subjects rested (Control) or performed 5 x 30 s maximal sprints (high intensity interval exercise). On day 2 and 3 subjects consumed high fat meals for breakfast and 3 h later for lunch. Blood samples were taken at various times and analysed for TG, glucose and TG+NRP (TRI) dependent LPL-TG hydrolysis (LTH). Flow cytometry was used to evaluate granulocyte, monocyte and lymphocyte CD11b and CD36 expression. On day 2 after high intensity interval exercise TG area under the curve was lower (P=0.05) (7.46±1.53 mmol/L) compared to the control trial (8.47±3.04 mmol/L), with no differences during day 3 of the trial. LTH activity was higher (P=0.05) after high intensity interval exercise, at 2 hours of day 2, compared to control. Granulocyte, monocyte and lymphocyte CD11b expression increased with time over day 2 and 3 of the study (P=0.0001). Lymphocyte and monocyte CD36 expression decreased with time over day 2 and 3 (P=0.06). There were no differences between trials in CD11b and CD36 expression on any leukocytes. A single session of high intensity interval exercise attenuated postprandial TG on day 2 of the study, with this effect abolished by day 3. The reduction in postprandial TG was associated with an increase in LTH. High intensity interval exercise had no effect on postprandial responses of CD11b or CD36.


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Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide, and is becoming more prevalent [1]. The most common CVD is coronary artery disease (CAD), which has atherosclerosis at the centre of its pathology. In recent years it has been established that atherosclerosis is a chronic inflammatory condition with immune cells present in the artery wall and plaque itself (for review see 2). Whilst there is clear data linking testing LDL levels to atherosclerosis [3] testing triacylglycerol (TG) levels have been found to be a poor, with non-fasting TG a strong, independent predictor of atherosclerosis and CAD [4]. Furthermore, as humans spend the majority of their day in a postprandial state it has been suggested that this postprandial phase is a strong contributor in atherosclerosis [5].

The precise mechanism linking postprandial TG levels with atherosclerosis are unknown. It is thought to result from a series of linked events including the generation of free radicals and activation of the immune system leading to endothelial dysfunction, an early event in the development of atherosclerosis [6]. Even a single high fat meal induces endothelial dysfunction, the magnitude of which associate strongly with the magnitude of postprandial TG [7]. Support for the role of oxidative stress as a cause of endothelial dysfunction has come from studies demonstrating that the
consumption of antioxidants (Vitamin C) can attenuate the magnitude of postprandial endothelial dysfunction [6-10].

Adiponectin, following a high fat meal, induces an increase in circulating TG postprandially with a lesser extent neutrophils, and an increase in production and accumulation of TG in adipose tissue, indicating concurrent activation of the immune system. Indeed, several studies have shown that TG-rich lipoproteins (TRL) activate monocytes (increased CD11c) and to a lesser extent neutrophils (increased CD11b and CD66b) via uptake of TG [11]. These findings have recently been enhanced by Guo et al. [12] with the observation that a high-fat meal monocytes internalise lipid, upregulate CD11c and increase adhesion to VCAM-1. This leukocyte activation has several consequences including an increase in pro-inflammatory cytokine production, oxidative stress, adhesion, activation of endothelial cells and ultimately an increase in migration of leukocytes and monocytes to the sub endothelium (for review see 14).

Leukocyte activation can also lead to the uptake of oxidised plasma low density lipoprotein (oxLDL) by monocyte-derived macrophages in the vascular wall, a well-established step in the development of atherosclerosis [15]. The scavenger receptor CD36 has been shown to be central in facilitating monocyte/macrophage uptake of oxLDL [16,17], and appears to be linked to atherosclerosis [18]. The effect of a high-fat meal on monocyte CD36 expression, however, remains to be established.

Several investigators have reported the beneficial effects of a single bout of exercise on postprandial TG with both endurance exercise and high intensity interval exercise (HIIE) being demonstrated to reduce postprandial TG the following day [20,21,22,23] with the latter altering insulin sensitivity and therefore decreasing TG concentrations [24]. What remains to be established is whether these effects remain the second day after HIIE, information which will have clear implications for the required frequency of such exercise and exercise prescription recommendations. Furthermore, the mechanisms underlying the reduction in TG after HIIE have yet to be investigated. Potential candidates are a reduction in hepatic VLDL secretion or an increase in lipoprotein lipase (LPL) activity. In our previous work we have shown that HIIE does not affect plasma VLDL or hepatic lipase activity in young healthy males [25]. The aim of this study was to determine whether the alteration in postprandial TG remained 2 days after a bout of HIIE. A secondary aim of the study was to determine the mechanisms underlying the reduction in postprandial lipemia observed after HIIE by measuring TRL-bound LPL-dependent TRL-TG hydrolysis (LTH).

