The effect of exercise in NAFLD and a GLP-1 receptor agonist in type 2 diabetes on lipid metabolism

By

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Doctor of Philosophy

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Abstract

**Background:** Hypertriglyceridaemia increases the risk of developing an atherogenic lipoprotein phenotype (ALP) in patients with type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD). An ALP is associated with an increased the risk of coronary heart disease (CHD) and cardiovascular disease (CVD).

**Objectives:** To determine the effects of exercise and a glucagon like peptide-1 (GLP-1) receptor agonists on hypertriglyceridaemia and high-density lipoprotein (HDL) metabolism in patients with altered lipid metabolism, by conducting two clinical trials using stable isotope trace labelling technique:

1. To determine the effect of exercise on HDL apolipoprotein A-I (apoA-I) and very low-density lipoprotein (VLDL) apoB-100 subgroups (VLDL$_1$-apoB-100 and VLDL$_2$-apoB-100) kinetics in NAFLD.
2. To determine the effect of the GLP-1 receptor agonist lixisenatide on postprandial triacylglycerol-rich lipoprotein (TRL) apo-B-100 and B-48 and HDL-apoA-I kinetics in T2D.

**Study design:** In the NAFLD study, participants were randomised into two groups for a period of 16 weeks. The first group received a supervised moderate-intensity exercise programme and the second group was the control group. Total HDL-apoA-I was measured using a primed constant intravenous infusion of $^{1-13}$C leucine for 9 hours in a total of 27 recruited participants; 15 participants in the exercise group and 12 in the control group.

In the lixisenatide study participants were randomised in a double-blinded two-period cross-over design (lixisenatide versus placebo). Participants received treatment with lixisenatide or placebo for four weeks followed by a four-week washout period then another four weeks with the other treatment. TRL-apoB-100, TRL-apoB-48 and total HDL-apoA-I were measured
using a primed constant intravenous infusion of $^{13}$C leucine for 8 hours during repeated meal feeding in a total of six participants.

**Laboratory protocol:** For both studies, hourly blood samples were taken during the study period. TRL-apoB-100, TRL-apoB-48 and total HDL-apoA-I fractions were isolated using ultracentrifugation. Fractions were delipidated and separated by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands from SDS-PAGE were hydrolysed, purified, and then derivatised. The isotopic enrichment of $^{13}$C leucine in TRL-apoB-100, TRL-apoB-48 and total HDL-apoA-I were measured using gas chromatography – mass spectrometry (GC-MS). Fractional catabolic rate (FCR) and production rate (PR) were calculated for TRL-apoB-100, TRL-apoB-48 and total HDL-apoA-I. TRL-apoB-100 and TRL-apoB-48 concentrations were measured using competitive ELISA. Total HDL-apoA-I, lipid profile including triacylglycerol (TG), cholesterol, and free fatty acids (FFA), also called non-esterified fatty acids (NEFA), and glucose concentrations were measured using automatic analysers.

**Results:** In the NAFLD study, sixteen weeks of exercise had no significant effect on HDL-apoA-I kinetics. However, the HDL-apoA-I pool size (PS) was significantly increased from [17.4±0.8 g/l to 18.9±0.75 g/l ($P$ =0.05)] after exercise in the exercise group. VLDL_1-apoB-100 FCR and PR were significantly increased between the exercise and control group; Exercise group FCR [(7.2±0.6) vs (10.9±1.5) pools/day $P$=0.02]; PR [(3.7±0.7) vs (5.5±0.5) mg/kg/day $P$= 0.003]. Fasting hypertriglyceridaemia was not significantly changed after exercise.

In the lixisenatide study, TRL-apoB-100 FCR significantly increased after lixisenatide treatment versus placebo; (6.3±0.4 vs 4.1±0.6 pools/day $P$=0.01). TRL-apoB-100 PR was increased with borderline significance after lixisenatide treatment ($P$=0.06) versus placebo.
TRL-apoB-48 and HDL-apoA-I kinetics were not significantly changed after lixisenatide treatment versus placebo. Fasting and postprandial plasma glucose concentrations were significantly lower after lixisenatide treatment (P=0.05 and P=0.001 respectively) versus placebo. Postprandial serum insulin concentration was significantly higher after lixisenatide treatment (P=0.001) versus placebo. Postprandial plasma TG, cholesterol and FFA concentrations were significantly lower after lixisenatide treatment (P=0.002, P=0.02 and P=0.05 respectively) versus placebo.

**Conclusion:** Exercise and lixisenatide were both effective in increasing VLDL-apoB-100 FCR which has the potential to reduce plasma TG concentrations in patients with altered lipid metabolism. However, exercise did not correct fasting hypertriglyceridaemia in patients with NAFLD due to increased VLDL<sub>1</sub>-apoB-100 PR. Liver fat was reduced by over 50% in the exercise group although was not normalised suggesting hepatic IR was maintained. To correct fasting hypertriglyceridaemia, accumulated liver fat must be further cleared to restore hepatic insulin sensitivity which would decrease VLDL<sub>1</sub>-apoB-100 PR. A longer exercise period is therefore needed to remove more liver fat and to correct fasting hypertriglyceridaemia. Postprandial hypertriglyceridaemia was lowered after lixisenatide treatment despite the fact that VLDL-apoB-100 PR was increased with borderline significance. A decrease in plasma TG concentration would be expected to reduce the rate of transfer of TG and CE between TRL and HDL via cholesteryl ester transfer protein (CETP) and increase HDL-cholesterol (HDL-C) concentration. The lack of effect of exercise on HDL kinetics in the NAFLD study reflects the failure to lower hypertriglyceridaemia. The lack of effect of lixisenatide to increase HDL despite lower plasma TG may be due to the study being underpowered.
Declaration

All the work, results and data collected mentioned in this thesis are the results of my own efforts except the following:

1. In Chapter five, the measurements of fractional HDL-apoA-I kinetics and related work, HDL-cholesterol and apoA-I concentrations were conducted by myself, and the other data results included in the chapter were performed by other personnel in the department.

2. In chapter six, the measurements of body fat and insulin concentrations were performed by other personnel in the department.

All the ideas, data, images or text related to other researchers (whether published or unpublished) are fully identified and attributed to their originator in the text and bibliography. This thesis has not been submitted in whole or in part for any other academic degree or professional qualification. I declare that the University of Surrey has the right to submit this thesis to the plagiarism detection service TurnitinUK for originality detection. Hence The University of Surrey reserves the right to require an electronic version of the final document (as submitted) for the originality assessment. A copy of this thesis will be available for The University of Surrey’s Library use on the understanding that it is a copyright material, and that no quotation from the thesis may be published without proper acknowledgement or consent.

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Date: 16/12/2016
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All the other people involved in the NAFLD and Lixisenatide study and all the participants.

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The Government of Saudi Arabia, for funding of this project and making all this possible.

All my friends, both here in the UK and in Saudi Arabia (or elsewhere in the world) who have a special place in my heart for each and every one of them.
# Statement of Contributions

<table>
<thead>
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<tbody>
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<td>Studies chair.</td>
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<td>Laboratory training and assistance. Stable isotope study day.</td>
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<td>Research facilities and general assistance, laboratory and GC-MS assistance.</td>
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<td>Blood sampling and stable isotope assistance during all clinical trials.</td>
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<td>AA</td>
<td>Amino acid</td>
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<tr>
<td>ABCA1</td>
<td>ATP-Binding cassette transporter A1</td>
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<td>ALP</td>
<td>Atherogenic lipoprotein phenotype</td>
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<td>The American Heart Association</td>
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<td>A-HDL</td>
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<td>FFA</td>
<td>Free fatty acids</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>FC</td>
<td>Free cholesterol</td>
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<tr>
<td>FCR</td>
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</tr>
<tr>
<td>GRP</td>
<td>gastrin-releasing polypeptide</td>
</tr>
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<td>Glycemic index</td>
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<tr>
<td>GIT</td>
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</tr>
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<td>GIP</td>
<td>glucose-dependent insulinotropic polypeptide</td>
</tr>
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<td>Haemoglobin A₁c</td>
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</tr>
<tr>
<td>IFCC</td>
<td>International Federation of Clinical Chemistry</td>
</tr>
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<td>Ion exchange chromatography</td>
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<td>Ischemic heart disease</td>
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<td>IR</td>
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<td>Alpha-ketoisocaproate</td>
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<td>Lecithin-cholesterol acyltransferase</td>
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<td>Lipoprotein lipase</td>
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<td>Myocardial infarction</td>
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<td>Magnetic resonance imaging</td>
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<td>Magnetic resonance spectroscopy</td>
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<td>N-Methyl-N-(tertbutyldimethylsilyl) trifluoroacetamide</td>
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<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>NCI</td>
<td>Negative chemical ionization</td>
</tr>
<tr>
<td>NH₄OH</td>
<td>Ammonium hydroxide</td>
</tr>
<tr>
<td>NHS</td>
<td>National health services</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OFN</td>
<td>Oxygen Free Nitrogen</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate-buffered saline-Tween</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>------------</td>
<td>----------</td>
</tr>
<tr>
<td>PCI</td>
<td>Positive chemical ionization</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>PR</td>
<td>Production rate</td>
</tr>
<tr>
<td>PV</td>
<td>Plasma volume</td>
</tr>
<tr>
<td>PLTP</td>
<td>Phospholipid transfer protein</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>RCT</td>
<td>Reverse cholesterol transport</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate – polyacrylamide gel electrophoresis</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>Sf</td>
<td>Svedberg flotation</td>
</tr>
<tr>
<td>SR-BI</td>
<td>Scavenger receptor B type 1</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TFAA</td>
<td>Trifluoroacetic acid anhydride</td>
</tr>
<tr>
<td>TG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TTR</td>
<td>Tracer/tracee ratio</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′,N′-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>VO\textsubscript{2max}</td>
<td>Maximum oxygen consumption during exercise</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organisation</td>
</tr>
</tbody>
</table>
Chapter 1: General Introduction

1.1 Triacylglycerols (TAG)

Triacylglycerol (TAG), also called triglycerides (TG), are one of the most important sources of energy in the body which can be utilised instantly or stored in adipose tissues to be used when energy supply is reduced. They play several significant roles such as the maintenance of the cell membrane structural integrity, cell signalling and hormonal functions (Frayn, 2010, Hussain, 2014). TGs are non-polar and hydrophobic, and are composed of three individual fatty acids (FAs), each linked by an ester bond to a glycerol molecule (Figure 1.0). TG derived from the diet is called exogenous TG. Endogenous TGs are mainly synthesised in the liver and derived from FAs derived from peripheral TG storage pools (such as adipose tissue) and de novo synthesised FAs. During digestion, TG is broken down (hydrolysed) into monoglycerides and FAs in the intestines by pancreatic lipase, and by lipoprotein lipase (LPL) in the circulation. TGs are transported in the blood in specialised particles called lipoproteins which contains TG, cholesterol, phospholipids and apolipoproteins (Frayn, 2010, Hussain, 2014).

![Glycerol and 3 Fatty acids](image)

Figure 1.0: The chemical composition of TG.
1.2 Lipoproteins

Lipoproteins are macromolecular complexes which carry several lipids and proteins in the human blood (Figure 1.2). The fats circulating in the blood, mainly free fatty acids (FFAs) (McQuaid et al., 2011, Hussain, 2014), TG and cholesterol are not water soluble and require specialised transport mechanisms. Plasma long-chain FFA binds to albumin (McQuaid et al., 2011, Hussain, 2014). The core of the lipoproteins, contains the hydrophobic cholesteryl esters (CE) and TG while the surface contains mainly the amphipathic phospholipids, proteins, and small amounts of free cholesterol (FC) (Figure 1.2). This particular structure enables the lipoprotein to interact with the aqueous environment of the human blood and transport the water insoluble lipids to and from the peripheral tissues (Frayn, 2010, Hussain, 2014).

Figure 1.2: General structure of a lipoprotein particle with its components.

1.3 Lipoprotein classification

Lipoproteins can be divided into five main types based on their size, ultracentrifugation flotation density as shown in Table 1.0 which illustrates lipoprotein density, weight, size and lipid content for each lipoprotein (Evans et al., 2002, Hussain, 2014, Ginsberg, 1998).
Table 1.0 Physical and chemical characteristics of the major lipoprotein classes (Ginsberg, 1998, Evans et al., 2002, Hussain, 2014).

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Density g/ml</th>
<th>MW Daltons</th>
<th>Diameter (nm)</th>
<th>Lipid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TG</td>
</tr>
<tr>
<td>CMs</td>
<td>0.95</td>
<td>400 × 10^6</td>
<td>75–1200</td>
<td>80–95</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019–1.063</td>
<td>2.3 × 10^6</td>
<td>18–25</td>
<td>5–15</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063–1.21</td>
<td>1–1.5 × 10^6</td>
<td>5–1</td>
<td>1–5</td>
</tr>
</tbody>
</table>

MW: Molecular weight; TG: triacylglycerols; CHOL: Cholesterol; PL: phospholipids. CM: chylomicron; VLDL: very low-density lipoprotein; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein; HDL: high-density lipoprotein.

1.3.1 Chylomicrons (CM)

CM are the largest and the least dense lipoprotein particles with Svedberg flotation (Sf) rate of > 400, and they form a creamy layer at the top of postprandial plasma when left to settle or spun (Hussain, 2014). CM size and density is related to the amount of TG and cholesterol absorbed from the diet, and the type of fat consumed in the food (Hussain, 2014). The principle role of CM is the delivery of dietary lipids to hepatic and peripheral cells. CM are synthesised in the enterocytes of the jejunum, and they represent exogenous TG in the blood (Bisgaier and Glickman, 1983, Hussain, 2014).

1.3.2 Very low-density lipoproteins (VLDL)

VLDL are approximately 30-80 nm in size with an Sf of 20-400, and divided into the larger TG-enriched VLDL₁ (Sf 60-400) and relatively smaller and denser VLDL₂ (Sf 20-60) (Adiels et al., 2005b). VLDL are enriched with TG and responsible for delivering hepatic-TG from the liver to the peripheral tissues, such as the adipose and muscle tissues. Therefore they represent
the endogenous TG in the blood. In the fasting state, VLDL account for most of the turbidity in plasma (Ginsberg, 1998). Some dietary TG besides CM appears soon after eating in VLDL (Ginsberg, 1998, Adiels et al., 2005b, Adiels et al., 2005a).

1.3.3 Intermediate density lipoproteins (IDL)

IDL are smaller than VLDL and denser with an Sf of 12-20, and they are considered to be the remnants of VLDL hydrolysis in the circulation, and the immediate precursor of LDL. An increase in the IDL concentrations has been shown to be proportional to an increase in the risk of cardiovascular disease (CVD) (Superko, 1996, Ginsberg, 1998, Carmena et al., 2004).

1.3.4 Low-density lipoproteins (LDL)

LDL are smaller and denser than IDL with an Sf of 0-12. LDL are the major cholesterol-carrying lipoprotein in the blood and are readily taken up by cells via the LDL-receptor in the liver and peripheral cells. The size and density of these particles are thought to be associated with plasma TG concentrations (Hevonoja et al., 2000). These particles especially the small dense LDLs (sLDL) are hypothesised to be atherogenic and play a significant role in increasing the risk of CVD as they can migrate to the arteries endothelial wall and initiate and/or increase the pathogenicity of atherosclerosis (McNamara et al., 1992, Carmena et al., 2004).

1.3.5 High-density lipoproteins (HDL)

HDL are the smallest and densest among all lipoproteins with an Sf of less than 0, and contain the highest protein composition of all lipoproteins. Newly synthesised HDL is discoidal in shape and has a high efficiency in removing cholesterol from the tissue cells. Once HDL acquires extra lipids, i.e. CE and TG from tissues, it transforms into a spherical
shaped HDL. Spherical HDL is divided into two major subclasses; HDL$_2$ (1.063-1.125 g/ml) and HDL$_3$ (1.125-1.21 g/ml), and these two subclasses can be divided further into five subfractions (Barter, 2002, Hussain, 2014). Spherical HDL is responsible for transporting cholesterol to the liver for bile acid synthesis (Morgan et al., 2004, Lewis and Rader, 2005, Hussain, 2014).

### 1.4 Apolipoproteins

Apolipoproteins (apo) are specific proteins associated with lipoprotein particles, and they are either an integral part of the lipoprotein or are located peripherally (Figure 1.3). They help in the solubilisation of core lipids and play essential roles in the process of regulating plasma lipids and lipoprotein transport. They facilitate the recognition of lipoproteins at cell surface receptors for lipid metabolism (Gursky, 2005). The major apos are classified into four main groups based on their weight and metabolic function as shown in Table 1.1; apo A, B, C, D and E, and each could be divided into further groups (Mahley et al., 1984, Gursky, 2005).

<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>MW</th>
<th>Metabolic functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA-I</td>
<td>28,016</td>
<td>LCAT activator, HDL Structural component</td>
</tr>
<tr>
<td>ApoA-II</td>
<td>17,414</td>
<td>Unknown</td>
</tr>
<tr>
<td>ApoA-IV</td>
<td>46,465</td>
<td>Enables transfer of apolipoproteins between HDL and CMs</td>
</tr>
<tr>
<td>ApoA-V</td>
<td>39,000</td>
<td>Linked with lower TG concentrations; unknown mechanism</td>
</tr>
<tr>
<td>ApoB-48</td>
<td>264,000</td>
<td>Essential for the assembly and secretion of CMs</td>
</tr>
<tr>
<td>ApoB-100</td>
<td>514,000</td>
<td>Essential for the assembly and secretion of VLDL from the liver</td>
</tr>
<tr>
<td>ApoC-I</td>
<td>6,630</td>
<td>May prevent hepatic uptake of CMs and VLDL remnants</td>
</tr>
<tr>
<td>ApoC-II</td>
<td>8,900</td>
<td>Activator of LPL</td>
</tr>
<tr>
<td>ApoC-III</td>
<td>8,800</td>
<td>Inhibitor of lipoprotein lipase and uptake of CM and VLDL</td>
</tr>
<tr>
<td>ApoE</td>
<td>34,145</td>
<td>Ligand for binding of several lipoproteins to the LDL receptor, LRP and proteoglycans</td>
</tr>
</tbody>
</table>

Apo: apolipoprotein; LCAT: Lecithin cholesterol acyltransferase; HDL: high-density lipoprotein; CM: chylomicron; TG: triacylglycerol; VLDL: very low-density lipoprotein; LPL: lipoprotein lipase; LRP: Lipoprotein receptor-related protein.
1.4.1 Apolipoprotein A-I (apoA-I)

ApoA-I is the main HDL structural component and is also found on CM and CMR (Segrest et al., 2000b). ApoA-I is synthesised in the intestine and the liver, and has a plasma concentration of 1-1.5 mg/ml. CM contains apoA-I, and during TG hydrolysis by lipoprotein lipase (LPL), apoA-I is transferred to HDL particles. ApoA-I plays a protective role against CVD in humans as an anti-atherogenic function of the HDL particles (Segrest et al., 2000b, Hattori et al., 2004). Furthermore, one of the primary roles of apoA-I is activating the lecithin-cholesterol acyltransferase (LCAT) enzyme which is essential for extracellular cholesterol esterification (Miida et al., 2004, Lewis and Rader, 2005).

1.4.2 Apolipoprotein B-100 (apoB-100)

ApoB-100 is essential for the generation of VLDL, and also found in IDL and LDL (Burnett and Barrett, 2002). ApoB-100 is a large apolipoprotein containing 4536 amino acids (AA) residues with a molecular mass of about 513 kDa. It comprises many hydrophobic areas that possibly serve as strong lipid-binding domains (Marshall and Bangert, 2008). It also has many binding domains that might act as binding sites for heparin-like particles and form the base for some of the cell surface interactions of the apoB-containing lipoprotein (Marshall and Bangert, 2008). It is synthesised in the rough endoplasmic reticulum (RER) of the human hepatocytes, and binds to the ligand-binding domain of the LDL receptor (Burnett and Barrett, 2002, Boren et al., 1992).

