DEVELOPMENT AND APPLICATION OF A NOVEL METHOD TO DETERMINE LARGE VERY LOW DENSITY LIPOPROTEIN (VLDL₁) KINETICS

by

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Author’s Declaration

Unless otherwise indicated by acknowledgment or reference to published literature, the presented work in this thesis is the author’s own and has not been submitted for a degree at another institution.

IQBAL ALSHAYJI ____________________________________ Date____________________

The findings of some of the studies have been published as follows:

Published Papers


Published Conference Communications


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To my Mother,

with the utmost love, respect and appreciation.
The more I know, the more I realise I don’t know.
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<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette A1</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ARF-1</td>
<td>ADP ribosylation factor 1</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary artery bypass graft</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesteryl esters</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CHOL</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>CM</td>
<td>Chylomicrons</td>
</tr>
<tr>
<td>CR</td>
<td>Clearance rate</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DNL</td>
<td>de novo lipogenesis</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERGIC</td>
<td>Endoplasmic reticulum-Golgi intermediate compartment</td>
</tr>
<tr>
<td>Exp</td>
<td>Exponential decay</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FC</td>
<td>Free cholesterol</td>
</tr>
<tr>
<td>FCR</td>
<td>Fractional catabolic rate</td>
</tr>
<tr>
<td>FSR</td>
<td>Fractional synthetic rate</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HL</td>
<td>Hepatic lipase</td>
</tr>
<tr>
<td>HOMA\text{IR}</td>
<td>Homeostasis model assessment-estimated insulin resistance</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycans</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoprotein</td>
</tr>
<tr>
<td>IL</td>
<td>Intralipid</td>
</tr>
<tr>
<td>IVFTT</td>
<td>Intravenous fat tolerance test</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin cholesterol acyltransferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>LDL receptor</td>
</tr>
<tr>
<td>LLTP</td>
<td>Large lipid transfer protein</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>LRP</td>
<td>LDL receptor-related protein</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MTP</td>
<td>Microsomal triglyceride transfer protein</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acid</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipids</td>
</tr>
<tr>
<td>PLTP</td>
<td>Phospholipids transfer protein</td>
</tr>
<tr>
<td>PR</td>
<td>Production rate</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>SAAM II</td>
<td>Simulation, Analysis And Modeling II</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>sdLDL</td>
<td>Small dense low density lipoprotein</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>S_f</td>
<td>Svedberg floatation rate</td>
</tr>
<tr>
<td>SR-BI</td>
<td>Scavenger receptor type B class I</td>
</tr>
<tr>
<td>SS</td>
<td>Steady State</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>TRL</td>
<td>Triglyceride-rich lipoprotein</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>VLDLR</td>
<td>VLDL receptor</td>
</tr>
<tr>
<td>VO₂max</td>
<td>Rate of maximal oxygen uptake</td>
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Figure 5.4: Possible mechanisms involved in moderate exercise-induced reduction in VLDL1. Control: once in the circulation, the TG content of the newly secreted VLDL1 particle from the liver, and consequently its size, are being reduced rapidly by hydrolysis by lipoprotein lipase (LPL) and the action of cholesteryl ester transfer protein (CETP), which promotes the transfer of TG from VLDL (and LDL) in exchange for cholesteryl ester (CE) from HDL. Exercise: A prior session of moderate exercise significantly reduces VLDL1 concentration by increasing its clearance from plasma, possibly by compositional changes to the VLDL1 particle which render it bigger in size and more TG-enriched, thereby increase its affinity for LPL-mediated lipolysis. The bigger particles are either produced directly by the liver and/or reduced CETP activity. Also, increased LPL activity is likely to play a role.

CHAPTER 8

Figure 8.1: Production rates of [A] VLDL1-TG and [B] VLDL1-apoB and [C] Intralipid-TG clearance rate for all subjects (n = 23) according to apoE phenotypes: E2/E3 (n = 3), E3/E3 (n = 11) and E4+ (n = 9). $P > 0.05$ for differences between phenotypes.
Abstract

High concentrations of large very low density lipoproteins (VLDL₁) give rise to atherogenic dyslipidaemia, which is usually associated with insulin resistant conditions (e.g. obesity) and increases the risk for cardiovascular disease (CVD). Isotopic tracer methods for determining VLDL₁ kinetics are costly, time-consuming, labour intensive and need experience and skill to calculate the kinetic parameters. The aim of this thesis was to develop a simpler and cost-effective method of obtaining triglyceride-rich lipoproteins (TRL) kinetic data, based on the fact that chylomicrons (CM) or CM-like particles (e.g. Intralipid) compete with large VLDL₁ for the same lipoprotein lipase (LPL)-mediated catalytic pathway. From this method, it was possible to determine VLDL₁-triglyceride (TG) and -apolipoprotein (apo) B production rates and the Intralipid-TG clearance rate (as a surrogate measure of CM clearance). Kinetic data obtained from this method agreed with values and relationships obtained from stable isotope methods. The protocol is relatively quick, inexpensive, and transferable to non-specialist laboratories.

As a first application, the ‘Intralipid method’ was used to investigate the effects of hyperinsulinaemia and hyperglycaemia due to glucose ingestion on VLDL₁-TG and -apoB production rates and Intralipid-TG clearance rate. This showed that hepatic VLDL₁ production is suppressed in response to hyperinsulinaemia and that the change in Intralipid-TG clearance rate with hyperinsulinaemia correlated significantly with HOMA-estimated insulin resistance (HOMA IR). In addition, alanine aminotransferase (ALT) concentrations (a marker for liver fat), within normal range, predicted the extent of hepatic VLDL₁ suppression.

Secondly, the Intralipid method was used to investigate the mechanisms responsible for the hypotriglyceridaemic effect of a moderate exercise session (120 min walking at 50% _VO₂₃₉₉₉₉₉₉₉₉₉) in overweight/obese middle-aged men; the section of the population at high risk of CVD in whom exercise-for-health guidelines are targeted. This showed that the exercise-induced reduction in plasma TG was due to increased VLDL₁-TG and -apoB clearance, rather than decreased hepatic production. Exercise
also increased Intralipid-TG clearance rate, but to a lesser extent than VLDL₁, suggesting an increased affinity of VLDL₁ for LPL-mediated lipolysis post-exercise.

Taken together, the Intralipid method is a relatively simple, safe and cost-effective method to determine in VLDL₁-TG and -apoB production rates and Intralipid-TG clearance rates. It is also sensitive enough to detect physiological changes in TRL kinetics.
1. Introduction and Literature Review

1.1 Introduction

This chapter aims to establish a scientific rationale for the experimental studies described in this thesis. It starts with a brief overview of lipoprotein classification and metabolism. This is followed by an in-depth review of very low density lipoprotein (VLDL) metabolism and regulation, with special emphasis on the effects of elevated concentrations of VLDL in the formation of atherogenic dyslipidaemia. In addition, the effects of hyperinsulinaemia and moderate exercise on VLDL production are also discussed. Finally, methods used for measuring VLDL kinetics are reviewed.

1.2 Lipoprotein Metabolism

1.2.1 Classification and Properties of Lipoproteins

Lipids, being insoluble in water, need to be combined with proteins for transport in the blood. The resulting particles are lipoproteins and the protein moieties are known as apolipoproteins (apo) (Gurr et al., 2002). The Lipoprotein particle consists of a hydrophobic core of triglycerides (TG) and cholesteryl esters (CE), surrounded by a monolayer surface of the amphipathic (both hydrophilic and hydrophobic) phospholipids (PL), small amounts of free cholesterol and proteins (Ginsberg et al., 2005). Lipoproteins differ according to the ratio of lipid to protein within the particle as well as the proportions of lipids, they form a continuum of particles varying in composition, size, density and function (Gurr et al., 2002; Ginsberg et al., 2005). It is convenient, therefore, to classify plasma lipoproteins into different density classes and to separate and isolate them by ultracentrifugation (Gurr et al., 2002). These are: chylomicrons (CM), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) (Table 1.1). It should be noted, however, that these classes are not homogenous and there is a wide variety of particle size and chemical composition within each class. The major apolipoproteins are described in Table 1.2.
Table 1.1: Composition and characteristics of the human plasma lipoproteins (Gurr et al., 2002; Ginsberg et al., 2005; Packard & Shepherd, 1997)

<table>
<thead>
<tr>
<th>Class</th>
<th>Density (g.ml⁻¹)</th>
<th>Svedberg Floatation Rate (Sₘ)</th>
<th>Diameter (nm)</th>
<th>Apolipoproteins</th>
<th>Function</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td>Chylomicrons</td>
<td>&lt;0.95</td>
<td>&gt;400</td>
<td>80-1000</td>
<td>AI, AII, AIV, B-48, CI, CII, CIII, E</td>
<td>Transport of dietary fat</td>
<td>2</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.95-1.006</td>
<td>20-400</td>
<td>30-80</td>
<td>B-100, CI, CII, CIII, E</td>
<td>Transport of endogenous fat</td>
<td>8</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006-1.019</td>
<td>12-20</td>
<td>25-35</td>
<td>B-100, CI, CII, CIII, E</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019-1.063</td>
<td>0-12</td>
<td>18-25</td>
<td>B-100</td>
<td>Transport of CHOL to periphery</td>
<td>22</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.210</td>
<td>–</td>
<td>5-12</td>
<td>AI, AII, AIV, CI, CII, CIII, D, E</td>
<td>Reverse transport of CHOL</td>
<td>40</td>
</tr>
</tbody>
</table>

* Free and esterified cholesterol (CHOL).
<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>MW</th>
<th>Major site of synthesis</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>apo A-I</td>
<td>28 000</td>
<td>Liver, intestine</td>
<td>Structural component of HDL; activates LCAT</td>
</tr>
<tr>
<td>apo A-II</td>
<td>17 000</td>
<td>Liver, intestine</td>
<td>May inhibit HL activity; inhibits AI/LCAT</td>
</tr>
<tr>
<td>apo A-IV</td>
<td>44 500</td>
<td>Intestine</td>
<td>Activates LCAT; possibly facilitates transfer of apos between HDL and chylomicrons</td>
</tr>
<tr>
<td>apo A-V</td>
<td>39 000</td>
<td></td>
<td>Associated with lower TG levels; facilitates LPL</td>
</tr>
<tr>
<td>apo B-48</td>
<td>241 000</td>
<td>Intestine</td>
<td>Structural component of chylomicrons</td>
</tr>
<tr>
<td>apo B-100</td>
<td>513 000</td>
<td>Liver</td>
<td>Necessary for secretion of VLDL from liver; structural protein of VLDL, IDL and LDL; ligand for the LDL receptor</td>
</tr>
<tr>
<td>apo C-I</td>
<td>6 600</td>
<td>Liver</td>
<td>Activates LCAT; may inhibit hepatic uptake of chylomicrons and VLDL remnants; may inhibit CETP</td>
</tr>
<tr>
<td>apo C-II</td>
<td>8 800</td>
<td>Liver</td>
<td>Activates LPL (essential cofactor)</td>
</tr>
<tr>
<td>apo C-III</td>
<td>8 800</td>
<td>Liver, intestine</td>
<td>Inhibits LPL and hepatic uptake of chylomicrons and VLDL remnants</td>
</tr>
<tr>
<td>apo E</td>
<td>34 000</td>
<td>Liver (60-80%), other tissues including adipose tissue</td>
<td>Ligand for LDL receptor, LDL receptor-related protein and proteoglycans</td>
</tr>
</tbody>
</table>
Lipoproteins can also be classified according to their major lipids content into TG-rich lipoproteins (TRL), which include chylomicrons and VLDL, and cholesterol-rich lipoproteins, which include LDL and HDL (Sparks & Sparks, 1994).

Lipoprotein metabolism is a complex process to which new insights are continually being added. TRL metabolism is divided into the exogenous and endogenous lipid transport pathways. The exogenous pathway involves the delivery of dietary lipids (cholesterol, TG and PL) from the small intestine to the liver and peripheral tissues, while the endogenous pathway involves the delivery of lipids from the liver to peripheral tissue. As far as cholesterol metabolism is concerned, the exogenous and endogenous pathways are collectively known as forward cholesterol transport, whereas HDL metabolism is also known as the reverse cholesterol transport as it removes cholesterol from peripheral tissues.

1.2.2 Exogenous Lipoprotein Metabolism

After ingestion of a meal, dietary TG and cholesterol are absorbed into the small intestine cells where they are packed into nascent apoB-48-containing chylomicrons (Ginsberg et al., 2005) with a Svedberg flotation rate (Sf) of >400 (Gurr et al., 2002) (Figure 1.1). The newly synthesised chylomicrons enter the circulation through the lymphatic system and via the superior vena cava, where they acquire apoC-II, apoC-III and apoE (Ginsberg et al., 2005). Chylomicrons also contain apoA-I and apoA-IV (Alaupovic, 1991). The TG content of chylomicrons is then hydrolysed by lipoprotein lipase (LPL) (section 1.2.5) situated on the capillary endothelium of predominantly adipose and muscle tissues (Fielding & Frayn, 1998; Goldberg, 1996). While apoC-II acts as activator for LPL (Olivecrona & Beisiegel, 1997), both apoC-III (Wang et al., 1985) and apoE (Jong et al., 1997) act to inhibit lipolysis by LPL. However, it is the balance of apoC-II and apoC-III that determines, in part, the efficiency with which LPL hydrolyses chylomicron TG (Ginsberg et al., 2005; Chan et al., 2008). The released non-esterified fatty acids (NEFA) from chylomicrons are either taken up by adipose tissues, re-incorporated into TG and stored, or by muscle tissue where they can be used for energy (Ginsberg et al., 2005). Some dietary NEFA will also ‘spillover’ into the circulation (Barrows et al., 2005). As a result of lipolysis, a new particle called ‘chylomicron remnant’ is formed with some attached LPL molecules (Saxena et al., 1989). However, it should be noted that the majority
of chylomicrons do not form small chylomicron remnants, rather they show signs of being marginated to the vascular endothelium and removed as very large chylomicron remnants long before they reach the size of VLDL (i.e. $S_r$ 60-400 or the $S_r$ 20-60 range) (Karpe et al., 1997). Chylomicron remnants are relatively enriched in CE, both diet- and HDL-derived, which is transferred in exchange for TG, mediated by cholesteryl ester transfer protein (CETP) (Cooper, 1997; Ko et al., 1994; Chung et al., 2004). They are also enriched in apoE and apoB and depleted of apoA-I and apoCs (Cooper, 1997; Ginsberg et al., 2005). The normal physiological function of chylomicron remnants is to return bile cholesterol to the liver by an enterohepatic circulation (Redgrave, 2004), while its residual TG content is an important source of hepatic fatty acids (FA), accounting for $\sim$73% of newly synthesised hepatic VLDL in mice (Jung et al., 1999).

Hepatic uptake of remnant particles (Figure 1.2) is thought to start when they are sequestered in the liver perisinusoidal space (space of Disse), where they undergo further processing by hepatic lipase (HL) and LPL (both of which may remain associated with the particle) and acquire apoE. LPL, HL and apoE can potentially serve as ligands for a group of hepatic scavenger receptors, which endocytoses the particles, leading to their lysosomal catabolism. Endocytosis of remnant particles occurs via the apoB/apoE-recognizing LDL receptor (LDLR) or LDL receptor-related protein (LRP) in association with heparan sulfate proteoglycans (HSPGs) or independently by proteoglycans that are secreted into the space of Disse (MacArthur et al., 2007; Havel & Hamilton, 2004; Mahley & Ji, 1999).

Another member of the LDLR family that functions as a peripheral apoE-recognising remnant lipoprotein receptor is the VLDL receptor (VLDLR). It is expressed abundantly in FA-active tissues (heart, skeletal muscle and adipose) and binds TRLs but not LDL (Takahashi et al., 2004). It is likely that VLDLR functions in concert with LPL and has been reported to play a major role in chylomicron metabolism by enhancing LPL-mediated TG hydrolysis in mice (Goudriaan et al., 2004).
Dietary triglycerides (TG) and cholesteryl esters (CE) are absorbed into the small intestine and packed as chylomicrons, which also contain apoB-48 and apoA-I proteins. Chylomicrons are secreted into the circulation through the lymphatic system, where they acquire apoCs and E (apoC-II acts as an activator for the lipoprotein lipase enzyme (LPL) and apoE is needed for receptor recognition by the liver). Chylomicron-TG is hydrolysed by LPL situated on the capillary endothelium of mainly adipose tissues and skeletal muscles, providing the underlying tissue with non-esterified fatty acids (NEFA). Some dietary NEFA may ‘spillover’ in the circulation during hydrolysis by LPL. Chylomicron-TG is also removed from the particle in exchange for CE from high density lipoprotein (HDL). This is catalysed by the action of cholesteryl ester transfer protein (CETP). The result is a slightly smaller and CE-enriched particle called ‘chylomicron remnant’. It is also depleted in apoCs and apoA-I. The majority of chylomicron remnants are removed from plasma long before they reach the size of VLDL particles range. Remnant particles are taken up by the liver by a number of receptors (see Figure 1.2).
Figure 1.2: Model of possible hepatic uptake of TRL remnants. Hepatocytes and endothelial cells produce membrane-bound heparan-sulfate (HS) proteoglycans (HSPGs) and secrete proteoglycans into the space of Disse. After lipolytic processing of lipoproteins in the circulation by lipoprotein lipase (LPL; blue triangles), apoE-enriched (black circles) remnant lipoproteins enter the space of Disse through fenestrations in the endothelium. Remnant lipoproteins are thought to be sequestered near the hepatocyte cell surface via apoE-HS binding or lipase-HS bridging on secreted HSPGs. Lipoproteins are further processed in the space of Disse by transfer of soluble apoE (grey circles) and by hepatic lipase (HL; red triangles) bound via HS. ApoE, HL, and LPL can potentially serve as ligands of TRLs. Endocytosis of lipoprotein particles occurs via LDL receptor (LDLR; blue) or LDL receptor-related protein (LRP; purple) in association with HSPGs or independently by proteoglycans. [Figure and legend reproduced with permission from Journal of Clinical Investigation (MacArthur et al., 2007).]
1.2.3 *Endogenous Lipoprotein Metabolism*

The major bulk of endogenous plasma TG is carried by VLDL, which is continually synthesised and secreted by the liver (Packard & Shepherd, 1997). The VLDL particle contains one molecule of apoB-100 (Elovson *et al.*, 1988) and approximately 5-25,000 TG molecules in its core (Bjorkegren *et al.*, 1998). It also contains apoCs and apoE (Packard & Shepherd, 1997). There are at least two major subclasses of VLDL particles: large TG-rich VLDL$_1$ (S$_r$ 60-400) and smaller, more dense VLDL$_2$ (S$_r$ 20-60), which have more cholesterol and a lower ratio of apoCs and apoE to apoB (Packard & Shepherd, 1997; Packard *et al.*, 1984). In normolipidaemic subjects, about 75% of the variation in plasma TG seems to be determined by the VLDL$_1$ concentration (Tan *et al.*, 1995). Once in the circulation, the TG content of VLDL$_1$ is rapidly removed by LPL-mediated hydrolysis (Karpe *et al.*, 2007) and the action of CETP, which facilitates the transfer of TG from TRLs (mainly VLDL) to HDL in exchange for CE from HDL to apoB-containing lipoproteins (VLDL, IDL and LDL) (Stein & Stein, 2005; Ko *et al.*, 1994). As VLDL$_1$ particles become progressively depleted in TG, they may either attain the same size and TG content as VLDL$_2$ particles, or continue its delipidation to form IDL. This is further aided by the lipolytic action of HL, which is also involved in the hydrolysis of IDL-TG. As a result, LDL appears as the terminal particle (Packard *et al.*, 1984). During the transition of VLDL to IDL and IDL to LDL, a portion of the surface PL, apoCs and apoE are transferred to HDL (Verges, 2005).

Approximately 10% of VLDL$_1$ particles undergo complete delipidation to LDL, while the remaining cease delipidation in the VLDL or IDL density range forming remnant particles that persist in the circulation for considerable periods before being removed from plasma (Packard & Shepherd, 1997; Packard *et al.*, 1984). On the other hand, almost all VLDL$_2$ particles will be effectively delipidated to LDL (Gaw *et al.*, 1995; Packard *et al.*, 1984). Similarly, not all IDL particles are delipidated to LDL, some are cleared by the liver (Packard & Shepherd, 1997) along with TRL remnant particles, possibly by the clearance pathway as chylomicron remnants (see Figure 1.2).

LDL is the main cholesterol-carrying lipoprotein in plasma with each particle containing one molecule of apoB-100. Generally about 65% of the total plasma
cholesterol is carried in LDL in man (Wang & Briggs, 2004) and its major role is to supply tissues with cholesterol, which is paramount for every cell for the synthesis and maintenance of cell membranes. LDL particles are removed from plasma by the LDLR with LDL2 particle exhibiting the highest affinity (Packard & Shepherd, 1997). While 70% of the LDLRs are located on hepatic cells, 30% are located on the other cells of the body (Verges, 2005).

1.2.4 HDL Metabolism

The movement of lipids via chylomicrons, VLDL, IDL and LDL provides the peripheral cells with FA (fuel) and cholesterol (necessary for synthesis and maintenance of cell membranes). While this ‘forward’ movement of TG is balanced by the metabolic consumption of NEFA as a source of fuel, there is no analogous (i.e. catabolic) mechanism for the metabolic disposal of cholesterol. This is accomplished by ‘reverse’ cholesterol transport by HDL particles (Tulenko & Sumner, 2002). Over the last several decades, epidemiological studies have revealed that levels of LDL-cholesterol are directly associated with risks of CVD, while an inverse relationship existed between HDL-cholesterol and the risk of the disease (Wang & Briggs, 2004).

HDL particles are synthesised and secreted, mainly by the liver, as nascent or lipid-poor disc-shaped particles (pre-β HDL) containing apoA-I and small amount of PL (Wang & Briggs, 2004; Tulenko & Sumner, 2002). This lipid-poor apoA-I avidly absorbs free cholesterol and PL from peripheral cells mediated by ATP-binding cassette A1 (ABCA1) transporter on the ‘donor’ cells plasma membrane (Wang & Briggs, 2004). The free cholesterol, being amphipathic, is absorbed onto the surface of the small HDL, which is progressively esterified by lecithin cholesterol acyltranferase (LCAT; bound to HDL and activated by apo A-I) and stored in the core of HDL (Tulenko & Sumner, 2002; Wang & Briggs, 2004). The continuous addition of cholesterol converts pre-β HDL to larger spherical HDL3. Together with the action of phospholipid transfer protein (PLTP) which transfers phospholipids to HDL (mostly HDL3) particles from VLDL/LDL (van Tol, 2002), HDL3 is converted to the much larger HDL2 particles (Wang & Briggs, 2004; Verges, 2005). This stage of cholesterol transfer from cells to the HDL3 is mediated either by cell-surface receptors scavenger receptor class B type I (SR-BI) or passive diffusion, both distinct
from that mediated by ABCA1 (Wang & Briggs, 2004). In addition, HDL accumulates apoC-II and apoE from VLDL and IDL, which serve as a reservoir of apolipoproteins, especially apoC-II which is needed for the activation for LPL (Tulenko & Sumner, 2002).

The TG content of HDL is hydrolysed by HL, while removal of cholesterol from HDL₃ particles occurs through CETP-mediated neutral lipid (TG and CE) exchange between HDL and apoB-containing lipoproteins. Because the bulk of cholesterol removal is accomplished by shuttling to these particles, their uptake by the liver is essential for the disposal of HDL cholesterol (Tulenko & Sumner, 2002; Wang & Briggs, 2004). The degradation of the larger HDL₂ occurs directly by CE-selective uptake by the liver mediated by SR-B1. The apoA-I from the degradation is either recycled for new HDL formation or cleared by the cubulin, a receptor highly expressed in kidney and yolk sac (Wang & Briggs, 2004).

**Figure 1.3** summarises the endogenous lipoprotein metabolism.

### 1.2.5 Lipoprotein Lipase

Lipoprotein lipase (*EC 3.1.1.34*) is a key enzyme for the hydrolysis of circulating TG (Fielding & Frayn, 1998; Preiss-Landl *et al.*, 2002). It is synthesized by parenchymal cells, then transferred to the luminal surface of endothelial cells, where it is bound to HSPG (Goldberg, 1996). LPL is distributed in a variety of tissues, with the highest concentrations occurring in adipose tissue and muscle (Wong & Schotz, 2002) [cardiac and skeletal (Preiss-Landl *et al.*, 2002)]. However, substantial LPL activity is also detectable in differential macrophages, brain, placenta, lung, spleen, pancreatic β-cells and steroidogenic tissue (Preiss-Landl *et al.*, 2002). LPL functions to supply the underlying tissue with FAs derived from the TG-rich core of circulating chylomicrons and VLDL (Olivecrona *et al.*, 1997). However, chylomicrons have been shown to be the preferred substrate for LPL (Potts *et al.*, 1991; Fisher *et al.*, 1995), as they may bind to LPL with an affinity of 50 times higher than VLDL (Xiang *et al.*, 1999). In addition, it has been shown that about forty LPL molecules may act on a TRL particle simultaneously to achieve the rates of TG hydrolysis observed (Scow & Olivecrona, 1977). After hydrolysis of TG, the TG-depleted particle detaches along with some LPL molecules that may dissociate from the
endothelium and leave attached to the remnant particle (Goldberg, 1996; Fielding & Frayn, 1998). There is a continual turnover of LPL at the endothelial site of action, with replacement of the dissociated LPL molecules by newly secreted molecules from within the tissue. LPL circulating attached to lipoprotein particles may play an important role in their eventual receptor-mediated uptake (Fielding & Frayn, 1998) (see Figure 1.2).

The apoC-II associated with the TRL particle acts as an activator of the enzyme (Goldberg, 1996; Olivecrona & Beisiegel, 1997) and apoE, which is a strong heparan-binding protein, anchors TRLs to the HSPG on the surface of the endothelial cells (Goldberg, 1996). In contrast, apoC-III is the principal plasma inhibitor of VLDL lipolysis; it inhibits LPL as well as interfering with remnant lipoprotein clearance (Shachter, 2001; Havel et al., 1973).

The regulation of LPL is tissue-specific in such a way that its expression correlates highly with both the need for, and the uptake of, lipid fuels by the tissue. For instance, in the fed state, when energy is abundant, LPL is downregulated in skeletal muscle and heart and activated in white adipose tissue which facilitates the lipolysis of TRL-TG in the latter tissue, directing released NEFA for esterification and storage (Fielding & Frayn, 1998). On fasting, however, the situation is reversed, with suppression in adipose tissue and upregulation in muscle, increasing TRL-TG lipolysis in muscle, so that the resultant NEFAs are directed to the tissue in which they are needed as an oxidative fuel (Fielding & Frayn, 1998; Ruge et al., 2005). Such changes in LPL expression are mediated predominantly through the action of hormones, such as insulin, glucocorticoid, and adrenaline (Mead et al., 2002) as well as cytokines, fatty acids and glucose (Preiss-Landl et al., 2002). Insulin is of particular interest because it plays a central role linking dyslipidaemia and insulin resistance, the defining feature of the metabolic syndrome (Howard, 1999). This will be discussed further in section 1.5.
1. Introduction and Literature Review

Figure 1.3: Overview of the general aspects of endogenous lipoprotein metabolism. The grey lines represent hepatic uptake of lipoproteins and lipoprotein remnants. The major bulk of endogenous triglycerides (TG) is secreted by the liver in the form of VLDL₁ particles, of which apoB-100 protein forms an integral part on the surface of the particle. Once in the circulation, TG in the core of VLDL₁ is hydrolysed by the action of lipoprotein lipase (LPL) which provides non-esterified fatty acids (NEFA) to adipose tissue and skeletal muscle. VLDL₁ also loses some of its TG content to HDL in exchange for cholesteryl esters (CE) via the action of cholesteryl ester transfer protein (CETP). The continual removal of TG from VLDL₁ gives rise to smaller VLDL₂, which is also produced by the liver. VLDL₂ is either taken up by the liver or delipidated further by the action of hepatic lipase (HL) to form IDL then LDL. Unlike other apolipoproteins, apoB-100 stays with the particles as they are delipidated. LDL is the main cholesterol (CHOL)-carrying lipoprotein which provides cholesterol to peripheral tissues. HDL, on the other hand, is also secreted by the liver and acts as a ‘reverse’ cholesterol carrier, from peripheral tissues to the liver. LDL is taken up by the liver by the apoB/E recognising LDL receptor (LDLR). Remnant (smaller) lipoprotein particles are also taken up by the liver in a similar manner to chylomicron remnants (Figure 1.2) by hepatic receptors, such LDLR and LDL receptor-related protein (LRP).
1.2.6 Integration of TRL Metabolism

In the fasted state, the continually secreted VLDL (particularly the larger VLDL₁) particles by the liver are the major substrates for LPL (Fisher et al., 1995), providing endogenous fat to peripheral cells. However, after ingestion of a meal, the newly formed chylomicrons, being the preferred substrate for LPL (Potts et al., 1991; Fisher et al., 1995), will compete with VLDL₁ for the same LPL-mediated catalytic pathway (Brunzell et al., 1973; Karpe et al., 1993a). As the percent TG hydrolysed by LPL in TRL subfractions was chylomicrons > VLDL₁ > VLDL₂, it was proposed that increasing the size and TG content of a lipoprotein particle increases its susceptibility to hydrolysis by LPL (Fisher et al., 1995). Another possible explanation for this preferential lipolysis of chylomicrons compared with VLDL might be due to transient changes in VLDL₁ composition in the postprandial state. Both VLDL₁ and VLDL₂ are enriched in cholesterol, apoE, apoC-I and apoC-III in the postprandial state, but depleted in apoC-II (Bjorkegren et al., 1997). While apoC-II acts as activator for LPL (Olivecrona & Beisiegel, 1997), both apoC-III (Chan et al., 2008) and apoE (Jong et al., 1997) act to inhibit lipolysis by LPL.

As a result of this competition, the delipidation of VLDL₁ particles is delayed and their concentration is increased as they accumulate in plasma after the appearance of chylomicrons (Karpe et al., 1993a) or chylomicron-like particles (Karpe & Hultin, 1995; Bjorkegren et al., 1996) (Figure 1.4). Indeed, the postprandial increase in the TRL particle number is mainly accounted for by VLDL, particularly VLDL₁ (Karpe et al., 1993a). VLDL₂ particles, on the other hand, are not affected by this competition and, in fact, their concentrations are often lowered or unchanged postprandially (Bjorkegren et al., 1996; Karpe et al., 2007). This is because, firstly, less VLDL₁ is converted into VLDL₂ and, secondly, VLDL₂ is not a particularly good substrate for LPL (Fisher et al., 1995; Karpe et al., 2007).

This competition and the preferential lipolysis by LPL between chylomicron-like particles and VLDL₁ form the basis of work described in this thesis.
1. Introduction and Literature Review

Figure 1.4: TRL metabolism in the [A] fasted and [B] postprandial state. VLDL₁ and chylomicrons (CM) are both cleared by lipoprotein lipase (LPL), situated on the capillary endothelium of adipose tissue and skeletal muscle. CMs are the preferred substrate for LPL. Thus, the presence of CM (or CM-like particles) inhibits VLDL₁ clearance by this pathway, causing VLDL₁ to accumulate in the circulation.
1.3 Hepatic Assembly and Secretion of VLDL

The assembly of VLDL in the liver involves a number of complex processes which are still not fully understood. VLDL assembly is believed to take place in two major steps within two different compartments of the cell, rough endoplasmic reticulum (ER) and Golgi apparatus, and involves three different particles, pre-VLDL, VLDL₂ and VLDL₁ (Olofsson & Borén, 2005). It commences during the synthesis of apoB which is an integral part of the VLDL structure.

1.3.1 Apolipoprotein B Structure

Unlike other plasma lipoproteins, apoB is an amphipathic high molecular weight glycoprotein which is insoluble in aqueous solutions (Segrest et al., 2001; Kane, 1983). Because it binds irreversibly to TRLs and LDL, it is not transferable between lipoproteins (Kane, 1983; Davidson & Shelness, 2000). There are two forms of apoB in mammals, which are encoded by the same gene (Davidson & Shelness, 2000). The full-length apoB, apoB-100, consists of 4536 amino acids, whereas apoB-48 corresponds to 48% of the protein from the N-terminal (Davidson & Shelness, 2000). ApoB-48 is produced by a site-specific cytidine-to-uridine RNA editing reaction which converts a glutamine codon to a stop codon causing translational termination of (intestinal) apoB mRNA at residue 2152 (Davidson & Shelness, 2000; Olofsson & Borén, 2005). In humans, apoB-100 is expressed in the liver, forming VLDL, while apoB-48 is expressed in the small intestine, forming chylomicrons.

The apoB protein has a pentapartite structure consisting of one globular N-terminal structure, followed by two amphipathic β-sheets alternating with two amphipathic α-helical domains (NH₂-βα₁-β₁-α₂-β₂-α₃-COOH) (Segrest et al., 2001). The βα₁ domain (the N-terminal 1000 residues) is a globular structure that bears structural homology with the lipid-binding pocket of microsomal TG transfer protein (MTP) (an ER luminal protein with lipid transfer activity – see below) and the lamprey vitellogenin (an egg yolk lipoprotein) (Segrest et al., 2001). Thus, apoB, MTP and vitellogenin are considered members of the same gene family collectively known as large lipid transfer proteins (LLTPs). Although vitellogenin was presumed to be the
ancestral member, recent evidence suggests that MTP may be the oldest LLTP family member (Shelness & Ledford, 2005).

The amphipathic β-sheet domains of apoB interact irreversibly with the neutral lipid core, while the α-helical domains, which are similar to those found in soluble apolipoproteins, may form strong but reversible binding to the lipoprotein surface (Davidson & Shelness, 2000). The β2-region contains the LDL receptor-binding domain, needed for binding and hepatic endocytosis of LDL particles, and resembles the receptor binding domain of apoE, another LDLR ligand. As this domain is in the truncated C-terminal region of apoB-48, chylomicrons depend on apoE to bind to the LDLR (Segrest et al., 2001).