Materials and Methods

Participants

Eight healthy male participants took part in this study (age 25±4 years, body fat 11.7±1.1%, weight 72.9±19.3 kg, height 1.77±0.13 m). All participants were regular physically active but none were specifically trained. Exclusion criteria for volunteers included a history of cardiovascular disease, smoking, hypertension (systolic/diastolic blood pressure >140/90 mmHg), diabetes, obesity (BMI >30 kg/m²) or subjects with any form of musculoskeletal injury. The power of the current proposal has been based on postprandial TG. The analysis is based on repeated measures design with 2 treatment groups (control and high intensity). Using the reduction in TG AUC 10 participants would provide 80% statistical power at α=0.05 to detect a difference in means of 3.26 mmol/l (observed in our previous work) assuming a common standard deviation of 3.5. Due to drop-out of 2 participants only eight are included in the final analysis.

Ethnic statement

The study conformed to current local guidelines, the declaration of Helsinki and was approved by the University of Aberdeen College Ethical Review Board. All participants were fully informed of the aims, risks and discomfort associated with the investigation before giving written informed consent.

Anthropometric measurements

Height was measured to the nearest 0.1 cm using a stadiometer (Stadiometer Ltd, Cymroch, Dyfed, Wales, UK). Weight was measured to the nearest 0.1 kg using a weighing scale (Ohaus cham-20, Ohaus Ltd., Leicester, England). Skinfold thickness was measured with callipers (Liaas, Harpenden skinfold callipers, England, UK) at 4 sites (biceps, triceps, subscapular & supra iliac) to the nearest 0.1 mm on the right side of the body. Percentage body fat was calculated using standard methods [26].

Experimental Protocol

Participants completed two three day trials in a randomised order. On day 1 (14.00 hours) subjects rested for 30 min (Control) or performed 5 x 30 sec maximal sprints with 4 min recovery between each sprint (HIIE). On day 2 (beginning at 06.00 hours) subjects arrived after an overnight fast and consumed a high fat meal for breakfast and 3 h later for lunch. This was repeated for day 3. Each trial was separated by at least 7 days. Participants were instructed not to ingest alcohol or caffeine in the 24 h period prior to day 1 up until the end of day 3. During this time they were also asked to refrain from exercise or strenuous physical activity other than that of the trials. Subjects were also asked to record their diet 24 hours before day 1 and during the trial and replicate this during the subsequent visit.

Day 1

High Intensity Interval Exercise. During the HIIE trials exercise was performed on a cycle ergometer (Monark 894, Wingate bike, UK). Subjects performed a 4 min warm-up with no load and then performed a 30 s maximal sprint against a load of 7.5% body weight following an unloaded cycling for 4 min. During the warm-up and unladen cycling phases participants were asked to maintain a cadence of ~70 rpm and accelerate after the 7.5% body weight resistance was applied. This sprint exercise was repeated a further four times, with 4
min unloaded cycling in between each sprint. During each 30 s sprint average power (Watts), peak power (Watts) and peak pedal rate (RPM) were recorded. Energy expenditure was estimated during the high intensity trial using the mean power output during the 30 s sprint and an estimate of mechanical efficiency of 8.6% [16].

Control. During the control trial participants sat and rested for 30 min.

Day 2

Subjects arrived at 3.45 am and rested for 15 min before a carnitine (200) was injected into a vein in the antecubital fossa and a baseline blood sample was collected. The carnitine was flushed regularly with saline throughout the day. A standardized high-fat meal was then consumed for breakfast. This consisted of white bread, mayonnaise, butter, whole milk, cheddar cheese and potato crisps. The meal provided 812±36 kcal energy with 59% from fat, 33% from carbohydrates and 11% from protein. The meal contained 0.7 g Na, 1 g carbohydrate, 0.3 g protein and 11 kcal per kg bodyweight. The mean macronutrient content of the meal was 51.2±10.1 g fat, 68.4±14.4 g CHO and 25.3±4.9 g protein. The mean time taken to consume the meal was 10 min 58 s±3 min 24 s.