1.4.3 Apolipoprotein B-48 (apoB-48)

ApoB-48 is a short form of apoB-100 representing 48% of the protein sequence of the aboB-100 C-terminal. It is required for the formation of CM at the small intestine, and cannot bind
to the LDL-receptor due to its short form (binding site close to C-terminal 3359-3369) (Powell et al., 1987, M.J. van Greevenbroek and W.A. de Bruin, 1998, Hussain et al., 2005). It consists of 2152 AA residues with a molecular mass of 241 kDa. It is synthesised in the RER in the enterocyte in the small intestine by post-transcriptional modification of the mRNA (Powell et al., 1987, Hussain et al., 2005, Hussain, 2014). In the process of premature CM particle formation, partly translocated membrane-bound apoB-48 polypeptide will be degraded if not bound to the lipid content of the RER membrane immediately (Milne et al., 2004, Morel et al., 2004). Therefore, the formation of CM is directly dependent on the availability TG, cholesterol and phospholipids (Morel et al., 2004, Milne et al., 2004).

1.4.4 Concentrations of apoB-100 and apoB-48 in human blood plasma

Plasma apoB-100 and apoB-48 concentrations vary with the feeding state. Karpe et al., 1993 and Karpe et al., 1996 measured apoB-100 and apoB-48 concentration as shown in Table 1.2 which shows the method of isolation, S floatation and fasting and postprandial concentration. The proportions of plasma concentrations of apoB-100 in fasting and postprandial states are higher when compared with minimum apoB-48 concentrations. The determination of apoB-48 concentrations in fasting and postprandial state is difficult (Karpe et al., 1996). The reason for this is that there is only one non-exchangeable apoB-48 per CM and CMR particles which remains an integral part of the CMR particle following lipolysis (Phillips et al., 1997). ApoB-48 concentration is only synthesised in the intestines during the fed state for CM formation. Conversely, apoB-100 can be found in VLDL, IDL and LDL particles after VLDL-TG hydrolysis in both feeding and fasting states. Therefore, a proper assessment of apoB-48 requires an increase in its concentration by administering a
standardised feeding protocol as shown in many studies (Duez et al., 2006, Duez et al., 2008b, Hogue et al., 2007a, Nogueira et al., 2012).

Table 1.2 Plasma concentrations of apoB-100 and apoB-48 in fasting and postprandial states (Karpe et al., 1996).

<table>
<thead>
<tr>
<th>Test meal</th>
<th>ApoB of interest</th>
<th>Method of lipoprotein isolation</th>
<th>Sf</th>
<th>Fasting Conc. (mg/l)</th>
<th>Peak postprandial Conc. (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat emulsion, 50 g fat/m² BSA</td>
<td>ApoB-48</td>
<td>U.C</td>
<td>Sf 60–400</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>ApoB-100</td>
<td></td>
<td>Sf 20–60</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sf 20–60</td>
<td>36</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sf 60–400</td>
<td>42</td>
<td>41</td>
</tr>
</tbody>
</table>

U.C: ultracentrifugation; Apo: apolipoprotein; Sf: Svedberg flotation; BSA: bovine serum albumin; Conc: concentration.

1.5 Essential enzymes and proteins involved in the lipid metabolic pathways

1.5.1 Microsomal triacylglycerol transfer protein (MTP)

MTP is a heterodimeric protein complex which is a member of a group of proteins that has lipid transfer activity between membranes (Hussain, 2014, Hussain et al., 2003). MTP is a dimer which comprises two subunits; a large M-subunit (97-kDa) and a small P-subunit (55-kDa), and both subunits are bound together by non-covalent interactions. This protein is primarily located in the endoplasmic reticulum lumen and plays an essential role in the maturation of endoplasmic reticulum, and its secreted proteins (Hussain, 2014, Hussain et al., 2003). It binds and shuttles individual lipids between membranes (Hussain, 2014, Hussain et al., 2003). Insulin downregulates the MTP promoter region and it is up-regulated by cholesterol. MTP has four binding sites; one neutral lipid binding site which binds TG and CE, and two or three phospholipid binding sites (Hussain, 2014, Hussain et al., 2003). MTP has been proposed to perform in a sequence cycle in which it transiently interacts with a membrane, extracts lipid molecules from it, dissociates from the membrane, delivers the lipids to another membrane and then returns for another cycle (Hussain, 2014, Hussain et al., 2003) as shown in Figure 1.3. MTP primarily transports ER membrane-bound lipid and
newly synthesised TG to recently translated apoB-48 in the small intestine and apoB-100 in the liver and facilitates their lipidation (Figure 1.3) (Hussain, 2014, Hussain et al., 2003). Both transportation steps to the RER lumen is considered as the first step in triacylglycerol-rich lipoproteins (TRL) (CM and VLDL) biogenesis before they are transported to Golgi apparatus to be secreted (Hussain, 2014, Hussain et al., 2003).

Figure 1.3: MTP performance in a sequenced cycle for lipidating nascent apoB. Figure obtained from Hussain et al., 2003. ApoB: apolipoprotein B; ER: endoplasmic reticulum; MTP: microsomal triacylglycerol transfer protein.

1.5.2 Lipoprotein lipase (LPL)

LPL is synthesised in two major sites; in parenchymal cells of the heart and skeletal muscles, mammary glands and in adipose tissues. The primary function of LPL is to hydrolyse the TG and phospholipids within CM and VLDL which transform them into remnants (Mead et al., 2002, Zilversmit, 1995). Therefore, it is synthesised in the sites in which FA are oxidised for energy or stored (Eckel, 1989, Frayn, 2010, Mead et al., 2002). LPL requires the presence of certain apolipoproteins to maintain its regular function (Mead et al., 2002). For instance, LPL is activated in the presence of apoCII and modulated by apoCIII (Mead et al., 2002, Wang et
LPL is located on the luminal surface of the endothelial cells (ECs) in which enzymes are attached with membrane-bound chains of heparin sulphate proteoglycans (HSPG) (Enerback and Gimble, 1993). Furthermore, LPL facilitates the binding of lipoproteins and their receptors such as VLDL and, LDL and Lipoprotein receptor-related protein (LRP) receptors (Mead et al., 2002, Chappell et al., 1993, Medh et al., 1996, Takahashi et al., 1995). Also, it is reported that LPL may play a pro-atherogenic role in atherosclerosis within muscles and adipose tissues (Mead et al., 1999, Mead and Ramji, 2002).

1.5.3 Hepatic lipase (HL)

HL synthesis takes place in the hepatocytes, and it is located on the surface of the hepatic ECs which is located predominantly on the extracellular surface in the space of Disse (Zambon et al., 2003a). HL plays a similar role to LPL in the remodelling of TRL and HDL remnants, and also plays a role in the reverse cholesterol pathway. HL binds with HSPG to facilitate the hydrolysis of TG and phospholipids in all lipoproteins, and can hydrolyze CE, monoglycerides and diglycerides (Zambon et al., 2003a). Furthermore, HL activity is associated with produced LDL size, and can be modulated by insulin resistance (IR), dietary fat intake, abdominal obesity, physical activity and certain drugs (Zambon et al., 2003b).

1.5.4 The LDL receptor and LDL receptor-related protein

The LDL receptor (LDLR) is a glycoprotein receptor which is present on the surface of all cells, and it binds with lipoprotein containing apoB-100 and apoE to facilitate their breakdown within the cell. LDL receptor-related protein (LRP) is expressed by the hepatocytes and has multiple ligands including apo-E, HL and LPL (Strickland et al., 1995). IDL and HDL-containing apoE binds to the LDLR and the lipid content (CE and TG) which are then taken up by the cell through endocytosis for oxidation or storage, and then the receptor is recycled back to the
surface (Koo et al., 1988, Go and Mani, 2012). The LDLR number is related to the concentration of circulating LDL in the plasma. Therefore, LDLR expression is controlled by feedback control by allowing sufficient cellular cholesterol uptake to maintain cellular cholesterol homoeostasis (Go and Mani, 2012). The LDL synthesis is regulated by CE cellular concentration as it affects the recycling process of LDLR to the cell surface (Koo et al., 1988, Brown and Goldstein, 1986, Go and Mani, 2012).

1.5.5 ATP-binding cassette transporter A1 (ABCA1)

The ABCA1 transportation system is predominantly found in hepatic cells, brain cells, macrophages and in other tissues (Segrest et al., 2000a, Singaraja et al., 2006, Zannis et al., 2006). The transportation system is one of the vital systems in the body in which molecules are transported via the breaking down of ATP for the movement of particles across the cellular membrane. ABCA1 plays a significant role in lipidating lipid-poor apoA-I through facilitating the efflux of cholesterol and phospholipids via the cell membrane. This efflux will result in the formation of discoidal apoA-I-containing HDL. This step is essential in synthesising HDL in the HDL metabolic pathway (Zannis et al., 2006, Barter, 2002).

1.5.6 Acyl-CoA: cholesteryl acyltransferase (ACAT)

The ACAT is responsible for facilitating the esterification of cholesterol (Chang et al., 2009). There are two recognised types; ACAT1, which is located in the ER of smooth muscle cells, and ACAT2 which is located predominantly in the ER of the enterocytes of the intestines and hepatocytes (Chang et al., 2009). The main function of ACAT1 is to saturate cellular membranes with cholesterol to an optimum concentration. ACAT2 acts mainly on the secretion of CE into VLDL-apoB100 in the liver and CM-apoB48 in the intestines (Chang et al., 2009).
1.5.7 Lecithin cholesterol acyltransferase (LCAT)

LCAT is synthesised in hepatocytes and is found bound to the HDL surface (Barter, 2002, Zannis et al., 2006). LCAT is activated by apoA-I (Frayn, 2010), and has many important functions such as the maturation of HDL particles and facilitating cholesterol transport from the peripheral tissues to the HDL particles. Also, it mediates CE esterification on the surface of HDL particles (Lewis and Rader, 2005).

1.5.8 Phospholipid transfer protein (PTP)

PTP is a plasma glycoprotein and plays a significant role in HDL metabolism (Colhoun et al., 2002). PTP is essential for HDL remodelling, the generation of Pre-βHDL, the transfer of lipids from triacylglycerol-rich lipoproteins (TRL) to HDL during intravascular lipolysis, and facilitates the efflux of cholesterol and phospholipids from cells to HDL (Colhoun et al., 2002, Lewis and Rader, 2005). Moreover, PTP activity increases in hypertriglyceridaemia and it can be modulated by IR (Colhoun et al., 2002).

1.5.9 Cholesterol ester transfer protein (CETP)

CETP is a glycoprotein that is predominantly found in the liver and adipose tissue (Barter, 2000, Bagdade et al., 1993). The primary function of CETP is to facilitate the relocation (redistribution) of TG and CE between HDL and TRL (VLDL and CM). As a result, TG from TRL are relocated within the HDL, and CE is relocated to the TRLs (TG is taken up by HDL from TRL and cholesterol is taken up by TRL from HDL) (Rye et al., 1999, Frayn, 2010) as shown in Figure 1.4. The concentrations and activity of CETP in the plasma are related to the state of either hypercholesterolaemia or hypocholesterolaemia in metabolic disorders (Barter, 2000, Bagdade et al., 1993, Frayn, 2010), and it is associated with plasma HDL-C concentrations
which is related to the CETP gene polymorphism (Freeman et al., 2003).

Figure 1.4: CETP facilitating the transfer of TG and CE between TRL and HDL. Figure obtained from Lewis and Rader, 2005. SR-B1: Scavenger receptor class B type 1; HL: hepatic lipase; HDL: high-density lipoprotein; apoA-I: apolipoprotein A-I; TG: triacylglycerol; CETP: cholesterol ester transfer protein; CE: cholesterol ester; PL: phospholipid; PLTP: Phospholipid transfer protein; VLDL: very low-density lipoprotein.

1.5.10 Scavenger receptor class B type 1 (SR-B1) protein

SR-B1 is a polypeptide protein that binds to some lipoproteins such as VLDL, HDL, LDL and oxidised LDL (oxLDL) (Connelly et al., 1999, Greene et al., 2001, Shen et al., 2014, Reboul et al., 2006, Ueda, 2001). SR-B1 is expressed in the hepatocytes and enterocytes in the intestines. SR-B1 binds to HDL and mediates the selective uptake of CE from the core of HDL particles to their plasma membranes without entailing the uptake and degradation of the whole HDL particle. This process is dependent on the presence and correct orientation of apoA-I (Connelly et al., 1999, Greene et al., 2001, Shen et al., 2014, Reboul et al., 2006, Ueda, 2001). SR-B1 also facilitates the efflux of unesterified cholesterol from cells to
lipoproteins. This means that SR-B1 is essential for both ends of the reverse cholesterol pathway; Initially with the efflux of FC from peripheral cells to HDL, and for delivery of CE and unesterified cholesterol to the liver for secretion into bile and synthesis of bile acids (Connelly et al., 1999, Greene et al., 2001, Shen et al., 2014, Reboul et al., 2006, Ueda, 2001).

1.6 Significant metabolic pathways involved in lipid metabolism

1.6.1 De-novo lipogenesis (DNL) pathway

DNL transforms carbohydrates to FA in the liver and adipose tissues (Hellerstein, 1999, Ameer et al., 2014). This pathway is activated in the feeding state in which it is controlled by many transcription factors and hormonal factors such as insulin (Ameer et al., 2014). These factors include sterol response elements (SRE) binding protein isoform one (SREBP1-c) (Horton et al., 2002) and the liver X receptor-α (LXRα) (Liu et al., 2007). It has been demonstrated that SREB1-c upregulates all enzymes in the FA synthesis pathway and enzymes that regulate the availability of acetyl-CoA units in the pathway (Horton et al., 2002). Also, LXRα regulates lipogenesis via inducing the expression of SREBP-1c (Liu et al., 2007). Lastly, Insulin increases the activity of LXRα, which hence induces SREBP-1c expression (Hellerstein, 1999, Ameer et al., 2014, Liu et al., 2007). Approximately 4% of FAs syntheses in the endogenous lipid pathway are derived from DNL in fasting state, and it increases to 8% in the feeding state in healthy population (Hellerstein, 1999, Ameer et al., 2014, Barrows and Parks, 2006). In individuals with obesity and IR, DNL significantly increases endogenous lipid synthesis (Ameer et al., 2014).

1.6.2 Exogenous and Endogenous lipoprotein pathways

The exogenous and endogenous lipoprotein pathways are shown in Figure 1.5.
1.6.2.1 The exogenous lipoprotein pathway

Figure 1.5 illustrates the exogenous lipid pathway. Large digested Lipids particles are emulsified into smaller units called micelles by bile acids through creating a liquid-crystalline interface at the surface of the emulsified particles, and hydrolysed as TG primarily by pancreatic lipase in the jejunum (Benzonana and Desnuelle, 1965, Rustow and Kunze, 1987). Then pancreatic lipase act on the TG molecule resulting in the release of monoacylglycerol (MAG) and FFAs (Mattson and Beck, 1956, Mattson and Volpenhein, 1968), and MAG is absorbed from the small intestine. Pancreatic lipase also hydrolyses MAG resulting in the formation of glycerol and FFA (Hofmann and Borgstrom, 1963); FFAs are taken up from the
intestinal lumen into the enterocytes and used for neutral fat biosynthesis. Glycerol is taken up via two ways; taken up bound to FA as MAG, and via specialised channels called aquaglyceroporins (Mukhopadhyay et al., 2014, Hara-Chikuma and Verkman, 2006). CD36 plays an important part in the uptake of FFAs (Abumrad, 2005, Bonen et al., 2007), and it is substantially expressed in the intestine (Chen et al., 2001). Specific binding proteins transport FFAs and MAG to the intracellular site where they will be used for the biosynthesis of TG. Once inside the enterocyte, the products of TG hydrolysis must cross the cytoplasm to reach the ER. Most TG biosynthesis in the enterocyte occurs via the MAG pathway in which fatty acyl-CoA and MAG are covalently joined to form diacylglycerol (DAG) in a reaction which is catalysed by monoacylglycerol acyltransferases (MGATs) (Coleman and Haynes, 1986, Yen et al., 2008). Further DAG acylation by diacylglycerol acyltransferase (DGAT) leads to TG synthesis which is used for CM synthesis (Black, 2007, Mansbach and Gorelick, 2007).

CM assembly is thought to be processed through two steps. The first step includes apoB-48 translation with the expulsion of the amino-terminal portion of the protein into the ER lumen. MTP lipidates apoB-48 and rescues it from degradation, which results in the formation a primitive CM. Also, MTP transfers lipid from the smooth ER membrane and other sites directly and bind to apoB-48 to endorse proper folding and lipid acquisition (Mahmood Hussain, 2000, White et al., 1998). Secondly, MTP mediates additional bulk lipidation of the primitive particle (Mahmood Hussain, 2000, White et al., 1998), in which apoA-IV is fused to the particle surface which appears to play a role in the process of CM assembly. The primitive CM is formed in a specialised vesicle in the smooth ER, which is called a pre-CM transport vesicle (PCTV), to be carried to the Golgi apparatus. In Golgi, lipid composition is altered, apoB-48 glycosylation is modified, and the particle acquires apoA-I. Finally, CM particles go through exocytosis at the basolateral membrane, and are secreted
into the intestinal lymph (lacteals) (Tso and Balint, 1986), and released into the circulation (Mahmood Hussain, 2000, Hussain et al., 2005, Iqbal and Hussain, 2009, Tso and Balint, 1986). CMs are macromolecules and cannot diffuse into the blood vessels. Instead, the lacteals transport them as chyle, and from which they diffuse readily through the lymphatic pores to the systemic circulation (Tso and Balint, 1986). After that, CM is secreted into the bloodstream through the lymphatic system (Hussain, 2014, Hussain et al., 2005). CM particles in the circulation interact with other lipoprotein fractions and exchange apolipoproteins other than apoB-48, comprising apoC-II, which mediates CM particles hydrolysis by LPL, and apoC-III, which inhibits LPL action. The hydrolysis of TG in CMs releases FAs, the majority of which will be taken up by the peripheral tissues (e.g. muscle, adipose tissue and liver) for oxidation or storage. The process of TG hydrolysis will result in significantly reduced CM particle size, with phospholipid transfer to HDL and movement of unesterified cholesterol from the core to the surface of the particle. Finally, chylomicron remnant (CMR) catabolism in the liver is carried out by binding of surface apoE to cell surface receptors in the liver (Cooper, 1997).

1.6.2.2 The endogenous lipoprotein pathway

In the liver as shown in Figure 1.5, VLDL synthesis, assembly and maturation can be described in two main steps. Firstly, nascent apoB-100 particles translocate through the smooth ER membrane, which results in rapid proteasomal degradation in the absence of sufficient neutral lipids. These lipid contents can be TG, cholesterol and phospholipids, which may be derived from fat stores or de novo synthesis. MTP facilitates the transportation to form primitive lipoprotein particles (Fisher and Ginsberg, 2002, Gordon and Jamil, 2000, Gordon et al., 1996). Secondly, apoB-100 is transported from the smooth ER to the Golgi as
primitive lipoproteins, and fuses with lipid droplets (cholesterol (from exogenous CMRs) and phospholipids) facilitated by the MTP enzyme. Also, apoC-II and apoE in the hepatocytes are packaged with the same primitive particle by MTP to produce mature VLDL-sized particles (Olofsson et al., 1999). VLDL is then released into the circulation. The TGs content of VLDL are hydrolyzed by LPL on endothelial tissue surfaces facilitating the release of glycerol and FFAs in adipose tissue and skeletal muscle. VLDL particles shrink in size as TGs, carried in VLDL, are hydrolysed by LPL forming IDL, and LDL (Iqbal and Hussain, 2009). Finally, the LDL particles formed are catabolised by the liver and other tissues.