1.3.2 Assembly of VLDL

The intracellular assembly of VLDL (Figure 1.5) starts in the rough ER during the biosynthesis of apoB, which is lipidated co-translationally; i.e. while the C-terminal portion of apoB is still being synthesised on the ribose of the ER, the N-terminal portion is translocated across the ER and is assembled as a small lipoprotein particle (Olofsson & Borén, 2005; Dashti et al., 2007). ApoB interacts co-translationally with MTP, which catalyses the addition of lipids [in the order of TG > CE > diacylglycerol > cholesterol > PL (Rava et al., 2005)] to the growing apoB molecule. This results in the formation of a primordial HDL-sized lipoprotein particle, pre-VLDL, which is retained in the cell by interaction with chaperones and other ER proteins (Olofsson & Borén, 2005; Shelness & Sellers, 2001). It has been recently shown that MTP is not required for the initiation of this step as the first N-terminal 1000 amino acid residue (apoB:1000) has the capacity to fold forming a PL-rich ‘lipid-pocket’, independently of the structural requirement and lipid-transfer activity of MTP (Manchekar et al., 2004; Dashti et al., 2007). It is currently unclear at which point in VLDL assembly that MTP is required. In addition to its lipid transfer activity, MTP facilitates the co-translational translocation of apoB across the ER lumen (Blasiole et al., 2007) and prevents its degradation as apoB will be sorted to proteasomal degradation if it misfolds or is underlipidated (Fisher & Ginsberg, 2002; Hussain et al., 2003). Pre-VLDL is lipidated further to become VLDL2, or sorted to degradation. This conversion of pre-VLDL to VLDL2 may explain why the MTP
activity is needed for the secretion of apoB-100 after the translation is completed (Rustaeus et al., 1998; Olofsson & Borén, 2005).

The apoB-100 containing VLDL\textsubscript{2} particle exits the ER at specific exit sites carried by two vesicle proteins: a GTPase referred to as SAR1 and a coat protein called coatamer protein II (COPII) (Gusarova et al., 2003; Olofsson & Borén, 2005), which fuse to become ER-Golgi intermediate compartment (ERGIC). It is converted to \textit{bona fide} VLDL\textsubscript{1} by post-translational acquisition of TG in the Golgi apparatus (Shelness & Sellers, 2001; Olofsson & Borén, 2005). This lipidation differs from that which gives rise to VLDL\textsubscript{2} and requires that apoB has reached the size of apoB-48. In addition, it is dependent on ADP ribosylation factor 1 (ARF-1), a small GTP binding protein which plays a role in the membrane trafficking between the ER and the Golgi apparatus (Asp et al., 2000; Asp et al., 2005). It has been suggested that this ‘maturation’ step of VLDL\textsubscript{1} may involve the formation of lipid droplets in the lumen of the secretory pathway, which fuse with apoB to form VLDL\textsubscript{1} (Olofsson & Borén, 2005). Further, this conversion of VLDL\textsubscript{2} to VLDL\textsubscript{1} seems an additional step for TG secretion from the liver as it is not necessary for the secretion of apoB (Olofsson & Borén, 2005). The liver has been shown to secrete both VLDL\textsubscript{1} and VLDL\textsubscript{2} particles (Packard et al., 2000). Interestingly, this stepwise assembly and secretion of VLDL explains the time delay between the biosynthesis of apoB-100 and the major addition of lipids to the VLDL\textsubscript{1} particles, which was estimated as \~15 min (Adiels et al., 2005a).

### 1.4 Regulation of VLDL Production

The secretion of VLDL has been shown to be mainly dependent on fatty acids and insulin.

#### 1.4.1 Role of Fatty Acids

The liver has the capacity to store TG in order to accommodate plasma fatty acids, which are surplus to immediate energy requirements, thereby neutralising any potential ‘lipotoxicity’ to peripheral tissue. There are three potential sources of fatty acids which enter the hepatic TG storage pool: \textit{de novo} lipogenesis (DNL), plasma NEFA [originating from adipose tissue and ‘spillover’ from dietary fat lipolysis (Barrows et al., 2005)] and remnant lipoproteins (Gibbons et al., 2000). A healthy
liver uses different sources of FAs in the fasted and fed states (Barrows & Parks, 2006). It is estimated that during fasting, ~77% of VLDL-TG is derived from recycling adipose FAs and ~4% from DNL. With feeding, 44% come from adipose FAs, 15% from uptake of chylomicron remnants, 10% from dietary NEFA spillover into plasma and 8% from DNL (Barrows & Parks, 2006), with dietary FAs appearing in VLDL within 90 min of food intake (Heath et al., 2003).

Figure 1.5: The Intracellular assembly of VLDL. (Numbers in parentheses refer to corresponding numbers in the figure.) The assembly process starts in the rough endoplasmic reticulum (ER) by the biosynthesis and concomitant (cotranslational) translocation of apoB-100 to the lumen of this organelle (1). ApoB-100 interacts cotranslationally with the microsomal triglyceride transfer protein (MTP) and is lipidated to form a primordial particle (pre-VLDL) (2). Alternatively, apoB-100 fails to be lipidated and misfolds resulting to degradation (3). The protein is unfolded and retracted to the cytosol, ubiquinated, and sorted to proteasomal degradation (3 and 4). Pre-VLDL is converted to VLDL₂ late in the ER compartment (5). VLDL₂ exits the ER at specific exit sites of this organelle by Sar1/Cop II vesicles (6), which fuse to become the ER–Golgi intermediate compartment (ERGIC) (7). ERGIC fuses with Cis-Golgi (8). In the Golgi apparatus, VLDL₂ is converted to VLDL₁ by the addition of a bulk load of triglycerides (9). This lipidation process differs from that which gives rise to pre-VLDL₁ and VLDL₂. The formation of VLDL₁ may involve the formation of a lipid droplet in the lumen of the secretory pathway (10), the mechanism of which may follow that of cytosolic lipid droplets (11 and 12). The formation of the cytosolic droplets also involves a fusion step (12). [Figure and legend with permission from Current Opinion in Lipidology (Adiels et al., 2006a).]
It has been suggested that this hepatic TG pool is metabolically active with a rapid turnover such that extracellular FAs entering the cell are either oxidised in the mitochondrion or esterified into TG in the ER and then transferred to the cytosolic storage pool. TG required for VLDL synthesis is recruited from this pool by a process of lipolysis to give FAs (Gibbons et al., 2000). This may explain the findings of Malmstrom et al. (1999) who reported that acute NEFA flux is not accompanied by concomitant changes in the production of total VLDL-apoB (Malmstrom et al., 1999). In fact, reducing NEFA concentrations using acipimox (an antilipolytic agent) caused a shift from VLDL₁ to VLDL₂ production, without affecting total VLDL-apoB production (Malmstrom et al., 1998), suggesting an important role of fatty acid availability for the assembly and secretion of VLDL₁.

Conversely, earlier studies which did not isolate VLDL subfractions indicate that acutely raising plasma NEFA concentrations could increase both VLDL-TG and VLDL-apoB production (Lewis et al., 1995), thus suggesting a role for FAs in the stimulation of TG and apoB synthesis. This assumption is supported by animal studies, where chronic administration of intravenous albumin-bound oleic acid doubled hepatic secretion of apoB-100 (without changes in apoB mRNA levels), suggesting that FAs might act as both a stimulus for TG synthesis, thereby driving assembly of VLDL, and as a ‘signal’ for apoB assembly (Zhang et al., 2004b). Indeed, increased liver fat has been shown to increase the secretion of VLDL₁-apoB-100, and more – but not larger – VLDL₁ particles are secreted (Adiels et al., 2006b).

### 1.4.2 Role of Insulin

Despite insulin’s induction of lipogenesis in the liver, it acutely suppresses hepatic VLDL production in normal weight, healthy subjects (Lewis et al., 1993) – specifically affecting the VLDL₁ subclass (Malmstrom et al., 1998; Adiels et al., 2007). This effect is partly attributed to its induced reduction of NEFA flux to the liver, thus decreasing substrate availability for VLDL assembly (Coppack et al., 1994). However, other mechanisms are likely to play a role as this suppressive effect of insulin on VLDL₁ production persisted even when compared with the action of acipimox (which caused a similar reduction in NEFA) (Malmstrom et al., 1998). Although these mechanisms are still elusive, some have been investigated. Insulin inhibits the secretion of apoB (Sparks & Sparks, 1994) and insulin signalling (via...
phospho-inositide 3-kinase) inhibits the maturation phase of VLDL assembly by preventing bulk lipid transfer to a VLDL precursor, thus enhancing the degradation of apoB (Brown & Gibbons, 2001). In addition, it inhibits transcription (Lin et al., 1995) and upregulation (Wolfrum & Stoffel, 2006) of the MTP gene, although it has been suggested that this is unlikely to account for the acute effect of insulin on VLDL production due to the long half-life of the MTP protein (4.4 days) (Lin et al., 1995; Blasiole et al., 2007).

In contrast, chronic hyperinsulinaemia, caused by insulin resistance, is associated with increased production of VLDL (Malmstrom et al., 1997a), specifically VLDL1 (Adiels et al., 2007). Possible mechanisms for this loss of the suppressive effect of insulin have been studied in a fructose-fed hamster model of insulin resistance (Taghibiglou et al., 2000). These included increased cellular secretion of total and VLDL-TG, enhanced stability of nascent apoB, thereby reducing its post-translational degradation and increased MTP mass (Taghibiglou et al., 2000). In addition, insulin resistance also leads to loss of the acute insulin-mediated inhibition of apoB secretion (Chirieac et al., 2004). In humans, central obesity and an increased liver fat content, which are usually associated with insulin resistance conditions, have been associated with the lack of insulin-induced suppression of VLDL1 production (Adiels et al., 2007) (see below).

1.5 Defective TRL Metabolism and Atherogenic Dyslipidaemia

The concept of atherogenicity of TG and TRL, especially in the postprandial state, was first introduced almost 30 years ago by Zilversmit (Zilversmit, 1979). Since then, an increasing body of evidence has implicated hypertriglyceridaemia as an independent risk factor for cardiovascular disease (CVD), particularly coronary heart disease (CHD) (Cullen, 2000). TRL are thought to have a role in the development and progression of atherosclerosis (Tanaka et al., 2001; Cullen, 2003) as TRL remnants have been found in human aortic atherosclerotic plaques (Nakano et al., 2008). In addition, elevated concentrations of VLDL1 particles are believed to start a chain of metabolic events that generate an ‘atherogenic lipoprotein phenotype’, also known as atherogenic dyslipidaemia.
1.5.1 Atherogenic Lipoprotein Phenotype

The atherogenic lipoprotein phenotype is a cluster of lipoprotein abnormalities associated with insulin resistant states (Ginsberg et al., 2005), such as obesity (Bamba & Rader, 2007; Marsh, 2003; Chan et al., 2004a), type 2 diabetes (Marsh, 2003; Taskinen, 2003) and the metabolic syndrome (Grundy, 2006; Kolovou et al., 2005), and significantly contributes to increased risk of CVD (Avramoglu et al., 2006; Watson et al., 2003).

Such atherogenic dyslipidaemia manifests as increased concentrations of hepatic VLDL (causing hypertriglyceridaemia), small dense LDL (sdLDL), TRL remnants and low HDL concentrations (Grundy, 2006; Taskinen, 2003; Avramoglu et al., 2006). Importantly, it is the excess hepatic production of VLDL$_1$ [the major determinant of plasma TG concentration (Hiukka et al., 2005)] but not VLDL$_2$ particles (Adiels et al., 2005b; Adiels et al., 2006b) that is associated with insulin resistance, indicating that VLDL$_1$ and VLDL$_2$ are independently regulated (Gill et al., 2004a; Packard & Shepherd, 1997).

1.5.2 Elevated Levels of VLDL$_1$, Insulin Resistance and Obesity

Hepatic overproduction of VLDL (particularly the large VLDL$_1$) has been associated with insulin resistance (Gill et al., 2004a; Johanson et al., 2004) and related conditions such as diabetes (Adiels et al., 2005b), the metabolic syndrome (Grundy, 2006) and obesity (Mittendorfer et al., 2003a). Of particular interest, abdominal obesity which is characterised by excess visceral and/or deep upper body subcutaneous adipose tissue accumulation and is strongly associated with obesity, insulin resistance and atherogenic dyslipidaemia (Després, 2007; Sniderman et al., 2007). Because visceral fat is more lipolytically active than subcutaneous fat in vitro (Mauriege et al., 1987; Arner, 1998; Rebuffe-Scrive et al., 1989), it has been hypothesised that visceral fat accumulation results in a markedly increased flux of NEFA via the portal vein to the liver (Bjorntorp, 1990; Chan et al., 2004a), thereby increase substrate availability for hepatic VLDL formation (Kissebah et al., 1976). However, Nielsen et al (2004) demonstrated that although the release of NEFA from visceral fat depots into the portal vein increases with increasing visceral fat, it was much smaller than the amount derived from lipolysis of upper-body subcutaneous fat.
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(Nielsen et al., 2004). Nevertheless, hepatic overproduction of VLDL\textsubscript{1} particles has been found to be driven by increased liver fat content in man (Adiels et al., 2006b) and reduction in visceral adipose tissue is associated with decreased hepatic production of VLDL (Riches et al., 1999). In addition, increased NEFA flux to the liver impairs hepatic extraction of insulin (Chan et al., 2004a). Normally, insulin inhibits hepatic VLDL\textsubscript{1} production, in the fed state, but this effect is lost in insulin resistant conditions and high liver fat content, leading to increased hepatic VLDL\textsubscript{1} secretion (Adiels et al., 2007).

Furthermore, the clearance of VLDL\textsubscript{1} and TRL particles and their remnants is impaired in insulin resistant conditions due to decreased LPL activity (Mead et al., 2002), excess of apoC-III (an inhibitor of LPL) (Florez et al., 2006) and failure to bind to LDLR and LRP (Packard, 2003) or suppression of LDLR (Mamo et al., 2001).

1.5.3 VLDL\textsubscript{1} and the Generation of Atherogenic Dyslipidaemia

Whether due to hepatic overproduction or defective clearance, elevated levels of VLDL\textsubscript{1}, which give rise to a plasma TG concentration $> 1.5$ mmol.l\textsuperscript{-1}, are believed to start a sequence of events that result in the atherogenic lipoprotein phenotype (Packard, 2003; Taskinen, 2003). Increased proportion of large LDL is a marker of efficient lipolysis of TG, which, in effect, will lower fasting and postprandial levels of TRL (Griffin, 1997). However, the presence of VLDL\textsubscript{1} gives rise to LDL particles ($\beta$LDL) with altered apoB-100 conformation which impedes binding of these particles to LDLR. In addition, impaired clearance of VLDL\textsubscript{1} particles (see above) will result in their accumulation in the circulation. As a result, VLDL\textsubscript{1} and $\beta$LDL particles have a prolonged residence time in the circulation, thereby increasing their likelihood of remodelling by CETP (Packard, 2003), which is increased in activity in insulin resistant states (Guerin et al., 2001). Under these conditions, CETP promotes the acquisition of TG by LDL and HDL in exchange for CE from HDL to VLDL\textsubscript{1} and LDL (Packard, 2003; Guerin et al., 2001). These TG-enriched LDL and HDL particles become favourable substrates for HL resulting in a reduction in particle size. HL also has an enhanced activity in obesity and insulin-resistant states (Deeb et al., 2003). This gives rise to (1) sdLDL with a prolonged residence time (~5 days) compared with the larger LDL derived from VLDL\textsubscript{2} and IDL, which
has a residence time of ~2 days (Packard, 2003) and (2) small unstable HDL particles that are cleared rapidly from the circulation, resulting in low HDL cholesterol and apoA-I (Rashid et al., 2003) and loss of its protective potential against CVD (Wang & Briggs, 2004). sdLDL is the most readily oxidised subfraction in the lipoprotein class (Tribble et al., 1992), increasing its atherogenic potential. **Figure 1.6** shows a schematic diagram of the mechanisms involved in the formation of atherogenic dyslipidaemia.
Figure 1.6: Schematic diagram of the mechanisms involved in the formation of atherogenic dyslipidaemia. Elevated concentrations of VLDL₁ particles, producing plasma triglyceride (TG) concentrations > 1.5 mmol.l⁻¹, are believed to start a chain of reactions to generate an atherogenic lipoprotein phenotype (hypertriglyceridaemia, small dense (sd)LDL and low HDL). VLDL₁ accumulates in the circulation due to hepatic overproduction, coupled with decreased clearance due to low lipoprotein lipase (LPL) activity and/or excess of apoC-III (an inhibitor of LPL). Lipolysis of VLDL₁ gives rise to βLDL particles which have an altered apoB confirmation and, thus, fail to bind well to the LDL receptor (LDLR). Consequently, VLDL₁ and βLDL have a prolonged residence time in plasma. This increases their likelihood of undergoing remodelling by cholesteryl ester transfer protein (CETP), which catalyses the transfer of TG from VLDL₁ to LDL and HDL in exchange for cholesteryl esters (CE). TG-enriched LDL and HDL become good substrates for hepatic lipase (HL) which hydrolyses the TG content of these particles. As a result, the atherogenic sdLDL and small unstable HDL particles are formed. sdLDL is readily oxidised, thus, increasing its atherogenic potential, while the smaller HDL particles are cleared rapidly from the circulation resulting in low HDL concentrations and losing its protective potential against CVD.
1.6 Effect of Exercise on TRL Metabolism

1.6.1 Exercise as a Therapy for Dyslipidaemia

Due to the well established role of TRLs in CVD in progression of atherosclerosis described above, interventions that have the potential to reduce fasting and/or postprandial TG concentrations have been investigated as valuable tools for lowering CVD risk. Exercise has long emerged as an affordable, non-pharmaceutical method of improving lipoprotein metabolism. Regular exercise training has been shown to reduce total TG [as well as VLDL-TG (Kraus et al., 2002)] concentrations, increase HDL cholesterol in adult men and women (Kraus et al., 2002; Kodama et al., 2007) and increase LDL particle size (Kraus et al., 2002; Altena et al., 2006). In addition, exercise improves insulin sensitivity in men and women (Gill et al., 2002b), sedentary overweight/obese subjects (Houmard et al., 2004), hypertriglyceridaemic men (Zhang et al., 2006) and subjects with the metabolic syndrome (Zhang et al., 2007) and type 2 diabetes (Alam et al., 2004). Certainly, sedentary lifestyle has been associated with increased risk of obesity, metabolic deterioration, type 2 diabetes, CVD, cancer and all-cause mortality (Slentz et al., 2007). In fact, a recent review on exercise training in patients with atheromatous CVD has found it to be a ‘true therapy’, reducing mortality by 25-35%, reducing clinical manifestations and complications (rhythm problems, thrombosis) and improving physical capacity, reintegration and quality of life (Casillas et al., 2007). The following section focuses mainly on the effect of exercise on TRL metabolism.

1.6.2 Impact of Endurance Training

Compared with their untrained peers, endurance-trained men have reduced levels of postprandial lipaemia (Cohen et al., 1989; Ziogas et al., 1997; Merrill et al., 1989). In addition, a period of endurance training has been shown to decrease fasting and/or postprandial TG concentrations in normolipidaemic subjects (Weintraub et al., 1989), overweight men and women with mild-to-moderate dyslipidaemia (Kraus et al., 2002) and older men and women (Halverstadt et al., 2007). However, evidence suggests that this TG-lowering effect of exercise is due to short-term metabolic responses to recent exercise which is lost after a period of detraining. For example, there was no difference between fasting or postprandial TG concentrations between endurance trained and untrained young adults (Herd et al., 2000) or untrained
middle-aged men (Tsetsonis et al., 1997) after at least 2 days of inactivity, or after an exercise training programme when post-training measurements were made 60 h after the last exercise session (Herd et al., 1998).

1.6.3 Impact of a Single Exercise Session

An increasing body of evidence shows that a bout of exercise (e.g. brisk walking, jogging, cycling etc) reduces fasting and/or postprandial TG concentrations in different population groups including young adult men (Tsetsonis & Hardman, 1996b; Zhang et al., 1998; Herd et al., 2001) and women (Tsetsonis & Hardman, 1996b; Gill et al., 2002a), middle-aged men (Gill et al., 2001a; Gill et al., 2001b) and women (Tsetsonis et al., 1997), postmenopausal women (Gill & Hardman, 2000) and individuals with the metabolic syndrome (Zhang et al., 2006) and type 2 diabetes (Tobin et al., 2008), although responses are more heterogeneous in patients with type 2 diabetes (Gill et al., 2007). The percentage reductions in plasma TG concentrations following exercise are broadly similar (15-25%) across these groups, however, subjects with higher TG concentrations (e.g. centrally obese men) had a greater decrease in postprandial TG compared with subjects with lower TG (Gill et al., 2004b). Furthermore, a single session of exercise has shown to acutely decrease VLDL (Gill et al., 2006) and VLDL-TG concentrations (Borsheim et al., 1999; Magkos et al., 2006; Morio et al., 2004).

A session of exercise conducted before (Katsanos & Moffatt, 2004; Tsetsonis & Hardman, 1996b; Tsetsonis & Hardman, 1996a; Gill & Hardman, 2000; Gill et al., 2001a; Gill et al., 2001b) or after (Katsanos & Moffatt, 2004; Hardman & Aldred, 1995) ingestion of a fat meal has been reported to attenuate postprandial lipaemia. However, the weight of evidence (Zhang et al., 2004a; Zhang et al., 1998; Ferguson et al., 1998; Borsheim et al., 1999) suggests that the maximal TG-lowering effect of exercise appears to occur after a delay of approximately 12-18 h, rather than during or immediately post-exercise (Malkova & Gill, 2006). For example, Zhang and colleagues (Zhang et al., 1998) reported a 21% greater reduction in postprandial TG when exercise was performed 12 h compared to 1 h prior to a meal and 49% lower than that performed after a meal. However, this TG-lowering effect seems to be ‘short-lived’ as exercising 24 h prior to the meal does not attenuate postprandial lipaemia (Zhang et al., 2004a).
1.6.4 Energy Expenditure and Energy Deficit

The energy expended during exercise is a key determinant of the TG-lowering effect: the higher the energy expenditure, the bigger the reduction in postprandial lipaemia (Petitt & Cureton, 2003). For instance, doubling energy expenditure by either doubling exercise intensity for the same duration [60 vs. 30% of maximal oxygen uptake (\(\dot{V}O_{2\text{max}}\)) for 90 min] (Tsetsonis & Hardman, 1996a) or by doubling exercise duration at the same intensity (120 vs. 60 min at 50% \(\dot{V}O_{2\text{max}}\)) (Gill et al., 2002a) essentially doubles the exercise-induced reduction to lipaemia. In addition, postprandial TG concentrations are similarly reduced by different exercise settings (e.g. 180 min of walking at 30% \(\dot{V}O_{2\text{max}}\) vs. 90 min at 60% \(\dot{V}O_{2\text{max}}\)) when the same amount of energy is expended (Tsetsonis & Hardman, 1996b). Similarly, a 2-h cycling at 60% \(\dot{V}O_{2\text{max}}\) significantly decreased VLDL-TG and -apoB concentrations (Magkos et al., 2006), whereas 1-h exercise of the same intensity had no effect (Magkos et al., 2007). Of note, recently, Gormsen et al (2006) reported a significant positive correlation between VLDL-TG production and resting energy expenditure, suggesting that it should be taken into account when VLDL-TG production comparisons between groups are made (Gormsen et al., 2006).

Furthermore, the exercise-induced improvement in insulin sensitivity appears to be related to duration and intensity of exercise (Houmard et al., 2004) and thus directly related to energy expenditure (Magkos et al., 2008). It was proposed that exercise-induced changes in a homeostasis model assessment-estimated insulin resistance (HOMA\(_{IR}\)) are curvilinearly related to exercise energy expenditure with a threshold of \(~3.77\) MJ (900 kcal) for improvements in HOMA\(_{IR}\) to be manifested (Magkos et al., 2008). However, an inverse association was observed between the exercise-reduced changes in baseline (i.e. resting) HOMA\(_{IR}\), suggesting that less exercise may be required to improve insulin sensitivity in insulin-resistant subjects than those with good insulin sensitivity at baseline (Magkos et al., 2008).

The mechanisms by which exercise energy expenditure attenuates postprandial lipaemia are currently not known. Interestingly, dietary-induced energy deficit of similar magnitude to that induced by a bout of exercise session results in a much smaller TG reduction (Gill & Hardman, 2000). This implies that either the TG-
lowering effect of exercise is not dependent on energy deficit and/or that exercise- and dietary-induced energy deficits are not metabolically equivalent. However, a recent study investigating the effects of exercise, with or without energy replacement, on fasting and postprandial TG metabolism, demonstrated that the exercise-induced TG-lowering effect was only evident with an accompanying energy deficit (Burton et al., 2008). This suggests that dietary-induced and exercise-induced energy deficits elicit different effects on postprandial metabolism. This may be related to specific body tissues in which the energy deficits occur as exercise induces quantitatively larger muscle and hepatic substrate deficits than energy intake restriction (Burton et al., 2008).

1.6.5 Intermittent versus Continuous Exercise

Current exercise-for-health guidelines recommend the accumulation of physical activity throughout the day (Haskell et al., 2007). Thus, it is important to understand whether small sessions of exercise spread throughout the day would be as beneficial in lowering TG concentrations as a single prolonged session of exercise. A number of studies have been conducted to investigate the effects of intermittent and continuous exercise, such as 90-min session vs. three 30-min sessions (Gill et al., 1998), 30-min vs. three 10-min exercise session (Murphy et al., 2000; Altena et al., 2004) and even 30-min continuous exercise vs. ten 3-min bouts performed throughout the day (Miyashita et al., 2006). All these studies consistently demonstrated that intermittent patterns of physical activity are effective in lowering TG concentrations as long as sufficient energy is expended, independently of the duration of the individual sessions.

1.6.6 Potential Mechanisms Responsible for the Exercise-Induced TG Reduction

Concentrations of TRLs in the circulation reflect the balance between rate of appearance and rate of clearance of intestinally-derived chylomicrons and hepatically-derived VLDL. Thus, the reduction in plasma TG concentration following exercise could be due to disturbance in the balance of appearance and clearance rates of chylomicrons and/or VLDL.
1.6.6.1 Effect of Exercise on Chylomicron Metabolism

The fact that maximal reduction in postprandial lipaemia is observed after a delay of number of hours, seems unlikely that the exercise-induced TG reduction is due to a reduction in gastrointestinal blood flow leading to potentially slower gut absorption and secretion of chylomicrons (Malkova & Gill, 2006). This is supported by three pieces of evidence: (1) the peak time for chylomicron-TG concentrations is not delayed post-exercise (Gill et al., 2001a; Gill et al., 2006), (2) gastric emptying time, evident by peak time of ingested paracetamol concentration, is unaffected by prior exercise (Gill et al., 2001a; Gill et al., 2001b) and (3) chylomicron particle number is not affected by prior exercise (James et al., 2007).

There is clear evidence that endurance-trained individuals have high clearance rates of chylomicron-like lipid emulsions compared with untrained peers (Cohen et al., 1989; Podl et al., 1994), which is likely to reflect the increased post-heparin plasma LPL activity (reflecting overall LPL activity from all body tissues) observed in them (Podl et al., 1994; Kantor et al., 1984). In addition, muscle LPL activity has been reported to increase over 200% in response to intense exercise sessions lasting for hours (Lithell et al., 1984; Sady et al., 1986).

On other hand, recent studies investigating the effects of a more moderate exercise on LPL activity are equivocal. While some studies reported a significant increase in plasma (Zhang et al., 2002), muscle and adipose tissue LPL activity after a moderate session of exercise (Perreault et al., 2004), others reported no significant difference in muscle (Herd et al., 2001) or post-heparin plasma LPL activity (Gill et al., 2003) or muscle LPL mass (Magkos et al., 2006) post-exercise. Interestingly, however, in the absence of an exercise-induced effect on muscle LPL mass, a significant ~20% increase in plasma LPL concentrations has been observed (Magkos et al., 2006). Similarly, a significant correlation between LPL activity and the exercise-induced changes in fasting and postprandial TG has been observed without an effect on post-heparin LPL activity post-exercise (Gill et al., 2003).

1.6.6.2 Effect of Exercise on VLDL Metabolism

Studies investigating the effect of moderate exercise on postprandial lipaemia demonstrated that the lipoprotein class most affected by prior exercise is VLDL
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(Malkova et al., 2000), specifically VLDL₁, rather than chylomicrons (Gill et al., 2001a; Gill et al., 2006). However, little is known about the mechanisms that regulate VLDL concentrations (i.e. production and clearance rates) in response to moderate exercise. Certainly, there is no information available regarding the effect of exercise on large VLDL₁.

Malkova et al (2000) examined the influence of a prolonged session of prior exercise (running at ~60% \( \dot{V}O_{2\text{max}} \)) on postprandial extraction of TG across the leg (Malkova et al., 2000). Although they found no significant increase in total, chylomicron- and VLDL-TG uptake across the leg, TG clearance (defined as uptake divided by arterial concentration) – a marker for the efficiency of TG removal - was greater following exercise. However, as absolute VLDL-TG uptake did not differ significantly between control and exercise conditions, it is unclear whether the lower VLDL-TG concentrations following exercise were due to increase efficiency of removal or a lower VLDL production rate (Malkova et al., 2000).

Following exercise, there is an increase in circulating NEFA (Burton et al., 2008), which, theoretically, can increase hepatic VLDL secretion. However, this is not the case, as there is no correlation between the change in NEFA concentration and VLDL-TG following exercise (Borsheim et al., 1999). This may be explained by the concurrent increase in 3-hydroxybutyrate following exercise (Malkova et al., 2000; Burton et al., 2008), which a marker of hepatic fatty acid oxidation (Williamson & Whitelaw, 1978), suggesting a shift of the hepatic fatty acid flux towards \( \beta \)-oxidation and ketone body production and away from re-esterification and VLDL synthesis (Malkova & Gill, 2006).

Due to the fact that chylomicrons are the preferred substrate for LPL and their presence prevents VLDL clearance (Fisher et al., 1995; Bjorkegren et al., 1997), it has been hypothesised that moderate exercise likely to reduce hepatic VLDL production, rather than increase its clearance (Malkova & Gill, 2006). However, recent evidence suggests that a session of moderate exercise (e.g. walking or cycling for more than 1 h at 60% \( \dot{V}O_{2\text{max}} \)) increases VLDL-TG clearance rates (Magkos et
1. Introduction and Literature Review

Magkos et al. also reported a decreased production of VLDL-apoB (reflecting the number of particles being produced) post-exercise (Magkos et al., 2006), which may contribute to the exercise-induced reduction in VLDL concentrations. It should be noted, however, that these findings may not necessarily be applicable to overweight or centrally obese individuals (at which exercise-for-health guidelines are targeted), as this condition is (1) associated with hepatic overproduction of VLDL due to their likely increased liver fat content (Adiels et al., 2006b) and (2) impaired ability to suppress hepatic VLDL production due to their likely insulin resistance (Adiels et al., 2007).

1.6.7 Effect of ApoE Phenotype

ApoE is a 299-amino acid glycoprotein which is an integral surface component of TRLs and some subclasses of HDL (Hatters et al., 2006). It primarily functions as a ligand for receptor-mediated uptake of TRLs, but also modulates the activity of LPL, LCAT and CETP (Leon et al., 2004). The apoE gene has three alleles (ε2, ε3, and ε4) that give rise to 6 different phenotypes (E2/2, E2/3, E2/4, E3/3, E3/4, and E4/4) (Davignon et al., 1988), with the E3 isoform being the most common (Hagberg et al., 2000). ApoE polymorphism has been shown to have a substantial influence on plasma lipids and lipoproteins, which could be explained by a number of mechanisms (Kolovou & Anagnostopoulou, 2007): (1) receptor-binding affinities of different apoE-containing lipoproteins, (2) dietary fat clearance, (3) differences in the clearance of LDL-apoB and (4) efficiency of intestinal cholesterol absorption.

In the general population, the E2 isoform is associated with elevated levels of TG and apoE compared with the E3 isoform (Hagberg et al., 2000), which is caused by impaired clearance of remnant particles bearing the apo E2 isoform probably due to defective receptor recognition (Havel et al., 1980). Homozygous E2/2 carriers can develop type III hyperlipoproteinaemia, which is characterised by the accumulation of chylomicron and VLDL remnants in fasting plasma (Kolovou & Anagnostopoulou, 2007). On the other hand, the apo E4 isoform is typically associated with low TG levels compared with the apo E3 individuals (Davignon et al., 1988). This is consistent with the fact that apo E4 subjects clear chylomicron
remnants into the liver more rapidly than E3/3 subjects and twice as fast as E3/2 subjects (Weintraub et al., 1987).

In contrast, studies investigating the effects of apoE polymorphism on \( \dot{V}O_{2\text{max}} \), lipids and lipoproteins in response to endurance exercise training are conflicting. While a cohort study reported an improvement in lipid and lipoprotein profile in subjects homozygous for the E3/3, despite a small reduction in \( \dot{V}O_{2\text{max}} \), compared with E2/3 and E3/4 (Thompson et al., 2004), another reported no significant influence of apoE polymorphism on \( \dot{V}O_{2\text{max}} \) in response to exercise (Leon et al., 2004). Conversely, in a study with smaller subject number, increases in \( \dot{V}O_{2\text{max}} \) (and HDL) in response to endurance exercise training, were in the order, apo E4 > apo E3 > apo E2 (Hagberg et al., 1999).

However, to the best of the author’s knowledge, there is only one study investigating the effect of apoE polymorphism on lipoprotein responses to moderate exercise. In a study including 38 men and 43 women, Gill et al (Gill et al., 2002b) reported no significant differences between subjects possessing the E3/2, E3/3 and E4/3 phenotypes on the magnitude of the exercise-induced reduction in fasting or postprandial TG.