Further blood samples were collected 1, 2, and 3 hours after the breakfast meal. A second identical meal was then consumed for lunch with subsequent blood samples taken 4, 5, 6, and 7 hours after the consumption of the first meal. Water was provided ad libitum throughout the day of the first trial and this volume of water was consumed during the subsequent trial. Upon completion of day 2 participants were asked to rest for the remainder of the day, consume an evening meal (which was recorded and replicated during the subsequent trial) and sleep from 10 pm (water allowed) before returning to the laboratory for day 3.

Day 3

Subjects arrived at 8.45 am and the protocol for day 2 was repeated as day 3.

Measurements

Blood handling and analysis. Blood samples were collected from sterile 6 ml K3-EDTA non-derided vacutainers (Vacutainer, greiner bio-one, Kremsmünster, Austria) and were centrifuged (Eppendorf Centrifuge 5810R, UK) at 3000 rpm at 4°C for 10 min. The plasma was retrieved and frozen at -80°C until analysis. Plasma TG (CV = 0.8%) and glucose (CV = 2.2%) concentrations were assessed using manual enzymatic colorimetric assays kits (Randox Crumlin, Co. Antrim, UK) using a spectrophotometer (Camspec M303B, Leeds, UK).

Ex vivo Immune Activation. Sample preparation and analysis was carried out on the day of sample collection with the cytokine settings the same for every analysis performed. The expression of CD11b on leukocytes was determined by Alexa Fluor 488 mouse anti-human CD11b and APC mouse anti-human CD64 used to determine CD64 expression. As negative controls Alexa Fluor 488 mouse IgG1, K isotype and APC mouse IgG1, K isotype controls were used. Measurements of leukocyte activation were performed on fasting, 2, 5 and 7 hours blood samples from days 2 and 3 of each trial. 5 µL of Alexa Fluor-488 and 20 µL of APC was added to a FACS tube prior to adding 100 µL of whole blood. Samples were then incubated for 30 min in a darkroom at room temperature. Nond blood cells were then lysed by adding 2 mL of precipitation solution and further incubated for 15 min. Samples were centrifuged for 5 min (250 x g, 4°C), washed in PBS (0.2% BSA) and centrifuged again before being fixed in 0.5 mL of 1% paraformaldehyde in PBS. For the negative controls 100 µL of whole blood was added along with each isotype control (CD11b and CD64) and 100 µL of whole blood was used as an unattended control. Samples were analysed using a FACS calibur (BD, Oxford, UK) and the data processed using FlowJo software. 50,000 cells were analysed for each sample. Granulocytes, monocytes, and lymphocytes were identified using forward and side scatter plots and mean fluorescent intensity (MFI) measured to indicate the surface expression quantity per leukocyte. The between day coefficient of variation for CD11b was 8.9% and for CD64 it was 9.6%.

Determination of LTH. A sample group of 6 subjects was used for this assay, where we had sufficient volume of frozen plasma to carry out this analysis. Analysis was carried out in a booth at the end of the study. Spontaneous (tolerant) activity in TLRs resulting from LPL bound on tissue surface was measured using the LTH assay as previously described [25]. TRLs were isolated by fast-protein liquid chromatography (FPLC) using a Superoxide 6 HR 10/30 column (Pharmacia) at 4°C to separate lipoproteins in TGL buffer containing 100U/ml heparin for stabilization of LPL during the procedure. 1 mL of filtered plasma was applied to the column and chromatographed at a flow rate of 0.3 ml/min under a pressure of 100 psi. Fractions corresponding to total TRLs, including both chylomicrons and VLDL (fractions 9 to 18), were pooled and immediately assayed for LPL activity. Aliquots of the pooled TRLs corresponding to 2.8 μmol of TG were incubated for one hour at 37°C of buffer and lipoprotein monitored over time. Blank were obtained by incubations of samples in the presence of 2 mMOL, Lipoxyn (Sigma), which totally blocks LPL activity. The resulting amount of non-esterified fatty acids (NEFA) released by LPL bound to TG rich lipoprotein were then measured in triplicate, and after correction for plasma TG concentrations. LTH was finally expressed as the amount of NEFA released per ml of plasma per hour. The coefficient of variation, for duplicate samples, for the LTH assay was 7.3%.