1.6.3 HDL metabolism (also known as reverse cholesterol pathway)

HDL synthesis starts after the secretion and release of apoA-I from both the liver and intestine into the plasma as shown in Figure 1.6 (Lewis and Rader, 2005, Frayn, 2010, Wang and Smith, 2014). Lipid-poor apoA-I in the intestines immediately obtains phospholipids and FC from the peripheral tissues by the help of LPL. In the liver, lipid-poor apoA-I receives similar lipid content from CM and VLDL remnants with the assistance of ATP-binding cassette transport A1 (ABCA1) (Wang and Smith, 2014, Hassan et al., 2007). The binding of these particles will form pre-mature HDL particles called nascent preβHDLs which are additionally enriched with cholesterol and phospholipids mediated via ABCA1 (Lewis and Rader, 2005, Frayn, 2010, Wang and Smith, 2014, Hassan et al., 2007). Enriched Pre-βHDL particles produced in the circulation interact with other HDL particles which carry LCAT which facilitates the esterification of enriched cholesterol to form CE. This process will facilitate the movement of CE to the core of these particles which will alter and modify the discoidal shape of these particles to form small spherical α-migrating particles called HDL₃. Free circulating HDL₃ are moreover exposed to LCAT which it causes further cholesterol
esterification and CE movement. Continued esterification enlarges HDL₃ and alters their shape to form large spherical α-migrating particles called HDL₂ (Lewis and Rader, 2005, Frayn, 2010). The final step is the remodelling of mature HDL particles which is done via two mechanisms. The first one takes place when circulating HDL interact with the TRL plasma pool in which CE molecules are transported and exchanged with TG molecules in TRL with the help of CETP, which controls forward and reverse cholesterol pathway. This transportation will result in the formation of enriched HDL-TG particles due to the process of TG enrichment and CE depletion. Furthermore, TG-enriched HDL particles will obtain phospholipids from TRL at the same time mediated by PLTP (Lewis and Rader, 2005, Frayn, 2010). Upon the arrival of these particles to the liver, HL in hepatocytes causes the modification of these particles to become HDL remnant (HDLR) and to release the lipid-poor apoA-I for degradation via the renal system. The other way of HDL remodelling takes place via hepatic uptake of mature HDL particles from the circulation to interact with SR-B1. During particle interaction with SR-B1, CE is taken up by the hepatocytes to be used in the formation of bile acids. Remaining HDL particles after interaction will become HDLR, which are degraded by the renal system along with lipid-poor apoA-I (Lewis and Rader, 2005, Frayn, 2010) (Figure 1.6).
1.7 Obesity and IR as CVD risk factors

1.7.1 Cardiovascular disease

The cardiovascular system comprises the heart, a muscular pump, and the systemic and pulmonary blood vessels. CVD occurs via disordered or altered function to any of these components (WHO, 2015a). CVD is the predominant cause of death globally. An approximately 17.5 million people died from CVD in 2012, representing 31% of all global deaths. About 7.4 million of them died due to coronary heart disease, and 6.7 million were due to stroke (WHO, 2015a). Most of the CVD deaths occur in developing countries more than the developed countries with low or intermediate income (WHO, 2015a). People with CVD or who are at high cardiovascular risk (due to the presence of one or more risk factors such as diabetes, hypertension, hyperlipidaemia) need early detection and management using counselling and medicines, as appropriate (WHO, 2015a). CVD represents many conditions such as coronary heart disease (CHD) also known as coronary artery disease, ischemic heart disease, cerebrovascular disease, peripheral arterial disease and many others.
(WHO, 2015a). CHD most common representations are angina and myocardial infarction which are caused by an arterial blockage that stops blood flow to the myocardium. Cerebrovascular disease as haemorrhagic or thrombolytic stroke is caused by blood vessel leakage or bleeding in the brain (WHO, 2015a).

In the UK, rates of cardiovascular mortality remain among the uppermost in the world (WHO, 2015a, British Heart Foundation, 2012). The approximate mortality rate is one in four deaths in men and one in five deaths in women. CVD is responsible for over 80,000 premature deaths, and there are 120,000 confirmed cases of CHD discharges from the hospital per annum. The total estimated cost associated with CHD was £7 billion in 2004 (British Heart Foundation, 2012). The WHO predicts an increase in the number of people who die from CVD mainly from CHD and stroke to reach to 23 million people by the year 2030 (British Heart Foundation, 2012, WHO, 2015a).

1.7.2 Atherosclerosis

Atherosclerosis (also known as atherosclerotic vascular disease or ASVD) is the condition in which an artery wall thickens as the consequence of a build-up of fatty material such as, cholesterol which results to an endothelial dysfunction as altered vasoconstriction and vasodilation (Rasmussen et al., 2009) (Figure 1.7). It is a chronic inflammatory response in the walls of arteries, in large part due to the accumulation of white blood cells (macrophages and lymphocytes) and promoted by LDL without sufficient removal of fats and cholesterol from the tissues by functional HDL (Weber et al., 2008, Griffin, 1999). Atherosclerosis commonly develops from LDL and \( \text{LDL} \) molecules adhesion and accumulation at an arterial wall (Griffin, 1999). LDL particles become oxidised by free radicals; particularly reactive oxygen species (ROS). During the oxidation process, a series of reactions occur and the area
becomes inflamed due to injury. Inflammatory cytokines such as tumour necrosis alpha (TNF-α) and interleukin-6 (IL-6) are secreted as a sign of inflammation (Weber et al., 2008, Libby, 2002, Hansson and Nilsson, 2009). White blood cells (macrophages) start to adhere, migrate and infiltrate the arterial wall intima at the inflammatory site to start the process of phagocytosis (Weber et al., 2008, Libby, 2002, Hansson and Nilsson, 2009). During phagocytosis, dead macrophages with engulfed oxLDL form foam cells which, along with the inflamed endothelium, secretes macrophage chemoattractant protein-1 (MCP-1) which attracts further monocytes to the inflammation site (Weber et al., 2008, Libby, 2002, Hansson and Nilsson, 2009). The ongoing process results in the accumulation of foam cells which results in the formation of a cholesterol plaque that causes the muscle cells to enlarge and form a hard cover over the injured area, which narrows of the artery, reduces the blood flow and increases blood pressure. This manifestation can lead to the development of CHD as angina, and a sudden rupture of this plaque leads to the formation of a blood clot which blocks the blood supply to a certain area causing infarction (i.e. myocardial infarction) which may result in death or heart failure (Weber et al., 2008, Libby, 2002, Hansson and Nilsson, 2009). The association between coronary risk factors and heart disease is well established. Risk factors such as hypercholesterolemia and hypertension promote the development of coronary atherosclerosis through two key mechanisms: endothelial dysfunction or injury, and accumulation and modification of cholesterol, primarily LDL and cholesterol. Coronary risk factors facilitate the atherosclerotic process by increasing LDL cholesterol concentrations, enhancing endothelial permeability to LDL cholesterol, and promoting cholesterol modification (oxidation, glycosylation, and acetylation). Modified cholesterol (e.g., oxLDL) produces endothelial dysfunction, which appears to precede even the earliest development of atherosclerosis (Hansson and Nilsson, 2009, Weber et al., 2008).
1.7.3 High LDL and low HDL concentrations in contribution to Atherosclerosis and CVD

High concentrations of plasma LDLs are considered one of the most important risk factors which contribute to increasing the risk of CHD (Hoogeveen et al., 2014). \( \text{sd}\text{LDL} \), a subfraction of LDL, is considered even more potent for CHD risk through many mechanisms via increasing the pathogenesis of atherosclerosis (Hoogeveen et al., 2014, Griffin, 1999). These mechanisms are as follows; \( \text{sd}\text{LDL} \) has a lower affinity for the LDL receptor which increases cholesterol delivery to cells (Hoogeveen et al., 2014). Therefore, this will facilitate their entry into the endothelial cells (EC) of the arteries and arterial wall. Once \( \text{sd}\text{LDL} \) penetrates the arterial wall, it strongly binds to the proteoglycans which increase their arterial retention (Hoogeveen et al., 2014). Finally, as a result of their arterial retention at the arterial wall, this will make them more susceptible to oxidation which results in increased availability of \( \text{ox}\text{LDL} \). It directly increases the progression of atherosclerosis in the formation of foam cells at the lesion site of the arterial wall (Hoogeveen et al., 2014) as shown in Figure 1.7.
On the other hand, HDL plays a vital role as an atheroprotective particle with an inverse association with CVD. In many studies, it has been shown that reduced plasma HDL cholesterol (HDL-C) plasma concentrations can be used as a predictive marker for the development of premature CHD (Frick et al., 1987, Barter and Rye, 1996, Kontush and Chapman, 2006). The relationship between HDL-C and CHD has been widely addressed in many studies. It was shown that HDL-C is significantly associated with CHD development and incidence (Gordon et al., 1977, Gordon et al., 1989). HDL has several protective role against CVD which all help to correct and/or manage atherosclerosis (Kontush and Chapman, 2006). HDL can mediate the cholesterol efflux from cholesterol-loaded macrophages. This step will help in reducing and/or stopping the deposition of cholesterol-loaded macrophages at the arterial injury site in response to the inflammatory process. This will results in the inhibition of foam cell formation at the injury site (Barter et al., 2004, Brewer, 2004). The removal of cholesterol by HDL is promoted via interaction with the SR-B1 receptor and binding with the ABCA1 transporter. Excessive cholesterol obtained by HDL from arterial macrophages will be delivered to the liver and used for bile acids synthesis and excretion (Barter et al., 2004, Brewer, 2004). Also, HDL can significantly reduce the progression of atherosclerosis by protecting LDL from oxidation. This particular function of HDL is due to by the action of apoA-I and certain enzymes which are carried and/or located on the surface of the HDL particles (Kontush et al., 2003). ApoA-I as the primary HDL carrying apolipoprotein plays a vital role in inhibiting LDL oxidation. ApoA-I eliminates LDL-derived oxidised phospholipids and lipid hydroperoxides from LDL, and from the arterial wall injury site (Navab et al., 2004, Barter et al., 2004). HDL carries enzymes such as paraoxonase and platelet activating factor acetylhydrolase which play a role in degrading oxidised phospholipids from LDL and inhibiting LDL oxidation (Wang and Briggs, 2004). Furthermore, HDL plays an anti-
inflammatory role in inhibiting the expression of adhesion proteins in EC needed for the development of the atherosclerosis lesion. Also, HDL reduces monocyte collection and grouping at the site of injury in the early stages of atherosclerosis (Barter et al., 2004, Kontush et al., 2003). Finally, HDL particles play a protective role by producing certain atheroprotective factors such as Nitric Oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF). NO and EDHF are essential for controlling vasoconstriction and vasodilation within the artery (Rasmussen et al., 2009). Maintaining this artery function is necessary for preventing and/or reducing EC dysfunction. EC dysfunction takes place in the first stages of atherosclerosis which promotes the arterial lesion (Wang and Briggs, 2004).

1.7.4 Obesity

Obesity has become a widespread health problem in recent years. It is especially worrying that the increase was most noticed in children and that it occurred both in developed and increasingly in developing countries with low or intermediate income (Knight, 2011, WHO, 2015c, Westphal, 2008). Worldwide obesity doubled since 1980 (WHO, 2015c). It is recorded by world health organization (WHO) that in 2014, more than 1.9 billion adults were overweight in which 600 million of them were obese (WHO, 2015c, Westphal, 2008). Also, it is recorded that 42 million children under five years old were overweight or obese in 2013 (WHO, 2015c). Obesity is a globally independent risk factor for CHD (WHO, 2015c, WHO, 2015a). Body mass is positively related to fasting TG concentration, plasma cholesterol and blood pressure, and inversely related to HDL-cholesterol (HDL-C) concentration (Westphal, 2008). The distribution of body fat appears to be particularly important. Central or visceral obesity that is most strongly linked to IR and CHD risk (WHO, 2015a, Knight, 2011, WHO, 2015c, Westphal, 2008). Obesity, especially central obesity, increases the risk for metabolic
syndrome which is a cluster of risk factor for type 2 diabetes (T2D) and CVD (WHO, 2015a, WHO, 2015c, Knight, 2011, Westphal, 2008). These factors include dysglycaemia, high blood pressure, elevated TG concentrations, low HDL-C concentrations, and obesity in particularly central obesity (Knight, 2011, WHO, 2015a, Alberti et al., 2009, Westphal, 2008). The metabolic syndrome is strongly related to an increased risk of developing many metabolic diseases such as T2D and non-alcoholic fatty liver disease (NAFLD) (Alberti et al., 2009, Westphal, 2008). Obesity, IR and hyperinsulinaemia are strongly associated with T2D (Alberti et al., 2009, Ogbera, 2010) and NAFLD (Kotronen et al., 2007, Hsiao et al., 2007).

1.7.5 Insulin, IR, atherogenic dyslipidaemia and hyperinsulinaemia

Insulin is a polypeptide hormone produced by the β cells of the islets of Langerhans (Greenfield and Campbell, 2004, Kriketos et al., 2004, Dimitriadis et al., 2011). These cells are embedded in the exocrine portion of the pancreas. Insulin is the most important hormone coordinating the use of fuels by tissues. It is composed of 51 (amino acid) AA arranged in two polypeptide chains, designated A and B. These chains are linked together by two disulphide bridges. Insulin is carefully coordinated with the release of glucagon from pancreatic α cells. The relative amounts of both hormones released by the pancreas are regulated so that the rate of hepatic glucose production is kept equal to the use of glucose by peripheral tissues (Greenfield and Campbell, 2004, Kriketos et al., 2004, Dimitriadis et al., 2011). The synthesis and release of insulin are decreased during the scarcity of dietary fuels and during stress. These latter effects are mediated by adrenaline which is a hormone secreted by the adrenal medulla in response to stress, trauma, or extreme exercise. On the other hand, insulin secretion and synthesis are increased by glucose, AA, FA and gut hormones (Greenfield and Campbell, 2004, Kriketos et al., 2004, Dimitriadis et al., 2011).
Insulin exerts important actions on metabolism as shown in Figure 1.8. For instance, in the liver and muscle tissues, insulin increases glycogen synthesis, and glucose breakdown by increasing glycogenesis and glycolysis respectively. In muscle and adipose tissue, insulin increases glucose uptake by increasing the number of glucose transporters (GLUT-4) in the cell membrane. Also, in the liver, insulin decreases the production of glucose through the inhibition of glycogenolysis and gluconeogenesis (Greenfield and Campbell, 2004, Kriketos et al., 2004, Dimitriadis et al., 2011). Insulin also decreases TG degradation by inhibiting lipolysis in adipose tissue. This is accomplished by inhibition of hormone-sensitive lipase (HSL) activity which reduces the concentration of circulating FFA. Insulin also increases TG synthesis in adipocytes by increasing the rate of transport and metabolism of glucose into adipocytes, providing the substrate glycerol 3-phosphate for TG synthesis. Insulin also increases LPL activity in adipose tissue by increasing its synthesis, which increases TG hydrolysis releasing FAs needed for TG synthesis. Also, insulin in the liver promotes de Novo Lipogenesis, AA cellular uptake and protein synthesis (Greenfield and Campbell, 2004, Kriketos et al., 2004, Dimitriadis et al., 2011).
Figure 1.8: Insulin action on various nutrients metabolism.

An altered cellular biological response to insulin action is known as IR, and it is associated with T2D, obesity and metabolic syndrome (Greenfield and Campbell, 2004, Kriketos et al., 2004). Therefore, IR is a strong sign of the risk of developing T2D especially in obese individuals who are presented with central or visceral obesity (Greenfield and Campbell, 2004, Kriketos et al., 2004). In obese individuals, FFA flux from accumulated visceral fat is transported directly to the hepatocytes which triggers cellular gluconeogenesis and interferes with lipid metabolism resulting in the development of hepatic IR (Westphal, 2008). This pathogenesis is typically found among patients with NAFLD. This state is initiated by increased FFA flux to the liver due to altered suppression of lipolysis as a result of peripheral IR and hyperinsulinaemia. That will lead to the accumulation of TG in the hepatocytes which eventually causes hepatic IR and NAFLD development (Westphal, 2008). This will potentially lead to the development of a state of dyslipidaemia which manifests increased LDL and TG concentrations, and decreased HDL concentrations.
IR is also related to the development of an atherogenic plasma lipid profile which is found among patients with metabolic syndrome, T2D and NAFLD (Westphal, 2008, Adiels et al., 2006, Guerin et al., 2001, Sobenin et al., 1996, Grundy, 1997, Bril et al., 2016, Carmena et al., 2004). This is reflected in altered lipid metabolism and altered LPL and HL activities in the peripheral tissues and the liver respectively. That is due to increased lipolysis, circulating FFA, and accumulation of TG in the hepatocyte which results in hypertriglyceridaemia and the increased size and production of TRL (Westphal, 2008, Adiels et al., 2006, Guerin et al., 2001, Sobenin et al., 1996, Grundy, 1997, Bril et al., 2016, Carmena et al., 2004). This results in an increase in IDL and LDL concentrations, both of which are atherogenic due to their role in enhancing the progression of atherosclerosis (Westphal, 2008, Adiels et al., 2006, Guerin et al., 2001, Sobenin et al., 1996, Grundy, 1997, Bril et al., 2016, Carmena et al., 2004). As a result, LDL particles interact with the expanded TG-rich plasma pool leading to an exchange of CE and TG facilitated by CETP (Westphal, 2008, Adiels et al., 2006, Guerin et al., 2001, Sobenin et al., 1996, Grundy, 1997, Bril et al., 2016, Carmena et al., 2004). This will lead to the production of SDLDL (Frayn, 2010, Barter, 2000). These particles are at more risk of oxidative damage as they lose their core lipids and fat-soluble antioxidant vitamins (Westphal, 2008, Adiels et al., 2006, Guerin et al., 2001, Sobenin et al., 1996, Grundy, 1997, Bril et al., 2016, Carmena et al., 2004). Also, SDLDL can infiltrate the arterial wall radially, oxidised, and taken up by macrophage scavenger receptors to initiate the process of foam-cell formation. As described earlier, foam-cell formation is a significant step in atherosclerosis. Hence, SDLDL particles are directly associated with increasing the risk of CHD and CVD (Westphal, 2008, Adiels et al., 2006, Guerin et al., 2001, Sobenin et al., 1996, Grundy, 1997, Bril et al., 2016, Carmena et al., 2004).
IR also leads to hyperinsulinaemia. Eventually, this can contribute to the development and increased risk of CVD via atherosclerosis (Bugianesi et al., 2005, Giovannucci, 2007). There is evidence that hyperinsulinaemia causes changes in the arterial wall by enhancing smooth muscle cell proliferation (Bugianesi et al., 2005, Giovannucci, 2007), and this is, as mentioned earlier, essential in the development of atherosclerosis. As the condition progresses, it will cause blood vessel inflexibility (altered arterial elasticity) which controls resistance in the peripheral circulation and regulates blood pressure (Bugianesi et al., 2005, Giovannucci, 2007). Further progression of this condition will lead to hypertension which is indeed a crucial factor for CHD and CVD (Giovannucci, 2007).

1.8 NAFLD and T2D Mellitus as CVD risk factors

These two diseases are both manifestations of the metabolic syndrome and are associated with an increased risk of CVD. In both conditions, three key major metabolic risk factors can be found; IR, obesity, hypertriglyceridaemia, atherogenic lipoprotein phenotype (ALP) and hyperinsulinaemia (Westphal, 2008, Giovannucci, 2007). Therefore, both diseases are an area of interest as major factors for increasing the risk of CVD.

1.8.1 Non-alcoholic fatty liver disease (NAFLD)

NAFLD is a disorder with elevated lipid deposition in the liver (steatosis) that can lead to the development of an inflammatory condition called non-alcoholic steatohepatitis (NASH) which could lead to an end-stage disease or death (Vernon et al., 2011). NAFLD is considered the most common liver disease as it is estimated that around 20-30% of the adult population in the developed countries have NAFLD (Bellentani et al., 2010). There are several stages of NAFLD progression starting with steatosis (Preiss and Sattar, 2008). The process of steatosis is the abnormal retention of TG within the cell cytoplasm indicating impairment in the
synthesis and clearance of cellular TG. Progression of hepatic steatosis to NASH can increase the risk of developing liver fibrosis, cirrhosis, and hepatocellular carcinoma leading to death (Musso et al., 2011).