### 1.7 Measurements of TRL Kinetics

Lipoprotein metabolism is a complex system of tightly regulated and coordinated dynamic processes. Measurements of plasma lipid and lipoproteins concentrations provide useful, yet limited information – ‘a snapshot of the various processes’ (Packard, 1995), which do not reveal the dynamics of the system. This is achieved in vivo by the use of kinetic studies, in which labelled precursors are used to help characterise the metabolic pathways of lipoprotein metabolism (Packard, 1995; Barrett et al., 2006). The system can be studied either in steady state (when the concentration of the substance of interest is constant) or in acute perturbation (non steady state, where a stimulus is applied and the return to steady state is monitored) (Packard, 1995).

There are basically two study protocols for investigating the metabolism of apoB-containing lipoproteins (i.e. VLDL1, VLDL2, IDL and LDL) (Demant & Packard,
1997). Lipoproteins can be tracer-labelled exogenously by isolating them from plasma, labelling them with radioactive substance (e.g. radioiodine). They are then re-injected into the donor subject and followed over time as they disappear from the plasma compartment. Alternatively, a stable isotope amino acid (e.g. D₃-leucine or D₅-phenylalanine) is injected, either as a bolus or as a primed constant infusion, and incorporated into the newly synthesised apoB protein serving as an endogenous label (Table 1.3 shows the characteristics of each tracer and labelling method). In either case, multiple plasma samples are collected during the first 12 h of tracer injection and daily thereafter in the fasted state for up to 10-14 days. ApoB-containing lipoprotein fractions are then separated at each timepoint using cumulative gradient ultracentrifugation in salt solutions (Lindgren et al., 1972) and apoB is isolated by selective precipitation (Egusa et al., 1983). In the case of radioactive tracer studies, the specific activity is calculated by measuring the radioactive tracer in a scintillation counter, whereas in the stable-isotope tracer studies, tracer enrichment is determined by gas chromatography mass spectrometry (GC-MS) (Demant et al., 1996). Multicompartmental modelling using the Simulation, Analysis And Modeling software (SAAM II; SAAM Institute, Seattle, WA) is used to calculate rates of production, delipidation and catabolism of apoB-containing lipoproteins (Packard et al., 1995).

Although such methods provide valuable information about lipoprotein metabolism, they are difficult, time-consuming, expensive and labour-intensive. Furthermore, the laboratory manipulations are complex and require the use of specialised equipment (see below). [Comprehensive reviews of kinetic methods can be found by (Barrett et al., 2006; Packard, 1995; Chan & Watts, 2006; Chan et al., 2004b).]

1.7.1 Using Stable-Isotopes to Measure VLDL₁ and VLDL₂ Kinetics

Recently, Adiels and colleagues (Adiels et al., 2005a) developed a multicompartmental model which permits the kinetics of apoB and TG to be assessed simultaneously in VLDL₁ and VLDL₂ fractions. The method involves administering D₃-leucine and D₅-glycerol as bolus injections in the fasted state. Several blood samples are taken before and for 8 h after the tracer injection while subjects are still fasting. Adiels et al. (Adiels et al., 2005a) reported a significant linear correlation
between TG and apoB production in VLDL₁ and VLDL₂, suggesting a coupling of the two processes governing the metabolism of these subfractions.

Table 1.3: Characteristics of tracers [modified from (Packard, 1995)].

<table>
<thead>
<tr>
<th>Exogenous</th>
<th>Endogenous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labels specific lipoprotein apolipoprotein subfractions</td>
<td>Labels all lipoprotein apolipoproteins</td>
</tr>
<tr>
<td>Labelled according to mass distribution</td>
<td>Labels according to production rate</td>
</tr>
<tr>
<td>Measures synthesis indirectly by inference from steady state calculation</td>
<td>Measures synthesis directly by tracer incorporation</td>
</tr>
<tr>
<td>Tracer does not recycle</td>
<td>Tracer may recycle</td>
</tr>
<tr>
<td>Urine data useful for assessing catabolism</td>
<td>Excretion data of little value</td>
</tr>
</tbody>
</table>

Radioactive versus stable-isotope endogenous tracers

<table>
<thead>
<tr>
<th>Radioactive</th>
<th>Stable isotope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiation hazard</td>
<td>Safe</td>
</tr>
<tr>
<td>Limited applicability</td>
<td>Applicable to all, including women and children</td>
</tr>
<tr>
<td>Limited repeatability</td>
<td>Can be repeated many times</td>
</tr>
<tr>
<td>Easy to measure</td>
<td>Difficult to measure with sufficient precision</td>
</tr>
<tr>
<td>Inexpensive apparatus and tracers</td>
<td>Capital cost high</td>
</tr>
</tbody>
</table>

For purposes of this thesis, it is essential to appreciate the complexity of the stable-isotope method, thus, Figure 1.7 shows a schematic summary of the laboratory techniques involved, which were developed and are still used in the Vascular Biochemistry Department (Glasgow Royal Infirmary, University of Glasgow). Briefly, VLDL₁ and VLDL₂ fractions are separated from plasma by cumulative gradient ultracentrifugation (Lindgren et al., 1972). ApoB protein is precipitated using absolute isopropanol and delipidated using ethanol-ether (Egusa et al., 1983). Samples are then prepared (derivatised and fragmentised) for analysis of leucine and D₃-leucine enrichment in hydrolysed apoB protein and plasma amino acids using the GC-MS (Demant et al., 1994). In order to determine glycerol enrichment in TG, the isopropanol and ethanol-ether supernatants are treated with zeolite to precipitate PL and obtain TG. Glycerol is then extracted by saponification using potassium hydroxide and ethanol (Witter & Whitner, 1972), then derivatised and fragmentated for enrichment determination by the GC-MS (Beylot et al., 1987).
1. Introduction and Literature Review

Plasma deproteinisation (Trichloroacetic acid)

D₃-Leucine bolus injection

D₅-Glycerol bolus injection

Plasma samples (for 48 h)

Protein precipitation (Heptane, H₂O & acetone)

Cumulative gradient ultracentrifugation

Ion exchange column

VLDL₁ and VLDL₂

ApoB precipitation (Isopropanol)

Non-apoB proteins & lipids

Lowry Method

ApoB-100 & associated lipids

PL precipitation (with zeolite)

Supernatant (TG & PL)

TG (supernatant)

Drying under N₂

Saponification (KOH & ethanol)

Glycerol

Centrifugal evaporation

Derivatisation & fragmentation of leucine & glycerol

GC-MS

Data analysis (SAAM II)

Supernatant

Glycerol extraction (Hexane & H₂O)

Aqueous phase

Plasma Glycerol

Figure 1.7: Schematic diagram of the stable-isotope laboratory protocol used to determine the kinetics of VLDL₁-TG and VLDL₂-TG and -apoB.

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Based on the enrichment of leucine and glycerol in plasma, VLDL₁ and VLDL₂, and the known injected amounts of labelled leucine and glycerol, the kinetic parameters are determined using the modelling software SAAM II as previously described (Adiels et al., 2005a). **Figure 1.8** shows the mathematical multicompartmental model used to assess the kinetics of glycerol and leucine in plasma and lipoprotein fractions, which is based on the apoB model originally described by Packard et al (Packard et al., 1995).

![Figure 1.8: A diagram of the mathematical multicompartmental model (using SAAM II software) used to simultaneously determine the kinetics of apoB and TG in VLDL₁ and VLDL₂ after a bolus injection of stable isotopes D₃-leucine and D₅-glycerol [Figure from (Adiels et al., 2005a)].](image)

### 1.7.2 Chylomicron Kinetics

#### 1.7.2.1 ApoB-48 Labelling with Stable-Isotopes

Kinetics of apoB-48 containing lipoproteins are difficult with only a few studies reported using endogenously labelled stable-isotope tracer (primed constant infusion of [5,5,5-²H₃] leucine) (Welty et al., 1999; Welty et al., 2004; Lichtenstein et al., 1992; Hogue et al., 2007). This is because in the fasting state, apoB-48 concentrations are generally too low for protein enrichment to be detected, whereas they vary postprandially (along with other lipoproteins) which makes interpretation
of tracer data very difficult (Barrett, 1998). However, these obstacles were first overcome by Lichtenstein et al. (1992) by providing small hourly feeds of identical composition to the subjects (over a 20 h period) to ensure an ‘elevated’ steady state of chylomicron concentrations. Recently, Bickerton et al. used a combination of stable isotope labeling to specifically label VLDL (using an intravenous infusion of \( ^2\text{H}_2\)palmitate) and chylomicrons (using a test meal containing \([U-^{13}\text{C}]\)palmitate) to quantify the TG extraction across human skeletal muscle and adipose tissue (Bickerton et al., 2007). However, it was reported that rapid recirculation of the label leads to loss of chylomicron specificity (Heath et al., 2003; Karpe et al., 2007).

### 1.7.2.2 Retinol Palmitate

Another commonly used, non-stable isotope, method to measure chylomicron metabolism involves the ingestion of retinol with a fat load, which is esterified into retinyl palmitate and secreted into the intestinal lymph in the core of the chylomicron particle. The palmitate within the core remains with the particle as it undergoes lipolysis and becomes a remnant particle. Thus, production and clearance rates of chylomicrons can be determined by monitoring the concentrations of plasma palmitate or chylomicron-associated palmitate (Barrett, 1998). However, a major disadvantage of this approach is the exchange of core components between chylomicrons and other lipoproteins [e.g. 25% of retinyl palmitate may also be found in apoB-100 containing TRLs (Cohn et al., 1993)].

### 1.7.2.3 Chylomicron-Like (Lipid) Emulsions

Intravenous lipid emulsions have been developed to supply patients with a balanced parenteral nutrition and to prevent or correct essential fatty acid deficiency. They are TG-rich particles that are modeled on the endogenous chylomicrons and consist of a TG core, traditionally derived from vegetable oils (e.g. soybean, safflower, coconut, olive). This TG is stabilised by a monolayer of PL derived from egg yolk (Olivecrona & Olivecrona, 1998; Carpentier & Dupont, 2000). However, unlike chylomicrons, lipid emulsions contain no cholesterol or apolipoproteins (Olivecrona & Olivecrona, 1998). Nevertheless, once in the circulation, these TG-rich particles rapidly acquire apoCs and apoE from plasma lipoproteins, mainly HDL (Iriyama & Carpentier, 1994); apoC-II is necessary for the action of LPL and apoE is important for receptor recognition.
Because the intravascular metabolism of these TG-rich particles resembles that of chylomicrons (Olivecrona & Olivecrona, 1998), they have been also used to study particular aspects of chylomicrons metabolism, both in vitro and in vivo. After binding of emulsion particles to LPL situated on the endothelial cells of adipose and muscle tissues, a substantial amount of the TG content of these particles is hydrolysed and the released NEFAs are either taken by the adjacent tissue or spilled into the circulation (Evans et al., 1999; Carpentier & Dupont, 2000). However, evidence suggests that lipolysis and particle removal may be simultaneous rather than sequential mechanisms like chylomicrons; i.e. emulsion particles may disappear from blood before they have become TG-depleted remnants. This is because some remnants are not likely to leave the site of lipolysis (endothelial site) but be internalized together with HSPG/LPL or be delivered to adjacent specific lipoprotein receptors (Olivecrona & Olivecrona, 1998), including VLDLR (Carpentier & Dupont, 2000).

Intralipid® (soybean oil) and Liposyn® (safflower oil) are two widely used commercial lipid emulsions, with the major difference in composition is their α-linolenic acid content. While Intralipid contains 8%, Liposyn has only 0.5% (Byrne, 1982). Intralipid emulsions have been used in intravenous fat tolerance tests to study TG clearance rate (see below). Recently, Park and colleagues (Park et al., 2000; Park et al., 2001) have reported a new method to measure chylomicrons kinetics by labelling Liposyn with radioactive tracers.

Redgrave and Maranhao (1985) prepared experimental lipid emulsions which, unlike the commercial ones, contain triolein, cholesteryl oleate, cholesterol and egg phosphatidyl-choline (Redgrave & Maranhao, 1985). The particles are usually smaller than those in commercial emulsions and they contain no PL vesicles (Olivecrona & Olivecrona, 1998). These emulsions have also been used in chylomicron kinetic studies, where the emulsion was double-labelled with radioactive cholesteryl esters and TG. After intravenous injection into the subjects, determination of the plasma decaying curves of the labeled lipids allows the 2-step metabolism of chylomicrons to be followed (Oliveira & Maranhao, 2002).
1.7.2.4 Intravenous Fat Tolerance Test

The intravenous fat tolerance test (IVFTT) provides a simple measure of postprandial TG clearance. It was first introduced in the early 1960s by Carlson and Hallberg (Carlson & Hallberg, 1963) in dogs then in humans in 1965 (Hallberg, 1965). In 1972, Carlson and Rössner (Carlson & Rossner, 1972) provided an IVFTT protocol which involved the injection of a bolus dose of Intralipid (0.1 g TG per body mass) into a forearm vein, with blood being drawn from a contralateral antecubita vein at 5-min intervals for 40 min. The decline in plasma Intralipid-TG concentrations followed first-order kinetics (i.e. the clearance rate was directly proportional to the concentration). Initially, Carlson and Rössner measured Intralipid-TG concentrations indirectly in plasma by measuring plasma turbidity using nephelometry (Carlson & Rossner, 1972), and although Rössner later reported a good reproducibility of this method for up to 6 months (Rossner, 1982), Sady and colleagues found better reproducibility using enzymatic TG quantification (Sady et al., 1986).

1.8 Summary and Objectives

This chapter attempted to briefly review current knowledge of lipid and lipoprotein metabolism, particularly TRLs. Special emphasis was placed on VLDL₁ assembly, metabolism, regulation and role in development of atherogenic dyslipidaemia. In addition, the role of exercise as a potential therapeutic option to reduce VLDL concentrations, in an attempt to improve the atherogenic lipoprotein phenotype, was discussed. Finally, the last section focused on the necessity of determining TRL kinetics; i.e. production and clearance rates, rather than concentrations, and the methods used to do so. Of note, despite the well-established heterogeneity of VLDL and the independent regulation of VLDL₁ and VLDL₂, the majority of kinetic studies, especially those aimed at investigating TG kinetics, have focused on total VLDL, rather than VLDL₁ and VLDL₂ separately. This is likely due to the difficulty in determining TG kinetics in VLDL subfractions compared with total VLDL. Although this obstacle has been recently overcome, the method still employed the use of stable-isotope tracers. Although these methods are considered the ‘gold-standard’ in kinetic studies and have been providing valuable information for a better understanding of lipoprotein metabolism, they are not widely available to most
laboratories due to their complexity and high cost. Therefore, the aims of the present thesis are to develop and validate a relatively easy and cost-effective method of determining VLDL\textsubscript{1} kinetics and use it to investigate the effects of hyperglycaemia and hyperinsulinaemia as well as moderate exercise on VLDL\textsubscript{1} kinetics. This is important as elevated concentrations of VLDL\textsubscript{1}, rather than VLDL\textsubscript{2}, are believed to be responsible for the generation of atherogenic dyslipidaemia. In addition, it is essential to elucidate the mechanisms by which moderate exercise, a potential tool for lowering CVD risk, reduces plasma TG in obese/overweight middle-aged men; a typical population at which exercise-for-health guidelines are targeted.
2. General Methods

This chapter is divided into three main parts. The first part describes the employed experimental procedures, many of which are common to several studies. The second part explains a study conducted to compare between a chemical manual reaction and an antibody-based automated method for apoB determination in VLDL fractions. Finally, the third part describes a pilot study conducted to determine the concentration and dose of a glucose drink needed to sustain a steady hyperinsulinaemic and hyperglycaemic state.

2.1 Subject Recruitment

Apparently healthy subjects were recruited from within the University of Glasgow, Glasgow Royal Infirmary and Greater Glasgow area by personal contact as well as by local advertising using posters, web-based and radio announcements. At least one week prior to the study, volunteers were interviewed and a subject information sheet about the study, including possible risks and discomforts (Appendices A1 & A2), was given and explained to them. Volunteers were also encouraged to ask any questions before signing an informed consent (Appendices A1 & A2) and screened according to a health screen questionnaire (Appendices B1 & B2). Their resting arterial blood pressure was measured and a venous blood sample was collected to test for their liver, renal, and thyroid functions. For the glucose and exercise studies described in Chapters 4 and 5, subjects were also tested for fasting glucose. Common exclusion criteria were used as follows:

- A history of known cardiovascular disease or abnormalities, including established CHD (e.g. MI, stroke, CABG), acute illness or active, chronic systemic disease
- Uncontrolled hypertension (>160/90 mmHg on anti-hypertensive medication)
- Taking any medication known to influence carbohydrate or lipid metabolism;
- Anaemia (Hb <12 g.dl\(^{-1}\) males, <11 g.dl\(^{-1}\) females)
- Current smoking (stopped for at least 6 months)
- Frank diabetes (fasting blood sugar \(\geq 7\) mmol.l\(^{-1}\)) (Chapters 4 and 5)
g. Abnormal renal, liver or thyroid function tests
h. Allergy to soybeans
i. Taking part in another study (within the last 3 months)

Inclusion criteria were different for different studies. These will be discussed in Chapters 4 and 5. All study protocols were approved by the Research Ethics Committee of the North Glasgow University Hospitals NHS Trust.

2.2 Anthropometry

2.2.1 Height and Weight

The heights and weights of subjects were measured using a weighing machine (D. Brash and Sons; Glasgow, UK) with an attached graduated metal plate. Subjects stood barefoot with their heels together and wearing light clothing. Height (m) and weight (kg) measurements were made to the nearest 0.1 unit.

2.2.2 Waist and Hip Circumferences

Waist and hip circumferences were measured using a flexible, inelastic tape measure (Supralip®160, West-Germany). The waist circumference was measured on the horizontal plane midway between the costal margin and iliac crest with the abdominal muscles relaxed and the subject breathing shallowly. The hip measurement was made horizontally around the maximum circumference of the buttocks. The reported values are means of two to three measurements.

2.3 Blood Sampling

Venous blood was obtained via an indwelling cannula (Biovalve, 18G/1.2 mm, Vygon, France) placed in an antecubital vein, to which a 10 cm three-way stopcock (Connecta Plus 3, BD, Sweeden) was attached. The cannula was kept patent by flushing with non-heparinised saline solution (0.9% NaCl). Blood samples were collected directly in 10-ml tubes containing K$_3$EDTA as an anticoagulant (BD Vacutainer Systems, Plymouth, UK). Blood samples were then placed immediately in ice and centrifuged (GS-6KR, Beckman Instruments, Inc, California, US) within 15-30 min of collection for 15 minutes at 3000 rpm and 4° C.
EDTA plasma was pipetted into aliquots of 200 μl in 0.5 ml Eppendorf tubes (Treff Lab, Switzerland) and 2 × 350 μl in Apex tubes (2 ml, Alpha Laboratory Ltd, UK) and frozen immediately at -70° C, for subsequent analysis of insulin, NEFA, glucose and lipid profile. The remaining EDTA plasma was used for lipoprotein separation. This was either started on the same day of blood collection or the next morning. In case of the latter, plasma was stored overnight at 4° C.

At the end of a successful lipoprotein separation, the remaining EDTA plasma was pipetted into aliquots of no less than 0.5 ml (to minimise any freeze drying effect) in 1.5 ml Eppendorf tubes (Treff Lab, Switzerland) and stored at -70° C.

2.4 Blood Samples Analysis

Lipoprotein fractions and EDTA plasma were separated and analysed in the Vascular Biochemistry Department of Glasgow Royal Infirmary, University of Glasgow. The methods and techniques employed were taught to the author by researchers in the Vascular Biochemistry Department.

2.4.1 Separation of VLDL₁ and VLDL₂ Lipoprotein Fractions

VLDL₁ (Sₚ 60-400) and VLDL₂ (Sₚ 20-60) fractions were isolated from plasma using a modification of the cumulative ultracentrifugation density gradient technique described by Lindgren et al. (Lindgren et al., 1972).

The density of 2 ml of plasma was adjusted to d 1.118 g.ml⁻¹ by the addition of 0.341 g NaCl. This was carefully layered over a cushion of 0.5 ml d 1.182 g.ml⁻¹ solution in an ultraclear Beckman SW 40 ultracentrifugation tube (Beckman Instruments Inc., UK) which had been coated with polyvinyl alcohol (Holmquist, 1982); this allowed the solutions to gravity feed down the side of the tubes smoothly without disturbing the formation of the gradient. A discontinuous gradient was formed by overlaying d 1.0988 g.ml⁻¹ (1 ml), d 1.0860 g.ml⁻¹ (1 ml), d 1.0790 g.ml⁻¹ (2 ml), d 1.0722 g.ml⁻¹ (2 ml), d 1.0641 g.ml⁻¹ (2 ml) and finally d 1.0588 g.ml⁻¹ (2 ml). The density solutions were prepared from stock solutions 1.006 g.ml⁻¹ and d 1.182 g.ml⁻¹ of NaBr in 0.195M NaCl, 0.001% Na₂EDTA and their densities were measured to 3 decimal places in a Paar Scientific densitometer (model DMA 35). The centrifugation was carried out using a Beckman SW 40 rotor (Beckman Instruments Inc., UK) in a
Beckman L8-60M ultracentrifuge for 1.38 h at 39K rpm and 23° C for separation of the VLDL₁ fraction. Rotors were decelerated without brakes and VLDL₁ was removed in the top 1 ml using a finely drawn glass Pasteur pipette. This volume was replaced by 1 ml of d 1.0588 g.ml⁻¹ and tubes were recapped and placed back in the centrifuge overnight for separation of VLDL₂. Various run times and speeds required for VLDL₂ separation were previously calculated as shown in Table 2.1. At the end of the run, 0.5 ml of VLDL₂ fraction was aspirated in the same way as VLDL₁. Fractions were kept in tightly capped 2 ml Apex tubes (Alpha Lab Ltd., UK) at 4° C for subsequent analysis.

Table 2.1: Various times and speeds required for the separation of lipoprotein fractions using Beckman L8-60M Ultracentrifuge and SW 40 rotor.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>RPM</th>
<th>Time (h)</th>
<th>RPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.38</td>
<td>39K</td>
<td>12.03</td>
<td>21.1K</td>
</tr>
<tr>
<td>14.52</td>
<td>19K</td>
<td>15.41</td>
<td>18.5K</td>
</tr>
<tr>
<td>16.34</td>
<td>18K</td>
<td>17.31</td>
<td>17.5K</td>
</tr>
<tr>
<td>18.08</td>
<td>17.2K</td>
<td>20.58</td>
<td>16K</td>
</tr>
</tbody>
</table>

2.4.2 Spectrophotometric Assays
Plasma analyses were carried out using commercially available enzymatic colorimetric kits. Plasma glucose (Glucose hexokinase, Randox Laboratories Ltd, UK) and total and HDL cholesterol (CHOL and HDL-C, Roche Diagnostics, UK, respectively) were analysed in the fasted state. LDL cholesterol was calculated in the fasted state using the Friedewald equation [LDL cholesterol (mmol.l⁻¹) = total cholesterol (mmol.l⁻¹) − HDL cholesterol (mmol.l⁻¹) − (TG (mmol.l⁻¹)/2.2)] (Friedewald et al., 1972). TG (TG, Roche Diagnostics Limited, UK), NEFA (NEFA C, Wako Chemicals, USA) and glycerol (GLY, Randox Laboratories Ltd, UK) were analysed at all time points.
2. General Methods

In Chapter 5, PL (Phospholipids C, Wako Chemicals, USA) and FC (Free Cholesterol, Wako Chemicals USA) were measured in the VLDL\textsubscript{1} fraction in all time points. In addition, VLDL\textsubscript{1}-apoC-II, -apoC-III, and -apoE were also measured in the fasted state using commercial automated trubidimetric immunoassay kits, supplied by Wako Chemicals.

All the above analyses were made by an automated clinical chemistry analyser (ILab\textsuperscript{TM} 600, Instrumentation Laboratory, USA) by Mrs. Josephine Cooney, Mrs. Elaine McDonald and Mrs. Elizabeth Murray (Department of Vascular Biochemistry, Glasgow Royal Infirmary).

Serum ALT concentrations were measured at screening (General Biochemistry Laboratory, Glasgow Royal Infirmary) using commercially available enzymatic colorimetric kit (Alanine Aminotranferase, Abbott Laboratories, USA).

2.4.3 Insulin ELISA

Insulin was measured in freshly frozen EDTA plasma using commercially available ELISA kits (Mercodia AB, Uppsala, Sweden). The method is a solid phase two-site enzyme immunoassay. The CV for the assay was < 4%.

2.4.4 Apolipoprotein E Phenotyping

ApoE phenotype was determined for each subject by Mrs Elizabeth Murray (Department of Vascular Biochemistry, Glasgow Royal Infirmary), by isoelectric focusing using Western blot techniques as described by Menzel (Menzel & Utermann, 1986) and Havekes (Havekes et al., 1987).

2.4.5 Total Protein Determination (Lowry Method)

Total protein content in lipoprotein fractions was measured using a modified Lowry method (Lowry et al., 1951). This method involved the addition of 1 ml of Biuret reagent [100 ml of 2% Na\textsubscript{2}CO\textsubscript{3} in 0.1 M NaOH (w/v), 1 ml of 2% NaK Tartrate (w/v), 1 ml of 1% CuSO\textsubscript{4} (w/v), and 1 ml of 10% (w/v) sodium dodecyl sulphate (SDS)] to a total volume of 200 μl of sample (100 μl VLDL\textsubscript{1} + 100 μl of distilled water or 50 μl VLDL\textsubscript{2} + 150 μl distilled water). Samples were diluted or concentrated as
required. One hundred microlitre of 1:1 Folins Ciocalteu reagent (Sigma-Aldrich Company Ltd., Irvine, Scotland) was then added and mixed immediately. After incubation at room temperature for 30-60 min, the developed colour intensity was measured at an optical density of 750 nm using a Beckman DU 70 Spectrophotometer. The protein concentration was calculated using a standard curve of known concentrations. Inter-assay precision was checked using 2 levels (100 μl and 200 μl) of human and bovine quality control (QC) materials. The coefficients of variation (CVs) for the low QC were (human: 1.8%, bovine: 1.3%) and high QC were (human: 1.0%, bovine: 1.4%).

2.4.6 Accuracy and Precision of Assays

The accuracy and precision of the automated assays described section 2.4.2, except for glycerol, were monitored using quality control sera (Wako QC abnormal and normal, Wako Chemicals GmbH, Denmark; distributed by Alpha Laboratories, UK). Glycerol quality control was obtained from Randox Laboratories Ltd, UK. In order to minimise intra-assay variation, all samples obtained for each subject were performed in the same analyser run for a given assay. The CVs for the assays were 2.9% for total cholesterol, 3.8% for TG, 2.8% for HDL-cholesterol, 5.2% for NEFA, 2.0% for glucose, 1.9% for glycerol, 2.2% for free cholesterol, and 3.8% for PL.

2.5 Bruce Protocol

All subjects recruited for the exercise study described in Chapter 5 underwent a modified (ACSM, 2006) Bruce protocol (Bruce et al., 1973) under the supervision of a medical doctor: Dr Nicholas Barwell or Dr Lesley Hall, at the Institute of Diet, Exercise and Lifestyle (IDEAL) at the University of Glasgow. A modified twelve lead electrocardiographic (ECG) monitoring was conducted throughout the test and arterial blood pressure was measured at rest and immediately after a Bruce protocol during recovery.

2.6 Submaximal Exercise Test

In the exercise study described in Chapter 5, subjects undertook a submaximal exercise test to estimate the gradient necessary to elicit an intensity corresponding to 50% $\bar{VO}_2\text{max}$. The test consisted of a four-stage treadmill (Woodway GmbH, Weil am
Rhein, Germany) walk (Figure 2.1) to determine the relationship between increasing gradient and the subjects’ \( \dot{V}O_2 \) at their self-selected walking speed. \( \dot{V}O_{2\text{max}} \) was estimated by extrapolation of the \( O_2 \) uptake/heart rate relationship up to the subject’s predicted maximum heart rate: \([220 – \text{age}] \text{ beat.min}^{-1}\) (ACSM, 2006). Each stage was five minutes long with expired air samples being taken during the last minute of each stage. Heart rates were recorded during the expired air collections as were perceived rates of exertion using the Borg scale (Borg, 1973). The first stage of the test was on a level treadmill with gradient increasing by 2.0 to 3.0% in each subsequent stage. The increase in gradient for each stage was established on an individual basis based on the subject’s heart rate response. The relationship between \( \dot{V}O_2 \) and treadmill gradient was determined for each subject and, together with the estimated \( \dot{V}O_{2\text{max}} \), the gradient necessary to elicit an intensity corresponding to 50% \( \dot{V}O_{2\text{max}} \) was calculated.

![Figure 2.1: A schematic diagram of a 4-stage submaximal exercise test performed for subjects in Chapter 5. The blue block represents an expired air sample and heart rate measurements.](image)

2.7 Monitoring of Heart Rate

The subject’s heart rate (Chapter 5) was monitored continuously during exercise and the recovery period by short range telemetry (Polar S610i, Polar Electro, Finland).

2.8 Measurement of Oxygen Uptake and Carbon Dioxide Production

Oxygen uptake and CO\(_2\) production in Chapter 5 were determined at rest before and during exercise. Samples of expired air were collected into 100 or 150 L Douglas
2. General Methods

bags. While wearing a nose clip, subjects breathed through a mouthpiece fitted to a lightweight large 2-way respiratory valve (2700 series, Hans Rudolph Inc. USA), which in turn was connected to a lightweight tube. The tubing was terminated at a two-way valve which opened and closed the Douglas bag. All equipment was supplied by Cranlea & Co. Birmingham, England.

An aliquot of expired air (measured using a flow meter) was removed from each Douglas bag to determine the fraction of O₂ and CO₂ using a gas analyser (Servomex 4100, Servomex Group Ltd., East Sussex, England). The analyser was calibrated before each use with certified reference gases (BOC Ltd, Surry, UK) and the reference gases were calibrated against a ‘gold standard’ reference gas to ensure consistency of results.

The remaining volume of expired air in each Douglas bag was measured by evacuation through a dry gas meter (Harvard apparatus, supplied by Cranlea & Co. Birmingham, England). The temperature of air in the Douglas bag was measured during evacuation using the same dry gas meter.

Barometric pressure was measured using a barometer and the measured expired gas volumes were corrected to standard temperature and pressure (STPD) for a dry gas using the universal gas equation. Inspired gas volumes were derived using the Haldane transformation (Consolazio *et al.*, 1963) and O₂ uptake, CO₂ production, minute ventilation, respiratory exchange ratio and the ventilatory equivalent for oxygen were calculated. Rates of substrate utilization expenditures were calculated via indirect calorimetry using the equations described by Frayn (Frayn, 1983) and energy expenditure was determined by multiplying the mass of substrates used by their respective energy densities.
2.9 Apolipoprotein B Measurements in VLDL₁ and VLDL₂ Fractions – Comparison of a Manual and an Automated Method

2.9.1 Introduction

In the Vascular Biochemistry laboratory, for kinetic studies, apoB is usually measured in lipoprotein fractions using a standardised manual method, which is applicable to VLDL, IDL and LDL fractions. ApoB is precipitated after the addition of an equal volume of absolute isopropanol (Egusa et al., 1983). The supernatant is aspirated and its protein content is measured using a modified version of Lowry method (Lowry et al., 1951). There is no apoB solubilisation in isopropanol and the precipitated apoB is virtually free of soluble apolipoproteins. ApoB protein concentrations are measured by the difference between total protein content in lipoprotein and the protein measured in supernatant. This method is standardised and widely used for the measurement of the relatively low concentrations of apoB in VLDL₁ and VLDL₂ fractions. However, it is laborious, time consuming and, being a manual technique, is subject to personal error.

Another method for measuring apoB concentrations is direct measurement using an automated in vitro immunoturbidimetric assay with the use of commercially available kits (see below). Being an automated method, the immunoturbidimetric assay saves time, effort and potentially improves reproducibility. However, these have been designed for apoB measurements in serum or plasma, where apoB concentrations are high and mainly represent apoB in the LDL fraction [85-90% of total apoB (Alaupovic, 1991)]. It was therefore unclear whether this method is suitable for the measurement of the low apoB concentrations in VLDL₁ and VLDL₂ fractions.

Therefore, the purpose of the present study was to compare the automated turbidimetry immunoassay method with the conventional precipitation one as an alternative method for the measurement of apoB protein in VLDL₁ and VLDL₂.
2.9.2 Materials and Methods

2.9.2.1 Specimens and Separation of VLDL₁ and VLDL₂ fractions

A total of 260 EDTA plasma samples were used to separate VLDL₁ (S₉ 60-400) and VLDL₂ (S₉ 20-60) fractions by a modification of the cumulative ultracentrifugation density gradient technique as previously described (section 2.4.1).

2.9.2.2 Isopropanol Precipitation Method (Egusa et al., 1983)

All VLDL₁ and VLDL₂ samples were brought to room temperature and 0.5 ml of absolute isopropanol was added to 0.5 ml of each sample in glass tubes (13/100 glass tubes, Labco Ltd., Buckinghamshire, UK). After immediate vigorous mixing, samples were then incubated for at least 24 h at 4°C, after which they were spun at 3000 rpm and 4°C for at least 30 min. Immediately, the supernatant was carefully aspirated using a drawn-out Pasteur pipette, without drawing out any of the precipitated apoB.

Total protein content in the VLDL₁ and VLDL₂ fractions and the non-apoB protein content of the supernatant were measured using the modified Lowry method as described earlier (section 2.4.5).