Statistical analysis

Data were analysed using the GraphPad Prism 5 software. Normality was checked using the Shapiro-Wilk test, with no data requiring transformation. Both total and incremental (taking into account changes in baseline concentrations) area under the curve (AUC) values for plasma TG and glucose concentrations were calculated using the trapezoidal rule. The AUC values were calculated to provide a summary of the TG and glucose responses during the 7 h test period on each day. Calculated incremental and total AUC values on each day were compared between trials using a two-way ANOVA. To compare differences between the trials over time a two-way
ANOVA with repeated measures was performed. Where a significant effect was observed Bonferroni’s Multiple Comparison Tests were performed to locate differences. Significance was taken at P<0.05. Results are presented as means±S.E.M.

Results

Power Output and Energy Expenditure

The average peak power output during HIE was 870.3 ± 143.2 W and the average mean power output was 484.3 ± 197.6 W. This corresponded to an estimated energy expenditure of 93.1 ± 20.1 kcal during HIE.

Plasma Glucose and Triglyceride

Plasma TG and glucose values over time during control and HIE trials are presented in Figure 1. There were no differences between the trials in plasma glucose total and incremental AUC. When comparing TG total AUC this was lower (P<0.05) on day 3 of HIE compared to day 2 of the control trial (Table 1). There were no differences in total AUC between HIE and control groups during day 3 of the trial. Analysis of variation revealed a significant (P<0.05) group effect for incremental TG AUC, however Bonferroni post hoc tests found no differences between HIE or control trials on either day 2 or day 3.

Ex Vivo Immune Activation

Analysis of variance revealed CD11b expression increased with time over day 2 and day 3 of the study in lymphocyte (P=0.0001) monocytic (P=0.0001) and granulocyte (P=0.0001) populations (Figure 2A). Lymphocyte and monocyte CD38 expression decreased with time over day 2 and day 3 of the study (P=0.05) (Figure 2B). There were no group or interaction effects noted in either CD11b or CD38 expression amongst all leukocyte subgroups.

LTTH Assay

Analysis of variance revealed an interaction and time (P<0.05) effect in LTTH activity, with no group effect (P=0.09) observed. Post hoc tests showed that LTTH activity was higher (P<0.05) at 2 hours on day 2 of HIE (204.3±16 nmol/HER/ml) compared to control trial (142.7±31 nmol/HER/ml) (Figure 3).

Discussion

The main findings of the present study were that HIE attenuated postprandial TG on day 2 of the study compared with a control trial, with this effect abolished by day 3. The attenuation of postprandial TG the day following HIE is supported by recent studies [22,23] and the current study extends this finding by demonstrating that this reduction in plasma TG is associated with an increase in LTTH. Prior to the current study the longevity of, or mechanisms underlying, these effects had not been investigated.

The novel finding of the current study, that the beneficial effect of HIE on postprandial TG is abolished by day 3, suggests that one must engage in HIE every 1-2 days to attenuate the rise in postprandial TG which is a chronic risk factor for CHD [4]. Previous studies have proposed that the effect of endurance exercise on postprandial TG is acute, rather than a training effect [27,28,29], with the current study indicating the same is true for HIE. On top of this benefit of HIE, in reducing postprandial TG, training studies ranging from two to six weeks in duration have shown that HIE can improve endurance performance, muscle oxidative capacity [30], insulin action [31] and endothelial function [32]. It is also possible that more chronic improvements in muscle sensitivity, observed after HIE, would also result in long term reductions in TG levels, however, this remains to be investigated. Whilst similar benefits have been found after more prolonged endurance training HIE may also be beneficial as a public health tool. As time is often cited as the primary barrier to exercise participation [24] the use of such a protocol has therefore been proposed to overcome this barrier. Whilst the total exercise time in the current study was only 2.5 min the session did last 25 min, which is slightly shorter than the current recommendations. It remains to be investigated whether this would increase exercise participation. In that regard, although using a different protocol (6% min at 90% VO2max), high intensity interval training has been found to be more enjoyable than standard endurance training [33] suggesting adherence may be improved, although this needs to be examined using the HIE protocol employed in the current study.