The major risk factor that contributes to NAFLD is obesity (Preiss and Sattar, 2008). It is the primary driver for the development and prevalence of NAFLD. Hepatic cellular lipid retention is strongly correlated with central obesity (Sung et al., 2012, Seo et al., 2012). Also, ethnicity and genetic predispositions are potential risk factors based on preliminary data (Petersen et al., 2006). Importantly, there is a close link between T2D and NAFLD as both are considered as a risk factor for the other. NAFLD is associated with peripheral and hepatic IR, as explained previously, and contribute to increasing the risk of CVD in patients with metabolic syndrome (Kotronen et al., 2008a, Hsiao et al., 2007).

1.8.1.1 Diagnosis

NAFLD patients are often asymptomatic with abnormal liver function results. The essential element of NAFLD is the accumulation of TG as fat droplets within the cytoplasm of hepatocytes. The cut-off value is the accumulation of more than 5-10% of fat droplets in hepatocytes in the absence of alcohol consumption, and liver biopsy is an evident of a prerequisite for subsequent events of NASH (Hsiao et al., 2007, Miyake et al., 2015). Also, a typical biochemical pattern of increased concentrations of alanine aminotransferase (ALT) exceeding those of aspartate aminotransferase (AST) is necessary for NAFLD diagnosis (Sorbi et al., 1999). This standard pattern is particularly suitable for differentiating between hepatic steatosis from NAFLD and alcoholic liver injury, with the latter frequently associated with a high AST: ALT ratio (Sorbi et al., 1999). However, AST concentrations increase with a resultant rise in the AST: ALT ratio in the progression of hepatic steatosis to NASH and
hepatic fibrosis (Sorbi et al., 1999). Gamma-glutamyltransferase (γGT) might likewise increase (Sorbi et al., 1999). NAFLD can also be identified using hepatic echogenicity on abdominal ultrasonography (Westerbacka et al., 2004). History of past results for T2D or fasting glucose and/or HbA1c, lipid profiles, alcohol intake, weight are needed to confirm NAFLD diagnosis (Fraser et al., 2009, Wong et al., 2006, Chatterton et al., 2012). Also, computed tomography (CT) scan, and magnetic resonance spectroscopy (MRS) imaging can assess liver lipid stores of the abdominal region. Liver biopsy is needed to determine NAFLD progression to NASH (Miyake et al., 2015).

1.8.1.2 NAFLD treatment and management;

Lifestyle intervention is essential for managing NAFLD. Healthy diets with low fat intake and high intake of fibre and carbohydrates are advised which can lead to weight loss and decreased lipid storage (Thoma et al., 2012). Also, physical activity increases fat metabolism and reduces lipid storage (Thoma et al., 2012, American Diabetes Association, 2016). The combination of both approaches is considered key in the management of NAFLD and it is essential if the patient has T2D (American Diabetes Association, 2016). Finally, regarding drug treatment, numerous trials of vitamin E, metformin and statins have failed to demonstrate evidence in reducing liver disease progression. However, some studies have shown some improvement in early stages of NASH in patients with NAFLD under treatment with insulin sensitizers to treat hepatic IR such as pioglitazone (Shyangdan et al., 2011). There is an evidence showing that long-chain n-3 polyunsaturated fatty acids (PUFA) is a potential treatment for NAFLD in which they stimulate HL activity in the liver to which suppresses lipogenesis and increases FA oxidation (Clarke and Jump, 1997, Clarke et al., 1998), and increased LPL activity in the adipose tissue which increasing TRL removal and TG
uptake (Jacobson, 2008, Khan et al., 2002, Harris et al., 1997). It is been shown that n-3 PUFA cause the redistribution of atherogenic sdLDL which reduces the intensity of ALP associated with NAFLD (Griffin, 2001)

1.8.1.3 The effect of Exercise on altered lipid metabolism in patients with NAFLD

It has been demonstrated in many studies that exercise can improve atherogenic dyslipidaemia in patients with NAFLD (Burton et al., 2008, Farah et al., 2010, Tsetsonis and Hardman, 1996, Zhang et al., 2002, Bril et al., 2016). Exercise has been shown to increase blood flow to the exercised muscle and the liver (Hurren et al., 2011). Increased skeletal muscle blood flow is necessary for postprandial TG clearance thereby increasing capillary blood volume and allowing an opportunity for more widespread hydrolysis of circulating TG (Hurren et al., 2011). Increased blood flow will increase hepatic and peripheral insulin sensitivity (Shojaee-Moradie et al., 2007, Borghouts and Keizer, 2000). Increased insulin sensitivity will increase the rate of hydrolysis in the hepatocytes and decrease adipocyte lipolysis (Patsch, 1998). An elevated hydrolysis rate in the liver will directly contribute to increasing insulin sensitivity by hydrolyzing cellular fat droplets which reduce fat cellular accumulation (Hurren et al., 2011, Cuthbertson et al., 2016, Shojaee-Moradie et al., 2007). Exercise increases whole body fat oxidation especially in skeletal muscles and the liver, and will lead to increase the activity of LPL and HL via decreasing insulin concentration and IR (Al-Shayji et al., 2012, Shojaee-Moradie et al., 2007, Cuthbertson et al., 2016). Also, an exercise-induced energy deficit may stimulate skeletal muscle LPL activity and hence increased TRL hydrolysis and FFA release for cellular uptake (Kantor et al., 1987, Wang and Eckel, 2009, Zhang et al., 2002). Ultimately this enhances the reduction in circulating FFA and hence improving overall insulin sensitivity (Shojaee-Moradie et al., 2007). There is evidence that
exercise decreases VLDL$_1$-TG particle size, an effect that may raise the affinity of these particles for LPL hydrolysis and thus clearance from the circulation (Al-Shayji et al., 2012). As a result, more TRL are hydrolyzed, and TG concentration will decrease in the plasma (Wang and Eckel, 2009). Also, this will lead to an increase in glucose uptake in the peripheral tissues due to decreased IR, and that will result in reducing insulin secretion and correct hyperinsulinaemia (Newsom et al., 2010, Tuominen et al., 1997, Shojaee-Moradie et al., 2007).

Several studies have shown that exercise increases HDL-C in patients with altered lipid metabolism (Escola-Gil et al., 2015). However the possible mechanisms behind this are not yet clearly understood and further investigations are required. One possible hypothesis for this is that exercise decreases plasma TRL (Bellou et al., 2013, Al-Shayji et al., 2012, MacEneaney et al., 2009) which will lead to decrease the rate of exchange of TG and CE between TRL and HDL facilitated via CETP (Barter, 2000, Frayn, 2010), which may reduce the formation of $s_{50}$LDL and increases HDL-C concentrations which are manifestations of the ALP (Toft-Petersen et al., 2011).

### 1.8.2 Type 2 Diabetes (T2D)

T2D is a chronic disorder which is associated with impaired glucose and lipid metabolism with severe clinical consequences if not prevented and/or managed (WHO, 2015b). In 2014, it was estimated that the global prevalence of diabetes was 9% among adults above 18 years old (WHO, 2015b). In 2012, about 1.5 million deaths were directly caused by diabetes (WHO, 2015b). Also, it is recorded that more than 80% of diabetes deaths occur in developing countries with low or intermediate income. The WHO have estimated that diabetes will be the seventh leading cause of death in 2030 (WHO, 2015b). Various complications of diabetes
can affect the body (WHO, 2015b) such as microvascular complications include neuropathy, nephropathy and retinopathy, and macrovascular complications which include stroke, ischaemic heart disease and peripheral CVD (WHO, 2015a, WHO, 2015b). The prevalence of T2D has been rising in the last few decades, accompanied by the worldwide increase in obesity (WHO, 2015c). T2D is a significant public health disorder associated with increased morbidity and reduced life expectancy. It imposes a financial burden on the patient and the health service provider (WHO, 2015b, American Diabetes Association, 2016).

1.8.2.1 Diagnosis of T2D

T2D classification and diagnosis has been a subject of much consultation, debate and revision. Expert teams such as the WHO and the American Diabetes Association (ADA) have established the diagnostic criteria for diabetes, which are based on the measurement of fasting or 2-h post-glucose load concentration (WHO, 2015b, American Diabetes Association, 2016). The aetiological classification of diabetes has now been widely accepted, with type 1 and T2D Mellitus being the two primary types of diabetes, and T2D accounting for the majority 90% of total diabetes prevalence (WHO, 2015b, WHO, 2015c, American Diabetes Association, 2016).

The diagnosis of T2D can be determined by measuring fasting plasma glucose concentration in a blood sample, and it can be determined by using an oral glucose tolerance test (OGTT) as recommended by the WHO (WHO, 2015b). T2D is diagnosed if fasting plasma glucose concentration is $\geq 7$ mmol/l, and $\geq 11$ mmol/l two hours after a 75g OGTT (WHO, 2015b, American Diabetes Association, 2016). In 2009, the ADA agreed to use haemoglobin A$_1$C (HbA$_1$C) in the diagnosis of diabetes in the United States of America (USA) but not it is yet used in the United Kingdom (American Diabetes Association, 2016). The USA decided that
with the greater accessibility of a standardised HbA1c assay, it is now possible to use HbA1c as a diagnostic test (American Diabetes Association, 2016, WHO, 2015b). The main advantages of using HbA1c are practical as a random blood sample is adequate, and there is no necessity for special patient preparation. The ADA has adopted the recommendation of the expert committee on a cut-off value for the diagnosis of diabetes. The cut-off value is an HbA1c of 6.5% or more. It equals 47.5 mmol/mol in International Federation of Clinical Chemistry (IFCC) unit or 7.8 mmol/l (The Global Diabetes community, 2011, American Diabetes Association, 2016).

1.8.2.2 Altered lipoprotein metabolism in T2D

T2D is characterised by hyperglycaemia, IR, and impaired insulin secretion. IR has a significant role in the development of the characteristic ALP associated with T2D including reduced HDL and increased LDL concentrations (Adiels et al., 2006, Sobenin et al., 1996, King et al., 2011, Matikainen and Taskinen, 2013, Pettersson et al., 2011). It is noted that patients with T2D have increased fasting and postprandial TG and apoB-48 (Lewis et al., 1991, Meng et al., 1983, Schaefer et al., 2002, Taniguchi et al., 2000, Curtin et al., 1996). Elevated concentrations of postprandial CM and CMRs in IR have been mainly attributed to impaired TRL clearance from the circulation (Shojaee-Moradie et al., 2013). Indeed, increased plasma apoB-48 is related to reduced catabolic rates of the CMRs in patients with T2D (Dane-Stewart et al., 2003, Hogue et al., 2007a). Therefore, clearance of CMRs could be impaired as a result of increased hepatic-VLDL secretion in IR (Lewis, 1995). That is because increased availability of TG and cholesterol in T2D increases VLDL-hepatic synthesis which might affect the LPL activity in clearing CMR (Lewis, 1995). Slow removal of CM and CMRs by reduced LPL activity is also addressed in IR (Kobayashi et al., 2007) due to the diminished
regulation of LPL by insulin (Patsch, 1998). Overproduction of intestinal lipoproteins has been recently recognised as a feature of IR. Studies have demonstrated that the intestine actively contributes to elevating TRL, as CM and VLDL particles, in the state of IR by increased CM production (Adelis and Lewis, 2008, Duez et al., 2008b). Several studies have shown high intestinal production of lipoproteins in IR and obesity in humans (Su et al., 2009, Mamo et al., 2001, Chan et al., 2002b, Cohn et al., 1999, Phillips et al., 1997, Shojaee-Moradie et al., 2013). Recent studies suggested that obesity and IR may stimulate the action of MTP, which is responsible for assembling the apolipoprotein to the lipid fraction, which would increase TRL intestinal production by increasing TG per particle (Xiao et al., 2012).

An important regulatory mechanism of apoB-100 production is the availability of TG and cholesterol in the liver (Sniderman and Cianflone, 1993, Thompson et al., 1996). Many studies have shown that obese and insulin resistant individuals have increased hepatic VLDL-apoB-100 secretion compared with non-obese individuals (Riches et al., 1998, Chan et al., 2002a). In IR, hepatic synthesis of cholesterol and TG substrates is elevated. IR also causes the impairment of LDL-receptor activity and expression needed for LDL-apoB-100 clearance (Taskinen, 2002, Ginsberg, 2003). Other studies have demonstrated that T2D patients have a significant increase in VLDL particle production as a result of improved hepatic secretion of VLDL1 apoB-100 (Adiels et al., 2006). IR effects can be partly mediated by visceral adipose fat mass accumulation, the elevated flux of FA from adipose tissue to the liver, and induced fat accumulation in the liver. Also, chronic hypertriglyceridaemia in T2D increases HDL interaction with expanded TG plasma pool resulting in the production of enriched TG-HDL particles and SDLDL. Collectively with hyperglycaemia, this leads to increased VLDL1 particles secretion and resulting in the formation of ALP (Adiels et al., 2006).
1.8.2.3 Prevention and management of T2D

High-risk individuals with impaired glucose tolerance (Hupe-Sodmann et al., 1995), or those with T2D, can be managed by intensive lifestyle intervention or drug therapy with glucose-lowering agents such as metformin (Wareham and Griffin, 2001). Also, insulin administration can be a treatment in T2D when medication fails to correct hyperglycaemia. Furthermore, lifestyle interventions comprise preservation of a healthy body weight through reducing sedentary behaviour, increasing physical activity and eating a healthy diet rich in fibre and low in fat (WHO, 2015b). Screening has been proposed in the anticipation that early detection and early treatment would reduce the long-term liability to individuals and the health services (WHO, 2015b). However, most authorities such as the National Health Service (NHS) have suggested additional research and limited screening targeting high-risk subgroups to find a better and/or improved treatment. There are many ways in which high blood glucose and associated dyslipidaemia can be reduced directly and/or indirectly through various drug actions and/or gastrointestinal bypass surgery (American Diabetes Association, 2016, WHO, 2015b, Pok and Lee, 2014, Bradley et al., 2012). Most of these drugs will control and/or manage diabetic associated hyperglycaemia without having a significant effect on controlling or correcting associated altered lipid metabolism. Glucagon-like peptide-1 (GLP-1) receptor agonists have been recently approved as a treatment for uncontrolled hyperglycaemia, and it is been shown that it has a secondary effect on lipoprotein metabolism (Xiao et al., 2012).
1.9 GLP-1 and T2D

1.9.1 GLP-1 and its actions

GLP-1 is an incretin hormone composed of 30 AA derived from the proglucagon gene, which is secreted by neuroendocrine enteroglucagon-producing cells in the lower gut (L cells). L cells are located in the duodenum, jejunum, ileum, and the colon. GLP-1 secretion begins at the L cells which are directly stimulated by digested nutrients (AA, FA and glucose) and digestive juices such as bile acids. Nutrients interact with their apical surface which promotes GLP-1 secretion which reaches its highest concentration in blood plasma within 10 min of consumption (Persson et al., 2000, Holst, 2007). GLP-1 secretion is also indirectly stimulated by other neural and endocrine factors such as a gastrin-releasing polypeptide (GRP), glucose-dependent insulinotropic polypeptide (GIP), and impulses transmitted by the vagus nerve through acetylcholine by interacting with neuronal and vascular tissues on the basolateral side of enterocytes (Fehmann et al., 1995, Holst, 2007, Baggio and Drucker, 2007). GLP-1 diffuses through the basal lamina into the lamina propria and enters into circulation by intestinal capillaries, draining into the hepatic portal vein to act primarily at the pancreas on β cells, and finally into the systemic circulation (Steinert et al., 2009, Steinert et al., 2010). Once secreted into the circulatory system, the enzyme dipeptidyl peptidase-4 (DPP-4) mainly breaks down GLP-1 within minutes (Meier et al., 2004), and its elimination occurs by renal clearance through glomerular filtration and catabolism (Baggio and Drucker, 2007, Takei and Kasatani, 2004). Due to very efficient removal mechanisms especially by DPP-4 activity, the concentration of the active GLP-1 constitutes only 20% of its overall concentration (Deacon et al., 1995).

GLP-1 binds to a particular receptor, GLP-1 receptor (GLP-1R), which shows a similar structure to the glucagon receptor (Wilmen et al., 1998). GLP-1R is widely expressed in
pancreatic islets, where GLP-1 stimulates insulin secretion, decreases glucagon concentration, and suppresses its release (Figure 1.9) (Takei and Kasatani, 2004, Taskinen, 2005, Holst et al., 2011, Baggio and Drucker, 2007). GLP-1R has the classic structure of adenylate-cyclase-coupled and G-protein-coupled receptors. GLP-1 activates the GLP-1R signalling pathway in a glucose-dependent way, i.e., only during hyperglycaemia (Baggio and Drucker, 2007, Portha et al., 2011). The molecular mass of the GLP-1R is about 65 kDa. GLP-1 stimulation results in an elevated intracellular cyclic adenosine monophosphate (cAMP) and calcium concentration, which in β cells is an indication of exocytosis of early synthesised insulin (Drucker et al., 1987). Also, protein kinase A activation promotes the biosynthesis of insulin and gene expression modification (Drucker, 2006) as shown in Figure 1.9. GLP-1R is also expressed in the gastrointestinal tract (GIT) such as gastric mucosal gland cells in the stomach and enterocytes of the intestines (Broide et al., 2013, Xiao et al., 2012). GLP-1 acts by decreasing the rate of gastric emptying and CM production respectively (Gutzwiller et al., 1999, Broide et al., 2013, Xiao et al., 2012). Also, GLP-1R is expressed in the brain, where GLP-1 functions as a neurotransmitter in the hypothalamus, which controls satiety (Neumiller, 2011).
In patients with T2D, it has been suggested that the activity of GLP-1 is diminished, but the numbers of GLP receptors are unaltered. The reason behind this is debatable; it has been demonstrated that diminished effects of endogenous GLP-1 are due to chronic hyperglycaemia, hyperinsulinaemia and IR features found in T2D (Meier and Nauck, 2010). In patients with T2D, GLP-1 secretion cannot cope with chronic hyperglycemia resulted from IR (Meier and Nauck, 2010). Therefore, B cells become exhausted primarily due to continual insulin production in response to hyperglycaemia besides the secondary response to the minimal concentration of endogenous GLP-1 which diminishes its action (Arulmozhi and Portha, 2006, Marre and Penfornis, 2011, Meier and Nauck, 2010).

Another explanation is that hyperleptinaemia and leptin resistance in obese individuals diminishes GLP-1 actions (Anini and Brubaker, 2003). Leptin resistance state could lead to the development of hyperleptinaemia (Anini and Brubaker, 2003). This results in the development of IR, hyperinsulinaemia, fat accumulation and obesity which are all
manifestations of T2D (Anini and Brubaker, 2003). There is growing evidence for the existence of an adipo-enteroendocrine axis which is responsible for the regulation of nutrient homoeostasis, genetic predisposition and stress responses (Anini and Brubaker, 2003). It is associated with decreased nutrient-stimulated GLP-1 secretion via leptin resistance and altered lipolysis in obese individuals (Anini and Brubaker, 2003).