2.9.2.3 Immunoturbidimetric Method

The apoB content of the same samples was measured directly using commercially available kits (WAKO Apolipoprotein B-HA, Wako Chemicals GmbH, Denmark; distributed by Alpha Laboratories). This method involved the specific combination of apoB in the sample with anti-human apoB antibodies in the reagent to yield an insoluble aggregate that causes increased turbidity. The degree of turbidity is measured optically using an autoanalyser (ILab™ 600, Clinical Chemistry System, Instrumentation Laboratory, USA). The method in Vascular Biochemistry has been optimised for apoB measurements in lipoprotein fractions by increasing the volume of sample used in the assay from 3 μl to 12 μl (results were then multiplied by a factor of 0.25). The supplied quality control was also diluted to account for the lower concentrations of apoB (WAKO 3 was diluted 1:5 to give a mean (range) concentration of 15.3 (12.3-18.4) mg.dl⁻¹, which is within the range of expected results).
2.9.2.4 Calculating ApoB %CV for Each Method

VLDL\textsubscript{1} and VLDL\textsubscript{2} were separated from two subjects and each fraction was pooled together. ApoB was measured in ten samples using the immunoturbidimetric method and the isopropanol-Lowry method. Means, SDs and CVs were calculated for each method.

2.9.3 Results

Figure 2.2 shows VLDL\textsubscript{1}- and VLDL\textsubscript{2}-apoB concentrations (mg.dl\textsuperscript{-1}) measured by the immunoturbidimetry method in 260 samples plotted against that measured by the isopropanol precipitation method with the line of equality; the line all points would lie on if the two methods always gave exactly the same measurement. The two methods correlate well for both fractions with $R^2$ (goodness-of-fit) of 0.88 for VLDL\textsubscript{1} and 0.93 for VLDL\textsubscript{2}. However, the isopropanol method measured values as \(\sim\) 1.3 times higher than the immunoturbidimetric method for both VLDL\textsubscript{1} and VLDL\textsubscript{2} fractions. This was particularly apparent as the apoB concentrations increased.

Figure 2.3 shows the percent difference between measurements by the two methods (isopropanol minus immunoturbidimetry) for each sample against their mean in both the VLDL\textsubscript{1} and VLDL\textsubscript{2} fractions. This is done to explicitly show extreme or outlying observations and any lack of agreement between the two methods (Bland & Altman, 1999). In addition, Figure 2.3 shows the 95% limits of agreement estimated as mean percentage difference $\pm$ 2 SD for each fraction, which define the range within which most differences between measurements by the two methods will lie (Bland & Altman, 1999). This was: 0.8 $\pm$ 110.3% for VLDL\textsubscript{1} and 9.1 $\pm$ 66.4% for VLDL\textsubscript{2}, giving rise to 95% limits of agreement between (-109.4% to +111.1%) for VLDL\textsubscript{1} and between (-57.3% to +75.5%) for VLDL\textsubscript{2}. From Figure 2.3, a cut-off value of \(\sim\)3.0 mg.dl\textsuperscript{-1} could be made, above which measurements between the two methods appeared to show better agreement for both VLDL\textsubscript{1} and VLDL\textsubscript{2} fractions compared to values less than 3 mg.dl\textsuperscript{-1}, where deviation around the mean was more scattered. This is better illustrated in Figure 2.4, which shows the %difference (isopropanol minus immunoturbidimetry) in apoB concentrations plotted against the mean of two methods for values $\geq$ 3.0 mg.dl\textsuperscript{-1}. In this case, the deviation around the mean is evenly scattered, especially in the VLDL\textsubscript{1} fraction, and the 95% limits of
agreement (mean ± 2SD) became smaller: between -33.3% and 73.7% (20.2 ± 53.5%, n = 65) for VLDL₁ and between -17.5% and 53.0% (17.7 ± 35.3%, n = 84) for VLDL₂. In addition, it is clear from Figure 2.4A that there are two outliers in the VLDL₁ fraction. Although excluding these two measurements did not influence the mean percentage difference, it reduced the SD value (20.7 ± 37.5%) and consequently, the 95% limits of agreement (-16.8 to 58.2%).

Finally, Table 2.2 shows the mean VLDL₁- and VLDL₂-apoB concentrations, SDs and CVs measured 10 times for each method in VLDL₁ and VLDL₂ fractions using pooled samples. The apoB concentrations were lower in VLDL₁ compared to VLDL₂ and it is clear that the immunoturbidimetric method was more reproducible than the isopropanol method at such low concentrations (CV 3.4% vs. 9.9% for VLDL₁ and 1.4% vs. 3.2% for VLDL₂, respectively).

Table 2.2: Calculated mean, SD and CV for apoB concentrations measured 10 times for the same sample using the two methods

<table>
<thead>
<tr>
<th>VLDL₁-apoB (mg.dl⁻¹)</th>
<th>VLDL₂-apoB (mg.dl⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoturbidimetric method</td>
<td>Isopropanol method</td>
</tr>
<tr>
<td>Mean</td>
<td>3.74</td>
</tr>
<tr>
<td>SD</td>
<td>0.13</td>
</tr>
<tr>
<td>CV (%)</td>
<td><strong>3.38</strong></td>
</tr>
</tbody>
</table>

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Figure 2.2: ApoB concentrations (mg.dl⁻¹, with linear-regression lines of ‘best-fit’ and equation of the line) in [A] VLDL₁ and [B] VLDL₂ fractions measured using an automated immunoturbidimetric method and by a manual isopropanol method with the line of equality. (n = 260)
Figure 2.3: A scatterplot of the percent difference in apoB concentrations (mg.dl^{-1}) between the two methods (isopropanol minus immunoturbidimetry) against their mean in [A] VLDL\(_1\) and [B] VLDL\(_2\), with 95% limits of agreement (mean ± 2SD): VLDL\(_1\) (0.8 ± 110.2%) and VLDL\(_2\) (9.1 ± 66.4%), n = 260.
2. General Methods

Figure 2.4: A scatterplot of the percent difference (isopropanol minus immunoturbidimetry) for apoB concentrations ≥ 3.0 mg.dL⁻¹ against the mean of the two methods in [A] VLDL₁ and [B] VLDL₂, with 95% limits of agreement (mean ± 2SD): VLDL₁ (20.2 ± 53.5%, n = 65) and VLDL₂ (17.7 ± 35.3%, n = 85).
2.9.4 Discussion

Due to the low concentrations of apoB in VLDL fractions, the manual precipitation of apoB using isopropanol (Egusa et al., 1983) has historically been the preferred choice, followed by a modification of another manual method (Lowry et al., 1951) to determine the apoB protein concentrations in the fractions. Being a chemical method, this technique is not affected by the size of the lipoprotein particle, the possible masking of the apoB epitopes or changes in time over standardisation of commercial apoB assays. However, this method is time-consuming and requires a high degree of precision and skill in separating the non-apoB supernatant from the precipitated apoB protein, which may sometimes be difficult in cases of very high or very low apoB concentrations.

Conversely, apoB can be measured directly using an automated immunoturbidimetric assay, with the use of commercially available kits. The fact that this method is automated, makes it faster, allows for a minimal involvement of human error, and, consequently, better precision. One possible shortcoming of this immunological method is that it relies on detecting certain epitopes on the apoB particle and, therefore, has the disadvantage of being dependent on the manufacturer’s choice of such epitopes, which could be subject to change at any time.

Because of the large number of samples per trial used in this thesis and the relative difficulty of using the manual isopropanol method for apoB measurements, the two methods were compared using 260 samples from the VLDL₁ and VLDL₂ fractions. This was done to assess the possibility of replacing the isopropanol precipitation method with the automated immunoturbidimetric method.

The present results show that the two methods agree well for both fractions with R² values of ~0.9. This is especially true for apoB concentrations ≥ 3.0 mg.dl⁻¹. Although the immunoturbidimetric method seems to give values ~20% lower than the isopropanol method, this is consistent and the deviation from the mean of the two methods still lies within the 95% limits of agreement. However, the immunoturbidimetric method allows better precision and reproducibility than the manual isopropanol method, with CVs of 3.4% and 1.4% (compared to 9.9% and 3.2%) in VLDL₁ and VLDL₂ fractions, respectively. In addition, being an automated
method, the immunoturbidimetry allows the feasibility and the ease of analysing large number of samples in a relatively short period of time, compared to the isopropanol method.

In conclusion, it is inevitable to find some lack of agreement between different methods, but what matters is the amount by which methods disagree (Bland & Altman, 1999). The automated immunoturbidimetry method correlated consistently and reasonably well with the manual isopropanol precipitation method, with better precision, reproducibility and ease of use for measurements of multiple samples. Therefore, the immunoturbidimetric method was chosen to measure apoB concentrations in this thesis.

2.10 Glucose Pilot Study

The purpose of this pilot study was to determine whether it was possible to maintain a ‘pseudo’ steady state of high circulating glucose and insulin concentrations by ingesting small amounts of a glucose solution at frequent intervals. If this approach was feasible, it would represent a more straightforward and less invasive method of inducing hyperinsulinaemia and hyperglycaemia than invasively infusing glucose and/or insulin. A number of different patterns of glucose ingestion were investigated to determine an appropriate protocol, which will be used in the study described in Chapter 4.

2.10.1 Subjects and Methods

Four subjects (2 women and 2 men – age (range) 20-33 years; body mass 55-95 kg; BMI 22.6-28.1 kg.m\(^{-2}\)) were included in this study after giving informed consent. Subjects reported in the morning to the metabolic suite at the Institute of Diet, Exercise and Lifestyle, at the University of Glasgow, after a 12-h fast. Subjects were cannulated in an anticubital vein and after a 10-min interval a baseline EDTA fasting blood sample was drawn. A second baseline sample was taken 10 min later. All blood samples were placed immediately in ice. EDTA plasma was obtained after centrifugation for 10 min at 3000 rpm and divided into 5 aliquots in 1.5 ml Eppendorff tubes. Aliquots were stored immediately at -20\(^{\circ}\) C for subsequent analysis of glucose, NEFA and insulin concentrations as previously described in sections 2.4.2 and 2.4.3.
The glucose drinks were prepared by dissolving the appropriate weight of glucose powder (Thornton and Ross Ltd., Huddersfield, England) in hot water and making the required volume up with cold water. Approximately 5-6 ml of lemon juice per 100 ml of water were added for taste.

2.10.2 Protocol I

The first protocol was designed to introduce a glucose concentration of 30% (w/v) (15 g in 50 ml) every 15 min for 2 h. Two female subjects participated in this protocol (Table 2.3). Blood samples were drawn every 15 min.

Table 2.3: Characteristics of the two female subjects who participated in Protocol I

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (y)</th>
<th>Body mass (kg)</th>
<th>BMI (kg.m(^{-2}))</th>
<th>HOMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>66.4</td>
<td>23.81</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>33</td>
<td>55</td>
<td>22.6</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Figure 2.5 shows glucose, insulin and NEFA responses for the two subjects. Glucose concentrations increased slowly in subject 1 and immediately in subject 2 before declining close to baseline at the end of 2 h. Consequently, insulin concentrations increased in response to the glucose drink, but differently in the two subjects: while subject 1 had a delayed insulin response corresponding to glucose concentrations, subject 2 had a dramatic increase in insulin concentrations for the duration of the trial. However, none of the two subjects reached any steady state during 2 h. Similarly, NEFA concentrations declined slowly in subject 1 but dramatically in subject 2 over the course of trial in response to insulin. NEFA concentrations were only suppressed at the end of the 2 h.

Despite different responses in glucose and insulin concentrations, neither subjects reached a ‘pseudo’ steady state. Because one subject had a delayed response, it was clear that an initial bolus glucose drink is needed to induce a more rapid response. Also, because the other subject had an exaggerated insulin response to the glucose drink, it might be beneficial to decrease its dose, and possibly increase its frequency to account for the reduced concentration.
2.10.3 Protocol II

The second protocol started with a bolus drink of 20% glucose (30 g in 150 ml) followed by 20% glucose drink (5 g in 25 ml) every 10 min (instead of 15 min) for a period of 2 h. Blood samples were taken at 15-min intervals. One male subject participated in this protocol (aged 29 years, body mass 95 kg, BMI 28.1 kg.m\(^{-2}\) and HOMA 1.4).

Figure 2.6 shows glucose, insulin and NEFA responses during this protocol. The bolus glucose drink successfully increased glucose and insulin concentrations shortly after ingestion. However, the glucose drink, thereafter, was not sufficient to maintain a steady state of glucose and insulin as they started to decline after 90 min. In addition, the drink did not induce a desirable suppression in NEFA concentrations before the 2 h.

Therefore, a bolus glucose drink seemed essential to induce a faster and immediate response in glucose and insulin concentrations. However, a dose of 5 g glucose every 10 minutes did not seem sufficient to maintain elevated glucose and insulin concentrations. Furthermore, it would be helpful to increase the observation time of the study to investigate whether a steady state could be reached and/or maintained for a longer period of time.

2.10.4 Protocol III

In this protocol, glucose drink concentration and the observation time were increased. It started with 30% bolus glucose drink (30 g in 100 ml) followed by 60% glucose drink (15 g in 25 ml) every 15 min for 4 h. These doses were chosen as it became obvious from the previous two protocols that a stronger glucose drink might be needed to achieve the desired ‘pseudo’ steady state and suppress NEFA at a time shorter than 2 h. One subject participated in this protocol (aged 22 years, body mass 80.5 kg, BMI 27.8 kg.m\(^{-2}\) and HOMA 4.0).

Figure 2.7 shows glucose, insulin and NEFA responses during the 4-h observation period for this protocol. Glucose and insulin concentrations increased quickly in response to the bolus glucose drink, which caused a desirable, fast reduction in NEFA concentrations. However, the glucose drink caused abrupt responses in both
2. General Methods

glucose and insulin concentrations. In addition, there was tendency for insulin concentrations to continually increase during the 4 h. It is uncertain, however, whether this increase is related to the subject’s HOMA of 4.0, which may indicate insulin resistance, or whether it was caused by the increased glucose drink concentration.

2.10.5 Conclusion

From these protocols, it was obvious that (1) a bolus glucose drink was needed to trigger an immediate insulin response, (2) providing 5 g of glucose every 10 minutes was not sufficient to maintain elevated levels of glucose and insulin, while providing 15 g glucose every 15 minutes produced abrupt responses and (3) although NEFA concentrations started to decline immediately in response to insulin, it took about 60 min to be suppressed almost completely in the last protocol.

In conclusion, taking these findings into account, it was decided that the final protocol for the glucose ingestion would be; a bolus glucose drink of 25% (w/v) (30 g of glucose in 120 ml), followed by 25% glucose drink (10 g in 40 ml) every 15 min for the duration of the trial. The glucose ingestion protocol would start an hour prior to the Intralipid infusion described in Chapter 4 to ensure adequate suppression of NEFA concentrations.
Figure 2.5: [A] glucose, [B] insulin and [C] NEFA response (n = 2) for the glucose pilot - Protocol I: 30% (w/v) glucose (15 g in 50 ml) every 15 min for 2 h. The solid lines represent subject 1, while the dotted lines represent subject 2.
Figure 2.6: [A] glucose, [B] insulin and [C] NEFA response (n = 1) for the glucose pilot - Protocol II: a bolus drink of 20% (w/v) of glucose (30 g in 150 ml) followed by a 20% glucose drink (5 g in 25 ml) every 10 min for 2 h.
Figure 2.7: [A] glucose, [B] insulin and [C] NEFA response (n = 1) for the glucose pilot - Protocol III: a bolus glucose drink of 30 g (in 100 ml) followed by 15 g of glucose (in 25 ml) every 15 min for 4 hours.
3. Development of a Novel Method to Determine VLDL₁ Kinetics

3.1 Introduction

A large body of evidence suggests that increased circulating concentrations of TRLs increase the risk of atherosclerosis (Malloy & Kane, 2001; Tanaka et al., 2001). This is particularly evident in the postprandial state (Karpe, 1999; Schneeman et al., 1993). However, the measurement of a high TRL concentration provides no information regarding the mechanisms responsible for this elevation; i.e. increased rate of synthesis and/or reduced rate of catabolism. As it is important to understand the mechanisms responsible for increased TRL concentrations in different metabolic states, both to advance basic scientific understanding and to help guide therapeutic treatments, studies investigating kinetics of TRL can yield useful data. Such an approach has, for example, revealed that the dyslipidaemia associated with insulin resistance and type 2 diabetes is largely due to an overproduction of hepatically derived large VLDL₁ (S₁ 60-400) (Watson et al., 2003; Adiels et al., 2005a). These studies typically use precursors labelled with stable or radioactive isotope tracers to measure the synthesis of lipids and apolipoproteins directly (Adiels et al., 2005a; Gill et al., 2004a; Packard et al., 2000; Packard, 1995). Although these techniques yield detailed kinetic data, they are costly, time consuming, labour-intensive and require the use of specialised equipment and techniques in research laboratories.

The aim of the present study was, therefore, to develop a relatively straightforward method of obtaining TRL kinetic data. The method relies on the fact that chylomicrons compete with VLDL₁ particles for the same catalytic pathway; i.e. hydrolysis of their TG content by the action of LPL. Previous studies (Karpe & Hultin, 1995; Björkegren et al., 1996) have shown that VLDL₁ accumulates in plasma after fat ingestion or intravenous infusion of a lipid emulsion (e.g. Intralipid) due to the presence of the newly secreted chylomicrons or chylomicron-like particles, which are the preferred substrate for LPL because of their larger size and TG content (Fisher et al., 1995). Indeed, using stable isotope methods, Björkegren et
3. Development of a Novel Method to Determine VLDL₁ Kinetics

al. demonstrated that infusion of Intralipid prevents over 90% of VLDL₁ catabolism (Bjorkegren et al., 1996). It was therefore hypothesized in the present study that it would be possible to calculate the production rates of VLDL₁-TG and -apoB from the rate of their accumulation during an infusion of Intralipid. The former would be a measure of lipid production while the latter would represent the rate of VLDL₁ particle production, as there is one apoB molecule per VLDL₁ particle (Elovson et al., 1988). Furthermore, using this approach, it is possible to calculate the rate of Intralipid-TG clearance (a surrogate measure of TRL-TG clearance) from either the steady-state Intralipid-TG concentration during infusion (Rang et al., 2003) or from the exponential decay in Intralipid-TG concentration post-infusion (Rossner, 1974). Here the author reports the development and validation of this ‘Intralipid method’ to determine TRL kinetics.

3.2 Material and Methods

3.2.1 Subjects

Ten non-smoking healthy subjects (7 males and 3 females) were included in this study after giving written informed consent. All subjects had normal thyroid, liver and renal function and none had acute illness, a history of known cardiovascular disease and hypertension, nor were under medication known to influence carbohydrate or lipid metabolism. The subject information sheet, consent form and health screen for the present study are shown in Appendices A1 and B1. Two subjects had the E3/2 apoE phenotype, seven had the E3/3 phenotype and one had the E4/3 phenotype. The subjects’ characteristics are shown in Table 3.1. Subjects were requested not to exercise for three days before their study days as this is known to affect TRL metabolism (Gill & Hardman, 2003). In addition, they were asked to weigh and record their dietary intake for two days prior to the Intralipid test and this diet was replicated in those subjects who underwent a second Intralipid test. The study protocol was approved by the Research Ethics Committee of the North Glasgow University Hospitals NHS Trust.
3. Development of a Novel Method to Determine VLDL\textsubscript{1} Kinetics

Table 3.1: Subjects’ physical and metabolic characteristics (n=10).

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>33.5</td>
<td>(20.0 – 55.0)</td>
</tr>
<tr>
<td>Body mass index (kg.m\textsuperscript{-2})</td>
<td>25.9</td>
<td>(20.8 – 34.7)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>85.1</td>
<td>(65.0 – 113.5)</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>0.84</td>
<td>(0.71 – 1.04)</td>
</tr>
<tr>
<td>Triglycerides (mmol.l\textsuperscript{-1})</td>
<td>1.36</td>
<td>(0.40 – 4.43)</td>
</tr>
<tr>
<td>Total cholesterol (mmol.l\textsuperscript{-1})</td>
<td>4.15</td>
<td>(2.85 – 5.90)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol.l\textsuperscript{-1})</td>
<td>1.30</td>
<td>(0.75 – 1.85)</td>
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<tr>
<td>LDL cholesterol (mmol.l\textsuperscript{-1})</td>
<td>2.20</td>
<td>(1.22 – 4.00)</td>
</tr>
<tr>
<td>Glucose (mmol.l\textsuperscript{-1})</td>
<td>5.5</td>
<td>(4.3 – 8.0)</td>
</tr>
<tr>
<td>Insulin (mU.l\textsuperscript{-1})</td>
<td>8.92</td>
<td>(2.78 – 24.81)</td>
</tr>
<tr>
<td>HOMA\textsubscript{IR}</td>
<td>2.41</td>
<td>(0.56 - 8.82)</td>
</tr>
<tr>
<td>NEFA (mmol.l\textsuperscript{-1})</td>
<td>0.51</td>
<td>(0.34 – 0.70)</td>
</tr>
<tr>
<td>ALT (U.l\textsuperscript{-1})</td>
<td>23</td>
<td>(16-38)</td>
</tr>
</tbody>
</table>

3.2.2 Intravenous Intralipid Test

Each subject reported to the Clinical Investigation Suite in the Department of Vascular Biochemistry in Glasgow Royal Infirmary after an overnight fast of 12 h. Transportation to the hospital was provided for the subjects, when needed, to ensure that they arrived in a rested state. A cannula was introduced into an antecubital vein in both arms; one for administration of Intralipid (purified soybean oil emulsion, Fresenius Kabi Ltd., Warrington, UK) and the other for blood sampling. The cannulae were kept patent by flushing with non-heparinised saline solution (0.9% NaCl). Ten min after cannulation a first baseline blood sample was obtained. A second baseline blood sample was obtained 10 min later.

The intravenous Intralipid test used was a modification of that described by Björkegren and colleagues (Bjorkegren et al., 1996). A bolus dose of 20% Intralipid (0.1 g.kg\textsuperscript{-1} body mass) was injected within one min. This was followed immediately by a constant continuous infusion of 10% Intralipid (0.1 g.kg\textsuperscript{-1}.h\textsuperscript{-1}). This dose was chosen as Björkegren et al. reported that the rate of rise of VLDL\textsubscript{1}-apoB during Intralipid infusion was no greater for a 0.2 g.kg\textsuperscript{-1}.h\textsuperscript{-1} infusion dose compared with 0.1
g.kg\(^{-1}\).h\(^{-1}\), suggesting that the lower dose was sufficient to saturate LPL and prevent measurable VLDL\(_1\) catabolism (Bjorkegren et al., 1996). However, experiments with the 0.2 g.kg\(^{-1}\).h\(^{-1}\) dose were also performed in the present study to confirm that this was the case in the author’s hands (see below). Initially, the infusion period was 120 min, however, during development of the technique this was subsequently shortened to 75 min after it became clear that a 75-min infusion was long enough to induce a sufficient measurable rise in VLDL\(_1\)-TG and -apoB. Figure 3.1 shows an example of a subject during an Intralipid trial.

Blood samples were obtained at 15-min intervals during the infusion. Further blood samples were drawn 2.5, 5, 10, 15, 20, 30, 45, 60 and 75 min post-infusion. Initially, the post-infusion period was 3.25 h. However, this was subsequently shortened to 75 min when it became clear that this was sufficient to calculate the Intralipid-TG clearance rate using the exponential decay. All samples were obtained directly into 10-ml potassium EDTA tubes (BD Vacutainer Systems, Plymouth, UK) and placed immediately in ice before centrifuging for 15 min at 3000 rpm and 4\(^\circ\)C.

Aliquots of plasma were frozen immediately at -70\(^\circ\)C, as described in section 2.3, for subsequent analysis of insulin, NEFA, glucose, TG, and total and HDL-cholesterol. The remaining plasma was stored overnight at 4\(^\circ\)C prior to separation of Intralipid and lipoproteins.

### 3.2.3 Increasing the Intralipid Infusion Rate

Five subjects (2 females and 3 males, age 23-47 y [range], BMI 20.8-28.7 kg.m\(^{-2}\), fasting TG 0.47-2.45 mmol.l\(^{-1}\)) underwent a second test using a higher infusion dose (0.2 g.kg\(^{-1}\).h\(^{-1}\)) of 10% Intralipid with the same 0.1 g.kg\(^{-1}\) bolus dose. This was carried out to determine whether an Intralipid infusion dose of 0.1 g.kg\(^{-1}\).h\(^{-1}\) was sufficient to completely prevent measurable lipolysis of VLDL\(_1\) by LPL, and therefore enable determination of VLDL\(_1\)-TG and -apoB production rates from their rises in concentration. If the infusion rate of 0.1 g.kg\(^{-1}\).h\(^{-1}\) was sufficient to saturate LPL and block lipolysis of VLDL\(_1\), the 0.2 g.kg\(^{-1}\).h\(^{-1}\) dose would not result in higher calculated production rates of VLDL\(_1\)-TG or -apoB compared to the 0.1 g.kg\(^{-1}\).h\(^{-1}\) dose. The order of testing was randomized. Other than the higher infusion dose, all conditions of the tests were the same.
Figure 3.1: A picture of a subject during an Intralipid trial. A cannula is placed in an antecubital vein in each arm; one for the Intralipid infusion of 0.1 g.kg⁻¹.h⁻¹ (right arm) using an IVAC Signature Edition Gold pump (ALARIS Medical Systems, Inc.) and one for blood sampling (left arm). An initial bolus Intralipid dose (0.1 g.kg⁻¹ body mass) was given in the right arm via a three-way stopcock immediately before starting the infusion. (Published with permission from the subject).
3.2.4 Intralipid (Sf > 400) Separation from Whole Plasma

Two ml of plasma were overlayed with 4 ml of 1.006 g.ml\(^{-1}\) density solution in ultra-clear centrifuge tubes and spun at 10,000 rpm and 4\(^\circ\) C for 30 min (Lindgren et al., 1972) using Beckman L8-60M Ultracentrifuge and Beckman 50.4 rotor (Beckman Instruments Inc., UK). Intralipid (d <1.006 g.ml\(^{-1}\)) was removed in the top 2 ml (IL-1) for subsequent measurements of TG using commercially available kits as described in section 2.4.2. TG concentration was also measured in the middle 1.5 ml fraction (IL-2) to verify complete separation of Intralipid. The final Intralipid-TG concentration was calculated as the addition of these two fractions \[\text{IL-1} + (\text{IL-2} \times 1.5/2)\]. In addition, glycerol was measured in these IL-1 and IL-2 fractions (section 2.4.2) to determine the amount of free glycerol. The final 0.5 ml of the density solution was discarded and the remaining 2 ml Intralipid-free plasma was used for separation of VLDL\(_1\) and VLDL\(_2\). The CV for the Intralipid-TG separation was 6.9%.

3.2.5 VLDL\(_1\) and VLDL\(_2\) Separation

VLDL\(_1\) (S\(_f\) 60-400) and VLDL\(_2\) (S\(_f\) 20-60) were isolated from plasma using a modification of the cumulative ultracentrifugation density gradient technique as previously described in section 2.4.1. TG concentrations were then measured in the VLDL\(_1\) and VLDL\(_2\) fractions in all time points and apoB concentrations were also measured directly by immunoturbidimetry as described in section 2.9.2.3. The CVs for separation of VLDL\(_1\)-TG and -apoB were 5.0% and 3.4%, respectively and for VLDL\(_2\)-TG and -apoB were 5.8% and 1.4%, respectively.

3.2.6 Fasting Plasma Analysis

Plasma glucose, insulin, total and HDL cholesterol concentrations were analysed in the fasted state and TG and NEFA concentrations were analysed at all time points. LDL cholesterol was calculated in the fasted state using the Friedewald equation (Friedewald et al., 1972). Serum ALT concentrations were measured at screening. All analyses were done as previously described in section 2.4.2.
3. Development of a Novel Method to Determine VLDL$_1$ Kinetics

3.2.7 Correction for Glycerol

Enzymatic kits for TG analysis measure the glycerol that is hydrolyzed from TG by LPL. As Intralipid contains free glycerol as an excipient, it has been reported that it overestimates the true TG concentrations of Intralipid (Howdieshell et al., 1995). Therefore, all Intralipid-TG measurements were corrected for free glycerol and are reported as ‘true’ TG concentrations [true TG concentration (mmol.l$^{-1}$) = measured TG (mmol.l$^{-1}$) - glycerol (mmol.l$^{-1}$)]. Glycerol concentrations were also measured in 5 subjects in the VLDL$_1$ fraction during infusion and were found to be negligible (influencing VLDL$_1$-TG concentrations by less than 1%).

3.2.8 Kinetic Data Calculations

The clearance rates of Intralipid-TG and production rates of VLDL$_1$-TG and -apoB were calculated as described below using examples from individual subjects.

3.2.8.1 Calculating VLDL$_1$-TG and VLDL$_1$-ApoB Production Rates

The production rates (mg.h$^{-1}$) of VLDL$_1$-TG and -apoB were calculated from the gradient of the linear rise in their pools (total mg in plasma) over time (min) multiplied by 60 min. Pool sizes (mg) were calculated as concentrations in mg.dl$^{-1}$ multiplied by plasma volume [4% of body mass (Packard et al., 1984)] in decilitres.

Figure 3.2A represents the linear increase in TG pool (mg) in the VLDL$_1$ fraction of subject No.3 (female, 55 y, 84.5 kg) with $R^2$ (goodness-of-fit) value of 0.97 and a gradient of 35.8. Thus, the VLDL$_1$-TG production rate of this subject would be (35.8 × 60) 2147.4 mg.h$^{-1}$ (609.9 mg.kg$^{-1}$.d$^{-1}$). Similarly, from Figure 3.2B, the VLDL$_1$-apoB production rate of the same subject was (0.85 × 60) 50.9 mg.h$^{-1}$ (14.5 mg.kg$^{-1}$.d$^{-1}$).
Figure 3.2: Changes in [A] VLDL₁-TG and [B] VLDL₁-apoB pools (mg) over time in a female subject No. 3 (55 yr, 84.5 kg) during infusion of 10% Intralipid (0.1 g.kg⁻¹.h⁻¹). An initial bolus dose of 20% Intralipid (0.1 g.kg⁻¹ body mass) was given at 0 min.
3. Development of a Novel Method to Determine VLDL₁ Kinetics

3.2.8.2 Calculating VLDL₁-TG and VLDL₁-ApoB Fractional Synthetic and Catabolic Rates

The fractional synthetic rate (FSR) is defined as the rate of incorporation of a precursor into a product per unit of product mass (Foster et al., 1993), which can be calculated as:

\[
FSR = \frac{\text{initial rate of change in product}}{\text{initial precursor concentration}} \quad (Eq. 1)
\]

In this method, the FSR of VLDL₁-TG and -apoB (pools.d⁻¹) can be calculated from the gradient of the linear rise in their pool (mg) over time (min) divided by fasting pool size (mg) then multiplied by 60 and 24 (to account for number of minutes in 1 day). However, since the system is in a steady state in fasting conditions, where synthesis of VLDL₁ equals its clearance, FSR will also be equivalent to the fractional clearance rate (FCR) in the fasted state (Foster et al., 1993).

Subject No. 3 had a VLDL₁-TG and -apoB fasting pool sizes of 1669.5 and 61.7 mg, respectively. According to the equation of the line in Figure 3.2A, the subject’s VLDL₁-TG FSR and FCR were \((35.8 \div 1669.5 \times 60 \times 24)\) 30.9 pools.d⁻¹. Similarly, from Figure 3.2B, VLDL₁-apoB FSR and FCR were 19.8 pools.d⁻¹.

3.2.8.3 Calculating Intralipid-TG Clearance Rate

Assuming that all TG clearance is Intralipid-TG clearance, it is possible to determine Intralipid-TG clearance rate in two ways:

3.2.8.3.1 The Steady-State (SS) Method

The clearance rate of Intralipid-TG can be calculated from the steady-state concentration during infusion using the following equation (Rang et al., 2003):

\[
\text{Clearance rate (ml.min}^{-1}) = \frac{\text{infusion rate (mg.min}^{-1})}{\text{steady state concentration (mg.ml}^{-1})} \quad (Eq. 2)
\]

In this method, steady state is defined as being achieved when the final 3 values of the Intralipid-TG concentrations differed by less than 13.8% (i.e. two times the CV for the separation of the Intralipid fraction and measurement of the TG. This
represents the 95% confidence interval for the measured value). Figure 3.3A shows the concentration of Intralipid-TG ± 13.8% during a 75-min infusion for subject No.8 (female, 23 y, 52.0 kg, BMI 20.8 kg.m⁻²). The steady state concentration, i.e. the mean of the last 3 points of infusion, is 0.86 mmol.l⁻¹ (0.76 mg.ml⁻¹). Since this subject’s body mass is 52.0 kg, the infusion rate of 0.1 g of 10% Intralipid per kg of body mass per h equals:

\[
\frac{0.1 \text{ g.h}^{-1}}{\text{kg}} \times 52.0 \text{ kg} = 5.2 \text{ g.h}^{-1} = 5200 \text{ mg.h}^{-1} = 86.67 \text{ mg.min}^{-1}
\]

From equation (2):

\[
\text{Clearance rate} = \frac{86.67 \text{ mg.min}^{-1}}{0.76 \text{ mg.ml}^{-1}} = 113.7 \text{ ml.min}^{-1}
\]

Assuming plasma volume [4% of body mass, (Packard et al., 1984)] for this subject was 2080 ml, the clearance rate can also be expressed in pools.d⁻¹ as follows:

\[
\text{Intralipid-TG clearance rate} = 113.7 \text{ ml.min}^{-1} \times \frac{60 \text{ min} \times 24 \text{ h}}{2080 \text{ ml}} = 78.9 \text{ pools.d}^{-1}
\]

3.2.8.3.2 The Exponential (Exp) Method

After stopping the intravenous infusion, Intralipid-TG declines exponentially following first-order kinetics as described by Rössner (Rossner, 1974). Figure 3.3B shows the Intralipid-TG concentrations (mmol.l⁻¹) post-infusion over time (min) curve for the same female subject (No.8) on a semi-log scale. (In this case, data for the first 45 min post-infusion were used for calculation, as beyond this Intralipid-TG concentrations approached zero increasing the error associated with an exponential curve fit). The equation of the fitted line is:

\[
y = k e^{-bt} \quad \text{(Eq. 3)}
\]
Figure 3.3: Intralipid-TG concentrations (mmol.l⁻¹) [A] during infusion and [B] post-infusion of 0.1 g.kg⁻¹.h⁻¹ of 10% Intralipid for a female subject (23 y, 52.0 kg). An initial bolus dose of 20% Intralipid (0.1 g.kg⁻¹ body mass) was given at 0 min. The Y-axis error bars are ± 13.8% (i.e. the 95% confidence interval for the measured Intralipid-TG concentration).
where \( k \) is the proportionality constant, \( t \) is the time, and \( b \) is the exponential decay constant, which in turn is defined as:

\[
b = \frac{\text{clearance rate (ml.min}^{-1})}{\text{plasma distribution volume (ml)}} \quad (\text{Eq. 4})
\]

Hence,

\[
\text{Clearance rate (ml.min}^{-1}) = b \times \text{plasma volume (ml)} \quad (\text{Eq. 5})
\]

or \( b \times 60 \text{ min} \times 24 \text{ h} = \text{in pools.d}^{-1} \)

Since the \( b \) value from Figure 3.3B is 0.056 and the subject’s plasma volume was 2080 ml, then from equation (5):

\[
\text{Intralipid-TG clearance rate} = 0.056 \times 2080 \text{ ml} = 116.5 \text{ ml.min}^{-1}
\]

or \( b \times 60 \text{ min} \times 24 \text{ h} = 80.6 \text{ pools.d}^{-1} \)

Figure 3.4 shows a schematic diagram of the Intralipid method protocol. (For comparison purposes with the stable-isotope method see Figure 1.7).