The mechanisms responsible for the reduction in postprandial TG concentrations after exercise remain to be elucidated. Previous studies using moderate intensity, continuous exercise have shown an upregulation in LPL activity for up to 13 hours, an observation associated with a fall in serum TG after exercise bouts lasting several hours [34-35]. Some studies have suggested that there is a threshold energy expenditure above which LPL activity is increased following moderate intensity exercise. A study comparing 4 separate bouts of moderate intensity exercise with energy expenditures of 800, 1,100, 1,300 or 1,500 kcal, found that the 800 kcal exercise induced a reduction of postprandial TG, but there was no increase in LPL activity [36]. On the other hand the 1,100, 1,300 and 1,500 kcal exercise bouts showed a significant increase in LPL activity 24 hours after the bout of exercise, again with a reduction of postprandial TG. Further studies have shown that moderate intensity, continuous exercise (215-1075 kcal energy expenditure) reduces postprandial TG but does not induce a statistically significant increase in LPL activity [37-38] [39]. In light of these studies showing a reduction of postprandial TG with no increase in LPL activity, it has been proposed that a decreased hepatic VLDL secretion also plays a role in reducing postprandial TG after moderate intensity exercise [41]. Support of this comes from the same laboratory where moderate intensity exercise had no effect on TG clearance, monitored after intralipid infusion [42]. On the other hand further studies have shown that there was no effect of moderate intensity exercise on hepatic VLDL-TG secretion rate.
Figure 1

A

Plasma Triacylglycerol (mmol/l)

Control day 2
Control day 3
HIIE day 2
HIIE day 3

Time (Hours)

B

Plasma Glucose (mmol/L)

Meal 1
Meal 2
Meal 3

Time (Hours)

Figure 1. Plasma TG(A) and glucose (B) concentrations on day 2 and 3 of the HIIE and control trials.
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Table 1. TG total and incremental AUC over the 7 h experimental period of day 2 and 3 of the HIIE and control trials.

<table>
<thead>
<tr>
<th>Control</th>
<th>HIIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incremental TG AUC</td>
<td></td>
</tr>
<tr>
<td>(mmol/L/24h)</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>Total TG AUC (mmol/L)</td>
<td>9.7 ± 0.7</td>
</tr>
</tbody>
</table>

*Denotes a significant difference (P < 0.05) from day 2 of the control trial.

It seems then, that moderate intensity exercise may attenuate postprandial TG via an increased LPL activity (possibility above a threshold energy expenditure), with conflicting evidence as to whether decreased hepatic VLDL-TG secretions play a major role. However, the mechanisms of reducing postprandial TG have not been studied after HIIE in humans. It is worth noting, at this point, that the current study measures LTTH in pre-heparin LPL which is more likely to reflect physiological processes rather than studies employing heparin titration where the majority of the LPL is released.

There is some evidence in rats that muscle contractile activity is the main regulator of LPL activity [45]. This study also indicated that the intensity of physical activity required to stimulate an upregulation of LPL activity may be fibre type specific with LPL activity, following high intensity exercise, increasing only in fast fibres. Additionally, increases in LPL activity seemed to be due to local contractile activity rather than hormonal changes [46], although these findings cannot be directly applied to humans. In our previous study, using HIIE [23], we found that β-hydroxybutyrate was not changed after high intensity exercise. This would suggest that a change in VLDL secretion rates was not responsible for the reduction in postprandial TG observed after HIIE, although LPL activity was not measured in the previous study. In the current study, tripotyrosine TRLs resulting from LPL bound on their surface was measured using the LTTH assay as previously described [29]. LTTH was greater after HIIE at 2 hours of day 2 of the trial, compared with the control group, indicating that the main mechanism through which HIIE reduces postprandial TG is an increase in TRL bound TG hydrolysis by LPL and subsequent peripheral tissue, most likely skeletal muscle, uptake. This increase in LPL-dependent TG hydrolysis could be due to an increase in LPL activity and/or an increased affinity of TRL for LPL. Indeed recent work has indicated that moderate intensity exercise may be compositional changes to VLDL, particles, by increasing particle size, that increase affinity to LPL for clearance from the circulation [47]. Whether such a change occurs after HIIE merits further examination.