1.9.2 GLP-1 receptor agonists

GLP-1 receptor agonists are used for T2D therapy based on its actions on plasma blood glucose concentrations (Sadrzadeh et al., 2007, Vilsboll et al., 2001). However, endogenous GLP-1 is limited due to the metabolic instability caused once DPP-4 enzyme rapidly cleaves its N-terminal. The rapid cleavage of the secreted GLP-1 is almost immediate, resulting in a short half-life of less than 2 min. DPP-4 is mainly found on the luminal surface of the ECs, which means that a large portion of the GLP-1 that leaves the gut is already degraded as an inactive metabolite (Baggio and Drucker, 2007, Takei and Kasatani, 2004), and only 25% of the GLP-1 secreted reaches the portal circulation. Additionally, 40-50% of GLP-1 that bypasses gut is metabolised in the liver, and only the remaining 10-15% enters the systemic circulation (Holst, 2007). Long-acting GLP-1 receptor agonists have been developed to overcome premature GLP-1 metabolism by DPP-4. Alternatively, DPP-4 inhibitors can be used alone and are also being considered for concomitant administration with GLP-1 receptor agonists (Unger, 2011). However, the action of GLP-1 receptor agonists is independent of the concentration of endogenous GLP-1 and provides much higher pharmacological concentrations than the DPP-4 inhibitors (Unger and Parkin, 2011, Garber, 2011).
In 2005 and 2010, two GLP-1 receptor agonists, Exenatide (Bydureon®) and Liraglutide (Victoza®), were approved by the Food and Drug Administration for the treatment of T2D (Li et al., 2011). Exenatide (4186.6 Da) is the synthetic form of exendin (Arulmozhi and Portha, 2006), a peptide isolated from the salivary gland of Gila monster (Heloderma suspectum), showing 53% homology to GLP-1. It has a half-life of 2.4 hours and acts by binding GLP-1R at least with the same affinity as native GLP-1 (Marre and Penfornis, 2011, Ahren, 2011, Garber, 2011, EMA, 2011). On the other hand, Liraglutide (3751.2 Da) shows 97% homology to GLP-1, being very similar to native peptide, and altered by only one AA substitution, being further linked by a FA side chain. This FA portion allows a non-covalent binding with serum albumin, which increases the half-life of the peptide for approximately 13 hours, resulting in slow degradation and clearance by DPP-4. Regardless of the modifications, the ability of binding with GLP-1R remains the same. Both Exenatide and Liraglutide are delivered subcutaneously and have been shown to improve glycemic control (Marre and Penfornis, 2011, Li et al., 2011, Deacon, 2009). Many other GLP-1 receptor agonists are in late clinical development (Kalra et al., 2009); thus new agents are anticipated to reach the market in the future.

Lixisenatide (Lyxumia®), is a GLP-1 receptor agonist that has been safety tested and was licensed for use in the EU in February 2013 for the management of glycemic control in T2D patients. It is recommended to be given as a once-daily dose due to its half-life of 3 hours with long duration of action (EMA, 2013). It is recommended that lixisenatide should be given as combined treatment with oral glucose-lowering medicinal products and/or basal insulin when adequate glycemic control is not achieved even with controlled diet and increased physical activity (EMA, 2013).
1.9.3 GLP-1 effect on lipoprotein metabolism

GLP-1 receptor agonist modulation of fasting glucose and HbA₁c concentrations in T2D patients have been well established by many studies (Drucker and Nauck, 2006). In a study conducted by Cummings et al. (2010), the administration of Liraglutide into a young male T2D rat model caused a reduction in fasting plasma TG concentrations by about 50–60% in Liraglutide-treated animals compared with the control and food-restricted rats. Also, liver TG content was found lower in both Liraglutide-treated and food-restricted animals compared with control animals (Cummings et al., 2010). Chronic administration of CNTO736, a novel GLP-1R agonist, was found to inhibit VLDL secretion in high-fat fed mice, whereas acute administration did not seem to affect this pathway (Parlevliet et al., 2009a). More recently, these findings were confirmed by a 2-week treatment of fructose-fed hamster and mouse models with exendin-4 which illustrated the significant decrease in intestinal CM secretion when compared to controls (Hsieh et al., 2010). In a recent study conducted in Japan, serum concentrations of TG were also decreased after three months of treatment in 188 T2D patients with 50 mg Sitagliptin, a DPP-4 inhibitor, for the first three months (Sakamoto et al., 2013). In two separate studies, exenatide treatment in T2D patients resulted in a significant reduction in postprandial TG concentrations (Schwartz et al., 2008, DeFronzo et al., 2008) and Vildaglaptin treatment, a DPP-4 inhibitor, decreased postprandial elevation of TG concentrations and apoB-48 in T2D patients (Matikainen et al., 2006). Interestingly, Meier et al. (2006) studied the effect of acute synthetic GLP-1 infusion on a test meal on TG concentrations in healthy participants. GLP-1 infusion during meal intake resulted in complete prevention of a postprandial increase in plasma TG by delaying in gastric emptying (Meier et al., 2006).
In a study published by Schwartz et al. (2010) which focused on the acute effects of GLP-1R modulation with exenatide on postprandial lipoprotein and TG concentrations in patients with recent onset T2D and impaired glucose tolerance (Schwartz et al., 2010a). Exenatide decreased the lipid content and the number of intestinally-derived lipoprotein particles (Schwartz et al., 2010a). Furthermore, the study showed that Exenatide treatment enhanced apoB-48 lipoprotein particle clearance, and decreased intestinal particle secretion into the circulation (Schwartz et al., 2010a). In a study by Xiao et al. which investigated the effect of exenatide treatment on postprandial production of CM-apoB-48, exenatide significantly suppressed TG concentration and the production rate (PR) of CM-apoB-48 (Xiao et al., 2012). These results suggest possible direct effects of Exenatide on the PR of intestinal lipoprotein particles (Xiao et al., 2012).

It is important to note that postprandial lipaemia reduction via correcting hypertriglyceridaemia is possibly anti-atherogenic (Greene et al., 2001), and may be a novel approach to hyperlipidaemia treatment associated with other conditions. Therefore, it is important to elucidate the role of GLP-1 receptor agonists on hepatic and intestinal lipoprotein PRs and to determine the mechanism of this effect. One well-established method to investigate lipid metabolism in humans in vivo is the use of isotope-labelled tracers for the quantitative evaluation of TG metabolism (Magkos and Mittendorfer, 2009).

T2D patients with improved metabolic control have less postprandial apoB-48-containing particles (Phillips et al., 2000), and an intact insulin signalling pathway acutely suppresses hepatic VLDL secretion and intestinal CM production in vivo (Levy et al., 1996). It is important to note that increased insulin sensitivity in T2D patients treated with GLP-1 receptor agonists may decrease the elevation of postprandial hepatic VLDL-apoB-100 synthesis and intestinal CM-apoB-48 production through direct and indirect effects (Levy et
al., 1996, Phillips et al., 2000). As a consequence, this will correct postprandial hypertriglyceridaemia and lessen the exchange rate of CE and TG transfer between HDL particles and the TRL-TG pool in the plasma via CETP (Barter, 2000, Diffenderfer et al., 2012, Frayn, 2010). This may lead to a decrease in TG-enriched HDL formation and hence reduces the clearance of HDL-apoA-I from the circulation (Zannis et al., 2006). Consequently, this will increase the concentration of functional HDL-C and apoA-I and decrease sLDL, which contributes to reducing the risk of CVD (Lamarche et al., 1999, Patsch, 1998, Greene et al., 2001, Barter, 2000, Diffenderfer et al., 2012).

1.9.4 GLP-1 potential direct effects on apolipoprotein-B and HDL-apoA-I

Direct effects of GLP-1 on the enterocytes can be observed in rats where recombinant GLP-1 inhibited lymph flow, lymph TG absorption, and lymph apob-48 secretion in response to the intraduodenal fat infusion (Qin et al., 2005). Also, GLP-1 caused a direct effect on apob-48 secretion in the enterocytes observed in hamsters and mice (Hsieh et al., 2010). In humans, a study proposes that exenatide decreases the lipid content and the number of intestinally-derived lipoprotein particles (Schwartz et al., 2010a). Also, the recent study conducted by Xiao et al. (2012) indicated possible direct involvement of GLP-1R signalling in intestinal lipoprotein particle secretion. The suggested effects of GLP-1 resulted in suppressing TRL intestinal production at least partly by directly suppressing CM assembly, and CM flow in the enterocytes (Xiao et al., 2012) but not hepatic VLDL-apoB100. The study also suggested that the decrease in CM-apoB-48 by exenatide not only by reducing apoB-48 protein synthesis but also CM packaging with neutral lipids, without suppressing and/or up-regulating MTP in the enterocytes (Xiao et al., 2012). Moreover, the study suggested that GLP-1 may suppress apoB-48 mRNA translation, apoB-48 intracellular trafficking, and stability in the enterocytes.
(Xiao et al., 2012). Although human liver cells contain GLP-1R, there are no clinical studies showing direct active signalling of GLP-1 on human hepatocytes regarding VLDL-apoB100 secretion. However, it is important to mention that MTP is expressed in human hepatocytes, and it is essential for VLDL-apoB100 synthesis. Therefore, GLP-1 might exert similar effects on VLDL-apoB100 in the liver which is seen in CM-apoB48 in the intestinal enterocytes.

Furthermore, GLP-1 can exert a direct impact on VLDL-apoB-100 and HDL-apoA-I metabolism. Many studies have investigated the effect of GLP-1 receptor agonist on HDL-C concentration but not HDL-apoA-I in humans. For instance, a study conducted by Chehade et al. (2013) investigated the direct impact of GLP-1 on apoA-I gene expression in HepG2 liver cells and Caco-2 intestinal cells (Chehade et al., 2013). This study showed that GLP-1 induces apoA-I gene expression in HepG2 liver cells but not Caco-2 intestinal cells (Chehade et al., 2013). Also, it was demonstrated in the same study that GLP-1 induces mRNA concentrations of ABCA1 which is strongly related to apoA-I and HDL metabolism (Wang and Smith, 2014). Chehade et al. (2013) found that GLP-1 induced apoA-I gene expression through an effect on the transcription factor SP1 and an insulin-responsive core element (IRCE) (Taylor et al., 1996, Lam et al., 2003, Zheng et al., 2001, Chehade et al., 2013, Rye et al., 2016). It is essential to confirm these effects of GLP-1 on apoA-I and ABCA1 gene expression in human enterocytes and hepatocytes. Therefore, to confirm these potential direct effects, more clinical studies need to be performed.

1.10 NAFLD, T2D and CVD;

Genetic factors in combination with obesity and poor lifestyle can lead to the development of IR (Pietilainen et al., 2005, Kechagias et al., 2008, Valtuena et al., 2006). In this condition, insulin’s action on tissues will be impaired. Increased lipolysis in IR will lead to the increase in delivery and synthesis of FFA in the liver (Fabbrini et al., 2008, Kotronen et al., 2008a,
Kotronen et al., 2008b). As a result, this will cause an increase in the synthesis of TG and the evolvement of hepatic steatosis (Dowman et al., 2010). Increased VLDL-TG synthesis will result in the presence of hypertriglyceridaemia and reduced HDL-C concentrations (Dowman et al., 2010). In IR, gluconeogenesis and glycolysis increases, and uptake of glucose by muscles and adipose tissue is reduced which will lead to hyperglycaemia and hyperinsulinaemia (Lewis et al., 1993, Adiels et al., 2006). All the previous co-existing events will give rise to the development of T2D and/or NAFLD (Rashid et al., 2003, Eckel et al., 2010). Also, an elevated postprandial TG plasma pool will increase TRL concentration which increases the risk of atherogenic dyslipidaemia in patients with T2D and NAFLD (Alcala-Diaz et al., 2014, Aathira and Jain, 2014, Tushuizen et al., 2010, Griffin et al., 1994). In both conditions, these co-existing factors take place although it is not necessary that a patient with NAFLD will develop T2D or vice versa. However, both diseases could be present in the same patient, and each disease if not treated and/or managed could lead to the development of the other (Eckel et al., 2010, Bulum et al., 2011). Also, both diseases are a manifestation of IR and obesity and lead to the increased incidence of CHD and CVD through atherogenic dyslipidaemia (Taskinen and Boren, 2015, Manoria et al., 2013, Hassing et al., 2014, Tushuizen et al., 2010, Nicholls and Lundman, 2004). Patients with NAFLD but without T2D have a slight advantage is that their pancreas β-cells are still intact (not exhausted like in patients with T2D) and capable of producing adequate insulin when IR is corrected. While patients with T2D do not necessarily develop liver steatosis and/or NASH, however, they need to maintain their normal glucose concentrations using glucose-lowering agents to balance reduced insulin secretion and IR condition.
1.11 Stable isotope technique for measuring CMs and VLDL

Investigation of lipid metabolism and its regulation requires information about the rates of lipid absorption into the body (from the intestine), production and transport in the body and utilisation by different tissues. In nature, there are three occurring isotopes of carbon: 12, 13, and 14; in which $^{12}\text{C}$ and $^{13}\text{C}$ are stable in a proportion of approximately 99:1 as seen in Figure 1.10. Stable isotope tracer molecules are naturally occurring, non-radioactive isotopes of a given atom that are less abundant in a particle within a biological system than the lightest naturally occurring isotope (Boren et al., 2012). The use of stable isotopes revolves around the concept of ‘enrichment’. Enrichment is defined as the tracer/tracee ratio (TTR) within a sample, with the ‘tracer’ being the labelled substance introduced into the system and the ‘tracee’ is defined as the substance of interest within the body to be traced. Isotopically labelled materials, for example, an AA tracer can be administered orally or by an intravenous infusion to trace apolipoproteins in TG particles. Apolipoproteins are essential for the synthesis and assembly of TG molecules in vivo as explained earlier. Repeated feeding protocol is used to establish a constant TG concentration in the blood or a TG ‘steady state’. This steady state enables the measurement of apolipoproteinB kinetics accurately within TRL particles. Enrichment is measured by gas chromatography-mass spectrometry (GC-MS) which can separate and quantify stable isotope tracers according to molecular mass (Boren et al., 2012). In this study, the rate of incorporation of infused $^{13}\text{C}$ tracer (1-$^{13}\text{C}$- leucine) in apoB-100 and apoB-48 moiety of VLDL and CM respectively, and the concentration of both apoB-100 and apoB-48 and $\alpha$-ketoisocoporate ($\alpha$-KIC), as a measurement of cellular precursor pool, will be used to determine the kinetics of VLDL-apoB-100 and CM-apoB-48. This determination will reflect the rate of VLDL and CMs metabolism, as each molecule of VLDL, or CM is assembled with one particle of apoB-100 or
apoB-48 respectively. The measurement of CM and VLDL kinetics has been undertaken in many studies as shown in Table 1.3 which shows key information from previous studies such as the meal content, meal intervals, study duration and stable isotope used.

![Figure 1.10: An example of a tracer labelled stable isotope used in metabolic research is 1-\textsuperscript{13}C-Leucine.](image)

Table 1.3 Feeding protocols used in published studies. The table lists the meal content of fat; protein and carbohydrates expressed as energy\% and/or amount of each content in grams. The meal intervals, study duration and isotope tracer were listed in each human study.

<table>
<thead>
<tr>
<th>Author, Year</th>
<th>Fat/Protein/CHO (%)</th>
<th>Meal intervals</th>
<th>Study duration (h)</th>
<th>Stable Isotope Infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wong et al., 2014</td>
<td>Meal 130g/17g/21g with total of 4800 kJ</td>
<td>-</td>
<td>10</td>
<td>\textsuperscript{2}H\textsubscript{3}-Leucine</td>
</tr>
<tr>
<td>Hermansen et al., 2013</td>
<td>Energy 61-65/19-16/20-19</td>
<td>-</td>
<td>42 days</td>
<td>\textsuperscript{13}C Octanoate orally added to the egg yolk</td>
</tr>
<tr>
<td>Xiao et al., 2012</td>
<td>49/13/38</td>
<td>Infusion of 40 mL/h for the 1\textsuperscript{st} 2 hours and 80 mL/h for the rest of the study</td>
<td>16</td>
<td>\textsuperscript{2}H\textsubscript{3}-Leucine</td>
</tr>
<tr>
<td>Hogue et al., 2007a</td>
<td>Energy 40/15/45</td>
<td>1/30\textsuperscript{th} every 30mins</td>
<td>15</td>
<td>L-[5,5,5-D\textsubscript{3}]\textsuperscript{2}H\textsubscript{3}-Leucine</td>
</tr>
<tr>
<td>Duez et al., 2006</td>
<td>Energy 18/20/62</td>
<td>Identical hourly meals</td>
<td>12</td>
<td>Deuterated L-[5,5,5-\textsuperscript{2}H\textsubscript{3}]-leucine</td>
</tr>
<tr>
<td>Zheng et al., 2006</td>
<td>Fat only (almond oil)</td>
<td>9g every 30 minutes</td>
<td>12</td>
<td>Tri deuterated leucine \textsuperscript{2}H\textsubscript{3}-Leucine</td>
</tr>
<tr>
<td>Duvillard et al., 2005</td>
<td>Energy 39/7/55</td>
<td>Identical meals every 2 hours</td>
<td>16</td>
<td>\textsuperscript{13}C-Leucine</td>
</tr>
<tr>
<td>Welty et al., 2004</td>
<td>Energy 36/15/49</td>
<td>Identical hourly meals</td>
<td>15</td>
<td>Deuterated [5,5,5,-\textsuperscript{2}H\textsubscript{3}]leucine</td>
</tr>
<tr>
<td>Batista et al., 2004</td>
<td>Energy 36/15/49</td>
<td>Identical hourly meals</td>
<td>15</td>
<td>Deuterated 5,5,5,-\textsuperscript{2}H\textsubscript{3}leucine</td>
</tr>
</tbody>
</table>
1.12 Stable isotope enrichment detection using Gas chromatography-mass spectrometry (GC-MS)

GC-MS is a complicated two-part machine which it enables the measurement of the enrichment of an isotopically labelled molecule such as $^{13}$C leucine (Figure 1.10). In the case of using AA as a stable isotope, the GC side of the instrument isolates AA from a mixture of protein hydrolysate AAs. The GC then present them to be identified and quantified by the MS, which is the other part of the instrument (Bodamer and Halliday, 2001, Boren et al., 2012, Rambal et al., 1992, Sunehag and Haymond, 2003). To measure AA isotopic enrichment, they need to be processed and prepared to be presented in a particular form of complexed molecules to be recognised by the GC-MS. Therefore, the sample containing a mixture of protein hydrolysate AAs go through a process called derivatization. Derivatized AAs are transformed into molecules that have the ability to evaporate at high temperatures (volatile products) (Bodamer and Halliday, 2001, Boren et al., 2012, Rambal et al., 1992, Sunehag and Haymond, 2003). These volatile products are then introduced to the injector of the GC at high temperature to evaporate them, and consequently, they are carried on the capillary column by the inert pressurised gas. This gas could be methane, hydrogen, nitrogen or helium based on the type of experiment chosen. AAs of interest are then separated from other molecules based on their volatility at certain temperatures. This complexed separation takes place in the column by the power of temperature-regulated interaction between the AA carried via the pressurised gas (also called mobile phase) and the coating of the inner column surface (also known as stationary phase). Separated AAs are then moved to the ionisation source of the MS to be ionised. Two different modes of source ionisation can be used to quantify and identify AAs; chemical ionisation (CI) and electron ionisation (EI) modes (Bodamer and Halliday, 2001, Boren et al., 2012, Rambal et al., 1992, Sunehag and Haymond, 2003). After the ionisation process, negatively charged electrons are transported
from the hot filament part and attach to the positive anode to the positively charged plate. Direct bombardment with electrons accomplishes the ionisation process in the EI mode in which they are broken into fragments by the power of applied energy. On the other hand, the ionisation by CI mode is different in which a gas such as methane is introduced as a reagent ion source for ionisation. This takes place by hitting released electrons by methane molecules to react with electrons and generate different methane ions. This can be achieved by either a positive chemical ionization (PCI) or by negative chemical ionization (NCI) to produce either positive or negative ions respectively based on the substance of interest (Bodamer and Halliday, 2001, Boren et al., 2012, Rambal et al., 1992, Sunehag and Haymond, 2003). The final step after ionisation is to analyse the generated ions based on their mass. This takes place via transporting the ions to a mass analyser by the help of focusing lenses to be filtered quickly depending on their mass. In the end, the mass of an interested ion with its quantity is detected and recorded with the help of a computer programme. Therefore, every AA has its mass to be identified and quantified to measure the isotopic enrichment and calculate the TTR (Bodamer and Halliday, 2001, Boren et al., 2012, Rambal et al., 1992, Sunehag and Haymond, 2003).