3.2.9 Intralipid Recovery

In order to assess the recovery of plasma-Intralipid in the Intralipid fraction (\( S_t >400 \)), EDTA plasma was spiked with Intralipid to produce an Intralipid-TG concentration in plasma of \( \sim1.5 \text{ mmol.l}^{-1} \) and \( \sim4 \text{ mmol.l}^{-1} \). These reflect approximate Intralipid-TG concentrations at the 0.1 g.kg\(^{-1}\).h\(^{-1}\) and 0.2 g.kg\(^{-1}\).h\(^{-1}\) infusion doses. For each Intralipid concentration, samples of spiked plasma were divided into 10 aliquots and the Intralipid fractions were separated as described in section 3.2.4. TG and glycerol concentrations were measured in plasma before and after addition of the Intralipid (to calculate the actual Intralipid-TG concentration), as well as in the separated Intralipid fractions. The Intralipid recovery was calculated as follows:

\[
\% \text{ recovery} = \frac{\text{separated Intralipid-TG}}{\text{actual Intralipid-TG}} \times 100 \quad (\text{Eq. 6})
\]

where actual Intralipid-TG = total TG (plasma with Intralipid) – TG (Intralipid-free plasma).

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3. Development of a Novel Method to Determine VLDL₁ Kinetics

Bolus Intralipid 0.1 g.kg⁻¹
Intralipid Infusion 0.1 g.kg⁻¹.h⁻¹
(75 min)

Post-infusion blood sampling
(60 min)

Total 18 EDTA blood samples

Intralipid (Sₚ >400) separation
(30 min at 10,000 rpm)

Intralipid-free plasma

Density Gradient Ultracentrifugation

VLDL₁ (Sₚ 60-200) fraction

VLDL₁-TG measurement
(automated spectrophotometry)

VLDL₁-apoB measurement
(automated immunoturbidimetry)

Kinetic calculations
(Excel workbook)

VLDL₁-TG production rate

VLDL₁-apoB production rate

Intralipid-TG clearance rate

EDTA blood samples

2x 0min + 5, 15, 25, 35, 45, 55, 65, & 75 min

77.5, 80, 85, 90, 95, 105, 115 & 135 min

Plasma analysis:
TG, glycerol & NEFA
(glucose, insulin etc)

IL₁ & IL₂ fractions
for TG & glycerol measurements

Kinetic calculations
(see below)

Figure 3.4: Schematic diagram of the 'Intralipid Method' protocol
3. Development of a Novel Method to Determine VLDL₁ Kinetics

3.2.10 Statistical Analyses

Statistical analyses were performed using MINITAB for Windows (Version 13.1, MINITAB Inc., State College, PA) and STATISTICA (Release 6.0, StatSoft, Inc, USA). Normality was checked for all the data using the Anderson-Darling test. When data did not approximate a normal distribution, these were log-transformed, specifically TG, glucose, insulin, HOMA₀, production rates of VLDL₁-TG (expressed in both mg·h⁻¹ and mg·kg⁻¹·d⁻¹) and VLDL₁-apoB (expressed in mg·h⁻¹), Intralipid-TG clearance rate and VLDL₁-apoB FSR required transformation. Time trends were tested using one-way ANOVA with repeated measures. Paired t-tests were used to compare between the Intralipid-TG clearance rates calculated from the steady state and the exponential decay and between the kinetic data obtained from the low and high Intralipid doses. HOMA [calculated as insulin (mU·l⁻¹) × glucose (mmol·l⁻¹)/22.5] was used as a validated surrogate measure of insulin resistance (Matthews et al., 1985). Relationships between HOMA-estimated insulin resistance (HOMA₀), NEFA, BMI, waist circumference and kinetic parameters were assessed using Pearson product-moment correlations. Significance was accepted at the \( P < 0.05 \) level. Data are presented as mean ± SEM unless otherwise stated. It was not possible to perform a power calculation for this study as the method was still in the developmental stage and thus no data on the reliability of the method were available at this stage.

3.3 Results

3.3.1 Plasma, Intralipid, VLDL₁- and VLDL₂-TG Concentrations During and Post-Infusion

Figure 3.5A shows the mean plasma-, Intralipid-, VLDL₁- and VLDL₂-TG responses in 10 subjects during 75-min Intralipid infusion (0.1 g·h⁻¹·kg⁻¹ body mass). Plasma-TG concentrations were increased to ~2-3 times the fasting value during the infusion. Similarly, mean Intralipid-TG concentrations increased in response to the bolus dose (\( P < 0.001 \)). VLDL₁-TG concentrations rose linearly during the infusion (\( P < 0.001 \)), but VLDL₂-TG did not change significantly during the course of the infusion (\( P = 0.14 \)).
After stopping the infusion, the plasma- and Intralipid-TG concentrations decreased exponentially (both $P < 0.001$, Figure 3.5B). VLDL$_1$-TG continued to rise for ~20 min ($P < 0.001$) before plateauing and subsequently decreasing. In subjects where the post-infusion period was extended, VLDL$_1$-TG returned to baseline concentrations within 105-135 min (an example of an earlier Intralipid trial is shown in Figure 3.6). The mean VLDL$_2$-TG concentrations remained unchanged for the 75 min post-infusion observation period.

Figure 3.5: Plasma-, Intralipid-, VLDL$_1$- and VLDL$_2$-TG concentrations (mmol.L$^{-1}$) [A] during infusion of 0.1 g.kg$^{-1}$.h$^{-1}$ of 10% Intralipid and [B] for 75 min post-infusion. An initial bolus dose of 20% Intralipid (0.1 g.kg$^{-1}$ body mass) was given at 0 min. N = 10, values are mean ± SEM.

### 3.3.2 VLDL$_1$- and VLDL$_2$-ApoB Concentrations During Infusion

The mean apoB concentration in the VLDL$_1$ (S$_r$ 60-400) fraction increased steadily from fasting levels throughout the infusion and was significantly higher than baseline within 15 min ($P < 0.001$). On the other hand, mean VLDL$_2$-apoB concentrations declined significantly ($P < 0.001$) during 75 min of infusion. Total (VLDL$_1$ + VLDL$_2$) VLDL-apoB concentrations rose slightly but significantly ($P < 0.05$) during infusion (Figure 3.7). There was no significant change in the VLDL$_1$ or VLDL$_2$ TG/apoB ratio (expressed in mol:mol) over the 75 min of infusion ($P = 0.21$ and $P = 0.16$, respectively) (Figure 3.8).
3. Development of a Novel Method to Determine VLDL₂ Kinetics

Figure 3.6: An example of a full 135min-Intralipid trial during development of the method. [Top] Plasma-, Intralipid-, VLDL₁- and VLDL₂-TG and [Bottom] VLDL₁- and VLDL₂-apoB concentrations during and post-infusion for a female subject (34 y, 55.2 kg). A bolus dose of 20% Intralipid (0.1 g.kg⁻¹ body mass) was injected at zero min, followed immediately by a constant infusion of 10% Intralipid (0.1 g.kg⁻¹.h⁻¹) for 120 min. The dotted line represents the end of infusion. VLDL₁ kinetics for this subject were calculated from the first 75 min of infusion: VLDL₁-TG and VLDL₁-apoB production rates were 711.2 and 20.7 mg.h⁻¹ and fractional catabolic rates were 56.3 and 20.0 pools.d⁻¹, respectively. Intralipid-TG clearance rate was calculated only from its exponential decay post-infusion as 146.9 pools.d⁻¹, as no steady state was achieved during infusion.
3. Development of a Novel Method to Determine VLDL₂ Kinetics

Figure 3.7: VLDL₁-, VLDL₂- and total VLDL-apoB concentrations (mg.dl⁻¹) during infusion of 10% Intralipid (0.1 g.kg⁻¹.h⁻¹). An initial bolus dose of 20% Intralipid (0.1 g.kg⁻¹ body mass) was given at 0 min. N = 10, values are mean ± SEM.

Figure 3.8: VLDL₁- and VLDL₂-TG/apoB ratios during infusion of 10% Intralipid (0.1 g.kg⁻¹.h⁻¹). An initial bolus dose of 20% Intralipid (0.1 g.kg⁻¹ body mass) was given at 0 min. N = 10, values are mean ± SEM.

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3.3.3 Kinetic Data

Table 3.2 shows the production rates and FSR of VLDL\textsubscript{1}-TG and VLDL\textsubscript{1}-apoB as well as the clearance rates of Intralipid-TG calculated for each subject (n = 10) as previously described. Fasting VLDL\textsubscript{1}-TG and VLDL\textsubscript{1}- and VLDL\textsubscript{2}-apoB concentrations are also presented.

3.3.3.1 VLDL\textsubscript{1}-TG and VLDL\textsubscript{1}-ApoB Production Rates

The mean ± SEM (range) production rates for VLDL\textsubscript{1}-TG and -apoB were 1076.7 ± 224.7 (446.0 – 2563.2) mg.h\textsuperscript{-1} and 25.4 ± 3.9 (12.0 – 50.9) mg.h\textsuperscript{-1}, respectively. These corresponded to 333.6 ± 49.1 (198.5 – 609.9) mg.kg\textsuperscript{-1}.d\textsuperscript{-1} and 8.1 ± 0.9 (4.4 – 14.5) mg.kg\textsuperscript{-1}.d\textsuperscript{-1}, respectively.

3.3.3.2 VLDL\textsubscript{1}-TG and VLDL\textsubscript{1}-ApoB Fractional Synthetic and Catabolic Rates

The mean ± SEM VLDL\textsubscript{1}-TG and -apoB FSRs, which are equal to the VLDL\textsubscript{1}-TG and -apoB FCRs in the fasted state (Foster et al., 1993), were 30.2 ± 5.7 (6.5 – 57.8) pools.d\textsuperscript{-1} and 21.1 ± 5.1 (2.2 – 56.2) pools.d\textsuperscript{-1}, respectively.

3.3.3.3 Intralipid-TG Clearance Rate

The Intralipid-TG clearance rates calculated for individual subjects by two methods as described above (i.e. SS and Exp) are shown in Table 3.2. Eight out of the 10 subjects reached the defined steady state during infusion. The mean ± SEM Intralipid-TG clearance rates in these 8 subjects did not differ significantly between the two calculation methods (52.4 ± 8.6 pools.d\textsuperscript{-1} for SS vs 55.3 ± 9.2 pools.d\textsuperscript{-1} for Exp, P = 0.45) and the values obtained were strongly correlated (r = 0.96, P <0.001). However, since not all subjects reached a steady state, the Intralipid-TG clearance rates mentioned hereafter will refer to that calculated using the exponential method.
Table 3.2: Fasting concentrations, apoE phenotypes and individual lipoprotein kinetic parameters calculated using the ‘Intralipid method’ (0.1 g.kg⁻¹.h⁻¹ infusion dose) (n=10).

<table>
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<tr>
<th>Subjects</th>
<th>Fasting Concentrations</th>
<th>Production Rates</th>
<th>Intralipid-TG Clearance Rate</th>
<th>FSR§</th>
</tr>
</thead>
<tbody>
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<td>(apoE phenotype)</td>
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<td>VLDL₁-ApoB</td>
<td>VLDL₂-ApoB</td>
<td>VLDL₁-TG</td>
</tr>
<tr>
<td></td>
<td>mmol.l⁻¹</td>
<td>mg.dl⁻¹</td>
<td>mg.dl⁻¹</td>
<td>mg.h⁻¹</td>
</tr>
<tr>
<td>1 (3/3)</td>
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<td>0.8</td>
<td>1.6</td>
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<tr>
<td>2 (3/2)</td>
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<td>1.1</td>
<td>2.9</td>
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</tr>
<tr>
<td>3 (3/2)</td>
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<td>1.8</td>
<td>6.8</td>
<td>2147.4</td>
</tr>
<tr>
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<td>0.8</td>
<td>0.9</td>
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<tr>
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<td>3.7</td>
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<tr>
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<td>0.5</td>
<td>2.9</td>
<td>798.2</td>
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</table>

Exp., exponential decay; FCR, fractional catabolic rate; FSR, fractional synthetic rate; SS, steady state.
Subjects 3, 5 and 8 were female subjects.
** Subject did not reach a steady state.
§ As FSR equals FCR in under steady state conditions, the VLDL₁-TG and -apoB FSR values are equal to the FCR values in the fasted state.
3.3.4 Effect of Increasing the Intralipid Infusion Rate

For the 5 subjects who underwent Intralipid infusion at the Low (0.1 g.kg\(^{-1}\).h\(^{-1}\)) and the High (0.2 g.kg\(^{-1}\).h\(^{-1}\)) doses, there were no significant differences in the mean VLDL\(_1\)-apoB production rates (Low dose: 23.8 ± 2.8 vs High dose: 22.0 ± 1.9 mg.h\(^{-1}\), \(P = 0.21\)) or VLDL\(_1\)-TG production rates (Low dose: 813.8 ± 127.0 vs High dose: 960.9 ± 136.8 mg.h\(^{-1}\), \(P = 0.10\)) between both doses, although there was a tendency for the VLDL\(_1\)-TG production rate to be higher at the High Intralipid dose. However, it was observed that separation of the large amount of Intralipid from plasma at the higher 0.2 g.kg\(^{-1}\).h\(^{-1}\) dose was technically quite difficult and it was suspected that the VLDL\(_1\) fraction in some samples may have become slightly contaminated with Intralipid at this dose (Figure 3.9). This suggestion is supported by the substantially lower recovery of Intralipid in the Intralipid fraction at high Intralipid concentrations (see below). Calculated FSRs for VLDL\(_1\)-TG (Low dose: 30.1 ± 8.8 vs High dose: 30.4 ± 8.1 pools.d\(^{-1}\), \(P = 0.94\)) and VLDL\(_1\)-apoB (Low dose: 28.5 ± 8.7 vs High dose: 33.4 ± 14.6 pools.d\(^{-1}\), \(P = 0.64\)) did not differ between the two doses. In addition, FSRs for VLDL\(_1\)-TG (\(r = 0.88, P = 0.05\)) and VLDL\(_1\)-apoB (\(r = 0.95, P = 0.01\)) between the two doses correlated highly with each other and, when plotted, followed the line-of-equality (Figure 3.10).

Furthermore, although a formal reproducibility test was not performed, the repeated two doses provided an opportunity to estimate the test/re-test reproducibility of VLDL\(_1\)-TG and -apoB production rates. Based on data from these 5 subjects, the within-subject CV for VLDL\(_1\)-TG production was 20.1% and for VLDL\(_1\)-apoB production was 12.7%.

3.3.5 Intralipid Recovery

Recovery of the Intralipid-TG in the Intralipid (S\(_f\) >400) fraction was 95 ± 7% (mean ± SD) for the 1.5 mmol.l\(^{-1}\) Intralipid-TG concentrations and 71 ± 4% for the ~4.0 mmol.l\(^{-1}\) Intralipid-TG concentrations.
3. Development of a Novel Method to Determine VLDL\textsubscript{1} Kinetics

Intralipid \textit{d} 1.006 g.ml\textsuperscript{-1}

**Figure 3.9**: [A] An example of Intralipid separation in the High dose of Intralipid infusion (0.2 g.kg\textsuperscript{-1}.h\textsuperscript{-1}). Intralipid separation is done by overlaying 2 ml of plasma with 4 ml of density solution 1.006 g.ml\textsuperscript{-1} and centrifuged at 10,000 rpm for 30 min. [B] A magnification of the bottom of the tube showing a thin layer of possible contamination of Intralipid at the top of plasma due to the high concentration of Intralipid.
Figure 3.10: Scatterplots (with the line of equality) illustrating the agreement between [A] VLDL₁-TG FSR (pools.d⁻¹) and [B] Ln VLDL₁-ApoB FSR between the Low (0.1 g.kg⁻¹.h⁻¹) and High (0.2 g.kg⁻¹.h⁻¹) doses. FSR is equivalent to FCR in the fasted state. N = 5, r and P values are for Pearson product-moment correlations between variables. VLDL₁-ApoB FSR was expressed in pools.d⁻¹ prior to log-transformation.
3. Development of a Novel Method to Determine VLDL\textsubscript{1} Kinetics

3.3.6 Interrelationships between VLDL\textsubscript{1}-TG and VLDL\textsubscript{1}-apoB Production and TG Clearance

Intralipid-TG clearance rate and VLDL\textsubscript{1}-TG and -apoB production rates (expressed in mg.h\textsuperscript{-1}) were significantly inter-related (Figure 3.11) with the expected negative correlation between Intralipid-TG clearance and VLDL\textsubscript{1}-TG ($r = -0.67$, $P = 0.04$) and VLDL\textsubscript{1}-apoB ($r = -0.69$, $P = 0.03$) production rates and a positive correlation between VLDL\textsubscript{1}-TG and -apoB production rates ($r = 0.85$, $P = 0.002$). There was also a very strong relationship between VLDL\textsubscript{1}-TG FSR (which equals the VLDL\textsubscript{1}-TG FCR in the fasted state) and Intralipid-TG clearance rate ($r = 0.90$, $P < 0.005$). The positive correlation between VLDL\textsubscript{1}-TG and -apoB production rates remained significant between production rates when values were expressed in mg.kg\textsuperscript{-1}.d\textsuperscript{-1} ($r = 0.73$, $P = 0.02$). However, the relationships between VLDL\textsubscript{1}-TG production rate expressed in mg.kg\textsuperscript{-1}.d\textsuperscript{-1} and Intralipid-TG clearance ($r = -0.46$, $P = 0.18$) and between VLDL\textsubscript{1}-apoB production rate expressed in mg.kg\textsuperscript{-1}.d\textsuperscript{-1} and Intralipid-TG clearance ($r = -0.28$, $P = 0.44$) were not statistically significant.

3.3.7 Relationships between Kinetic Variables and Subjects Characteristics

Figure 3.12 shows the relationships between the calculated kinetic variables and subjects characteristics, with VLDL\textsubscript{1}-TG and -apoB production rates expressed in mg.h\textsuperscript{-1}. VLDL\textsubscript{1}-TG and -apoB production rates (mg.h\textsuperscript{-1}) correlated strongly and significantly with waist circumference and fasting TG concentration. VLDL\textsubscript{1}-TG production rate also correlated significantly with HOMA\textsubscript{IR}. Similarly, Intralipid-TG clearance rate was significantly and inversely correlated with waist circumference, fasting TG concentrations and HOMA\textsubscript{IR}. In addition, BMI correlated significantly and positively with VLDL\textsubscript{1}-TG ($r = 0.83$, $P = 0.003$) and VLDL\textsubscript{1}-apoB ($r = 0.81$, $P = 0.004$) production rates and inversely with Intralipid-TG clearance rate ($r = -0.60$, $P = 0.07$). Fasting NEFA concentrations were not significantly correlated with any of the kinetic variables. The relationships between VLDL\textsubscript{1}-TG and -apoB production rates expressed in mg.kg\textsuperscript{-1}.d\textsuperscript{-1} units with BMI, waist circumference, fasting TG concentration and HOMA\textsubscript{IR} are shown in Table 3.3. The correlations between VLDL\textsubscript{1}-TG production and all of these variables remained strong and statistically significant, however the correlations between VLDL\textsubscript{1}-apoB production rate and
waist circumference and fasting TG were not statistically significant when the production rates were normalized for body mass.

### 3.3.8 Relationships between Kinetic Variables and ALT Concentrations

Figure 3.13 shows the relationships between serum ALT concentrations and kinetic variables. Although all subjects had ALT concentrations within the normal range, one subject was on antibiotics shortly prior to participating in the study and was excluded from the following correlations. ALT concentrations correlated significantly with VLDL$_1$-apoB production rate ($r = 0.73$, $P = 0.03$) and Intralipid-TG clearance rate ($r = -0.79$, $P = 0.01$). This indicates that, within normal range, ALT concentrations explained 53% ($0.73^2$) and 62% ($0.79^2$) of the variance in VLDL$_1$-apoB production rate and Intralipid-TG clearance rate, respectively. There was a tendency toward a positive correlation with VLDL$_1$-TG production rate, but it did not reach statistical significance ($r = 0.59$, $P = 0.09$).

<table>
<thead>
<tr>
<th>Table 3.3: Correlations between VLDL$_1$-apoB and -TG production rates (expressed in mg.kg$^{-1}$.d$^{-1}$) and subjects’ characteristics.</th>
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<tr>
<td><strong>BMI</strong></td>
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<td><strong>Ln Fasting TG</strong></td>
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<td><strong>Ln HOMA$_{IR}$</strong></td>
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BMI, body mass index; HOMA$_{IR}$, HOMA-estimated insulin resistance; WC, waist circumference.

N = 10, $r$ and $p$ values are for Pearson product-moment correlations between values.
Figure 3.11: Scatterplots (with linear-regression lines of ‘best-fit’) illustrating the interrelationships between [A] Ln Intralipid-TG clearance rate and Ln VLDL_{1}-apoB production rate, [B] Ln Intralipid-TG clearance rate and Ln VLDL_{1}-TG production rate, and [C] Ln VLDL_{1}-apoB and Ln VLDL_{1}-TG production rates. N = 10, $r$ and $P$ values are for Pearson product–moment correlations between variables. Production rates and Intralipid-TG clearance rate were expressed in mg.h^{-1} and pools.d^{-1} prior to log-transformation, respectively.
Figure 3.12: Scatterplots (with linear-regression lines of ‘best-fit’) illustrating the relationships between the kinetic variables [Ln VLDL$_1$-TG production rate (left), Ln VLDL$_1$-apoB production rate (middle) and Ln Intralipid-TG clearance rate (right)] and subjects’ characteristics: waist circumference (top), Ln fasting TG concentrations (middle) and Ln HOMA-estimated insulin resistance (HOMAIR) (bottom). N = 10, r and p values are for Pearson product-moment correlations between variables.
3. Development of a Novel Method to Determine VLDL₂ Kinetics

Figure 3.13: Scatterplots (with linear-regression lines of ‘best-fit’) illustrating the relationships between serum ALT concentration (mU.l⁻¹) and [A] Ln VLDL₁-apoB production rate, [B] Ln Intralipid-TG clearance rate and [C] Ln VLDL₁-apoB production rate. N = 9, r and P values are for Pearson product-moment correlations between variables.
3.4 Discussion

In the present study, a relatively straightforward method of determining TRL kinetics was developed. The method relies on the fact that chylomicrons or chylomicron-like particles, such as Intralipid, compete with hepatically-derived large VLDL\textsubscript{1} particles for clearance by a common saturable pathway – i.e. hydrolysis of their TG content by LPL (Björkgren \textit{et al.}, 1996; Karpe & Hultin, 1995) – and that chylomicrons or chylomicron-like particles are the preferred substrate for LPL (Fisher \textit{et al.}, 1995). Thus, the presence of a sufficient concentration of chylomicrons or chylomicron-like particles in the circulation will almost entirely prevent clearance of VLDL\textsubscript{1} by LPL (Björkgren \textit{et al.}, 1996) and the rates of VLDL\textsubscript{1}-TG and -apoB production can therefore be calculated from their rates of rise in concentration. The present work builds on the findings of Björkgren\textit{et al.} who, in studies designed to evaluate the effects of Intralipid infusion on VLDL\textsubscript{1} (S\textsubscript{f} 60-400) and VLDL\textsubscript{2} (S\textsubscript{f} 20-60) kinetics, found that individual rates of VLDL\textsubscript{1}-apoB production calculated from the rate of rise of VLDL\textsubscript{1}-apoB during infusion were virtually identical to those calculated from the ‘gold-standard’ stable isotope method (see below) (Björkgren \textit{et al.}, 1996). The ‘Intralipid method’ described in the present study enables the determination of the rate of VLDL\textsubscript{1}-TG (i.e. VLDL\textsubscript{1} lipid) and VLDL\textsubscript{1}-apoB (i.e. VLDL\textsubscript{1} particle) production as well as the clearance rate of chylomicron-like particles.

The Intralipid method specifically measures the production rate of large VLDL\textsubscript{1} (S\textsubscript{f} 60-400) rather than total VLDL (i.e. S\textsubscript{f} 20-400). VLDL is a metabolically heterogeneous class of lipoproteins, and it is the larger VLDL\textsubscript{1} subclass which competes with chylomicrons/chylomicron-like particles for LPL-mediated clearance and would have its clearance blocked by the presence of Intralipid (Björkgren \textit{et al.}, 1996). In contrast, catabolism of the smaller VLDL\textsubscript{2} subclass would not be completely blocked by Intralipid as its clearance can occur via the action of hepatic lipase as well as LPL (Packard & Shepherd, 1997). Indeed, as one source of VLDL\textsubscript{2} is from catabolism of VLDL\textsubscript{1} (the other being direct hepatic production), and this process was blocked by Intralipid infusion, mean VLDL\textsubscript{2}-apoB concentrations decreased slightly during the infusion although individual responses were more heterogeneous than that observed in VLDL\textsubscript{1}-apoB; a finding also reported by Björkgren \textit{et al.} (Björkgren \textit{et al.}, 1996). This heterogeneity in individual VLDL\textsubscript{2}-
3. Development of a Novel Method to Determine VLDL₁ Kinetics

apoB responses meant that it was not possible to perform any kinetic analyses using the VLDL₂ data.

To validate the calculation of VLDL₁-TG and -apoB production rates using the ‘Intralipid method’ it was necessary to consider a number of issues. The first was to determine whether infusing a higher Intralipid dose would influence the calculated VLDL₁-TG and -apoB production rates. This was necessary to establish whether the proposed Intralipid infusion dose of 0.1 g.kg⁻¹.h⁻¹, was sufficient to saturate LPL and block clearance of VLDL₁: if the 0.1 g.kg⁻¹.h⁻¹ dose was sufficient, infusing a higher Intralipid dose should not affect calculated production rates. In agreement with the findings of Björkergren et al. (Björkergren et al., 1996), the calculated VLDL₁-apoB production rate in the present study was not changed when a higher, 0.2 g.kg⁻¹.h⁻¹ Intralipid infusion dose was used. Similarly, FSRs, which correspond to the FCRs in the fasted state, for VLDL₁-TG and -apoB did not differ between the two doses (Figure 3.10). Although there was tendency for the calculated VLDL₁-TG production rate to be higher with the 0.2 g.kg⁻¹.h⁻¹ dose, this was not statistically significant. However, the author feels that the slightly higher apparent VLDL₁-TG production rate at the High dose was a methodological, rather than a physiological, issue caused by the difficulty in separating Intralipid at the High dose, leading to the potential contamination of the VLDL₁ fraction with Intralipid. This is supported by the fact that Intralipid recoveries at high Intralipid doses were relatively low (71% at an Intralipid concentration of 4 mmol.l⁻¹). This contrasts with the near complete recovery of Intralipid at lower Intralipid doses (95% at an Intralipid concentration of 1.5 mmol.l⁻¹). This, of course, would not influence the VLDL₁-apoB production rate calculations, as Intralipid particles do not contain apoB.

A further issue to consider is whether, following lipolysis by LPL, Intralipid ‘remnant’ particles may have appeared in the VLDL₁ fraction, thereby increasing the measured VLDL₁-TG concentration and the apparent VLDL₁-TG production rate. However, the author does not believe that this would have had a substantial effect on calculated VLDL₁-TG production rates for a number of reasons. Firstly, evidence from the literature suggests that for large TG-rich particles, particularly chylomicron-like particles, lipolysis and particle removal from the plasma is likely to occur simultaneously, rather than by sequential mechanisms (Olivecrona & Olivecrona,
1998; Hultin et al., 1996), with the majority of particles removed from the plasma before conversion to smaller VLDL-sized remnant particles (Karpe et al., 1997). Secondly, as Intralipid contains TG but not apoB, appearance of Intralipid remnants in the VLDL₁ fraction would lead to a disproportionate rise in VLDL₁-TG compared with VLDL₁-apoB, leading to an increase in the VLDL₁ TG/apoB ratio. There was no significant increase in this ratio during the infusion \((P = 0.21)\). Thirdly, if the rise in VLDL₁-TG was influenced by the appearance of Intralipid remnant particles, then a positive correlation between Intralipid clearance and VLDL₁-TG production would be evident (i.e. increased Intralipid clearance would lead to increased VLDL₁-TG production). Instead, a negative relationship between Intralipid clearance and VLDL₁-TG production (expressed in mg.h⁻¹) was observed (i.e. subjects with slow Intralipid clearance also had high VLDL₁-TG production) \((r = -0.67, P = 0.04)\). Furthermore, the relationship between VLDL₁-apoB production, which would be unaffected by the presence of Intralipid remnant particles, and VLDL₁-TG production was very strong, with 71% of the variance in the VLDL₁-TG production rate explained by the VLDL₁-apoB production rate \((r = 0.85, P = 0.002)\).

Furthermore, it is important to ascertain whether the results obtained are comparable with data obtained using the ‘gold standard’ stable isotope tracer method. An internal validation of this method was previously undertaken by Björkegren and colleagues (Björkegren et al., 1996) in 3 subjects. They reported VLDL₁-apoB production rates of 20.0, 25.6 and 7.2 mg.h⁻¹ calculated from the Intralipid infusion method with corresponding rates calculated from a stable isotope method of 23.8, 21.6 and 8.0 mg.h⁻¹, respectively, indicating that data obtained from the two methods were comparable. In addition, from Table 3.4, it is clear that the values for VLDL₁-apoB production in the present study are of the same order as those obtained from a number of studies which determined VLDL₁-apoB production using staple isotope techniques. Determination of VLDL₁-TG production rates using stable isotope tracer methods is technically more difficult than determination of VLDL₁-apoB production, and the author is only aware of one group of workers who have evaluated this (Adiels et al., 2005a; Adiels et al., 2006b). The values obtained for VLDL₁-TG production in the present study are of the same order as those published by Adiels et al. (Adiels et al., 2005a; Adiels et al., 2006b).
This Intralipid method enabled the Intralipid-TG clearance rate to be calculated in two different ways: from the steady state concentration of Intralipid-TG during the infusion, which was defined in the present study as the mean of the final 3 values if these differed by less than 13.8% (i.e. two times the CV for the separation of the Intralipid fraction and measurement of the TG), and from the exponential decrease in Intralipid-TG post-infusion (Rossner, 1974). In subjects where a steady-state Intralipid-TG concentration was achieved, the Intralipid-TG clearance rates calculated from the steady state concentration and from the post-infusion exponential decrease agreed closely (see Table 3.2). However, not all subjects achieved a steady-state Intralipid-TG concentration in 75 min of infusion and it is not possible to determine whether a steady-state was achieved for a given subject until sample analysis was completed. Therefore, in practice, it may be easier to use the post-infusion values to determine Intralipid-TG clearance rates, as this ensures that the Intralipid infusion can be kept as short as is necessary to enable calculation of VLDL₁-TG and -apoB production rates.

Moreover, the author sought to determine whether this Intralipid method revealed the physiologically expected differences in TRL kinetics between subjects with differing physical and metabolic profiles. As expected, there were strong positive correlations between fasting TG concentrations and VLDL₁-TG production rates expressed in either absolute terms or normalized according to body mass and between fasting TG and VLDL₁-apoB, production expressed in mg.h⁻¹ units, with a strong negative correlation between fasting TG and the Intralipid-TG clearance rates, indicating that those with high TG concentrations exhibited a combination of enhanced VLDL₁ production and diminished TG clearance. VLDL₁-TG FCR in the fasted state (i.e. with no Intralipid present) was ~45% of the Intralipid-TG clearance rate (30.2 ± 5.7 pools.d⁻¹ vs. 66.2 ± 11.7 pools.d⁻¹, see Table 3.2) and there was a very strong correlation between the two variables (r = 0.90, P <0.0005), indicating that clearance rates for VLDL₁ and chylomicron-like particles within an individual are very tightly linked, consistent with the fact that these particles are cleared by the same pathway. The expected positive correlations between indices of body fatness (waist circumference and BMI) and insulin resistance (HOMA IR) and VLDL₁-TG and VLDL₁-apoB production rates were also observed, in agreement with findings previously reported using stable isotope tracer methods (Gill et al., 2004a).
addition, significant negative relationships were observed between Intralipid-TG clearance and HOMA IR and waist circumference. Thus, the Intralipid method appears to be sensitive enough to detect physiologically relevant differences in TRL kinetics between individuals across the normal and moderately hypertriglyceridaemic range.