If it is the case that LPL activity is increased by HIIE then this would be somewhat surprising if HIIE is viewed in light of the hypothesized energy expenditure threshold proposed for moderate intensity exercise. The estimated energy expenditure of HIIE in the current study is only 92 ± 20.1 kcal. Even allowing for a large error in estimation or post-exercise oxygen consumption this is well below the proposed threshold of 1,100 kcal for moderate intensity exercise. It is, therefore, possible that LPL activation is fibre type specific in humans, as well as rats, and that if exercise is of sufficient intensity/duration to recruit fast fibres then LPL activity will be increased. Further work is clearly required to firstly confirm the hypothesised increase in LPL activity after HIIE and secondly to investigate the potential for this to be a fibre type specific effect.

As many of the deleterious effects of elevated postprandial TG are thought to be due to leukocyte activation we investigated whether HIIE could attenuate this. We found that there was no difference in any of the markers of leukocyte activation used in the current study between trials, although effects of time were noted. The rise in CD11b during day 2 and 3 of the study in monocyte, lymphocyte and granulocyte populations is likely due to the rise in postprandial TG. Increased TG levels after a high-fat meal have previously been shown to activate leukocytes and increase CD11b expression [48]. This may be caused by endothelial inflammation which is enhanced in response to lipid exposure. The majority of this increase was seen in day 3 and it may be that this more prolonged, compared to a single high fat meal (e.g. in day 2), level of exposure to lipids is required for this immune activation to occur. Further work designed to specifically investigate this is needed. To the best of our knowledge, the current study is the first to assess CD36 expression in human leukocytes after a high-fat meal. The current study showed that CD36 expression was decreased in monocyte and monocyte populations over day 2 and 3 of the study. This decrease in CD36 surface expression might increase increased CD36 internalization as Zammors et al. [22] have shown that CD36 can become internalized in response to a pro-inflammatory environment. Furthermore, it is believed that decreased expression of CD36 in macrophages, induced by various cytokines, is linked to the development of atherosclerosis [49,50], possibly due to Toll-like receptor (TLR) pathway activation arising from the increase in cytokines, such as TNF-α. This can occur during pathogenic conditions, often seen after a high fat meal [10], where CD36 takes up and internalises oxLDL and may eventually lead to foam cell formation in macrophages [46]. While the current study found no effect of HIIE on the expression of leukocyte activation marker, a limited number of markers, from a wide number available, were monitored and clearly further work is needed in this area. Indeed a recent study found that moderate intensity exercise blunted the postprandial rise in the markers of leukocyte activation CD11a and CD18 [50], however this has not been investigated after HIIE.

In conclusion, the beneficial effects of HIIE on postprandial TG are abolished 2 days after exercise. This suggests that although high intensity exercise may be a time-efficient tool that is useful in the prevention of CVD, it will have to be performed 3-4 times a week to be effective. Furthermore, whilst the current study demonstrates a reduction in postprandial TG after HIIE, this is in young healthy participants. Whether this exercise may be of benefit in this group in the prevention of future disease there is a need to carry out such studies in populations who are at risk of CVD, such as in obesity or type 2 diabetes. This reduction in postprandial TG after HIIE was...
Figure 2

A

- HIIE Granulocytes
- Control Granulocytes
- HIIE Monocytes
- Control Monocytes

B

- HIIE Monocytes
- Control Monocytes
- HIIE Lymphocytes
- Control Lymphocytes

Figure 2. CD11b (A) and CD36 (B) Mean fluorescence intensity on gated cell populations on day 2 and 3 of the control trials. (A) *** denotes an effect of time for granulocytes and monocytes (P<0.001) (B) * denotes an effect of time for monocytes (P<0.005) and lymphocytes (P<0.05). ■ denotes laboratory meal times.

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associated with an increase in LTTH activity, with no difference in markers of leukocyte activation between trials.

Author Contributions

Conceived and designed the experiments: MG AR SG. Performed the experiments: MG AR SG JP VP-D. Analyzed the data: MG SRG JP PM VP-D. Contributed reagents/materials/analysis tools: SRG PM AR. Wrote the manuscript: MG JP VP-D PM AR SG.

References