In this project, $^{13}$C-leucine was used as a stable isotope to be infused intravenously for specified time periods in each clinical trial to measure apoA-I, apoB100 and apo-B48 kinetics in three different clinical studies. NCI mode was used by introducing methane as a reagent gas for generating negatively charged ions in ordered to measure TTR of $^{13}$C-leucine/$^{12}$C-Leucine with an ion mass of 210/209 respectively (Sunehag and Haymond, 2003).
1.13 Establishment of a postprandial TG steady state using repeated feeding protocol

There have been several research studies involved in elucidation the role of GLP-1 receptor agonists in lipid metabolism in healthy subjects in a fasted state with an established fasting state there is a steady state of blood plasma TG due to the absence of exogenous TG which disrupts the steady state (Sakamoto et al., 2013). On the other hand, few studies have investigated the effect of GLP-1 receptor agonists on postprandial lipid metabolism required for the production of CM-apoB-48 (Xiao et al., 2012). One explanation for this is that it is hard to maintain a steady state of TG in blood in the fed state. Investigators have used a variety of feeding protocols and a wide range of meals with different nutrient compositions to maintain postprandial TG steady state in the blood as shown in Table 1.3. In some postprandial studies, administration of a meal bolus at the start of feeding protocol may or may not be required to achieve a postprandial steady state. Also, the administration of the meal bolus is directly dependent on the meal composition. Feeding protocols with the different nutrient composition to maintain postprandial TRL steady state in the blood from many published studies are shown in Table 1.3.
1.14 Hypotheses

Central obesity, IR, hyperinsulinaemia and especially hypertriglyceridaemia are important manifestations of metabolic syndrome seen in T2D and NAFLD. These events can be treated, and/or managed to reduce the risk of CVD as follows;

A. It is hypothesised that physical exercise will correct altered lipoprotein metabolism and reduce liver fat in patients with NAFLD by increasing hepatic and peripheral insulin sensitivity, reducing fasting hypertriglyceridaemia and increasing low HDL-C. This will be achieved by the exercise action on reducing the PR of VLDL-apoB-100 or increasing VLDL-apoB-100 FCR, and directly increasing the PR of HDL-apoA-I or indirectly reducing FCR of HDL-apoA-I. This will contribute to reducing CVD risk.

B. Lixisenatide treatment administration for patients with T2D will correct altered lipoprotein metabolism by increasing hepatic and peripheral insulin sensitivity and reducing postprandial hypertriglyceridaemia. This will be accomplished by reducing plasma postprandial TG concentration by decreasing CM-apoB-48 PR as a result of indirect and direct effects of lixisenatide on intestinal enterocytes, and will also reduce VLDL-apoB-100 PR from the liver as a consequence of an improvement in insulin sensitivity. The latter will indirectly increase the PR of HDL-apoA-I. These effects will contribute to reducing CVD risk.

1.15 Aims

1. To conduct a clinical trial that will study the effects of a physical exercise programme on HDL-apoA-I kinetics and concentration, fasting hypertriglyceridaemia, liver fat, IR and lipid profile in patience with NAFLD using stable isotope trace-labelling technique.

2. To conduct a clinical trial that will study the effects of a 4-week treatment with lixisenatide (a GLP-1 receptor agonist) versus placebo on postprandial lipoprotein...
metabolism, hypertriglyceridaemia, IR and lipid profile in patients with T2D using a constant feeding protocol and a stable isotope trace-labelling technique.

1.16 Objectives

1. To develop a constant feeding protocol that will maintain an elevated plasma TG concentration to provide a postprandial TG steady state which is essential for the calculation of postprandial lipoprotein kinetics (TRL-apoB-100 and TRL apoB-48).

2. To develop a laboratory protocol that will facilitate apoB-100, apoB-48 and apoA-I isolation, precipitation, measurement enrichment using GC-MS.

3. To develop a clinical protocol to be used in a clinical trial that will facilitate the study time points for blood sample collection and administration of the trace-labelling stable isotope $^{13}$C Leucine for the measurement of apoB-100, apoB-48 and apoA-I concentration and TTR enrichment

4. To validate the developed constant feeding, laboratory and clinical protocols by conducting a pilot study for the measurement of fasting and postprandial lipoprotein kinetics (CM-apoB-48, VLDL-apoB-100 and HDL-apoA-I) using stable isotope trace-labelling technique.

5. To develop two sensitive and specific techniques that will facilitate the measurement of apoB-100 and apoB-48 concentrations separately using competitive ELISA.
Chapter 2: Clinical protocols and methodology

2.1 The methods used for participant recruitment in conducted clinical trials

Many different ways of contact were applied in the recruitment procedure for the three clinical trials. The primary method was through the Primary Clinical Research Network South East (PCRN SE) with GP surgeries in Surrey, which searched for suitable patients using BMI, plasma lipids, blood glucose, blood pressure, current health, prescribed medication and distance of home from Guildford. Another important method was the use of newspaper advert which covered both the Surrey area and nearby counties to attract participants from a wider distance. Other methods that were applied included advertisement on the University of Surrey website (SurreyNet), and at local hospitals, public and private sector organisation by either poster leaflets or via email.

2.2 Pilot feeding study (PFS) to develop a feeding protocol to measure apoB-100 and apoB-48 in blood samples using a stable isotope technique (two parts):

2.2.1 Participant recruitment for both parts of the PFS

Both parts of the PFS were given a favourable opinion by the University of Surrey Ethics Committee in October 2013 EC/2013/98/FHMS. Caucasian men aged 40-65 with body mass index (BMI) 30-35 were recruited for the PFS. Participants had a screening visit in which they were asked to attend after an overnight fast, in the Clinical Investigation Unit (CIU) at the faculty of health and medical sciences (FHMS), University of Surrey. At the screening visit, the following was performed and recorded: medical history, current medication, physical examination, diastolic and systolic blood pressure, weight, height, age, tobacco smoking status, alcohol intake and haematocrit. Fasting blood samples were collected to measure TG, total cholesterol, blood glucose and insulin concentrations.
Subjects who were smokers and had cardiovascular and endocrine disease (such as unstable ischemic heart disease), diabetes and diabetic complications, uncontrolled hypertension, hepatic and renal disorders, gastrointestinal illness or surgery other than minor endoscopic procedures such as peptic ulcer injection, unstable weight in the past three month, mental incapacity, alcohol consumption of more than 2 units/day (more than 12 to 15 g of alcohol/day), had been taking any medication known to affect lipid and/or glucose metabolism, allergies to dairy products, and hormone therapy were excluded from the study. Potential participants who expressed interest and fulfilled the required criteria were recruited for the PFS as seen in Figure 2.0. A total of n=7 participants were screened and n=4 were recruited for this pilot study; n=3 for the first part and n=1 for the second part (Figure 2.0).

2.2.2 Development of a feeding protocol to maintain postprandial plasma TRL concentrations at a constant concentration (part one);

2.2.2.1 Liquid meal composition and preparation

Based on the meal protocols described previously and shown in Table 1.3 in Chapter one section 1.11, a liquid meal was optimised to be used for the postprandial study to maintain a steadily elevated concentration of plasma TG. Three meal flavour options were developed, and each was a 100 ml liquid meal drink which could be chosen by the participant on the study day. Although they were different in the content added, they all had the same macronutrient content and caloric value as shown in Table 2.0 which shows the meal ingredient’s amounts in each meal option, total volume, and energy percentage with total calorie per drink.
Figure 2.0: The sequential steps performed in both parts of the PFS. N: number; PFS: pilot feeding study; TG: triglycerides.
Table 2.0: The three meal options offered to the participants with an equal amount of energy and calories.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>1st meal option</th>
<th>2nd meal option</th>
<th>3rd meal option</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulated sugar (Tate Lyle)</td>
<td>6.5 g</td>
<td>8.6 g</td>
<td>6 g</td>
</tr>
<tr>
<td>Boiling water.</td>
<td>45 ml</td>
<td>45 ml</td>
<td>45 ml</td>
</tr>
<tr>
<td>Unflavoured whey powder (Natures Best, UK)</td>
<td>7 g</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Vanilla flavoured whey powder (instant whey-PRO.com) as 80% protein,</td>
<td>X</td>
<td>8.75 g</td>
<td>8.75 g</td>
</tr>
<tr>
<td>Extra virgin olive oil (Tesco own brand, UK)</td>
<td>3.5 ml</td>
<td>3.5 ml</td>
<td>3.5 ml</td>
</tr>
<tr>
<td>Sunflower oil (Tesco brand, UK)</td>
<td>3.5 ml</td>
<td>3.5 ml</td>
<td>3.5 ml</td>
</tr>
<tr>
<td>Double cream (Tesco brand, UK)</td>
<td>10 ml</td>
<td>10 ml</td>
<td>10 ml</td>
</tr>
<tr>
<td>Raspberry or chocolate flavouring (CRUSHA milkshake)</td>
<td>10 ml</td>
<td>X</td>
<td>10 ml</td>
</tr>
<tr>
<td>Cold water</td>
<td>X</td>
<td>10 ml</td>
<td>X</td>
</tr>
<tr>
<td>Final meal flavour</td>
<td>Chocolate or Raspberry</td>
<td>Vanilla</td>
<td>Vanilla mixed with chocolate or raspberry</td>
</tr>
<tr>
<td>Total volume per drink</td>
<td>100ml</td>
<td>100ml</td>
<td>100ml</td>
</tr>
<tr>
<td>Energy % and total calorie per drink</td>
<td>CHO 22%, Fat (saturated and non-saturated) 66.1%, Protein 11.9% / 100ml /drink.</td>
<td>2280 Kcal/drink</td>
<td></td>
</tr>
</tbody>
</table>

2.2.2.2 Preparation of the liquid meal;

Based on the selected meal option, granulated sugar (Tate Lyle) was weighed out in the required amount into a plastic beaker. Then 45ml boiled water was added and stirred with an electric mixer until the sugar was completely dissolved. Then, the necessary amount of whey powder was added, and mixed with an electric mixer to homogenise the components of the meal. Afterwards, the oil was added; olive oil (Tesco brand, UK) and sunflower oil (Tesco brand, UK), and mixed with an electric mixer to homogenise the components of the meal. Ten ml of cold double cream (Tesco brand, UK) was then added and mixed with the electric mixer. Finally, 10 ml of flavouring (CRUSHA raspberry milkshake) or cold water was added and mixed with an electric mixer. For better palatability and taste, the liquid meal was cooled down in the refrigerator until served.
2.2.3 Study design to achieve a steady state of TRL postprandial concentrations (part one).

On the study day, weight and blood pressure was measured, and then a doctor inserted an intravenous cannula into an antecubital vein for blood sampling at -240 minutes (this was the first time point of the study instead of 0). After taking a fasting blood sample, participants were asked to drink hourly the prepared liquid meals. Blood samples (13 ml) were taken at hourly intervals for the measurement of exogenous and endogenous TRL for 12 hours as; -240, -180, -120, -60, 0, 60, 120, 180, 240, 300, 360, 420 and 480 minutes as illustrated in Figure 2.1. Two feeding protocols (A and B) were tested to determine if a postprandial TG steady state could be achieved between 0 and 480 minutes as shown in Figure 2.1;

1. In feeding protocol A hourly consumption of liquid meals were given in equal amounts to gradually elevate the plasma TG and keep it elevated in a steady state.

2. Feeding protocol B was similar to protocol A except for the 1st liquid meal at -240 minutes which was consumed as twice the quantity and volume of the hourly drinks, i.e. the 1st liquid meal drink was considered as a bolus meal and used to trigger the TG plasma elevation. Then, equal hourly meals were given afterwards to maintain the elevated steady state of plasma TG.

2.2.4 Study design for the pilot study with stable isotope infusion (part two)

The results of PFS part one suggested that equal hourly feeding was better at achieving a plasma TG steady state. Therefore, this feeding protocol was chosen for conducting PFS part two of the study. Participants were asked to arrive at the CIU having fasted from 22:00 the previous evening. A doctor then inserted two cannulas into the antecubital vein. Four hours
later (t=0 minutes) after a plasma TG steady state was achieved, an intravenous primed (1mg/kg) constant infusion of 1-¹³C leucine at 1 mg/kg/min was administered. Hourly blood samples (13 ml) were taken for the measurement of TRL apoB (apoB-100 and apoB-48) kinetics for 8 hours as follows; 0, 60, 120, 180, 240, 300, 360, 420 and 480 minutes as illustrated in Figure 2.2.

![Figure 2.1: Illustration of PFS part one time points without (A) and with (B) the liquid bolus meal. Upward arrows indicate hourly blood sampling for 12 hours, and downward arrows indicate hourly liquid meals consumed.](image)

![Figure 2.2: Sampling protocol for PFS part two with tracer infusion is shown here. Liquid meals were given hourly and blood samples collected before and after the injection of the 1-¹³C leucine tracer bolus at (t=0) of 1mg/kg and constant infusion between 0 – 480 minutes of 1mg/kg/min for 8 hours.](image)
2.3 The effect of GLP-1 receptor agonist (Lixisenatide) on postprandial TG metabolism against placebo in patients with T2D

2.3.1 Participant recruitment

This study was given a favourable opinion by three committees; the University of Surrey Ethics Committee with reference number EC/2013/104/FHMS, the RSCH research and development Ethics Committee with a reference number 13/SC/0378, and Medicines and Healthcare products Regulatory Agency (MHRA) at the national health services (NHS) clinical trial authorisation team with a reference number 2013-002826-22. Also, this clinical trial was registered at www.clinicaltrials.gov with a reference number NCT02049034. This study was un-blinded at 01/11/2016 which indicated the order of the lixisenatide and placebo treatments to define the participants into two groups to perform the statistical analysis tests.

Caucasian men aged 40-65 with BMI 27-37 kg/m² diagnosed with T2D with fasting blood glucose (FBG) of above 7 mmol/L and HbA₁C between 7.5-9.5% were recruited. The recruitment procedure using an initial theoretically planned BMI range of 30-37 kg/m² was not efficiently effective for recruiting participants in this study as the required number of patients with this BMI range could not be found at the designated searching area. Therefore, the BMI range was adjusted to 27-37 kg/m² to help in recruiting more participants needed for this study. Inclusion criteria included a stable diabetes management with uncontrolled hyperglycaemia over the previous three months with a stable Metformin dose, able and willing to self-administer placebo/lixisenatide injection (once daily (10µg for 14 days followed by an increase to 20µg for 14 days), perform self-blood glucose monitoring, and wear a Continuous Glucose Monitoring System (CGMS) for three days.
Subjects who were smokers and had cardiovascular and cerebrovascular disease (such as unstable ischemic heart or brain disease), endocrine disease, uncontrolled hypertension or under β-Blockers treatment, hepatic and renal disorders, gastrointestinal illness or surgery other than minor endoscopic procedures such as peptic ulcer injection, unstable weight in the past three month, mental incapacity, alcohol consumption of more than 2 units/day (more than 12 to 15 g of alcohol/day), been taking any medication known to affect lipid and/or glucose metabolism except only Metformin and statins, allergic to dairy products and any GLP-1 receptor agonist or meta-cresol, under hormonal therapy were excluded from the study. Potential participants who expressed interest and fulfilled the required criteria were recruited for this study as seen in Figure 2.3. A total of six participants were recruited for this study and samples were used for all the laboratory measurements including TRL-apoB-100 and TRL-apoB-48 kinetics (Figure 2.3). After the study was initiated and samples from the first two participants were analysed, an introduced objective of studying the effect of lixisenatide on HDL-apoA-I kinetics was added to this study’s objectives. Therefore, samples from only the last 4 out of 6 recruited participants were used to measure HDL-C and HDL-apoA-I concentration and calculate HDL-apoA-I kinetics (Figure 2.3).

2.3.2 Study power

The power calculation used was obtained from Kane, (2011). There are no published data on the determination of lixisenatide effects on postprandial glucose and TG metabolism against placebo in patients with T2D by measuring postprandial glucose, TRL-apoB-100, TRL-apoB-48 and HDL-apoA-I kinetics. The study power calculated for this study was originally based on postprandial plasma glucose rate of appearance (RaT) (measured on visit 3 and 7). Therefore, the sample size was based on the primary endpoint of total RaT following the
breakfast meal. This was calculated using data from a previous study by Cersosimo et. al 2011, which measured the effect of two weeks treatment with exenatide (a GLP-1 receptor agonist) on glucose RaT following a meal in 17 type 2 diabetic patients (Cersosimo et al., 2011). The glucose RaT (mean ± SD) in patients before treatment was 23.7±3.0 mcmol/kg/minute which was significantly reduced to 14.3±3.5 mcmol/kg/minute after two weeks of treatment. The correlation between measurements in the same person was unknown so this was assumed to be zero. Based on this, completing the study with a data set of 12 subjects would have the power of 80% at the 5% concentration to detect a difference of 20% in which glucose RaT would be considered clinically significant.

Another power calculation was conducted based on postprandial TRL-apoB-48 kinetics (Kane, 2011). The sample size was based on the primary endpoint of TRL-apoB-48 PR after elevation of plasma FFA. This was calculated using data from a previous study by (Duez et al., 2008a which measured effect of short-term acute elevation of plasma FFA on TRL-apoB-48 kinetics using a feeding protocol and a stable isotope technique for 12 healthy obese subjects. TRL-apoB-48 PR 5.95±1.13 mg/kg/day (mean ± SD) at baseline was significantly increased (P=0.03) after Intralipid/heparin (IH) treatment 3.53±0.58 mg/kg/day (Duez et al., 2008a). The correlation between measurements in the same person was unknown so this was assumed to be zero. Based on this, completing the study with a data set of 18 subjects would have the power of 80% at the 5% concentration to detect a difference of 25% which would be considered clinically significant as shown in Figure 2.3.

Another power calculation was conducted based postprandial HDL-apoA-I kinetics (Kane, 2011). The sample size was based on the primary endpoint of HDL-apoA-I FCR after exercise. This was calculated using data from a previous study by Thompson et al., 1997 which measured the effect of 12 months of exercise intervention on HDL-ApoA-I kinetics using a
feeding protocol and radioactive tracer technique for 17 overweight males. HDL-apoA-I FCR 0.23±0.03 pools/day (mean ± SD) before exercise at baseline was significantly decreased (P=0.01) after exercise treatment 0.21±0.03 pools/day (Thompson et al., 1997). The correlation between measurements in the same person was unknown so this was assumed to be zero. Based on this, completing the study with a data set of 14 subjects would have the power of 80% at the 5% concentration to detect a difference of 20% which would be considered clinically significant (Figure 2.3).

Figure 2.3: A consort diagram to show participant recruitment number for the lixisenatide study. N: number; TRL: triglyceride-rich lipoprotein; HDL: high-density lipoprotein; PR: production rate; FCR: fractional catabolic rate; apoB: apolipoprotein B; apoA-I: apolipoprotein A-I.
2.3.3 Study design and clinical protocol

This study was a randomised double-blind crossover study. This clinical trial was conducted to investigate the effect of lixisenatide vs. placebo (Sanofi, UK) treatment on postprandial glucose and TRL metabolism in patients with T2D. This was achieved by measuring the kinetics of TRL-apoB-100, TRL-apoB-48 and HDL-apoA-I. Patients were randomised into two groups. One group received a once daily subcutaneous injection of 10 µg for the first 14 days followed by 20µg for the remaining 14 days lixisenatide or placebo for four weeks followed by a four-week washout then once daily subcutaneous injection of 10 µg for the first 14 days followed by a 20µg for the remaining 14 days of the other treatment (Figure 2.4).

Subjects were asked to fill in a seven-day diet diary and to monitor their blood glucose during the 3rd week of each treatment period. They were studied on two separate days at the end of each treatment period (Visits 3, 4, 7 and 8). At all visits, subjects were asked to attend the Centre for Diabetes, Endocrinology and Research (CEDAR), Royal Surrey County Hospital, Guildford (RSCH).