Finally, liver fat has been reported to be independently associated with insulin (Westerbacka et al., 2004) and predicted VLDL1 production (Adiels et al., 2006b). It was proposed that insulin resistance induces an increase in the fatty acid flux from adipose tissue to the liver, which leads to the accumulation of fat in the liver, resulting in overproduction of VLDL1 particles (Adiels et al., 2006b). As serum ALT concentrations, within normal range, have been shown to be a marker for liver fat (Westerbacka et al., 2004) and has been shown to predict the onsets of type 2 diabetes (Sattar et al., 2004; Sattar et al., 2007), the metabolic syndrome (Schindhelm et al., 2007b) and CVD events (Schindhelm et al., 2007a), the author investigated whether serum ALT concentrations, would predict hepatic VLDL1 production rate in the present study. Indeed, ALT concentrations correlated significantly with VLDL1-apoB production rate and Intralipid-TG clearance rate, explaining 53% and 62% of their variance; respectively. This suggests that ALT concentrations are significant predictors of hepatic VLDL1 production and further validates the sensitivity of the ‘Intralipid Method’.

In conclusion, this chapter describes the development of a novel method to determine TRL kinetics. The ‘Intralipid method’ provides a relatively straightforward and cost-effective way of determining VLDL1-TG and VLDL1-apoB production rates, and the clearance rate of chylomicron-like particles, which does not require specialised equipment or skill in multicompartmental modelling. The author believes that this method will increase the scope for the study of TRL kinetics, particularly in circumstances where issues related to funding or equipment availability preclude the use of more traditional isotopic tracer methods.
Table 3.4: Comparison of values for VLDL₁-TG and VLDL₁-apoB production rates (range) calculated in the present study (Intralipid Method) and in previously published studies using the stable-isotope method.

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Production rates</th>
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<tr>
<td></td>
<td>N (m/f)</td>
<td>BMI kg.m⁻²</td>
</tr>
<tr>
<td>(Bjorkegren et al., 1996)</td>
<td>16 (m)</td>
<td>20.0 - 25.8</td>
</tr>
<tr>
<td>(Demant et al., 1996)</td>
<td>6 (m)</td>
<td>-</td>
</tr>
<tr>
<td>(Pietzsch et al., 1996)</td>
<td>6 (3/3)</td>
<td>20.5 - 25.0</td>
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<tr>
<td>(Gill et al., 2004a)</td>
<td>16 (8/8)</td>
<td>19.6 - 32.9</td>
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<tr>
<td>(Adiels et al., 2005a)</td>
<td>17</td>
<td>22.4 - 30.1</td>
</tr>
<tr>
<td>(Zheng et al., 2006)</td>
<td>5 (f)</td>
<td>22.0 – 27.0</td>
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<tr>
<td>(Adiels et al., 2006b)</td>
<td>18</td>
<td>22.0 – 30.0</td>
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<tr>
<td>Intralipid Method</td>
<td>10 (7/3)</td>
<td>20.8 - 34.7</td>
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*Data are mean ± SD.
4. Effects of Acute Hyperinsulinaemia and Hyperglycaemia on VLDL\textsubscript{1}-TG and VLDL\textsubscript{1}-ApoB Production and Intralipid-TG Clearance in Normoglycaemic Subjects

4.1 Introduction

Diabetic dyslipidaemia is believed to be initiated by high concentrations of large VLDL\textsubscript{1} particles, which, consequently, cause the accumulation of remnant atherogenic particles and the generation of small dense LDL and small dense HDL (Taskinen, 2003). Interestingly, this profile of atherogenic lipoprotein abnormalities along with insulin resistance and hyperinsulinaemia is detectable in both the fasting (Tilly-Kiesi et al., 1996) and postprandial (Johanson et al., 2004) states years before the diagnosis of diabetes and appears to be related more to insulin resistance than hyperglycaemia. Therefore, it is important to understand the role of insulin in the regulation of hepatic VLDL\textsubscript{1} production.

Insulin plays a pivotal role in the regulation of hepatic VLDL production and acute hyperinsulinaemia has been shown to suppress VLDL production in normal subjects (Lewis et al., 1993; Malmstrom et al., 1997b). Insulin directly inhibits VLDL assembly and secretion in the liver through a number of mechanisms; e.g. it enhances intracellular apoB degradation (Sparks & Sparks, 1990) and inhibits the maturation process of VLDL assembly (Brown & Gibbons, 2001). In addition, it suppresses the NEFA flux to the liver, thereby reducing substrate availability for VLDL formation (Coppack et al., 1994). However, these suppressive effects may be attenuated or lost in insulin resistant conditions. It has been reported that type 2 diabetic (Malmstrom et al., 1997a; Bioletto et al., 2000) and obese individuals (Lewis et al., 1993; Bioletto et al., 2000) fail to suppress hepatic VLDL production and concentrations (Bioletto et al., 2000) to the same extent as their normal-weight, non-diabetic counterparts.
It is important to recognise that VLDL is a heterogeneous particle consisting of at least two major subclasses - large TG-rich VLDL₁ (S₁ 60-400) and small, denser VLDL₂ (S₁ 20-40) (Packard & Shepherd, 1997) - which have been shown to be independently regulated (Packard & Shepherd, 1997; Malmstrom et al., 1998; Gill et al., 2004a). Thus, the apparent effects of regulators on VLDL₁ metabolism may be masked or attenuated in studies examining the kinetics of total VLDL without subdivision into VLDL₁ and VLDL₂ subclasses. Indeed, VLDL₁, but not VLDL₂, particles have been shown to be the major determinant of plasma TG (Tan et al., 1995) and the target for insulin-mediated VLDL suppression (Gill et al., 2004a; Malmstrom et al., 1998).

Furthermore, it is important to differentiate between VLDL-apoB and VLDL-TG production rates. As there is only one apoB molecule per VLDL particle (Elovson et al., 1988), VLDL-apoB reflects particle number, whereas VLDL-TG is a measure of the major lipid component of VLDL particle. VLDL-apoB production rate may be dissociated from that of VLDL-TG (Kissebah et al., 1981), giving rise to variable TG-to-apoB ratios and, hence, VLDL particle size.

To the best of the author’s knowledge, studies examining the effects of acute hyperinsulinaemia on VLDL have either (1) addressed VLDL heterogeneity but considered only the production of VLDL₁-apoB, but not VLDL₁-TG (Malmstrom et al., 1997b; Malmstrom et al., 1998; Bioletto et al., 2000; Annuzzi et al., 2001); (2) investigated both apoB and TG production rates, but in total VLDL (Lewis et al., 1993; Lewis et al., 1994; Lewis & Steiner, 1996; Mittendorfer et al., 2003b; Lewis et al., 1995), rather than VLDL₁ specifically; or (3) studied the composition of VLDL subfractions rather than production rates (Annuzzi et al., 2001; Bioletto et al., 2000). However, at the start of the present study, no information existed on the acute effects of insulin on both VLDL₁-TG and -apoB production rates in normoglycaemic subjects. Recently, a non-steady-state stable isotope study by Adiels et al. (Adiels et al., 2007) was published, which investigated the acute effect of insulin on the production rates and pool sizes of VLDL₁-TG and -apoB in relation to liver fat content in normal and diabetic subjects.
Therefore, the aims of the present study initially were to investigate (1) whether acute hyperinsulinaemia inhibits VLDL$_1$-TG and VLDL$_1$-apoB production to the same extent and (2) factors that might influence the extent to which VLDL$_1$ is suppressed in a group of normoglycaemic individuals with a wide range of adiposity, fasting TG and insulin resistance. This was conducted using a novel, non-stable isotope method to determine VLDL$_1$-TG and -apoB kinetics (Al-Shayji et al., 2007), the development of which was previously described in Chapter 3.

4.2 Subjects and Methods

4.2.1 Subjects

Eight non-smoking healthy subjects (6 men and 2 women) were included in this study after giving written informed consent. All subjects had normal thyroid, liver and renal function and none had acute illness, a history of known cardiovascular disease or hypertension, nor were under medication known to influence carbohydrate or lipid metabolism. The subject information sheet, consent form and health questionnaire were the same as those used for the ‘Intralipid Method’ study (Appendices A1 & B1). Two subjects had the E3/2 phenotype, five had the E3/3 phenotype and one had the E4/4 phenotype. The subjects’ characteristics are shown in Table 4.1. The study protocol was approved by the Research Ethics Committee of the North Glasgow University Hospitals NHS Trust.

4.2.2 Study Design

Each subject participated in 2 Intralipid trials (Control and Glucose) in random order at an interval of 2-3 weeks for men and 4 weeks (to control for menstrual cycle) for women. In the Glucose trial, subjects were given an oral bolus of a glucose drink (30 g in 120 ml) an hour before an Intralipid infusion (see below) followed by 10 g of glucose (in 40 ml) at 15-min intervals throughout the duration of the trial. This was substituted with equivalent volumes of water in the Control trial (Figure 4.1). The dose and frequency of the glucose drink were previously determined, as explained in section 2.10, to elucidate sufficient suppression in NEFA concentrations, before administering the Intralipid infusion, and maintain, as far as possible, a steady state of hyperinsulinaemia and hyperglycaemia throughout the trial.
Subjects were requested not to exercise for three days before their study days as this is known to affect TRL metabolism (Gill & Hardman, 2003). In addition, they were asked to weigh and record their dietary intake for two days prior to the first Intralipid test and this diet was replicated for the second trial.

Table 4.1: Subjects’ physical characteristics and mean fasting concentrations (n=8).

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
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<tr>
<td>Age (years)</td>
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<tr>
<td>Body Mass (kg)</td>
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<td>Body mass index (kg.m⁻²)</td>
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<td>(21.0-34.7)</td>
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<td>Waist circumference (cm)</td>
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<td>Waist/hip ratio</td>
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<tr>
<td>ALT (U.l⁻¹)</td>
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<td>(15-26)</td>
</tr>
<tr>
<td>Triglycerides (mmol.l⁻¹)*</td>
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<td>(0.52-2.36)</td>
</tr>
<tr>
<td>Total cholesterol (mmol.l⁻¹)*</td>
<td>4.21</td>
<td>(3.37-5.65)</td>
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<tr>
<td>HDL cholesterol (mmol.l⁻¹)*</td>
<td>1.37</td>
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<tr>
<td>LDL cholesterol (mmol.l⁻¹)*</td>
<td>2.29</td>
<td>(1.47-3.63)</td>
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<tr>
<td>Glucose (mmol.l⁻¹)*</td>
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<td>(4.50-5.91)</td>
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<tr>
<td>Insulin (mU.l⁻¹)*</td>
<td>7.15</td>
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<td>HOMAIR*</td>
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<td>NEFA (mmol.l⁻¹)*</td>
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</tr>
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<td>Fasting VLDL₁-TG (mmol.l⁻¹)*</td>
<td>0.47</td>
<td>(0.12-1.31)</td>
</tr>
<tr>
<td>Fasting VLDL₁-apoB (mg.dl⁻¹)*</td>
<td>1.51</td>
<td>(0.38-4.04)</td>
</tr>
</tbody>
</table>

*Values are the mean of Control and Glucose trials

Figure 4.1: Study Protocol. In the Glucose trial a bolus drink of 30 g of glucose in 120 ml water was given at -60 mins, followed by 10 g of glucose in 40 ml of water at 15-min intervals thereafter. In the Control trial, equivalent volumes of water were given at the same intervals.
4. Effects of Hyperinsulinaemia & Hyperglycaemia on VLDL₁ Production Rates

4.2.3 Intralipid Infusion

TRL kinetics were determined using the ‘Intralipid Method’ (Al-Shayji et al., 2007) as previously described in Chapter 3. Briefly, subjects reported to the Clinical Investigation Suite in the Department of Vascular Biochemistry in Glasgow Royal Infirmary after an overnight fast of 12 h where they were given a bolus dose of 0.1 g.kg⁻¹ of 20% Intralipid followed immediately by an intravenous infusion of 0.1 g.kg⁻¹.h⁻¹ of 10% Intralipid for 75 min. Multiple EDTA blood samples were drawn at baseline, before and at 15-min intervals during infusion as well as at 2.5, 5, 10, 15, 20, 30, 45, 60 and 75 min post-infusion.

4.2.4 Analytical Methods

Plasma total and HDL cholesterol concentrations were measured in the fasted state and glucose, TG and NEFA concentrations were measured in all time points. LDL cholesterol was calculated in the fasted state using the Friedewald equation (Friedewald et al., 1972). Plasma insulin concentrations were analysed in the fasted state and every 30 min throughout the trial. Serum ALT concentrations were measured at screening and the apoE phenotype was determined for each subject. All analyses were performed as previously described in sections 2.4.2 to 2.4.4.

Intralipid (Sₐ >400) and VLDL₁ (Sₐ 60-400) fractions were separated from whole plasma as previously described in sections 3.2.4 and 2.4.1, respectively (Al-Shayji et al., 2007). TG concentrations (corrected for glycerol, section 3.2.7) were measured in the Intralipid and VLDL₁ fractions in all time points and apoB concentrations were measured directly in the VLDL₁ fraction by immunoturbidimetry as previously described in section 2.9.2.3.

4.2.5 Calculations and Statistical Analyses

VLDL₁-TG and VLDL₁-apoB productions rates were calculated from their linear rises in the VLDL₁ fraction during infusion. Intralipid-TG clearance rate was calculated from its exponential decay post-infusion as described in Chapter 3 (Al-Shayji et al., 2007). The production ratio of TG/apoB (mol:mol) was calculated as an index of the size of the VLDL₁ particle being produced.
4. Effects of Hyperinsulinaemia & Hyperglycaemia on VLDL1 Production Rates

Time-averaged concentrations for glucose, insulin and NEFA were calculated from their respective areas under the curve from the start of the Intralipid infusion to 75 min post-infusion.

Statistical analyses were performed using MINITAB for Windows (Version 14.0, MINITAB Inc., State College, PA) and STATISTICA (Release 6.0, StatSoft, Inc, USA). Normality was checked for all the data using the Anderson-Darling test. When data did not approximate a normal distribution, these were log-transformed, specifically fasting TG, kinetic data and the time-averaged concentrations of glucose and insulin. Time-trends were tested using two-way ANOVA (trial × time) with repeated measures on both factors. Post hoc Fisher LSD tests were used to identify exactly where any differences lay. Paired t-tests were used to compare fasting concentrations and kinetic variables between the Glucose and Control trials. HOMAIR was used as a validated surrogate measure of insulin resistance (Matthews et al., 1985) and was calculated as insulin (mU.l⁻¹) × glucose (mmol.l⁻¹) / 22.5. To determine factors which predicted the change in VLDL₁-TG and -apoB production and Intralipid-TG clearance rates between Control and Glucose trials, Pearson product-moment correlations were performed between the changes (Control minus Glucose) in kinetic variables on one hand and HOMAIR, ALT, NEFA and the differences in the time-averaged concentrations of glucose and insulin on the other hand. Significance was accepted at the $P < 0.05$ level. Data are presented as mean ± SEM unless otherwise stated. There were no data available to base a formal power calculation on for the present study. However, in a previous study investigating the effects of hyperinsulinaemia on VLDL₁-apoB production using stable isotope methods, a significant effect was seen with five subjects (Malmstrom et al., 1998), suggesting that studying eight subjects in the present study would be sufficient to detect a significant effect of hyperinsulinaemia on VLDL₁ metabolism, should such an effect exist.
4.3 Results

4.3.1 Fasting Plasma Concentrations

There was no significant difference in fasting concentrations between the Glucose and Control trials. Table 4.1 therefore shows the mean of fasting plasma lipid, glucose, insulin, HOMAIR and NEFA concentrations in both trials.

4.3.2 Glucose, Insulin and NEFA Concentrations during and Post-Infusion

Mean plasma glucose concentrations were significantly higher throughout the Glucose trial compared with the Control trial ($P = 0.005$, Figure 4.2A). In the Glucose trial, glucose concentrations increased significantly within 30 min of the initial glucose ingestion (i.e. 30 min prior to the Intralipid infusion) (from $5.35 \pm 0.18$ to $7.92 \pm 0.59$ mmol.l$^{-1}$, $P < 0.001$) and averaged $7.80 \pm 0.12$ mmol.l$^{-1}$ during infusion. In the Control trial, glucose concentrations decreased slightly from baseline during the first hour before infusion (from $5.28 \pm 0.15$ to $5.06 \pm 0.11$ mmol.l$^{-1}$, $P = 0.01$), but remained unchanged at an average of $5.08 \pm 0.08$ mmol.l$^{-1}$ ($P = 0.44$) during infusion.

As expected, insulin concentrations were significantly higher in the Glucose trial compared to the Control trial ($P = 0.03$, Figure 4.2B). Like glucose concentrations, insulin concentrations increased significantly 30 min after the start of the glucose drinks in the Glucose trial (from $7.25 \pm 1.80$ mU.l$^{-1}$ to $47.61 \pm 8.78$ mU.l$^{-1}$, $P = 0.02$) and remained elevated ($P = 0.47$) throughout the trial with an average concentration of $64.91 \pm 9.7$ mU.l$^{-1}$. This is in contrast to the Control trial, where plasma insulin concentrations did not change significantly from baseline ($P = 0.91$) with an average of $6.87 \pm 0.17$ mU.l$^{-1}$.

In the hour prior to the start of the Intralipid infusion, mean plasma NEFA concentrations declined rapidly in the Glucose trial from $0.46 \pm 0.07$ at baseline to $0.16 \pm 0.04$ mmol.l$^{-1}$ immediately before infusion ($P < 0.001$, Figure 4.2C), but did not change significantly from baseline in the Control trial ($0.55 \pm 0.01$ mmol.l$^{-1}$, $P = 0.27$). When the Intralipid infusion started in both trials, NEFA concentrations
Figure 4.2: Mean [A] glucose, [B] insulin and [C] NEFA concentrations before and during Intralipid infusion (left panel) and post-infusion (right panel) between the Control (●) and Glucose (○) trials. The dotted line represents the beginning of a bolus Intralipid dose of 0.1 g.kg⁻¹ followed immediately by a constant Intralipid infusion of 0.1 g.kg⁻¹.h⁻¹. Significant differences between the two trials were tested using two-way ANOVA with (*) representing \(P < 0.05\) and (**) \(P < 0.001\).
increased about 2.5-fold and leveled during infusion at an average concentration of 0.85 ± 0.04 mmol.l⁻¹ in the Control trial and 0.34 ± 0.02 mmol.l⁻¹ in the Glucose trial. Overall, NEFA concentrations were significantly lower throughout the Glucose trial compared to the Control trial (P <0.001).

### 4.3.3 The Change in Production and Clearance Rates

Individual and mean VLDL₃-TG and -apoB production and Intralipid-TG clearance rates in the Control and Glucose trials according to apoE phenotypes are shown in Figure 4.3. The VLDL₃-TG production rate was lower in the Glucose trial in 6 out of 8 subjects with an overall mean production rate of 805.7 ± 264.0 mg.h⁻¹ compared to 969.3 ± 181.6 mg.h⁻¹ in the Control trial (P = 0.05, Figure 4.3A). Similarly, VLDL₃-apoB production rate was lower in the Glucose trial in the same 6 subjects with an overall mean production rate of 21.8 ± 5.3 mg.h⁻¹ in the Glucose trial versus 25.8 ± 4.1 mg.h⁻¹ in the Control trial (P = 0.04, Figure 4.3B). Overall, there was a 25.4% and 20.9% reduction in VLDL₃-TG and -apoB production rates, respectively, in the Glucose trials. It is unclear why these two subjects responded differently. However, of note, one of them had the highest HOMAIR value (3.59) among all subjects.

On the other hand, the Intralipid-TG clearance response differed widely amongst subjects (Figure 4.3C). While four subjects showed a slight increase in Intralipid-TG clearance rate in the glucose trial, one subject showed a dramatic increase and the remaining three subjects showed a decreased Intralipid-TG clearance rate. The reason for these different responses is unclear. However, it is possibly due to differences in insulin resistance: the subject who had the highest increase in Intralipid-TG clearance rate, had the lowest HOMAIR of 0.5 (mean between the Control and Glucose trials), while the three subjects who had decreased clearance rates in the glucose trial, exhibited the highest HOMAIR concentrations amongst the 8 subjects (1.97, 3.47 and 3.59). This suggestion is supported by a strong correlation between HOMAIR and the change in Intralipid-TG clearance rate (see below). There was no significant difference in Intralipid-TG clearance between the Control and Glucose trials (69.4 ± 12.7 and 103.5 ± 49.8 pools.d⁻¹ respectively, P = 0.95), although clearance was 16.9% higher in the Glucose trial. However, this numerical
increase in Intralipid-TG clearance was entirely due to one subject who showed a
dramatic increase in Intralipid-TG clearance following glucose ingestion. If this
subject was excluded from analysis, the Intralipid-TG clearance rates between the
Control and Glucose trials were almost identical (58.4 ± 7.3 and 54.8 ± 11.9 pools.d
1, respectively, \( P = 0.600 \)).

4.3.4 \( \text{VLDL}_1 \) Particle Size

The size of the \( \text{VLDL}_1 \) particle being produced (estimated as TG/apoB molar ratio)
in response to hyperinsulinaemia and hyperglycaemia varied widely among subjects:
five subjects exhibited a decrease in \( \text{VLDL}_1 \) particle size (range 7.4 - 50.6%),
whereas 3 subjects showed an increased \( \text{VLDL}_1 \) particle size (range 10.9 - 38.2%).
Unlike the Intralipid-TG clearance rate, the reason for this variable response in
\( \text{VLDL}_1 \) particles size is unclear and could not be explained by differences in
\( \text{HOMA}_{\text{IR}} \). In total, there was a mean reduction of 7.6 ± 10.2% in \( \text{VLDL}_1 \) particle
size with glucose ingestion. However, this change was not statistically significant (\( P
= 0.29 \)).

4.3.5 Predictors of the Change in Production and Clearance Rates

The change (Control minus Glucose) in NEFA concentrations at zero min (i.e. after
the first hour of the start of the drink, and just before the Intralipid infusion)
correlated significantly with the change in \( \text{VLDL}_1 \)-TG production rate (\( r = 0.79, P = 0.02 \)), but not with the change in \( \text{VLDL}_1 \)-apoB production rate (\( r = 0.59, P = 0.13 \),
\textbf{Figure 4.4A}). Overall, the change in NEFA concentrations explained 62% (0.792) of
the variance in the change in \( \text{VLDL}_1 \)-TG production rate.

In addition, the author investigated whether ALT concentrations within normal range
would predict the change in hepatic \( \text{VLDL}_1 \)-TG and -apoB production in response to
hyperinsulinaemia and hyperglycaemia. It was noted that serum ALT concentrations
correlated inversely and significantly with the change in \( \text{VLDL}_1 \)-TG (\( r = -0.83, P = 0.01 \)) and \( \text{VLDL}_1 \)-apoB (\( r = -0.74, P = 0.04 \)) production rates, explaining 69% and
55% of the variance in change in these responses, respectively (\textbf{Figure 4.4B}).

\( \text{HOMA}_{\text{IR}} \) did not correlate significantly with the change in \( \text{VLDL}_1 \)-TG and -apoB
production rates. However it correlated significantly with the change in Intralipid-
TG clearance rates ($r = 0.72, P = 0.04$, Figure 4.4C), explaining 52% of the variance of the change in response. This indicates that subjects who had the lowest HOMA\textsubscript{IR} (i.e. the more insulin sensitive individuals) had the biggest increase in Intralipid-TG clearance rate in response to hyperinsulinaemia.

There was no apparent effect of apoE phenotype on the change in VLDL\textsubscript{1}-TG and VLDL\textsubscript{1}-apoB production rates or Intralipid-TG clearance rate (Figure 4.3).
Figure 4.3: Individual (according to apoE phenotype) and mean (bars) [A] VLDL\(_1\)-TG and [B] VLDL\(_1\)-apoB production rates and [C] Intralipid-TG clearance rate in the Control and Glucose trials (n = 8). (#) \( P = 0.05 \), (*) \( P < 0.05 \).
4. Effects of Hyperinsulinaemia & Hyperglycaemia on VLDL₁ Production Rates

Figure 4.4: Scatterplots (with linear-regression lines of ‘best-fit’) illustrating the relationships between the change (Control minus Glucose) in [A] NEFA concentrations at 0 min (just before the start of the Intralipid infusion) and the change in VLDL₁-TG (left) and VLDL₁-apoB production rates (right); between [B] serum ALT concentrations and the change in VLDL₁-TG (left) and VLDL₁-apoB production rates (right); and [C] HOMA IR and the change in Intralipid-TG clearance rate. N = 8, r and P values are for Pearson product–moment correlations between variables.
4. Effects of Hyperinsulinaemia & Hyperglycaemia on VLDL\textsubscript{1} Production Rates

4.4 Discussion

This study aimed to investigate the acute effects of hyperinsulinaemia and hyperglycaemia on the production rates of both VLDL\textsubscript{1}-TG and -apoB and Intralipid-TG clearance rate in healthy normoglycaemic subjects. Firstly, the present results indicate that insulin acutely suppressed VLDL\textsubscript{1}-TG and -apoB production rates by 25\% and 21\%, respectively. However, as there was no significant difference in the size of the VLDL\textsubscript{1} particle being produced (i.e. TG/apoB ratio), this suggests equal suppression of VLDL\textsubscript{1}-TG and -apoB production. This is consistent with the recent findings of Adiels \textit{et al.} (Adiels \textit{et al.}, 2007), who showed that insulin acutely induced a 60\% suppression in both VLDL\textsubscript{1}-TG and VLDL\textsubscript{1}-apoB production rates in normal, non-diabetic subjects. The work of Adiels \textit{et al.} was concomitant with the present study and, to-date, these are the only two studies that specifically investigated the effect of hyperinsulinaemia on VLDL\textsubscript{1}-TG and VLDL\textsubscript{1}-apoB production rates.

The suppression in VLDL\textsubscript{1} production in response to insulin in the present study (at 21-25\%) was less than the 50-60\% reduction reported in VLDL\textsubscript{1}-TG (Adiels \textit{et al.}, 2007) and VLDL\textsubscript{1}-apoB (Malmstrom \textit{et al.}, 1997b; Malmstrom \textit{et al.}, 1998) production rates. This could be due to the time of exposure to acute hyperinsulinaemia: in these studies, exogenous insulin was administered for 5-8.5 h, whereas in the present study, VLDL\textsubscript{1} production rates were determined during the first 135 min of exposure to hyperinsulinaemia. This is supported by Malmstrom \textit{et al.} (Malmstrom \textit{et al.}, 1997b), who reported a significant detectable reduction in VLDL\textsubscript{1}-apoB synthesis after 0.5 h of insulin infusion at an average of \~9\% per h (range 0\% – 24\%).

Hepatic assembly of VLDL is a complex process (see \textit{section 1.3.2}). Briefly, it starts in the rough ER with the co-translational lipidation of apoB-100 forming a ‘lipid pocket’ and the subsequent interaction with MTP, which catalyses the transfer of TG to the continually synthesised apoB-100 and allowing apoB to fold on a core of neutral lipids. This gives rise to a partially lipidated primordial particle (pre-VLDL), which is retained in the cell by interaction with chaperones and other ER proteins until it is further lipidated to form VLDL\textsubscript{2} or sorted to degradation. If apoB fails to be lipidated and misfolds, it is unfolded, retracted to the cytosol, ubiquinated, and
VLDL\textsubscript{2} is converted to VLDL\textsubscript{1} in the Golgi apparatus by the uptake of a defined lipid load from a fairly rapidly mobilised cytosolic TG pool (Gibbons et al., 2000). Insulin controls a number of enzymatic steps in the assembly and secretion of VLDL by the hepatocyte (Gibbons et al., 2002; Sparks & Sparks, 1994). It downregulates MTP gene expression (Lin et al., 1995), enhances apoB degradation (Sparks & Sparks, 1990), inhibits the secretion of newly synthesised apoB as well as inhibiting VLDL maturation by suppressing transfer of cytosolic TG to pre-VLDL particles (Brown & Gibbons, 2001). This is likely to explain the reduction in VLDL\textsubscript{1}-TG and -apoB production rates observed in the present study. In addition, insulin decreases NEFA flux to the liver, likely due to inhibition of hormone-sensitive lipase (Lewis et al., 1993) and adipose tissue triglyceride lipase (Kershaw et al., 2006). This consequently decreases substrate availability for VLDL assembly (Coppack et al., 1994), as the influx of NEFA from adipose tissue to the liver normally accounts for 60-80\% of hepatic TG (Donnelly et al., 2005). Indeed, in this study, a rapid decrease in plasma NEFA concentrations occurred in the first hour, shortly after induction of hyperinsulinaemia in response to glucose ingestion, which correlated significantly and positively with the suppression in VLDL\textsubscript{1}-TG and -apoB production in response to insulin: subjects who had the biggest reduction in NEFA concentrations, had the biggest suppression in VLDL\textsubscript{1}-TG and -apoB production. It should be noted that although NEFA concentrations were significantly lower throughout the Glucose trial compared to the Control trial in response to insulin, NEFA concentrations increased during Intralipid infusion in both trials due to the ‘spill-over’ from Intralipid produced by LPL-mediated TG hydrolysis taking place directly into the circulation (Evans et al., 1999). Therefore, NEFA concentrations during the infusion were not included in the correlations with changes in VLDL\textsubscript{1} production rates as they do not reflect the ‘true’ insulin-mediated suppression of NEFA.

Secondly, in the present study, hyperinsulinaemia did not significantly influence Intralipid-TG clearance rate. This is in contrast to the findings of Preiss-Landl et al. who reported that insulin upregulates LPL activity in adipose tissue (Preiss-Landl et al., 2002). One likely explanation could be due to the heterogeneity of subjects’ insulin resistance in the present study as there was a significant correlation between HOMA\textsubscript{IR} and the change in Intralipid-TG clearance rate. While glucose ingestion
caused an upregulation in clearance in the more insulin sensitive subjects, it induced a paradoxical downregulation of TG clearance in the more insulin resistant individuals. Indeed, it was previously reported that the more resistant an individual is to insulin-mediated glucose uptake, the lower will be the plasma post-heparin LPL activity and adipose tissue LPL mRNA levels (Maheux et al., 1997).

Finally, the author investigated whether serum ALT concentrations, a marker for liver function and hepatic fat accumulation (Westerbacka et al., 2004), would also predict the change in VLDL₁ production in response to hyperinsulinaemia. The present results demonstrate that serum ALT is a strong predictor of the suppression in VLDL₁-TG and -apoB production, accounting for 69% and 55% of the variance in the change in production rates, respectively. This finding was recently supported by Adiels et al. (Adiels et al., 2007) who showed that individuals with high liver fat failed to suppress VLDL₁ production in response to insulin. Whether this reduced suppression of VLDL₁ production by insulin is a result of liver fat, a consequence of hepatic insulin resistance or both remains to be uncertain (Adiels et al., 2007). Similarly, it is unclear, in the present study, whether ALT (i.e. liver function) per se is the mediator of the change in VLDL₁ production or whether ALT is just a marker for liver fat.

In summary, this study demonstrated that, in normoglycaemic apparently healthy individuals, (1) insulin suppressed VLDL₁-TG and -apoB production; and (2) the more insulin sensitive the subject is, the bigger the increase in Intralipid-TG clearance rates due to hyperinsulinaemia and hyperglycaemia. In addition, ALT concentration – a marker of liver function and associated with liver fat content – strongly predicted individuals’ ability to suppress VLDL₁-TG and -apoB in response to hyperinsulinaemia. Not only do these findings help the basic scientific understanding of the effect of insulin on VLDL₁ production, they may also help identifying individuals at higher risk of developing atherogenic dyslipidaemia which is believed to be initiated by insulin resistance, liver fat and hepatic overproduction of VLDL₁ particles.
5. Effects of Moderate Exercise on VLDL₁ Kinetics in Overweight/Obese Middle-Aged Men

5.1 Introduction

CVD is the main cause of death in the UK, with CHD, being the most common form, accounting for about 101,000 deaths each year (Allender et al., 2007). Elevated plasma concentrations of TG are considered an independent risk factor for CHD (Cullen, 2000). High concentrations of large very low density lipoprotein (VLDL₁, S_f 60-400) are the major determinant of plasma TG levels (Tan et al., 1995; Hiukka et al., 2005) and believed to initiate a chain of reactions that generate the atherogenic lipoprotein phenotype associated with insulin resistance conditions (e.g. obesity, type 2 diabetes and the metabolic syndrome) (Bloomgarden, 2007; Taskinen, 2003; Ginsberg et al., 2005). Thus, reducing VLDL₁ concentration is likely to induce clinically important changes to the atherosclerotic risk profile.