Before screening took place, subjects were provided with verbal and written information about the trial and the procedures involved. The subjects were fully informed of their responsibilities and rights while participating in the trial, as well as of possible disadvantages in being treated with the trial product. They were given the opportunity to ask questions and an enough time for proper consideration of participating in the study. Subjects who wished to participate in the trial were required to sign and date an informed consent form before any trial-related activities took place. All subjects were provided with a copy of the Subject Information Sheet, and they signed and dated an informed consent Form.
At the screening visits, participants were asked to attend after an overnight fast, and the following were performed and recorded; medical history, current medication, physical examination, diastolic and systolic blood pressure. In addition to the measurements of weight, height, age, tobacco smoking status, alcohol intake, haematology (haemoglobin, total leukocyte count, and thrombocytes), clinical chemistry (sodium, potassium, creatinine, total protein, albumin, AST, ALT, and ALP), amylase, calcitonin, lipase, TG, total cholesterol, HbA1c, and blood glucose. The study involved multiple visits that took place in the CEDAR at RSCH as follows as seen in Figure 2.4;
Figure 2.4: The sequential steps and visits took place in the Lixisenatide clinical study.
**Visit 1:** Patients who were eligible for the study after screening were invited to the CEDAR where they had a one to one training session with a research nurse skilled in diabetes, on injection technique using the study drug device. They were randomised into two groups using computer randomisation. Depending on the randomization code the patient was prescribed either Lixisenatide or the placebo injection for 28 days. Patients were provided with a trial specific diary in which to record drug administration, any concomitant medication and any adverse events. Patients were made aware of symptoms suggestive of hypoglycaemia and were issued with (and instructed on) the use of a capillary glucose monitor.

**Visit 2:** This took place after 21 days. Patients were provided with a standardised meal to eat the evening before Visit 3.

**Visit 3:** Patients were asked to attend after an overnight fast on the morning of day 25 of the first four-week treatment period. They were requested to bring their glucose monitor to this visit. The following were measured; weight and metabolic profile, and postprandial glucose kinetics. Patients were provided with a standardised meal to eat the evening before Visit 4.

**Visit 4:** Visit 4 and 8 were identical, and they were the primary focus of this research. Patients were asked to attend after an overnight fast on the morning of day 28. The following were measured; blood biochemistry (as visit 1), physical examination and vital signs. Twenty µg of treatment (either Lixisenatide or placebo) were administered subcutaneously 30 minutes before the consumption of the first meal (the meal composition used in the PFS part two). Patients were given the option to choose which flavour of the meal they desired. Then equal liquid meals were given hourly for 11 hours to achieve an elevated steady state of plasma TG Figure 2.2. Four hours later, a primed continuous
infusion of $^{13}$C leucine (1mg/kg, 1mg/kg/h) was administered for 8 hours. Blood samples were taken hourly to measure the kinetics of TRL-apoB-100, TRLapoB-48 and HDL-apoA-I for 8 hours at 0, 60, 120, 180, 240, 300, 360, 420 and 480 minutes (Figure 2.2).

**Visit 5:** This visit was on day 56. The patient switched to either Lixisenatide or the placebo injection for 28 days. Patients were provided with a trial specific diary in which to record drug administration, any concomitant medication and any adverse events.

**Visit 6:** This visit was on day 77 and was identical to visit 2.

**Visit 7:** This visit was on day 81 and was identical to visit 3.

**Visit 8:** This visit was on day 84 and was identical to visit 4. In this visit, patients had successfully finished this clinical trial.

### 2.4 The effects of exercise training on visceral fat, insulin sensitivity, β-cell function and TG kinetics in patients with non-alcoholic fatty liver disease (NAFLD)

#### 2.4.1 Participant recruitment

This study was given a favourable opinion by two committees; the University of Surrey Ethics Committee with reference number 08/H1/008/165/FHMS, the RSCH research and development Ethics Committee with a reference number 09/H1109/97. Also, this clinical trial was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) with a reference number NCT01834300.

Caucasian men aged 18 and over with BMI 27-37 kg/m$^2$, diagnosed with NAFLD with more than 5% of liver fat droplets using imaging tests such as ultrasound, magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS). The recruitment procedure using an initial theoretically planned BMI range of 30-37 kg/m$^2$ was not efficiently effective for recruiting participants in this study as the required number of patients with this BMI...
range could not be found at the designated searching area. Therefore, the BMI range was adjusted to 27-37 kg/m² to help in recruiting more participants needed for this study. Inclusion criteria included positive NAFLD diagnosis with appearance suggestive of a fatty, echo-bright liver with no evidence of cirrhosis (in some cases, the diagnosis was confirmed histologically after liver biopsy) were recruited. In addition, motivation and willingness to engage in following an exercise programme were required for the study.

Subjects who were smokers and unable to exercise, and had T2D and diabetic complications, cardiovascular and cerebrovascular disease (such as unstable ischemic heart or brain disease), endocrine disease, uncontrolled hypertension, hepatic disease (besides NAFLD) and renal disorders, gastrointestinal illness or surgery other than minor endoscopic procedures such as peptic ulcer injection, unstable weight in the past three month, mental incapacity and claustrophobia, alcohol consumption of more than two units/day (more than 12 to 15 g of alcohol/day), been taking any medication known to affect lipid and/or glucose metabolism (except statins), been prescribed with drugs known to cause secondary steatohepatitis such as corticosteroids, hormone therapy were excluded from the study. A total of n=30 participants were recruited; n=15 were randomly distributed each group (the supervised exercise and control groups), and n=3 were dropped out from the study (Figure 2.5)

2.4.2 Study power

The primary endpoint for this study was VLDL apoB-100 secretion rate which was calculated from a previous study. There was one study with published data on the measurement of VLDL apoB-100 kinetics (but not HDL-apoA-I kinetics) in patients with NAFLD (Sullivan et al., 2012). However, this study was not chosen to calculate our study power and sample size as
there was no significant difference of VLDL-apoB-100 secretion rate before and after the intervention period (Sullivan et al., 2012). However, another study conducted by Alam et al. 2004, was used for the power calculation. In this study, eighteen patients with T2D were enrolled to determine the effect of a 6-month exercise program on VLDL-apoB-100 secretion rate (Alam et al., 2004). The study showed significant results including reduced VLDL-apoB-100 secretion as mean±SEM (11.3±2.5 mg/kg/day at 0 months which reduced by 48% to 5.5±2.0 mg/kg/day) which was detectable with nine patients in the supervised exercise group. Based on this, a data set of 15 patients in each exercise group (n=30 as n=15 in the supervised exercise group and n=15 for the lifestyle advice group) would have the power of 80% at the 5% level to detect at least 20% reduction in VLDL-apoB-100 production difference between the two groups which would be considered clinically significant (Figure 2.5).

The power calculation used to determine the sample size for measuring the effect of exercise on HDL-apoA-I kinetics was the same calculation used in the lixisenatide study mentioned earlier in section 2.3.3. Based on this, completing the study with a data set of 14 subjects would have the power of 80% at the 5% concentration to detect a difference of 20% which would be considered clinically significant (Figure 2.5).
2.4.3 Study design

A randomised controlled, four-month exercise intervention study of 30 patients with NAFLD was conducted as 15 patients in the supervised exercise group and 15 patients in lifestyle advice group. All patients were examined at two-time points: at baseline (before commencing the exercise programme) and after the four-month intervention period (Figure 2.6) which included;

1- Measurement of the patient’s physical fitness ($\text{VO}_{2\text{MAX}}$).
2- The measurement of dietary habits via seven-day diet diaries.
3- Measurement of liver and muscle fat by magnetic resonance spectroscopy (MRS), and whole body fat by magnetic resonance imaging (MRI) at Hammersmith Hospital in London.

4- The measurement of bioelectrical impedance analyses of body fat and anthropometry.

5- The measurement of VLDL apoB-100, HDL apoA-I, and VLDL-TG kinetics.

2.4.4 Study clinical protocol

During the study, participants attended the CEDAR at RSCH for seven visits (Figure 2.6) as follows;
Figure 2.6: The sequential steps and visits in the NAFLD study. (MRI); magnetic resonance imaging, (MRS); magnetic resonance spectroscopy, VO$_{2\text{max}}$; physical fitness test.
**Visit 1:** this was the screening visit. Patients were asked to complete a Physical Activity Readiness Questionnaire (PAR-Q), which is designed to identify the patient’s physical ability, commitment and readiness to enrol in an exercise program prior commencing the study. The doctor reviewed and discussed the patient’s medical history and performed a physical examination, including height, weight, pulse and blood pressure. Then an electrocardiograph (ECG) was carried out to identify the participants’ current heart rates and ensure they had no heart disease. Blood samples were taken for plasma liver function and lipid profile tests. If the patient wished to continue in the study, written consent was given to him. Following this, a seven-day diet diary was distributed, and they were instructed on how to record their intake in the food diary. All patients had an assessment of dietary habits by filling a seven-day food diary before the start of the intervention. A dietician examined the diet diary, and caloric intake was recorded. A seven-day activity recall was also administered. After that, all participants were randomly allocated to either the supervised exercise group (group 1) or the standard lifestyle advice group (group 2) by computer software.

The participants were advised to be in the CEDAR at the RSCH for this visit. The following was recorded; weight, height, body mass index (BMI), waist circumference, blood pressure, resting heart rate and a short questionnaire to be used for eligibility determination for this study. Also, randomization was taken place for both groups.

**Visit 2:** In this visit, measurement of fitness by \( \text{VO}_{2\text{max}} \) was determined (maximum oxygen consumption during exercise) (Dlugosz et al., 2013). The measurement of the \( \text{VO}_{2\text{max}} \) was performed using an electronically braked bicycle ergometer (Lode; Excaliber Sport, Groningen, the Netherlands) equipped with a computerised breath (oxygen \([\text{O}_2]\)/ carbon dioxide \([\text{CO}_2]\)) analyser system (Medical Graphics, St Paul, MN, USA). An ECG was also carried out during the exercise test to monitor the participants’ heart rates and ensure they
had no latent ischaemic heart disease. The VO\textsubscript{2max} measurements were performed within four days of the metabolic study.

**Visit 3:** In this visit, the patient had an MRI and MRS scanning at the Hammersmith Hospital in London.

**Visit 4:** This visit together with visit 7 were the primary focus of this research. The patients were given a standardised evening meal before the study day which was consumed at 20:00. On the morning of the study day, the patients were asked to come to the CEDAR at the RSCH having fasted from 20:00 the previous night. Patients were allowed to drink water only during the study period. The doctor then inserted two cannulas into the antecubital vein of each arm, one for obtaining 20 blood samples from the study. The other one was for the administration of a stable isotope of leucine ([1\textsuperscript{-13}C]-Leucine) at 1mg/kg body weight followed by an immediate infusion of leucine at 1mg/kg/hr for 9 hours. T=0 was the baseline time point in which a blood sample was taken before the infusion of the leucine isotope. Blood samples were taken at hourly intervals for the measurement of the pre-exercise kinetics of hepatic VLDL apoB-100 and HDL apoA-I for 9 hours at 0, 60, 120, 180, 240, 300, 360, 420, 480 and 540 minutes as shown in Figure 2.7.

**Visit 5:** This visit which took place after the four-month intervention period. This visit was identical to visit 2.

**Visit 6:** This visit was identical to visit 3.

**Visit 7:** This visit was identical to visit 4. Post-exercise measurement of VLDL apoB-100 and HDL apoA-I kinetics were made.
Figure 2.7: The NAFLD study day protocol with tracer infusion is shown here. Blood samples were collected hourly before and after the injection of the $^{13}$C leucine tracer bolus at (t=0) of 1mg/kg and constant infusion between 0 – 540 minutes of 1mg/kg/min for 9 hours.

### 2.4.5 Exercise programmes involved in this study

Participants were randomised to either a four months supervised exercise programme group or conventional lifestyle advice group.

#### 2.4.5.1 Supervised exercise programme group (Group 1):

Participants allocated to the supervised exercise programme group (group 1) were encouraged to exercise 4-5 times weekly moderate-high intensity exercise, including cycling and/or walking on automated treadmills, for 20 minutes progressing to 60 minutes per session under 40-60% $\text{VO}_{2\text{MAX}}$ equivalent to 65-80% $\text{HR}_{\text{MAX}}$ for 4 months. This dose of prescribed exercise is in correspondence with the American College of Sports and Medicine guidelines for sedentary or disease populations. This exercise regime was used and validated by a previous study investigating the effect of supervised exercise on insulin sensitivity in liver and muscle fat (Shojae-Moradie et al., 2007). Exercise was either conducted singly or in groups at the gym or in their own environment. Their activity was carefully monitored, encouraged and they were motivated continually to continue in the exercise programme. An exercise physiologist was available to give support as necessary, and to be in contact with the participants throughout the study. The gym-based exercise used a variety endurance exercises which were guided by the heart rate responses on heart monitors. Borgs perceived exertion (RPE) scale (a method of RPE on a scale of 11 or 15 points) was used to measure a
participant's concentration of intensity in training by evaluating their body's physical signs such as heart and breathing rates, perspiration, and sweating) (Borg, 1982), and resistance exercises (involving x 2-3 sets of 10-12 repetition of a resistance circuit using the major muscle groups i.e. legs, back, abs, chest, and shoulders).

2.4.5.2 Conventional lifestyle advice group (Group 2):

Participants allocated to this group received no further communication or support from the exercise physiologist. However, they were asked to adhere to a healthy diet as much as possible and advised to undertake regular exercises. Throughout that period, any participant had the ability to contact the primary investigator to address issues concerning NAFLD or their position within the study. At the end of the four-month study, after the repeated measurements, those volunteers in group 2 were given the opportunity to enrol in the same exercise programme as group 1 i.e. weekly exercise consultations for another four months upon their request without any further measurements.

2.4.6 Individual and group supervisions performed in this study

Individual supervision: an induction session took place and allowed the participants to familiarise themselves with the exercise equipment. Also, they selected a programme that was tailored to an appropriate concentration of exertion and exercise preference. During the session, the exercise physiologist provided individually supervised sessions at regular intervals. Also, the exercise physiologist gave a minimum support every week along with telephone supervision in case of weekly sessions were not possible. At each supervised session, the participant had an opportunity to raise any issues, and the instructor provided proplastid feedback throughout the session. After that, both the instructor and the participant agreed on a plan to maintain their exercise activity for the following week. The
elements of consultation were repeated at regular intervals in which they enhanced the adherence, and re-addressed issues provided by the participant’s feedback.

**Group supervision:** there was some allocated group exercise slots at convenient times, to enable participants to exercise together. Each week throughout the study, participants had the opportunity to exercise as part of a group to encourage them to meet and talk to each other. They were additionally invited to exercise in pairs with an exercise buddy. As a result, participants were enjoyed and motivated during their period of study.
Chapter 3: Laboratory protocols and methods

3.1 Materials and equipment used

The stable isotope levorotary Leucine (L-Leucine) \([1^{-13}C]\) (15mg/ml, \(^{13}\)C enrichment 99%) was obtained from Cambridge Isotopes (USA) and prepared by the Pharmacy Department, St Thomas’ Hospital (UK). The sterile tubes containing different anticoagulants for collecting blood samples were purchased from Becton Dickinson UK Limited (Oxford, UK).

Chemical reagents used in the lab including hydrochloric acid (HCL), acetic acid, ethanol, diethyl ether, acetonitrile, toluene, ethyl acetate, ammonia solution S.G.0.88 (35%), and sodium dodecyl sulphate were obtained from Fisher Scientific UK Ltd (UK). Methanol (for HPLC) was purchased from Agros Organics (UK). Chemical reagents including 3-Methylbutanol, N,N,N’,N’-Tetramethylethylenediamine (TEMED), glycine (for electrophoresis ≥99%), phosphate buffered saline (pH 7.4), Decane, bromophenol blue, trizma base (Primary standard and buffer ≥99.9%), sodium chloride, ammonium persulfate, β-mercaptoethanol, trifluoroacetic acid (TFA), trifluoroacetic anhydride (TFAA), N-tert-butyldimethylsilyl-N-methyl trifluoroacetamide (>97%) and O-phenylenediamine were obtained from Sigma-Aldrich Ltd (UK). Coomassie brilliant (Blue R-250), calcium lactate and tween 20 were purchased from BDH, VWR International Ltd (UK). AG 50W-X8 resin, acrylamide (Bis Solution, 40%) fixative enhance concentration, development accelerator reagent, Silver Stain Plus kit (silver complex solution, reduction moderator solution, and image development reagent), AP conjugate substrate kit were obtained from Bio-Rad Laboratories (USA).

Plasma and fraction TG, cholesterol, HDL-C, and apoA-I concentrations were measured using ABX Cobas MIRA auto analyser (Horiba ABX, France). The sample kits, reagents, sample cuvettes, and reagent racks and tubes were also obtained from (Horiba ABX, France).
3.2 Laboratory methods developed

The following methods were used in sequential order to enable us to detect the isotopic enrichment of $^{1-13}\text{C}$ leucine in apoB and apoA-I in blood samples obtained from clinical trials as seen in Figure 3.0.

![Diagram of sequential steps of methods used for laboratory analysis](image)

**Figure 3.0:** Illustration of sequential steps of methods used. SDS-PAGE: sodium dodecyl sulphate – polyacrylamide gel electrophoresis, GC-MS: gas chromatography – mass spectrometry, AA: amino acid.

3.2.1 Sequential flotation ultracentrifugation of blood plasma samples to obtain CMs & VLDL (TRL) and HDL fractions

For obtaining a TRL (Sf 20-400) lipoprotein fraction (CMs and VLDL) from plasma, three ml from each time point was placed into the respective labelled Optiseal tubes (Beckman, USA) pre-coated with polyvinyl alcohol (PVA), then the sample was carefully overlaid with a density 1.006 g/dl solution of Saline (sodium chloride) / ethylenediaminetetraacetic acid
(EDTA) up to 4.5 ml. The tubes were transferred to a pre-chilled rotor (Type 50.4 Ti rotor, Beckman Coulter, USA) for ultracentrifugation for 16 hours at 4°C at 37,000 revolutions per minute (rpm), corresponding to average relative centrifugal force (RCF) of 183, 935 x g for the inner row and 218, 180 x g for the outer row of the rotor respectively. The lipoprotein fraction in the top of the ultra-tube was isolated by the use of a tube cutting apparatus. The blade of this apparatus acts as a physical barrier, preventing upper and bottom fractions to mix inside the tube. The one ml retrieved was transferred into a two ml volumetric flask and accurately adjusted to two ml using 1.006 g/dl Saline/EDTA density solution. Only one ml was transferred to a 10ml hydrolysis tube for apo-B100 and apo-B48 protein precipitation.

New Optiseal tubes were prepared (not PVA coated) with 0.5 ml of 1.519 g/dl density solution. Then, the remaining sample in the previous Optiseal tube (TRL PVA Optiseal tube), after cutting the TRL fraction, was added and mixed well. The mixed solution was overlaid with 1.063 density solution to the top of the newly prepared Optiseal tube, and the tube transferred into the pre-chilled rotor for ultracentrifugation for 22 hours at 4°C at 37,000 rpm.

The next day, IDL and LDL was removed from the obtained fraction using the tube cutting apparatus. Then new Optiseal tubes were prepared, with 1.5 ml of 1.504 g/dl density solution. The remaining sample in the current Optiseal tube was mixed (IDL and LDL Optiseal tube after cutting) ensuring that the jelly-sediment at the bottom of the tube is moving freely. Three ml was taken from the remaining sample from the current Optiseal tube (including the sediment) and mixed with the 1.5 ml solution in the newly prepared Optiseal tube. The total mixture was overlaid with 1.21 g/dl density solution. These tubes were transferred into the pre-chilled rotor for the last ultracentrifugation for 24 hours at 4°C at 40,000 rpm.
The next day, the top one ml which contained the HDL fraction was removed, and the remaining solution in the Optiseal tube was discarded. The HDL fraction was transferred into a two ml volumetric flask and accurately adjusted to two ml using 1.006 g/dl Saline/EDTA density solution. Five hundred µl was transferred into a 10 ml hydrolysis tube and stored at -80°C for apoA-I delipidation and precipitation. The remaining amount in the flask was divided into two sets of two ml Eppendorf tubes for the measurements of apoA-I concentration and storage purposes.