While patient groups often undergo pharmacological treatments for these lipoprotein disorders, obesity and insulin resistance are growing problems in the population: over half of UK adults are overweight (BMI > 25 kg.m⁻²) and over 20% are obese (BMI > 30 kg.m⁻²) (Allender et al., 2006) and estimated prevalence of the metabolic syndrome in Scottish middle-aged men is ~25% (Sattar et al., 2003). Therefore, it is unfeasible and probably undesirable to subject large sections of the population to long-term pharmacological therapies. Moderate exercise is one potential non-pharmacological therapy for elevated VLDL₁ concentrations. There is clear evidence that exercise of this nature can lower fasting and postprandial TG concentrations by 20-25%, mostly in the VLDL₁ fraction (Gill et al., 2006), in population groups at increased risk of cardiovascular disease, such as centrally obese middle-aged men (Gill et al., 2004b) and postmenopausal women (Gill & Hardman, 2000). This effect is seen following a single exercise session, so is not mediated by weight loss (Gill et al., 2004b; Gill & Hardman, 2000; Gill et al., 2006). However the mechanism(s)
responsible for this TG-reduction are unclear and require further elucidation as it could reflect reduced hepatic VLDL$_1$ production and/or increased LPL-mediated VLDL$_1$ clearance. Interestingly, a prior session of moderate exercise induces a larger TG-reduction in the VLDL$_1$ fraction than the chylomicrons fraction (Gill et al., 2001a; Gill et al., 2006). Because chylomicrons are preferred substrates to LPL above VLDL$_1$ (Karpe et al., 1993a; Fisher et al., 1995), this reduction in VLDL$_1$ was hypothesised to be the result of an exercise-induced suppression of hepatic VLDL$_1$ production rather than increased clearance. However, two recent stable-isotope kinetic studies have demonstrated that, in a group of lean recreationally active young men, a single session of a 90-120 min of moderate intensity exercise (at 60% of V O$_{2\text{max}}$) resulted in an increased clearance of total VLDL-TG (Magkos et al., 2006; Tsekouras et al., 2007) and a significant decrease in hepatic VLDL-apoB production (Magkos et al., 2006) with no significant change in hepatic VLDL-TG production (Magkos et al., 2006; Tsekouras et al., 2007). However, studies investigating the effects of moderate exercise on large VLDL$_1$ kinetics, especially in overweight/obese middle-aged men, a typical population at which exercise-for-health guidelines are targeted, are lacking. This is important as moderate exercise may have a considerable potential to be used as a first-line therapeutic option for preventing and treating the primary defect in obesity/insulin resistance-related dyslipidaemia.

Kinetics of VLDL are usually determined using stable-, or radio-, isotopic tracer methods, but these techniques are expensive and time-consuming which limits the scope of studies to which these methods can be applied. Earlier in this thesis (Chapter 3) the development of a relatively easy method to determine VLDL$_1$-TG and -apoB production and clearance rates, as well as Intralipid-TG clearance rate (as a surrogate measure of chylomicron-TG clearance) was described (Al-Shayji et al., 2007), which is used in the present study to elucidate the mechanisms by which moderate exercise induces TG lowering.

The aim of the present study was to investigate the effects of a 120-min session of prior moderate exercise on VLDL$_1$-TG and -apoB kinetics and Intralipid-TG clearance rate in a group of overweight/obese middle-aged adults using the Intralipid method.
5. Effects of Moderate Exercise on VLDL1 Kinetics

5.2 Materials and Methods

5.2.1 Subjects

Twelve overweight/obese, middle-aged men participated in this study; their characteristics are shown in Table 5.1. All subjects were apparently healthy, normotensive, normoglycaemic, non-smokers who displayed no symptoms of coronary heart disease during a clinical exercise stress test (section 2.5). None was taking any drugs known to affect lipid or carbohydrate metabolism. The subject information sheet, consent form and health questionnaire are shown in Appendices A2 & B2. One subject had E3/2 apoE phenotype, 4 had E3/3 phenotype, 5 had E4/3 phenotype and 2 had E4/4 phenotype. The study was conducted with the approval of North Glasgow University Hospitals NHS Trust Ethics Committee and subjects gave written informed consent prior to participation.

Table 5.1: Characteristics of the participants (n=12).

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<th>Characteristic</th>
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<tbody>
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<td>Age (years)</td>
<td>44 ± 8.4</td>
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<tr>
<td>Body Mass (kg)</td>
<td>95.0 ± 17.1</td>
</tr>
<tr>
<td>Body mass index (kg.m⁻²)</td>
<td>31.3 ± 5.1</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>106.9 ± 13.2</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>0.98 ± 0.05</td>
</tr>
<tr>
<td>Maximal oxygen uptake (ml.kg⁻¹.min⁻¹)</td>
<td>37.9 ± 7.2</td>
</tr>
<tr>
<td>ALT (U.l⁻¹)</td>
<td>39 ± 10</td>
</tr>
</tbody>
</table>

5.2.2 Study Design

Each subject underwent two Intralipid infusions (see below) within an interval of 2 weeks. Preconditions (i.e. control and exercise) were different and administered in random order. On the afternoon prior to one Intralipid infusion subjects walked 120 min on a treadmill at an intensity of ~50% \( \dot{V}O_{2\text{max}} \) (Exercise trial). In the other trial, subjects performed no exercise on the day preceding the other Intralipid infusion (Control trial). They were asked to weigh and record their dietary intake for two days prior to their first Intralipid infusion and replicate this prior to the second test and were instructed to refrain from alcohol consumption on those two days. No
exercise other than the treadmill walk in the Exercise trial was performed during the three days prior to each Intralipid infusion.

5.2.3 Treadmill Walk

A preliminary submaximal incremental treadmill test was performed as previously described in section 2.6 at least 1 week prior to the first Intralipid test to estimate $\dot{V}O_2_{max}$ and determine the speed and gradient required to elicit 50% $\dot{V}O_2_{max}$. The 120-min walk in the Exercise trial was completed ~16-18 h prior to the Intralipid test. Heart rate, $O_2$ uptake and $CO_2$ production were measured as previously described in section 2.8. Ratings of perceived exertion (Borg, 1973) were obtained at 15-min intervals during the walk.

5.2.4 Intralipid Infusion

$VLDL_1$ kinetics were determined using the 'Intralipid Method' as previously described in Chapter 3 (Al-Shayji et al., 2007) with a slight modification in the frequency of blood sampling during infusion. Briefly, subjects reported to the Clinical Investigation Suite in the Department of Vascular Biochemistry at Glasgow Royal Infirmary in the morning after an overnight fast of 12 h and they were given a bolus dose of 0.1 g.kg$^{-1}$ of 20% Intralipid followed immediately by an intravenous infusion of 0.1 g.kg$^{-1}$.h$^{-1}$ of 10% Intralipid for 75 min. Multiple EDTA blood samples were drawn at baseline, before and at 10-min intervals during infusion as well as at 2.5, 5, 10, 15, 20, 30, 40 and 60 min post-infusion. The post-infusion period was reduced from 75 to 60 min when it became clear that 60 min was sufficient to calculate the Intralipid-TG clearance rate.

5.2.5 Analytical Methods

Plasma glucose, insulin, total and HDL cholesterol and apoC-II, apoC-III and E concentrations were analysed in the fasted state. TG and NEFA concentrations were analysed in all time points. LDL cholesterol was calculated using the Friedewald equation (Friedewald et al., 1972). Serum ALT concentrations were measured at screening. ApoE phenotype was determined for each subject by isoelectric focusing.
5. Effects of Moderate Exercise on VLDL₁ Kinetics

using Western blot techniques. All analytical methods were performed as previously described in sections 2.4.2 to 2.4.4.

Intralipid (Sₘ >400) was separated from whole plasma as previously described in section 3.2.4 with a minor modification. In the present subjects (typical candidates for hyperlipidaemia), it was found that the Intralipid fraction was technically easier to separate at 23° C instead of 4° C. The VLDL₁ (Sₘ 60-400) fraction was separated as previously described in section 2.4.1. TG concentrations were measured in the Intralipid and VLDL₁ fractions in all time points and were corrected for glycerol as described in section 3.2.7 (Al-Shayji et al., 2007).

The composition of the VLDL₁ fraction (i.e. TG, cholesterol, FC and PL) was measured as described in section 2.4.2. Total protein content was measured manually using a modified Lowry assay (section 2.4.5). CE concentration was determined by multiplying the difference between total cholesterol and free cholesterol concentrations (in mg.dl⁻¹) by 1.68 to account for the difference in mass between cholesterol and CE. VLDL₁ concentration was calculated by summing the concentrations of these components in mg.dl⁻¹ (i.e. TG, FC, CE, PL and protein). ApoB concentrations were measured directly in the VLDL₁ fraction by automated immunoturbidimetry (see section 2.9.2.3). In four subjects, where VLDL₁-apoB was not detectable by the immunoturbidimetry method, apoB concentrations were measured manually, as described in section 2.9.2.2 by precipitation with Isopropanol and subsequent protein measurement by Lowry method (section 2.4.5).

5.2.6 Calculations and Statistics

VLDL₁-TG and -apoB production rates and FCRs, as well as Intralipid-TG clearance rates were determined as previously described in Chapter 3 (Al-Shayji et al., 2007).

Net energy expenditure during the 120-min walk was calculated using indirect calorimetry assuming no protein oxidation (Frayn, 1983). HOMA was used as a validated surrogate measure of insulin resistance (IR) (Matthews et al., 1985).

The number of apoC-II, apoC-III and apoE molecules per VLDL₁ particle were calculated, in the fasted state, by dividing the apolipoprotein concentrations in
mmol.l\(^{-1}\) by the apoB concentration in mmol.l\(^{-1}\). In addition, VLDL\(_1\) TG/apoB ratio (mol:mol), representing the size of the particle in the circulation; CE/TG ratio (mol:mol), representing the composition of its core; CE/apoB ratio (mol:mol), representing the CE content per VLDL\(_1\) particle; and FC/PL ratio (mg:mg), representing the composition of its surface layer, were also calculated in the fasted state.

Statistical analyses were performed using MINITAB for Windows (Version 14.0, MINITAB Inc., State College, PA) and STATISTICA (Release 6.0, StatSoft, Inc, USA). Normality was checked for all the data using the Anderson-Darling test. When data did not approximate a normal distribution, these were log-transformed, namely insulin, HOMA\(_{IR}\), VLDL\(_1\)-apoB fasting concentrations, TG/apoB ratios, FC/PL ratio, Intralipid-TG clearance rate, VLDL\(_1\)-apoB FCR and production rate, VLDL\(_1\)-TG production rate, and the exercise-induced change in VLDL\(_1\)-apoB FCR and production rate required transformation. Comparisons of fasting values, summary responses and kinetic data between Control and Exercise trials were made using paired \(t\)-tests and changes over the trial period were assessed by two-way ANOVA (trial × time) with repeated measures on both factors. Post hoc Fisher LSD tests were used to identify exactly where any differences lay. Significance was accepted at the \(P < 0.05\) level. Data are presented as mean ± SEM unless otherwise stated.

To determine factors which predicted the change in VLDL\(_1\)-TG and -apoB production and clearance rates as well as Intralipid-TG clearance rates between Control and Exercise trials, Pearson product-moment correlations were performed between the exercise-induced changes in kinetic variables on one hand and ALT, the exercise-induced changes in glucose, insulin, HOMA\(_{IR}\) and NEFA on the other hand.

### 5.2.7 Power Calculations

The pilot data from 5 subjects who underwent two Intralipid infusions at the 0.1g.kg\(^{-1}\).h\(^{-1}\) Intralipid and 0.2 g.kg\(^{-1}\).h\(^{-1}\) Intralipid doses (described in Chapter 3), indicate that the standard deviation for the within-subject variability in VLDL\(_1\)-apoB and VLDL\(_1\)-TG production are 12.7% and 20.1%, respectively, when preceding diet, exercise and alcohol intake are well-controlled. Based on these data, 12 subjects
would enable detection of an 11% difference in VLDL<sub>1</sub>-apoB production and an 18% difference in VLDL<sub>1</sub>-TG production, with 80% power at the 0.05 level. Groups of 8-12 subjects clearly show significant effects of a single moderate exercise session on postprandial TG metabolism where a 20-25% reduction in plasma TG concentration is typically observed (Gill & Hardman, 2003).

5.3 Results

5.3.1 Treadmill Walk

Subjects walked at a speed of 4.5 ± 0.2 km.h<sup>-1</sup> up a gradient of 6.0 ± 0.6%. All subjects completed the 120-min walk without difficulty, with an average perceived exertion of 12.2 ± 0.4 (between ‘fairly light’ and ‘somewhat hard’) on the Borg scale of 6-20 (Borg, 1973). Mean ŔO<sub>2</sub> was 18.6 ± 0.8 ml.kg<sup>-1</sup>.min<sup>-1</sup> (49.2 ± 0.7% ŔO<sub>2</sub>max), mean heart rate was 123 ± 3 beat.min<sup>-1</sup> and net energy expenditure for the walk was 3.5 ± 0.1 MJ (837 ± 35 kcal).

5.3.2 Fasting Concentrations in the Control and Exercise Trials

Mean plasma and VLDL<sub>1</sub> composition and concentrations in the fasted state are shown in Table 5.2. In four subjects, VLDL<sub>1</sub>-apoB was not detectable directly using the immunoturbidimetry method in at least one trial, and, therefore, was measured manually for both the Control and Exercise trials. In addition, fasting apoE concentrations were below limits of detection in 2 subjects in one or two trials, and, therefore, these two subjects were omitted from all apoE calculations. Exercise significantly (P < 0.05) reduced fasting plasma TG and glucose concentrations by 21.9 ± 6.0% and 3.0 ± 1.0%, respectively, and increased NEFA by 23.8 ± 12.1%. There was a reduction of 12.6 ± 6.7% in insulin concentrations and of 14.8 ± 6.9% in HOMAIR post-exercise, but these did not reach statistical significance (P = 0.080 and 0.059, respectively). Fasting VLDL<sub>1</sub> concentrations were significantly lower by 32.6 ± 9.7% after exercise (P = 0.006), as were fasting concentrations of VLDL<sub>1</sub>-TG (29.6 ± 9.3%), VLDL<sub>1</sub>-Cholesterol (43.7 ± 11.4%), VLDL<sub>1</sub>-apoB (38.2 ± 9.8%), VLDL<sub>1</sub>-apoC-II (28.0 ± 10.7%), VLDL<sub>1</sub>-apoC-III (25.0 ± 12.1%) and VLDL<sub>1</sub>-apoE (27.6 ± 11.4%, n = 10), all P <0.05. There were no significant differences in fasting concentrations of total, HDL- and LDL-cholesterol (Table 5.2).
5. Effects of Moderate Exercise on VLDL₁ Kinetics

5.3.3 TRL Kinetics in Responses to Exercise

Figure 5.1 shows the mean percentage changes in VLDL₁-TG and -apoB kinetics and Intralipid-TG clearance rate and Figure 5.2 shows the absolute mean and individual production and clearance rates in response to exercise according to apoE phenotypes. There was a significant increase after exercise in Intralipid-TG clearance rate (Control: 47.6 ± 6.8, Exercise: 68.1 ± 9.7 pools.d⁻¹, \( P < 0.001 \)), VLDL₁-TG FCR (Control: 16.0 ± 2.1, Exercise: 29.1 ± 4.4 pools.d⁻¹, \( P = 0.002 \)) and VLDL₁-apoB FCR (Control: 10.4 ± 2.0, Exercise: 25.6 ± 5.1 pools.d⁻¹, \( P = 0.015 \)). However, there was no significant difference between the Control and Exercise trials in the production rates of VLDL₁-TG (Control: 1271.5 ± 155.7, Exercise: 1431.7 ± 148.3 mg.h⁻¹, \( P = 0.104 \)) and VLDL₁-apoB (Control: 37.2 ± 7.4, Exercise: 41.5 ± 5.4 mg.h⁻¹, \( P = 0.335 \)).

5.3.4 VLDL₁ Compositional Responses to Exercise

Table 5.3 shows the exercise-induced compositional changes in VLDL₁ in the fasted state – reflecting the composition of circulating VLDL₁ particles. There was a tendency for a bigger VLDL₁ particle (TG/apoB ratio) in the circulation in the Exercise trial (Control: 15392 ± 2178, Exercise: 19449 ± 3150). However, this did not reach statistical significance (\( P = 0.059 \)). On the other hand, the CE/TG ratio, reflecting the neutral lipid composition of the VLDL₁ core, was significantly lower in the Exercise trial (Control: 0.17 ± 0.02, Exercise: 0.12 ± 0.02, \( P = 0.007 \)). Consequently, the CE/apoB ratio, reflecting CE content per VLDL₁ particle, was not significantly different between both trials (Control: 2487 ± 353, Exercise: 2276 ± 564, \( P = 0.537 \)). PL was not detectable for one subject and, consequently, the FC/PL ratio in both trials for this subject were not included. There was no significant difference in the FC/PL ratio, which reflects the composition of the VLDL₁ surface layer, between the Control (0.32 ± 0.01) and Exercise (0.32 ± 0.02) trials (\( P = 0.606 \), n = 11).

Moreover, there was no significant difference in the fasting ratios of apoC-II/apoB (Control: 32.6 ± 5.0, Exercise: 42.8 ± 8.6, \( P = 0.23 \)), apoC-III/apoB (Control: 64.1 ± 7.9, Exercise: 83.8 ± 13.9, \( P = 0.10 \)) or apoE/apoB (Control: 0.83 ± 0.20, Exercise: 1.12 ± 0.33, \( P = 0.409 \), n = 10).
Table 5.2: Plasma and VLDL₁ concentrations in the fasted state

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exercise</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma TG (mmol.l⁻¹)</td>
<td>1.54 ± 0.16</td>
<td>1.21 ± 0.15</td>
<td>0.004</td>
</tr>
<tr>
<td>Total Cholesterol (mmol.l⁻¹)</td>
<td>5.17 ± 0.25</td>
<td>5.14 ± 0.24</td>
<td>0.843</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol.l⁻¹)</td>
<td>1.05 ± 0.06</td>
<td>1.08 ± 0.07</td>
<td>0.233</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol.l⁻¹)</td>
<td>3.38 ± 0.31</td>
<td>3.48 ± 0.29</td>
<td>0.409</td>
</tr>
<tr>
<td>Glucose (mmol.l⁻¹)</td>
<td>5.76 ± 0.09</td>
<td>5.58 ± 0.09</td>
<td>0.010</td>
</tr>
<tr>
<td>Insulin (mU.l⁻¹)</td>
<td>6.99 ± 1.30</td>
<td>6.29 ± 1.46</td>
<td>0.080</td>
</tr>
<tr>
<td>HOMAIR</td>
<td>1.81 ± 0.35</td>
<td>1.58 ± 0.37</td>
<td>0.059</td>
</tr>
<tr>
<td>NEFA (mmol.l⁻¹)</td>
<td>0.55 ± 0.04</td>
<td>0.64 ± 0.03</td>
<td>0.026</td>
</tr>
<tr>
<td>VLDL₁ concentration (mg.dl⁻¹)</td>
<td>94.9 ± 14.1</td>
<td>62.9 ± 11.4</td>
<td>0.006</td>
</tr>
<tr>
<td>VLDL₁-TG (mmol.l⁻¹)</td>
<td>0.67 ± 0.10</td>
<td>0.46 ± 0.08</td>
<td>0.007</td>
</tr>
<tr>
<td>VLDL₁-C (mmol.l⁻¹)</td>
<td>0.47 ± 0.07</td>
<td>0.28 ± 0.06</td>
<td>0.006</td>
</tr>
<tr>
<td>VLDL₁-apo B (mg.dl⁻¹)</td>
<td>2.77 ± 0.44</td>
<td>1.55 ± 0.30</td>
<td>0.004</td>
</tr>
<tr>
<td>VLDL₁-apo CII (mg.dl⁻¹)</td>
<td>1.22 ± 0.18</td>
<td>0.88 ± 0.16</td>
<td>0.017</td>
</tr>
<tr>
<td>VLDL₁-apo CIII (mg.dl⁻¹)</td>
<td>2.52 ± 0.40</td>
<td>1.84 ± 0.37</td>
<td>0.031</td>
</tr>
<tr>
<td>VLDL₁-apo E (mg.dl⁻¹)*</td>
<td>0.16 ± 0.06</td>
<td>0.11 ± 0.05</td>
<td>0.049</td>
</tr>
</tbody>
</table>

* n = 10. All values are mean ± SEM. P values are for the difference between the Control and Exercise trials.

Figure 5.1: The mean percentage change in VLDL₁-TG and VLDL₁-apoB production rates (PR) and fractional catabolic rates (FCR) and Intralipid (IL)-TG clearance rate (CR) in response to a single session of 120-min brisk walking at ~50% VO₂max. (* P <0.05, ** P <0.001).
Figure 5.2: Individual (according to apoE phenotype) and mean (bars) VLDL$_1$-TG and VLDL$_1$-apoB production rates (top panel) and fractional catabolic rates (FCR, middle panel) and Intralipid-TG clearance rates (bottom) in response to a single session of 120-min brisk walking at ~50% VO$_{2\text{max}}$ (* $P < 0.05$, ** $P < 0.001$ for the difference in mean response between the Control and Exercise trials).
Table 5.3: Exercise-induced compositional changes to circulating VLDL₁ particles

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exercise</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG/apoB ratio (mol:mol)</td>
<td>15392 ± 2178</td>
<td>19449 ± 3150</td>
<td>0.059</td>
</tr>
<tr>
<td>CE/TG ratio (mol:mol)</td>
<td>0.17 ± 0.02</td>
<td>0.12 ± 0.02</td>
<td>0.007</td>
</tr>
<tr>
<td>CE/apoB ratio (mol:mol)</td>
<td>2487 ± 353</td>
<td>2276 ± 564</td>
<td>0.537</td>
</tr>
<tr>
<td>FC/PL ratio (mg:mg)*</td>
<td>0.32 ± 0.01</td>
<td>0.32 ± 0.02</td>
<td>0.606</td>
</tr>
</tbody>
</table>

* n = 11. All values are mean ± SEM.

5.3.5 Predictors of the Exercise-Induced Changes in TRL Kinetics

There was a significant correlation ($r = 0.61$, $P = 0.035$) between the exercise-induced change in Intralipid-TG clearance rate and the exercise-induced change in VLDL₁-TG FCR (Figure 5.3A), which suggests that a single mechanism (likely LPL-mediated clearance) was likely to be responsible for both effects.

Although there was no statistically significant effect of exercise on fasting insulin concentrations and HOMA$_{IR}$, a significant inverse relationship was found between the exercise-induced change in VLDL₁-apoB FCR and the exercise-induced change in insulin concentrations ($r = -0.65$, $P = 0.022$), the exercise-induced change in glucose ($r = -0.58$, $P = 0.049$), and, consequently, with the exercise-induced change in HOMA$_{IR}$ ($r = -0.68$, $P = 0.016$, Figure 5.3B). This shows that those who had the biggest reduction in HOMA$_{IR}$ had the biggest increase in VLDL₁-apoB FCR.

No significant correlations were found between ALT or the exercise-induced change in NEFA and any of the exercise-induced changes in VLDL₁-TG and -apoB FCR or production rates or Intralipid-TG clearance rate (all $P > 0.05$). In addition, there was no significant effect of apoE phenotype on the response of the above kinetics to moderate exercise.
5. Effects of Moderate Exercise on VLDL₁ Kinetics

Figure 5.3: Scattergrams indicating the relationships between the exercise-induced changes (Exercise minus Control) in [A] Intralipid-TG and VLDL₁-TG FCR (pools.d⁻¹) and [B] VLDL₁-apoB FCR (pools.d⁻¹) and HOMA(IR).

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5.4 Discussion

To the best of the author’s knowledge, this is the first study to investigate the possible mechanism(s) underlying the hypotriglyceridaemic effect of a prior session of moderate exercise (120-min brisk walk at ~50% \( \dot{\text{V}}\text{O}_{2\text{max}} \)) in overweight/obese middle-aged men, a group typically targeted in exercise-for-health guidelines.

The results of the present study confirm and expand on earlier observations that a prior session of moderate exercise induces a significant reduction in plasma TG and influences VLDL\(_1\) to a greater extent than chylomicrons (Gill et al., 2006). Indeed, in the present study, the change in VLDL\(_1\)-TG clearance was greater than the change in Intralipid change (91.0% vs. 43.3%, respectively). Because chylomicrons are preferred substrates for LPL (Fisher et al., 1995) and, therefore, compete with VLDL\(_1\) particles for a LPL-mediated clearance (Karpe et al., 1993b), it was hypothesised earlier that the exercise-induced TG reduction was likely to be the result of a reduced hepatic VLDL\(_1\) production rather than increased clearance (Malkova & Gill, 2006). However, this does not seem to be the case. The present results demonstrate that this exercise-induced reduction in TG is due to a significant 91% increase in VLDL\(_1\)-TG FCR (i.e. clearance), with no significant effect on VLDL\(_1\)-TG production rate. This is consistent with two recent stable-isotope studies by Magkos et al. (Magkos et al., 2006) and Tsekouras et al. (Tsekouras et al., 2007) who reported an increased total VLDL-TG clearance, rather than decreased VLDL-TG production following a session of 120-min cycling and 90-min walking (at 60% \( \dot{\text{V}}\text{O}_{2\text{max}} \)), respectively.

The novelty of this study, however, is that it allowed the simultaneous measurements of VLDL\(_1\)-TG FCR and Intralipid-TG clearance rate in response to a prior session of moderate exercise. This is important in providing a better understanding of the major possible mechanism(s) involved in the exercise-induced hypotriglyceridaemia as it was difficult to measure the clearance of VLDL\(_1\)-TG in the presence of chylomicrons/Intralipid because of their competition for the same catabolic pathway (Bjorkegren et al., 1996; Karpe & Hultin, 1995). Importantly, the present results show a significant correlation \((r = 0.61, P = 0.035)\) between the exercise-induced
changes in Intralipid-TG and in VLDL\textsubscript{1}-TG indicating that exercise is upregulating their clearance by the same mechanism/pathway (i.e. LPL). However, it is unclear why VLDL\textsubscript{1} appears to develop a higher affinity following exercise for clearance by LPL relative to Intralipid, but this could be due to the compositional changes in the VLDL\textsubscript{1} particle post-exercise: the particles in the circulation tended to be \textasciitilde 26\% bigger following exercise and more TG-enriched, which may render them more favourable substrates for LPL-mediated lipolysis (Fisher \textit{et al.}, 1995). There are two possible explanations as to why this might be the case. Firstly, the liver might be producing a bigger, more TG-enriched VLDL\textsubscript{1} particle following exercise. Consistant with this, circulating NEFA concentrations were 16\% higher in the exercise trial than the Control trial, increasing fatty acid availability to the liver, which could lead to larger secreted VLDL\textsubscript{1} particle. Secondly, under normal conditions, once in the circulation, the TG content of the newly secreted VLDL\textsubscript{1} particle, and consequently its size, are being reduced rapidly by hydrolysis by LPL (Karpe \textit{et al.}, 2007) and the action of CETP, which promotes the transfer of TG from apoB-containing particles (VLDL and LDL) in exchange for CE from HDL (Barter, 2002). The present results show a significant \textasciitilde 29\% reduction in VLDL\textsubscript{1} CE/TG ratio in the Exercise trial, which is supported by a similar 18\% post-exercise reduction in CE/TG ratio reported by Gill \textit{et al} (Gill \textit{et al.}, 2006). This may be suggestive of a reduced \textit{in vivo} CETP activity following moderate exercise, rendering the VLDL\textsubscript{1} particle more TG-enriched. However, this explanation is less likely to occur as the CE/apoB ratio, representing the CE content per VLDL\textsubscript{1} particle, in the present study was not significantly different between the two trials (Control: 2487 \textpm 353, Exercise: 2276 \textpm 564, \textit{P} = 0.537). Unfortunately, conclusive results about the effect of moderate exercise on CETP activity or mass are currently lacking in the literature. While some studies found an increase in CETP mass following exercise (Thomas \textit{et al.}, 2001), others reported an exercise-induced decrease in CETP mass (Magkos \textit{et al.}, 2006). Two other studies found no significant difference in CETP activity post-moderate exercise (Thomas \textit{et al.}, 2004) even after 24 h (Zhang \textit{et al.}, 2002).

Furthermore, although there was no significant difference in the number of apos C-II, C-III and E per VLDL\textsubscript{1} between the Exercise and Control trials in this study, a reduced apoC-III and apoE enrichment of VLDL\textsubscript{1} was reported in a similar group of
participants following moderate exercise (Gill et al., 2006). These apolipoproteins are acquired by VLDL from circulating HDL and apoC-III (Wang et al., 1985) and apo E (Jong et al., 1997) both act to inhibit lipolysis by LPL. This could conceivably have increased the potential of post-exercise VLDL1 particle as an LPL substrate. It is unclear why the present findings differ from the earlier report.

The present results also show a significant 43% increase in Intralipid-TG clearance rate post-exercise, which is likely to reflect increased LPL activity and/or mass. Although early studies of prolonged and vigorous exercise, such as marathon running, clearly demonstrated that this type of exercise increased LPL-mediated TG-clearance (Sady et al., 1986), recent studies of a more moderate exercise are conflicting. While some studies show no significant difference in muscle (Herd et al., 2001) or post-heparin plasma LPL activity (reflecting overall LPL activity from all body tissues) ~16h after a moderate session of exercise (Gill et al., 2003), others reported a significant increase in plasma (Zhang et al., 2002), muscle and adipose tissue LPL activity post-exercise (Perreault et al., 2004). In addition, although Magkos et al. (Magkos et al., 2006) found no significant difference in muscle LPL mass, the group reported a significant ~20% increase in plasma LPL concentrations in response to acute moderate exercise. It should be noted, though, that even in the absence of a significant increase in post-heparin LPL activity, the exercise-induced changes in LPL activity was reported to correlate significantly with the exercise-induced changes in fasting and postprandial TG (Gill et al., 2003). Furthermore, if exercise increased the affinity of VLDL1 for clearance, this could have occurred without an increase in measured LPL action using a standard substrate. This further highlights the importance of LPL as a mediator of the TG-lowering effect of moderate exercise.

In addition to increased VLDL1-TG FCR, exercise significantly increased VLDL1-apoB FCR by 211% with no significant influence on VLDL1-apoB production rate. This is in contrast with Magkos et al. (Magkos et al., 2006) who reported a significant reduction in total VLDL-apoB production rate with no significant change in VLDL-apoB clearance. A likely explanation for these different findings could be due to the different characteristics of participants. Unlike the normal weight subjects who participated in Magkos et al. study (Magkos et al., 2006), the participants in the
present study were overweight/obese men with increased abdominal obesity, which makes them likely candidates for insulin resistance and high liver fat content (Karpe & Tan, 2005; Haffner, 2007; Després, 2007; Adiels et al., 2006b). Similar group of subjects with increased liver fat failed to suppress VLDL$_1$ production in response to insulin (Adiels et al., 2007). Indeed, the participants in the present study had higher insulin concentrations compared with the study subjects of Magkos et al. (Magkos et al., 2006) (7.0 vs. 5.4 mU.l$^{-1}$, respectively) and no significant reductions in insulin or HOMA$_{IR}$ concentrations were observed following exercise in the present study. Interestingly, however, there was a significant correlation in the present study between the exercise-induced change in HOMA$_{IR}$ and the exercise-induced change in VLDL$_1$-apoB FCR, indicating that subjects who had a bigger decrease in HOMA$_{IR}$ following exercise had a bigger increase in VLDL$_1$-apoB FCR (clearance). These exercise-induced changes in insulin sensitivity may augment changes in VLDL$_1$-apoB clearance, but this relationship was not observed for VLDL$_1$-TG.

In summary, a single session of moderate intensity exercise significantly reduced fasting plasma TG, mainly in the VLDL$_1$ fraction, by increasing the VLDL$_1$-TG and VLDL$_1$-apoB FCR (i.e. clearance). The increase in VLDL$_1$ was greater than the increase in Intralipid clearance, suggesting that exercise may have increased the affinity of VLDL$_1$ for LPL-mediated lipolysis (Figure 5.4). In contrast, exercise did not decrease hepatic VLDL$_1$-TG or VLDL$_1$-apoB production. However, further studies are needed elucidate the mechanism(s) underlying this increased affinity for clearance of VLDL$_1$, such as in vitro studies investigating the affinity of VLDL$_1$ for LPL as well as the change in CETP activity both under control and exercise conditions. This may help improve understanding of the nature of these exercise-induced changes.
5. Effects of Moderate Exercise on VLDL₁ Kinetics

Figure 5.4: Possible mechanisms involved in moderate exercise-induced reduction in VLDL₁.

**Control:** Once in the circulation, the TG content of the newly secreted VLDL₁ particle from the liver, and consequently its size, are being reduced rapidly by hydrolysis by lipoprotein lipase (LPL) and the action of cholesteryl ester transfer protein (CETP), which promotes the transfer of TG from VLDL (and LDL) in exchange for cholestryol ester (CE) from HDL. **Exercise:** A prior session of moderate exercise significantly reduces VLDL₁ concentration by increasing its clearance from plasma, possibly by compositional changes to the VLDL₁ particle which render it bigger in size and more TG-enriched, thereby increasing its affinity for LPL-mediated lipolysis. The bigger particles are either produced directly by the liver and/or reduced CETP activity. Also, increased LPL activity is likely to play a role.

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6. General Discussion

6.1 Summary

There is a growing body of evidence implicating elevated VLDL concentrations, particularly the larger VLDL₁ (S₁ 60-200) particles, as the primary cause of the atherogenic lipoprotein phenotype in insulin resistance conditions; such as type 2 diabetes (Taskinen, 2003), metabolic syndrome (Grundy, 2006), obesity (Aguilera et al., 2008) and, eventually, coronary heart disease (Abbasi et al., 2002; Tanaka et al., 2001). The fact that this atherogenic dyslipidaemia is detectable years before the onset of the disease (Tilly-Kiesi et al., 1996; Johanson et al., 2004) makes the measurement and regulation of hepatic VLDL₁ production of great importance. However, mere measurements of VLDL₁ concentrations provide limited information and do not reflect the dynamics of the continual synthesis and clearance of VLDL₁ particles. Radioactive and stable-isotope tracers have been successfully used to measure the kinetics of different lipoproteins, including VLDL₁. Although such methods yield a large amount of useful information, they are expensive, labour-intensive, time consuming and require the use of specialised laboratories, equipment and skills in multicompartmental modelling. Of note, these methods measure generally either total VLDL-TG and -apoB, or VLDL₁- and VLDL₂-apoB, but not VLDL₁- or VLDL₂-TG. It is only recently that Adiels and colleagues (Adiels et al., 2005a) have developed a multicompartmental stable-isotope method to determine both VLDL₁- and VLDL₂-TG and -apoB kinetics. Thus, this thesis was designed to develop and validate a simpler and cost-effective method – the ‘Intralipid Method’ – to determine VLDL₁ kinetics and use this method to study the effects of insulin and moderate exercise on the regulation of hepatic VLDL₁ kinetics.

The method builds on the work of Björkergren et al. (Bjorkegren et al., 1996) and relies on the fact that Intralipid (chylomicron-like particles) compete with VLDL₁ for the same catalytic pathway (Bjorkegren et al., 1996; Karpe & Hultin, 1995), which allows the accumulation of VLDL₁ in the circulation (Bjorkegren et al., 1996) during a constant Intralipid infusion. By measuring the linear rise in VLDL₁-TG and -apoB concentrations, it is possible to determine their production rates. In addition, because...
the method is employed in the fasted state in which there is a steady state where production equals clearance, it is also possible to determine the fractional catabolic rate (FCR, i.e. clearance) of VLDL\textsubscript{1}-TG and \textit{-apoB} by dividing their linear rises during the Intralipid infusion by their fasting concentrations. Furthermore, the Intralipid method combines the findings of Rössner (Rossner, 1974), who showed that Intralipid is cleared following first-order kinetics, which allows the determination of Intralipid-TG clearance rate as a surrogate measure of chylomicron-TG clearance.

In addition to the fact that the kinetic data obtained using the Intralipid method are similar to that obtained by the ‘gold standard’ stable-isotope method (Bjorkegren \textit{et al.}, 1996), this newly developed method has a number of strengths. First, it provides valuable information about TRL kinetics: both VLDL\textsubscript{1}-TG and VLDL\textsubscript{1}-apoB production rates \textit{and} Intralipid-TG clearance rate. In addition, under steady state conditions, it allows the simultaneous determination of large VLDL\textsubscript{1}-TG (rather than total VLDL-TG) and Intralipid-TG clearance rates. This is important for two reasons: (1) it is the high concentrations of large VLDL\textsubscript{1} particles that are implicated in atherogenic dyslipidaemia and the progression of atherosclerosis (Taskinen, 2003; Packard, 2003) and, unlike VLDL\textsubscript{2}, VLDL\textsubscript{1} are the target for insulin-induced hepatic suppression (Gill \textit{et al.}, 2004a); (2) due to the fact that Intralipid/chylomicrons and VLDL\textsubscript{1} compete for the same LPL-mediated catalytic pathway, it has previously been difficult to determine the clearance rate of VLDL\textsubscript{1} in the presence of Intralipid or chylomicrons. As a result, the Intralipid method helped to elucidate the hypotryglyceridaemic effects of exercise on TRL kinetics (Chapter 5). Second, the Intralipid method is sensitive enough to detect physiological changes in hepatic VLDL\textsubscript{1} production and Intralipid-TG clearance, such as the effect of insulin on TRL kinetics (Chapter 4). Third, the method is relatively easy and simple enough to be used in any laboratory equipped with only one ultracentrifuge without the need for any other specialised equipment (such as a mass spectrometer). Fourth, estimation of production and clearance rates using the Intralipid method is relatively easy to carry out and can be automated using Excel. Fifth, the protocol for the Intralipid method takes less time to complete than the stable-isotope method, which is beneficial both for the subject and the researcher. While a standard stable-isotope method to measure VLDL\textsubscript{1} kinetics requires ~48 h of the subject’s time, the Intralipid infusion
and post-infusion period of the present method lasts only for about 3.5 h. In addition, unlike the time required (at least 2 weeks) for laboratory techniques and sample and kinetic analyses using the stable-isotope method, the Intralipid method protocol, including lipoprotein separation, sample analysis and kinetic calculations, takes approximately 2-3 days. Sixth, the Intralipid method is cheaper than the stable-isotope method and suitable for laboratories with limited funding or resources as each trial (including investigator/technician time at £20 per hour) costs approximately £750 and 2-3 days to complete compared to the ~£2500 and about two weeks using the stable-isotope method. Finally, because the Intralipid method is safe as well as both time- and cost-effective, it can be used for a large number of subjects, with even multiple trials for each subject.

As a first application, the Intralipid method was used to study the effect of hyperinsulinaemia and hyperglycaemia on VLDL\textsubscript{1}-TG and -apoB production rates in 8 normoglycaemic subjects (Chapter 4). This was done using a simple frequent oral administration of glucose to provide a pseudo-steady hyperinsulinaemic state as the use of a conventional hyperinsulinaemic normoglycaemic clamp was not possible due to lack of constant medical cover. The result demonstrated that insulin acutely suppressed VLDL\textsubscript{1}-TG and -apoB production, a finding recently supported by Adiels et al. (Adiels et al., 2007). Although hyperinsulinaemia had no apparent effect on Intralipid-TG clearance rate, the change seemed to be related to insulin sensitivity, i.e. the more insulin sensitive the subject was, the biggest the increase in Intralipid-TG clearance rate. One possible limitation of this study is the number of subjects, especially with such a wide range of insulin resistance, which may have diluted the effect of insulin on Intralipid-TG clearance rate. However, the technique would make it very easy to increase the numbers in future studies.

Because moderate exercise has been shown to decrease plasma TG by 20-25%, mostly in the VLDL\textsubscript{1} fraction (Gill et al., 2006), the second application of the Intralipid method was to investigate whether this decrease is due to decreased hepatic VLDL\textsubscript{1} production, increased clearance or a combination of both (Chapter 5). The present results showed that a session of moderate exercise significantly increased the clearance of VLDL\textsubscript{1}-TG and -apoB rather than suppress their hepatic production. Although there is no information in the literature about the effect of exercise on
VLDL$_1$ kinetics specifically, these results are consistent with recent findings using stable-isotopes which investigated the effect of moderate exercise on total VLDL-TG (Magkos et al., 2006; Tsekouras et al., 2007) and VLDL-apoB (Magkos et al., 2006) using the stable-isotope method. In addition, because of the nature of the Intralipid method, it provided the opportunity to simultaneously study both Intralipid and VLDL$_1$ clearance, which was previously unfeasible. As a result, a novel finding is observed that this increased exercise-induced clearance of VLDL$_1$ is likely to be the result of increased affinity of VLDL$_1$ to LPL-mediated lipolysis. Furthermore, this study is the first to investigate the kinetics of such effects of exercise in overweight/obese middle-aged men, a group typically targeted in exercise-for-health guidelines.

Adequate liver function is important for VLDL$_1$ homeostasis. Serum ALT concentrations are used clinically as an indication of liver function and, within normal range, have been shown to be a marker for liver fat (Westerbacka et al., 2004). It was recently shown that hepatic overproduction of VLDL$_1$ particles is driven by liver fat accumulation (Adiels et al., 2006b). Results from this thesis show a significant relationship between serum ALT concentrations and hepatic VLDL$_1$ production and regulation. Even within normal range, ALT concentrations correlated significantly with VLDL$_1$ production (Chapter 3) and inversely with the extent to which VLDL$_1$ is suppressed in response to hyperinsulinaemia and hyperglycaemia (Chapter 4): subjects who had higher ALT concentrations, produced more VLDL$_1$ particles and failed to suppress VLDL$_1$ secretion in response to insulin. It is unclear, however, whether this correlation with ALT concentrations reflects liver function per se or hepatic fat content, as ALT is a marker for both.

In all studies described in this thesis, 3 subjects were E2/3 phenotype, 11 subjects were E3/3 phenotype, 6 subjects were E3/4 phenotype and 3 subjects were E4/4 phenotype. Previous studies showed that apoE phenotypes influence plasma lipids (Eto et al., 1986) and particularly large VLDL$_1$ concentrations (Bioletto et al., 1998) as well as VLDL-apoB kinetics (Watts et al., 2000; Welty et al., 2000; Demant et al., 1991). However, no apparent effect of apoE phenotype on VLDL$_1$-TG and -apoB production rates or Intralipid-TG clearance rate was observed in this thesis (Appendix C). It is uncertain, however, whether this is a true lack of influence of
apoE on VLDL₁-TG and -apoB production rates and Intralipid-TG clearance rate or whether it may be due to lack of sufficient numbers for each phenotype. Fortunately, because of the relative ease and cost-effectiveness of the Intralipid method, a bigger study can be conducted which may have not been feasible otherwise using the stable-isotope method.

6.2 Limitations of the Intralipid Method

There are two main limitations to the Intralipid method. The first is that it only allows the determination of VLDL₁ kinetics but not VLDL₂. This is because VLDL₂ is hydrolysed by HL; i.e. Intralipid does not compete with VLDL₂ for LPL-mediated lipolysis and, consequently, VLDL₂ will not accumulate in the circulation like VLDL₁ particles. Although VLDL₁ and VLDL₂ are regulated independently (Packard & Shepherd, 1997; Gill et al., 2004a), it has been recently reported that while insulin acutely reduced VLDL₁ secretion, it increased VLDL₂ secretion (Adiels et al., 2007). Although this acute suppression of VLDL₁ secretion by insulin was detected using the Intralipid method, it was not possible to investigate this effect on VLDL₂. Thus, the addition of VLDL₂ kinetics might have helped to complete the picture of the effect of insulin on total VLDL hepatic regulation. The second limitation of the Intralipid method is that the FCR can only be calculated if the subject is in a steady state. For example, it was not possible to determine VLDL₁-TG and VLDL₁-apoB FCRs in Chapter 4 where VLDL₁ production was investigated in response to hyperinsulinemia and hyperglycemia. This is because the steady state value used for FCR calculation is made in the fasted state before the glucose ingestion, which does not represent the steady state value for VLDL₁ concentration during glucose ingestion.

Furthermore, the lack of direct comparison with either the stable-isotope or radioactive tracer methods within the same subjects represents a limitation to the validation of the method. Although Björkegren et al. (1996) reported a close agreement between the two methods, ideally, further studies on a larger scale should be performed in the future to validate the Intralipid method against the currently used ‘gold-standard’ methods.
6.3 Future Experiments and Applications of the Method

Results from Chapter 5 suggest that moderate exercise is likely to increase VLDL₁ clearance by increasing its size and TG-enrichment, thereby increasing its affinity for LPL-mediated lipolysis. Reports from the literature with respect to the effect of moderate exercise on LPL and CETP activities, which are responsible for VLDL₁ TG-enrichment, are conflicting. Therefore, it would be helpful to conduct the following two studies. The first is an in vitro study investigating the suggested higher affinity of VLDL₁ to LPL post-exercise. This could possibly be done by incubating LPL (obtained commercially or post heparin from a donor) with different concentrations (and possibly at different durations) of the subject’s native VLDL₁ particles separated from the Control and Exercise trials. VLDL₁ lipolysis is then determined by measuring the released NEFA from VLDL₁ by LPL over time. In addition, Intralipid could also be incubated with native VLDL₁ and LPL to investigate the relative competition between Intralipid and VLDL₁ particles for LPL-mediated lipolysis in both the Control and Exercise trials.

Secondly, it is important to compare the activity of CETP in control and post-exercise as data from Chapter 5 suggest an increased TG-enrichment of VLDL₁ particles post-exercise. However, current commercial methods that measure CETP activity use the same concentration for donor and accepter particles in both exercise and control trials, which does not necessarily reflect physiological conditions as in vivo concentrations of VLDL particles differ between both conditions. Therefore, it would be better to measure CETP activity in an assay in which native VLDL₁ from the Exercise and Control trials are used as donor and accepter particles.

These two experiments would aid a better understanding of the effect of exercise on VLDL₁ and test the hypothesis that the increased clearance of VLDL₁ post-exercise is due to the increased size and TG-enrichment of the VLDL₁ particle.

Finally, previous studies have shown that the relationships between obesity, insulin resistance, metabolic syndrome and risk for cardiovascular disease are subject to ethnic variations (Joshi et al., 2007; Lear et al., 2003; Whincup et al., 2002). For example, compared with Caucasian men, South Asian men have excess subcutaneous abdominal adiposity, in spite of similar BMI, which renders them more susceptible to
insulin resistance than white men (Chandalia et al., 2007). In addition, although Afro-Caribbean men in the United Kingdom are reported to be as insulin-resistant as South Asian men, they have been found to be less susceptible to the dyslipidaemia that accompanies insulin resistance (Zoratti et al., 2000). Interestingly, Zoratti et al. (2000) reported that this favourable lipoprotein profile in Afro-Caribbean may relate to an effective VLDL metabolism. However, there is currently no information available on the effect of ethnicity on VLDL₁ kinetics. Therefore, an important application of the Intralipid method would be to investigate VLDL₁ kinetics in a different ethnic population. In addition, the method could be used to study VLDL₁ kinetics in response to moderate exercise in Kuwaiti subjects and compare that with results from those obtained in Scottish men. Kuwait (the author’s homeland) has a high prevalence of obesity (Al-Kandari, 2006), insulin resistance (Al-Shaibani et al., 2004) and type 2 diabetes (Al-Adsani et al., 2004) and ischemic heart diseases are a major cause of death in the country (El-Shazly et al., 2004).

6.4 Conclusion

In conclusion, this thesis describes the development and validation a novel method to determine the kinetics of large VLDL₁, and the application of this method to investigate the effects of hyperinsulinaemia/hyperglycaemia and moderate exercise on VLDL₁ kinetics. The method is relatively straightforward and cost-effective which means it has potential for future widespread use in situations where isotopic tracer methods are not feasible because of technical or cost limitations.
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7. References


7. References


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7. References


8. Appendices

Appendix A1: Subject information sheet and consent form – Chapters 3 and 4
Appendix A2: Subject information sheet and consent form – Chapter 5
Appendix B1: Health screen questionnaire – Chapters 3 & 4
Appendix B2: Health screen questionnaire – Chapter 5
Appendix C: Kinetic results for all subjects according to apoE phenotypes
Title: Development of novel method to determine very low density lipoprotein kinetics

Lay title: A new method of measuring the metabolism of blood fats

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.
Frequently Asked Questions

What is the purpose of the study?
The aim of this study is to develop a new method of measuring how fast your liver produces fatty particles (called lipoproteins) and how fast these particles are cleared from your bloodstream. This will help us to determine how effective different treatments are at influencing fat metabolism in future studies.

Why have I been chosen?
You have been chosen because you are a healthy adult aged between 18 and 65 years.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?
In the first instance you will be asked to attend for a screening visit in which we will discuss with you and complete confidential questionnaires regarding your health and family history, measure your blood pressure and provide an opportunity for you to ask questions about the study. We will then ask you to help us in one of two different ways:

To provide a fasting blood sample: We will ask you to come to the Clinical Investigation Suite after an overnight fast and provide a simple blood sample. We will take up to 60 ml (4 tablespoons) of blood from you. Your blood will be used to help us to develop laboratory methods for analysing the fat in your bloodstream.

OR

To help us to develop a new method for assessing the metabolism of blood fats: We will ask you to perform either one, two or three experimental trials to help us to develop a new method for assessing the metabolism of lipoproteins. Each trial will involve coming to our Clinical Investigation Suite after an overnight fast. We will then introduce two small plastic tubes called cannulas into a vein in your arms (one in each arm). This is no more painful than giving a normal blood sample. We will take blood from one cannula and use the other to pump small amounts of a fatty substance called Intralipid® into your bloodstream. Intralipid® is used in hospitals to feed people through a ‘drip’ when they are unable to eat normally. The Intralipid® we give you will cause the level of fat in your blood to rise about the same amount as it does after you eat a fatty meal and it will be pumped into your bloodstream for between one and three hours. We will take two blood samples from the cannula before giving you the Intralipid® and then take blood samples at intervals during the Intralipid® infusion and for a maximum of two hours after the Intralipid® pump is stopped. We may ask you to sip small amounts of a glucose
drink throughout the trial. We will take a maximum of 250 ml of blood during each trial – this is half the amount of blood that you give when you donate a 'pint' of blood. During the experimental trial you will only be able to drink water (or the glucose drink), but we will provide you with some food to eat when the trial is finished. The total duration of each trial will vary between 2½ and 6 hours but we will tell you specifically how long each of your own trials will take. You will be able to watch TV or videos, listen to music or read to keep yourself occupied during the experiment.

What do I have to do?
We will ask you to weigh and record everything that you eat and drink and to avoid alcohol and planned exercise for the two days before each Intralipid® test. We will provide you with scales and record sheets to enable you to record your diet easily. If you do more than one test, we will ask you to eat the same diet on the two days leading up to each test. (You will not need to do this if you are just giving us a fasting blood sample).

What are the possible disadvantages and risks of taking part?
Blood sampling via the cannula may cause minor bruising, an inflammation of the vein or haematoma (a small accumulation of blood under the skin). Good practice, however, minimises this risk. Some people may feel faint when they give blood. There is a small risk that you could have an allergic reaction to Intralipid® (less than one chance in a million). This usually only occurs in people are allergic to soya protein or eggs, so if you have these allergies you will not be able to participate in this study. In the unlikely event that you do have an allergic reaction to Intralipid®, the appropriate medicines and equipment are in place for you to be treated immediately. There is a small possibility that taking part in this study will reveal a health problem that you already have such as high cholesterol or high blood pressure. If such a problem is revealed, we will inform your GP to ensure that you receive appropriate treatment.

What are the possible benefits of taking part?
The purpose of this study is to develop a new method of determining how fast lipoprotein particles are produced by the liver and how fast they are broken down and cleared from the bloodstream. We will be able to use this method in future research to help to determine how effective different treatments are at improving the way in which the body handles fat.

What if something goes wrong?
The chances of something going wrong are extremely small. All of the procedures involved in this study are low risk and our screening tests are designed to ensure that you will only participate if it is safe for you to do so. In the unlikely event that you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you.
Will my taking part in this study be kept confidential?
All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the University or hospital will have your name and address removed so that you cannot be recognised from it.

Who has reviewed the study?
This study has been reviewed and approved by the North Glasgow NHS Trust Research Ethics Committee.

Contact for Further Information
Any questions about the procedures used in this study are encouraged. If you have any doubts or questions, please ask for further explanations by contacting either:

Iqbal AlShayji
Mobile: 07799353689  E-mail: ialshayji@yahoo.com
Tel: 0141 2114595

Dr Jason Gill
Tel: 0141 3302916  E-mail: j.gill@bio.gla.ac.uk

You will be given a copy of this information sheet and a signed consent form to keep for your records.
CONSENT FORM

Title of Project: Development of novel method to determine very low density lipoprotein kinetics

Lay title: A new method of measuring the metabolism of blood fats

Name of Researcher: ________________________________

Please initial box

1. I confirm that I have read and understand the information sheet dated 18 March 2004 (version 3) for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I agree to take part in the above study.

Name of Patient Date Signature

Name of Person taking consent Date Signature (if different from researcher)

Researcher Date Signature

Blue for patient
Pink for researcher

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Appendix A2: Subject Information Sheet and Consent Form - Chapter 5

VOLUNTEER INFORMATION SHEET

Title: Effects of Moderate Exercise on Very Low Density Lipoprotein Kinetics

Lay title: How does exercise affect the metabolism of the liver’s fatty particles?

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.
**What is the purpose of the study?**

Heart disease is the leading cause of death in Scotland and being even slightly overweight, particularly when fat is stored in the tummy region, increases your risk. Many factors contribute to a person’s risk of heart disease, but it is thought that the ability to cope with fat contained in food plays a role. A single session of moderate exercise decreases blood fat concentrations following a meal, but is unclear whether this is due to an increase of the clearance of fatty particles by the blood, or their reduced production from the liver. This study will help to investigate the effects of a single brisk walking session lasting from 90 to 120 minutes on the production and clearance of fatty particles. This is important as exercise could be used as a treatment option rather than drugs to prevent and treat disturbances in blood fat metabolism.

**Why have I been chosen?**

You have been chosen because you are a man or a postmenopausal woman aged between 30-60 years who is heavier than the ideal weight for your height.

**Do I have to take part?**

It is up to you to decide whether or not to take part. If you do decide, you will be given this information sheet to keep and be asked to sign a consent form. If you do this you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

**What will happen to me if I take part?**

In the first instance you will be asked to attend for a screening visit in which we will:

- discuss with you and complete confidential questionnaires regarding your health, family history and physical activity level
- measure your blood pressure
- take your height, weight and waist measurements
- take two small blood samples (20 ml or 4 teaspoons) to check the fat and sugar levels in your blood. We will also check if you are anaemic or have any abnormalities in the function of your liver, kidneys and thyroid gland
- provide an opportunity for you to ask questions

These preliminary procedures will enable us to determine whether you fall into the group of people we wish to study and will also ensure that it is perfectly safe for you to take part. In addition, we will inform your GP of your participation in the study and send them a copy of your screening results.

**Experimental procedures**

**A. Preliminary Exercise Test**

At the beginning of the study, an exercise test will be undertaken. This will involve walking on a motorised treadmill. If you are not used to walking on a treadmill, we will familiarise you with this before any ‘real’ sessions are performed. The test is designed to estimate your body’s ability to use oxygen and enables us to find the correct speed and gradient for you to walk at during your treadmill walks. This will **not** require a maximal effort and the test will last for about 20 minutes. Heart rate
will be monitored and recorded throughout using a heart rate monitor and expired air will be collected at intervals using a mouthpiece and respiratory valve. For safety reasons, the test will be stopped if your heart rate exceeds 85% of your predicted maximum.

**B. Body Composition**
The amount and distribution of your body fat will be determined by measuring body girths and by using callipers to measure skin fold thickness at four different sites (a sophisticated version of "pinch an inch"). Your height, weight and waist and hip circumferences will also be recorded. You will need to wear only underclothing for these measurements which will be made in private. These measurements only take a few minutes and can be made on the same day as other tests.

**C. Main Trials**
We will ask you to undertake 2 Intralipid tests, approximately 1-2 weeks apart, in random order. On the day prior to one Intralipid test, a controlled exercise session of 90- to 120-minutes walk will be undertaken (Exercise test). Other than this, conditions (such as alcohol consumption, food eaten, etc) in the days leading up to each trial will be EXACTLY the same. This is explained in detail in the “What do I have to do?” section.

**Control Test** – We will ask you to come to our Clinical Investigation Suite at Glasgow Royal Infirmary after an overnight fast (i.e. having eaten nothing and with only water to drink for 12 hours). We will then introduce two small plastic tubes called cannulas into a vein in your arms (one in each arm). This is no more painful than giving a normal blood sample. We will take blood from one cannula and use the other to pump small amounts of a fatty substance called Intralipid into your blood stream. Intralipid is used in hospitals to feed people through a ‘drip’ when they are unable to eat normally. This is a quick way for us to give you a fatty meal. The Intralipid we give you will cause the level of fat in your blood to rise about the same amount as it does after you eat a fatty meal and it will be pumped into your bloodstream for 60 to 75 minutes. We will take a maximum of 250 ml of blood during each trial – this is half the amount of blood that you give when you donate a ‘pint’ of blood. During the test you will not be able to eat or drink anything except water, but we will provide you with some food to eat when the trial is finished. The total duration of each test will be about 3 hours. You will be able to watch TV or videos, listen to music or read to keep yourself occupied during the experiment.

**Exercise Test** – This will be identical to the control test, except that we will ask you to come to the University to walk on the treadmill for 90 to 120 minutes on the day before the test.

**What do I have to do?**
We also ask you to maintain your usual lifestyle (i.e. don’t change your diet or exercise habits) for the duration of this study. However, before each Intralipid test, we ask you to do the following:
1. For 3 days before each Intralipid test, refrain from planned or strenuous exercise, other than for personal transportation and the 90- to 120-minute treadmill walk in our laboratory.

2. Weigh and record everything you eat and drink for 2 days before each test. We will provide you with weighing scales and diet sheets to do this.

3. Refrain from alcohol consumption on the day before each test.

4. For the 2 days leading up to the second test, we will ask you to eat the same diet as you did on the two days leading up to the first test. Therefore, we would advise you to eat meals that you will be able to easily repeat during the days preceding both tests.

**What are the possible disadvantages and risks of taking part?**

- Exercise testing will not be at a maximal level but the possibility exists that, very occasionally, certain changes may occur during or shortly after the tests. They include abnormal blood pressure, fainting or a change in the normal rhythm of the heartbeat.
- Blood sampling via the cannula may cause minor bruising or an inflammation of the vein. Good practice, however, minimises this risk. Some people may feel faint when they give blood.
- There is a small risk that you could have an allergic reaction to Intralipid (less than one chance in a million). This usually only occurs in people who are allergic to soya protein or eggs, so if you have these allergies you will not be able to participate in this study. In the unlikely event that you do have an allergic reaction to Intralipid, the appropriate medicines and equipment are in place for you to be treated immediately.
- There is a small possibility that taking part in this study will reveal a health problem that you already have such as high cholesterol or high blood pressure. If such a problem is revealed, we will inform your GP to ensure that you receive appropriate treatment.

**What are the possible benefits of taking part?**

There may be no benefits to you but as a result of being involved in this study you will receive health and fitness information about yourself including fitness tests, dietary assessment, body fat measurement and your cholesterol and blood sugar levels. This study will help us to determine how exercise can improve risk factors for heart disease and diabetes. The findings will be published in scientific journals so that understanding of the way in which exercise decreases the risk of heart disease and diabetes can be increased. This information may help make up better exercise guidelines, particularly for people who are overweight or obese.

We will provide you with feedback about the main study findings and also about your own results and would be delighted to explain results and discuss the implications with you.

**What if something goes wrong?**

The chances of something going wrong are extremely small. We have recently conducted a similar project and there were no problems. All of the procedures involved in this study are low risk and our screening tests are designed to ensure that you will only participate if it is safe for you to do so. In the unlikely event that you...
are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you.

**Will my taking part in this study be kept confidential?**

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the University or hospital will have your name and address removed so that you cannot be recognised from it. In addition, your records, samples and results will be identified by a number and not your name.

**What will happen to the results of the research study?**

The results from this study will be presented at scientific meetings and published in scientific journals. A copy of the published results will be sent to you upon request. You will be informed which part of the study you were in, as this information will be confidential and no one else will know your name and which part you participated in.

**What will happen to my samples after the study has finished?**

The blood samples that you provide for this study may be useful for future research into the prevention and treatment of diabetes and heart disease; this may involve investigating new biochemical markers that are not yet identified. Samples will be analysed anonymously and will require a new ethics application before they would be used for future research. If you do not wish your samples to be used for future research, please indicate this on the consent form.

**Who has reviewed the study?**

This study has been reviewed and approved by the North Glasgow NHS Trust Research Ethics Committee.

**Who is organising and funding the research?**

This study is being funded by the British Heart Foundation and the Glasgow Royal Infirmary R&D Endowment Fund (Chest, Heart and Stroke).

**Contact for Further Information**

Any questions about the procedures used in this study are encouraged. If you have any doubts or questions, please ask for further explanations by contacting either:

**Iqbal AlShayji**  
*Mobile: 07799353689  E-mail: i.alshayji.1@research.gla.ac.uk*  
*Tel: 0141 211 4595 or 4596*

**Dr Jason Gill**  
*Tel: 0141 3302916  E-mail: j.gill@bio.gla.ac.uk*
You will be given a copy of this information sheet and a signed consent form to keep for your records.

Thank You for Your Time and Participation
CONSENT FORM

Title of Project: Effects of moderate exercise on very low density lipoprotein kinetics

Lay title: How does exercise affect the metabolism of the liver’s fatty particles?

Name of Researcher: ____________________________________________

Please initial box

1. I confirm that I have read and understand the information sheet dated 21/02/2006 (Version 3) for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I agree to take part in the above study.

4. I agree that my GP is to be informed of my participation in this study.

5. I agree for my samples to be used for future research into the prevention and treatment of diabetes and heart disease. This may involve analysis of new biochemical markers not yet identified.

Name of Subject ____________________________ Date ____________ Signature ____________

Name of Person taking consent ____________________________ Date ____________ Signature ____________

(If different from researcher)

Re­searcher ____________________________ Date ____________ Signature ____________

Copy for subject
Copy for researcher
Appendix B1: Health Screen Questionnaire – Chapters 3 & 4

HEALTH SCREEN FOR STUDY VOLUNTEERS

Name: _____________________________________________________

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

1. **At present**, do you have any health problem for which you are:
   (a) on medication, prescribed or otherwise yes [ ] no [ ]
   (b) attending your general practitioner yes [ ] no [ ]
   (c) on a hospital waiting list yes [ ] no [ ]

2. **In the past two years, have you had any illness which required you to:**
   (a) consult your GP yes [ ] no [ ]
   (b) attend a hospital outpatient department yes [ ] no [ ]
   (c) be admitted to hospital yes [ ] no [ ]

3. **Have you ever had any of the following:**
   (a) Convulsions/epilepsy yes [ ] no [ ]
   (b) Asthma yes [ ] no [ ]
   (c) Eczema yes [ ] no [ ]
   (d) Diabetes yes [ ] no [ ]
   (e) A blood disorder yes [ ] no [ ]
   (f) Digestive problems yes [ ] no [ ]
   (g) Hearing problems yes [ ] no [ ]
   (h) Disturbance of balance/co-ordination yes [ ] no [ ]
   (i) Numbness in hands or feet yes [ ] no [ ]
   (j) Disturbance of vision yes [ ] no [ ]
   (k) Thyroid problems yes [ ] no [ ]
   (l) Kidney or liver problems yes [ ] no [ ]
   (m) Chest pain or heart problems yes [ ] no [ ]
   (n) Any other health problems yes [ ] no [ ]
   (o) An allergy to soya protein or eggs yes [ ] no [ ]
4. **For female volunteers only**
   (a) Are you pregnant or think that you might be pregnant  
       yes [ ] no [ ]
   (b) Do you take the contraceptive pill or other hormone-based contraceptives  
       yes [ ] no [ ]
   (c) Are you postmenopausal  
       yes [ ] no [ ]
   (d) Are you receiving Hormone Replacement Therapy (HRT)  
       yes [ ] no [ ]

5. **Have any of your family (parents, grandparents, brothers, sisters, children, aunts, uncles, cousins)** ever had any of the following: (if yes please give details including age of first diagnosis)
   (a) Any heart problems  
       yes [ ] no [ ]
   (b) Diabetes  
       yes [ ] no [ ]
   (c) Stroke  
       yes [ ] no [ ]
   (d) Any other family illnesses  
       yes [ ] no [ ]

6. Do you currently **smoke**  
   yes [ ] no [ ]
   Have you ever smoked  
   yes [ ] no [ ]

   If so, for how long did you smoke and when did you stop? ......................

7. How many units of alcohol do you typically drink in a week? ......................

   If YES to any question, please describe briefly if you wish (e.g. to confirm whether problem was short-lived, insignificant or well controlled.) (Use a separate sheet if necessary)

   ………………………………………………………………………………………………..
   ………………………………………………………………………………………………..
   ………………………………………………………………………………………………..
   ………………………………………………………………………………………………..

   Name and address of GP
   ………………………………………………………………………………………………..
   ………………………………………………………………………………………………..

   Blood pressure measured at screening ....................... mm Hg
Appendix B2: Health Screen Questionnaire – Chapter 5

HEALTH SCREEN FOR STUDY VOLUNTEERS

Name: _____________________________________________________

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

1. **At present**, do you have any health problem for which you are:
   (a) on medication, prescribed or otherwise   yes [ ] no [ ]
   (b) attending your general practitioner    yes [ ] no [ ]
   (c) on a hospital waiting list    yes [ ] no [ ]

2. **In the past two years**, have you had any illness which required you to:
   (a) consult your GP      yes [ ] no [ ]
   (b) attend a hospital outpatient department   yes [ ] no [ ]
   (c) be admitted to hospital      yes [ ] no [ ]

3. **Have you ever had any of the following:**
   (a) Convulsions/epilepsy     yes [ ] no [ ]
   (b) Asthma        yes [ ] no [ ]
   (c) Diabetes      yes [ ] no [ ]
   (d) A blood disorder     yes [ ] no [ ]
   (e) Digestive problems    yes [ ] no [ ]
   (f) Disturbance of balance/co-ordination yes [ ] no [ ]
   (g) Numbness in hands or feet     yes [ ] no [ ]
   (h) Disturbance of vision     yes [ ] no [ ]
   (i) Thyroid problems      yes [ ] no [ ]
   (j) Kidney or liver problems     yes [ ] no [ ]
   (k) Chest pain or heart problems     yes [ ] no [ ]
   (l) Any other health problems    yes [ ] no [ ]
   (m) An allergy to soya protein or eggs yes [ ] no [ ]
   (n) An allergy to nuts

4. **For female volunteers only**
   (a) Are you pregnant or think that you might be pregnant [ ] no [ ]
   (b) Do you take the contraceptive pill or other hormone-based contraceptives [ ] no [ ]
   (c) Are you postmenopausal [ ] no [ ]
   (d) Are you receiving Hormone Replacement Therapy (HRT) [ ] no [ ]

5. **Have any of your family (parents, grandparents, brothers, sisters, children, aunts, uncles, cousins) ever had any of the following: (if yes please give details including age of first diagnosis)**
   (a) Any heart problems [ ] no [ ]
   (b) Diabetes [ ] no [ ]
   (c) Stroke [ ] no [ ]
   (d) Any other family illnesses [ ] no [ ]

6. **Do you currently smoke** [ ] no [ ]
   Have you ever smoked [ ] no [ ]
   If so, for how long did you smoke and when did you stop? …………………

7. How many units of alcohol do you typically drink in a week? …………………

8. Have you taken part in a research study in the last 3 months? yes [ ] no [ ]
   **If YES to any question, please describe briefly if you wish (e.g. to confirm whether problem was short-lived, insignificant or well controlled.) (Use a separate sheet if necessary)**
   ………………………………………………………………………………………………………
   ………………………………………………………………………………………………………
   ………………………………………………………………………………………………………
   ………………………………………………………………………………………………………
   Name and address of GP
   ………………………………………………………………………………………………………
   ………………………………………………………………………………………………………
   Blood pressure measured at screening…………………..mm Hg
Appendix C: Kinetic Results For All Subjects According To ApoE Phenotypes

Figure 8.1: Production rates of [A] VLDL₁-TG and [B] VLDL₁-ApoB and [C] Intralipid-TG clearance rate for all subjects (n = 23) according to apoE phenotypes: E2/E3 (n = 3), E3/E3 (n = 11) and E4+ (n = 9). P > 0.05 for differences between phenotypes.