3.2.2 TRL-apoB and HDL-apoA-I delipidation, precipitation and isolation.

3.2.2.1 TRL-ApoB-100 and B-48

This method was modified from Egusa et al., 1983. To precipitate apolipoprotein B from a TRL fraction, one ml of the retrieved TRL fraction was mixed with 1 ml of propan-2ol (isopropanol) in hydrolysis tubes. The samples were mixed by pulse vortexing, then further vortexed using a multi vortexer for 1.5 minutes. These samples were stored at 4°C in a spark-free fridge/cold cabinet overnight or for at least 16 h. The samples were then centrifuged for 60 minutes at 4°C at 4000 rpm (corresponding to RCF of 1,792 x g) (Centra GP8R, Thermo UK). The supernatant was removed, and the precipitate from each sample was kept for further purification of apo B from lipids. Three ml Ethanol: diethyl Ether (in a ratio of 3:1) was added to each sample tube and stored in a spark-free -20°C freezer for at least 48 hours. The samples were then centrifuged at 4000 rpm (RCF of 1,792 x g) for 60 minutes at 4°C. The solvent was removed from each tube, and 3ml of diethyl Ether was added as a final step for complete delipidation. The samples were stored at -20°C for a minimum of 48 hours. The purified protein moiety of the particles was then centrifuged at 4000 rpm (RCF of 1792 x g) for 60 minutes. After centrifugation, samples were left to dry. If
necessary, Oxygen Free Nitrogen (OFN) (Nitroflow, Parker Filtration, and separation, Parker Hannifin Ltd, Maidstone, UK) was used for complete dryness. The dried pellet was reconstituted in sample buffer, and the samples were ready for preparation for separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

### 3.2.2.2 HDL-apoA-I

This method was obtained from a previous study in which the kinetics of HDL-apoA-I was measured (Li et al., 2012). To specifically precipitate apolipoprotein A-I from the fraction, 400 µl of the isolated HDL fraction was mixed with pre-cooled 8 ml mixture of Methanol and Diethyl Ether (4 ml each added separately) in hydrolysis tubes. Then the samples were mixed vigorously by pulse vortexing and multi vortexer for 1.5 minutes. These samples were then centrifuged at 4000 rpm (RCF of 1,792 x g) for 40 minutes at 1°C, the supernatant was removed, and the precipitate from each sample was kept. Another 4 ml of pre-cooled Diethyl Ether was added to each sample tube which was mixed vigorously by pulse vortexing. Then the samples were centrifuged at 4000 rpm (RCF of 1,792 x g) for 20 minutes at 1°C. After centrifugation, the solvent was removed from each tube, and the remaining precipitate in each sample tube was left to dry. If necessary, OFN was used for complete dryness. The dried pellet was reconstituted in sample buffer ready for preparation for separation by SDS-PAGE.

### 3.2.3 Preparation of protein samples in sample buffer for SDS-PAGE

The dried apoB and apoA-I pellets in hydrolysis tubes were reconstituted in 200µl and 75µl sample buffer PH 6.8 respectively for SDS-PAGE. The sample buffer contained 0.15 M sodium phosphate, 12.5% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.001% bromophenol blue. The samples were left in sample buffer for a minimum of 24 hours at 4°C prior SDS-PAGE.
ApoB sample tubes were then heated on a heating block for 10 minutes at 100°C for protein denaturation while apoA-I sample tubes were denatured for five minutes at 120°C. After heating, samples were immediately placed on ice for 10 minutes to stop further protein denaturation and structural damage. The samples were then centrifuged at 2500 rpm (RCF of 1,400 x g) for 15 seconds to spin down the condensation in the tubes after the heating stage. Following this, the samples were ready to be loaded on the SDS-PAGE.

3.2.4 Separation of TRL-apoB and HDL-apoA-I using SDS-PAGE

3.2.4.1 Resolving gel

Glass plates with 1.5 mm thick vertical slab gel separators were mounted on the apparatus for the preparation of the gels. The glass plates, slab gel separators, and Combs were soaked in 4M HCL or Decon Solution and cleaned with 70% ethanol/ deionized H₂O before the start of gel electrophoresis. The resolving gel for apoB and apoA-I was prepared by adding the components shown in Table 3.0 and 3.1 respectively which show specific amounts and the total volume per one gel. The gel was poured immediately after preparation taking care not to make bubbles. A thin layer of Isoamyl alcohol was placed across the top of the gel to seal the gel from oxidation and helped to remove any bubbles formed. The resolving gel for apoB was allowed to set for 1-1.5 hours at room temperature while the resolving gel for apoA-I took 15-25 minutes to settle as it contained a higher percentage of acrylamide which solidifies quicker than the apoB gel.
Table 3.0: Composition of a resolving gel solution for apoB gel electrophoresis

<table>
<thead>
<tr>
<th>Substance to add</th>
<th>Volume in ml for one gel preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized water</td>
<td>25.2 ml</td>
</tr>
<tr>
<td>40% Acrylamide (Bio-Rad) added as 4%</td>
<td>4 ml</td>
</tr>
<tr>
<td>Buffer A (1.5M Tris = 181.72 g/l, PH 8.8)</td>
<td>10 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>10% Ammonium persulfate (APS) 0.1 g/ml. Freshly made and added before use</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>Tetramethylethylenediamine (TEMED). To be added as the last component before use.</td>
<td>0.032 ml</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>40 ml</strong></td>
</tr>
</tbody>
</table>

SDS: sodium dodecyl sulphate; TEMED: Tetramethylethylenediamine; APS: Ammonium persulfate.

Table 3.1: Composition of a resolving gel solution for apoA-I gel electrophoresis

<table>
<thead>
<tr>
<th>Substance to add</th>
<th>Volume in ml for one gel preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized water</td>
<td>10 ml</td>
</tr>
<tr>
<td>40% Acrylamide (Bio-Rad) added as 10%</td>
<td>10 ml</td>
</tr>
<tr>
<td>Buffer A (Tris base 0.75M = 90.86 g/l, SDS 0.2%,PH 8.8)</td>
<td>20.2 ml</td>
</tr>
<tr>
<td>10% APS 0.1 g/ml. Freshly made and added before use</td>
<td>200 µl</td>
</tr>
<tr>
<td>TEMED. To be added as the last component before use</td>
<td>112 µl</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>40.5 ml</strong></td>
</tr>
</tbody>
</table>

SDS: sodium dodecyl sulphate; TEMED: Tetramethylethylenediamine; APS: Ammonium persulfate.

3.2.4.2 Stacking gel procedure

The Isoamyl alcohol was poured off the top of the gel and washed twice with Buffer B of either apoB or apoA-I as shown in Table 3.2 and 3.3 respectively which show specific amounts and the total volume per one gel. The stacking gel was then prepared, apoB for (Table 3.2) and for apoA-I (Table 3.3), and poured on the freshly prepared resolving gel. A comb was gently inserted into the stacking gel avoiding the formation of any air bubbles. This comb formed fifteen 65 µl wells at the top of the stacking gel for sample loading. The stacking gel for apoB was left to settle for two hours at room temperature while it took 30-
50 minutes for the apoA-I stacking gel to settle. Both the resolving and stacking gels were set up at room temperature, and they could be stored at 4°C until usage for a duration up to 24 hours.

Table 3.2: Composition of one stacking gel solution for apoB gel electrophoresis

<table>
<thead>
<tr>
<th>Substance to add</th>
<th>Volume in ml for one gel preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized water</td>
<td>7.35 ml</td>
</tr>
<tr>
<td>40% Acrylamide (Bio-Rad) added as 0.75%</td>
<td>0.75 ml</td>
</tr>
<tr>
<td>Buffer B (1.0M Tris = 121.14 g/l, PH 6.8)</td>
<td>1.67 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>10% APS 0.1 g/ml. Freshly made and added before use.</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>TEMED. To be added as the last component before use.</td>
<td>0.01 ml</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>10 ml</strong></td>
</tr>
</tbody>
</table>

SDS: sodium dodecyl sulphate; TEMED: Tetramethylethylenediamine; APS: Ammonium persulfate.

Table 3.3: Composition of one stacking gel solution for apoA-I gel electrophoresis

<table>
<thead>
<tr>
<th>Substance to add</th>
<th>Volume in ml for one gel preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized water</td>
<td>3.9 ml</td>
</tr>
<tr>
<td>40% Acrylamide (Bio-Rad) added as 1%</td>
<td>1.05 ml</td>
</tr>
<tr>
<td>Buffer B (Tris base 0.25M = 30.29 g/l, SDS 0.2%,PH 6.8)</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>10% APS 0.1 g/ml. Freshly made and added before use.</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED. To be added as the last component before use.</td>
<td>20 µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>10 ml</strong></td>
</tr>
</tbody>
</table>

SDS: sodium dodecyl sulphate; TEMED: Tetramethylethylenediamine; APS: Ammonium persulfate.

3.2.4.3 Sample loading and SDS-PAGE initiation

It is important to note that apoB and apoA-I were separated using two separate gels which had different compositions. After the stacking gel settlement, the complete SDS-PAGE apparatus was taken to be uploaded inside a tank. The Running buffer containing Glycine 10%, Tris 5%, SDS 1.0% (Bio-Rad, USA) was added to the reservoir to saturate negative and positive electrodes to generate an electric current required for the SDS-PAGE. Electrode saturation took place by filling both inner and outer compartments of the SDS-PAGE gel apparatus. After assuring that the gel apparatus was not leaking, the green Combs in the top of the stacking gel were removed carefully. The generated 15 wells were rinsed with the
running buffer to remove any solid particles which could interfere with sample loading and sample adhering to the stacking gel. Twenty µl of pre-stained ladder proteins (Bio-Rad, USA) with known molecular weight were loaded into the gel as markers for protein bands (Figure 3.1B). Then 55µl of TRL-ApoB or HDL-ApoA-I fraction proteins in sample buffer were pipetted into the wells. Spare wells also received 60 µl of sample buffer. This helped the samples run down the gel in straight columns when the electrophoresis was performed. ApoA-I samples were loaded in every other well to avoid overlapping of large apoA-I protein bands, and the SDS-PAGE run was at 80v for 16-20 hours overnight using a PowerPac basic power supply (Bio-Rad, USA). SDS-PAGE for apoB samples was run at 80v for 1 hour using the same PowerPac supply (Bio-Rad, USA) (Figure 3.1A) and then the voltage was changed to 60V to run for 18-20 hours overnight. The SDS-PAGE apparatus temperature was cooled using cold running tap water to prevent damage of separated proteins from over-heated electrodes during electrophoresis.

Figure 3.1: SDS-PAGE for TRL-apoB and HDL-apoA-I separation by SDS-PAGE. (A) PowerPack supply used. (B) Pre-stained Control ladder protein (250-10 kDa).
Figure 3.2: TRL-apoB-100, TRL-apoB-48 and HDL-apoA-I separation using the SDS-PAGE method. (A) A black arrow indicates HDL-apoA-I bands. (B) other black arrows indicate TRL-apoB-100 and TRL-apoB-48. The standard control ladder which starts at 250 kDa is also indicated in bold.
3.2.5 Silver stain and protein hydrolysis

After the completion of the SDS-PAGE, the gel was washed off the plate into the staining container using 200 ml of fixative solution (Methanol 50%, Acetic Acid 10%, Fixative enhancer concentrate (Bio-Rad, USA) 10%, deionised H₂O 30%) for 20 minutes. Then the gel was washed three times with 400 ml of deionized water. Finally, the gel was stained with 100 ml of freshly prepared staining solution (development accelerator solution (Bio-Rad, USA) 50%, Silver complex solution, Reduction moderator solution, and Image development reagent (Bio-Rad, USA) as 5% each, deionised H₂O 35 %). Once the bands on the gel were developed and visible after approximately 10-20 minutes, the staining was stopped by addition of 5% acetic acid (50ml) for a minimum of 15 minutes. The gel was photographed, and the bands were cut using a special gel cutter. Each sample band was then placed in a labelled hydrolysis tube, and 1ml of 6M HCL was then added. The tubes were then capped, vortexed and placed into a heating block for 24 hours. Protein moieties from the gel bands were hydrolyzed into AAs by incubation at 120 °C for 24 hours in the presence of 6M HCL.

3.2.6 Identification of apoB and apoA-I protein by western immunoblotting

3.2.6.1 Electrophoretic transfer procedure (wet transfer)

After SDS-PAGE, the bands were identified by Western immunoblotting. This procedure was followed immediately by electrophoresis. Two rectangular pieces of filter paper and one piece of 0.2um nitrocellulose membrane were prepared. The membrane and filter paper were soaked together with the fibre pads in transfer buffer pH 8.3 (Glycine 0.29%, Tris base 0.58%, SDS 0.037%, Methanol 20%) for 10 minutes, and the gel was immersed in the transfer buffer immediately. Then a wet sandwich was prepared for the transfer as follows; two pieces of filter paper were firstly put on a fibre pad, and the gel was then gently placed onto
the filter paper, then the wet nitrocellulose membrane was overlaid onto the gel and then covered with another piece of filter paper (Figure 3.2), and finally, another fibre pad was put on top to complete the sandwich. Then the sandwich was placed into a plastic sandwhiching cassette which was fitted into the mini transfer-blotting cell (Bio-Rad) as seen in Figure 3.3. The gel has been put on negative polarity and the membrane on the positive polarity. The electrophoretic transfer was run at 30v for 24 hours with running water to maintain a cold temperature.

Figure 3.3: Wet transfer “Sandwich” set-up. The pictures were obtained from Bio-Rad website [http://www.bio-rad.com/en-us/product/tetra-blotting-module].

3.2.6.2 Immunolocalisation procedure;

Once the transfer of proteins onto the nitrocellulose membrane was completed, the membrane was placed into a clear plastic box containing 100 ml of blocking buffer pH 7.5 (dried non-fat milk 5%, Tris base 10mM, NaCl 0.05M, Tween 20 0.5%) and shaken for 1 hour at room temperature. This has been shown to reduce nonspecific binding of the antibody to the membrane. Then the blocking buffer was poured off, and the membrane was washed with 100 ml PBST solution pH 7.4 (PBS (phosphate buffered saline) 1pack (sigma), Tween 20 0.05%) for 10 minutes. Table 3.4 shows the primary (1°) and secondary (2°) antibodies (AB) with their concentration to detect: total apoB, apoB-100, apoB-48 and apoA-1 protein bands by western immunoblotting. The membrane was incubated with a primary antibody AB prepared in the blocking buffer for 1 hour at room temperature (Table 3.4). The membrane
was then washed with 100 ml PBST solution for 10 minutes, and the wash repeated three times to remove excess antibody. The membrane was then incubated with a secondary antibody prepared in the blocking buffer for 1 hour at room temperature (Table 3.4). The membrane was then washed with 100 ml PBST solution for 10 minutes, and the wash was repeated three times to remove excess secondary antibody. The final step was staining the membrane with 50 ml alkaline phosphatase (AP) staining reagent (AP colour development buffer 25x 4% + AP colour Reagent A 2% + AP colour Reagent B 2% + H₂O). The apolipoprotein bands (either apoB-100, apoB-48 or apoA-I) appeared and were identified after 2-10 minutes. Upon visualisation of the bands, the staining reaction was terminated by adding 50 ml deionized H₂O (Figure 3.4).

Table 3.4: Primary and secondary antibody (AB) used in Western Immunoblotting to identify total apoB, apoB-100, apoB-48, and apoA-I.

<table>
<thead>
<tr>
<th>AB used</th>
<th>Total ApoB</th>
<th>ApoB-100</th>
<th>ApoB-48</th>
<th>ApoA-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>1° AB and concentration used</td>
<td>1D1 mouse anti-apoB monoclonal. Used at 1mg/ml as 10µl in 80ml blocking buffer.</td>
<td>4G3 mouse anti-apoB-100. Used at 1mg/ml as 10µl in 100ml blocking buffer.</td>
<td>151 JAP mouse anti-apoB-48. Used at 0.2mg/ml as 75µl in 100ml blocking buffer.</td>
<td>1. 4H1 mouse anti-apoA-I. Used at 0.5mg/ml. 2. AB6713 goat anti-apoA-I at 1mg/ml. Both prepared as 10µl in 100ml blocking buffer.</td>
</tr>
<tr>
<td>2° AB and concentration used</td>
<td>Donkey anti-mouse AP. Used at 1mg/ml as 200µl in 100ml blocking buffer.</td>
<td>Donkey anti-mouse AP. Used at 1mg/ml as 200µl in 100ml blocking buffer.</td>
<td>Donkey anti-mouse AP. Used at 1mg/ml as 200µl in 100ml blocking buffer.</td>
<td>Donkey anti-mouse AP at and Donkey anti-goat AP. Both used at 1mg/ml as 200µl in 100ml blocking buffer.</td>
</tr>
</tbody>
</table>

1°: primary; 2°: secondary; AB: antibody; apo: apolipoprotein; AP: Alkaline Phosphatase.

3.2.7 Purification of AAs by ion exchange chromatography (IEC)

AAs from hydrolysed apoB-100, apoB-48 and apoA-I were purified by ion exchange chromatography (IEC). IEC is based on the ionic interactions between the charged molecules in the mobile phase and the charged solid support in the immobile phase. Regarding cation exchange chromatography which was used, positively charged AAs in 6M HCl (at low pH) bound to the negatively charged solid support AG-50W Resin (Bio-
Figure 3.4: TRL-apoB-100, apoB-48 and HDL-apoA-I characterisation using western immunoblotting method. HDL-apoA-I bands identified and characterised using (A) AB6713 apoA-I specific antibodies, (B) 4H1 apoA-I antibodies. TRL-apoB bands identified and characterised using (C) TRL-apoB-100 using 4G3 mouse anti-apoB-100 and (D) TRL-apoB-48 using 151 JAP antibodies. SDS-PAGE and western immunoblotting methods were used respectively. The standard control ladder which starts at 250 kDa is also indicated in bold.
Rad). To elute the AAs, 4M ammonium hydroxide (NH₄OH) was added to increase the pH of the mobile phase which results in an ultimate reduction of the ionic interaction between AAs and the resin. As a result, the AAs were released from the resin, and all unwanted materials were removed.

The hydrolyzed samples from SDS-PAGE were cooled on ice before they were applied to IEC columns (Evergreen, UK). Then they were centrifuged at 2500 rpm (RCF of 1400 x g) rpm at 4°C to precipitate unwanted gel material which can block the column during IEC. Samples were then loaded onto the IEC columns with 1ml acid washed resin at pH 7.0 measured by instant pH 0-14 indicator paper (GE-Whatman, UK). The low pH which developed in the IEC column after sample pouring was restored to pH 7.0 by washing the column three to four times with 1ml deionised H₂O. The samples were eluted by the addition of 3 ml of 4M NH₄OH. The eluted samples were collected in labelled glass vials, and then frozen and concentrated by freeze-drying (ModulyoD Freeze Dryer, Thermo Electron Corporation, UK) to remove the solvent.

### 3.2.8 Leucine derivatisation

Freeze dried samples containing purified AAs were derivatized for the determination of 1-¹³C leucine enrichment by gas chromatography-mass spectrometry (GC-MS). Leucine was converted into a volatile and thermally stable trifluoromethyl oxazolinone (Oxazolinone) derivative (Figure 3.5) (Dwyer et al., 2002). The oxazolinone derivative was analysed by negative ion chemical ionisation GC-MS. Oxazolinone was formed in a rapid single step procedure by adding a mixture of 50µl TFAA and 50µl TFA. The samples were then mixed by vigorous vortexing and heated at 110°C for five minutes. Then 500µl toluene and 1ml deionised H₂O were added respectively for the extraction of the volatile oxazolinone derivative. The samples were then capped, vortexed, and centrifuged at 2500 rpm (RCF of
1,400 x g) for 10 minutes at 4°C to separate the mixture into two layers. Finally, the top toluene layer in each sample was taken into a GC vial (03-FIV C201 Chromacol LTD, UK) and capped for GC-MS analysis which contained the oxazolinone leucine derivative.

![Chemical structure of oxazolinone derivative of leucine obtained from Dwyer et al., 2002.](image)

**3.2.9 Determination of leucine isotopic enrichment of apo B-100, apo B-48, and apoA-I by GC-MS**

**3.2.9.1 Standard Curve for Leucine enrichment**

It was essential to confirm and verify that the GC-MS detector was functioning properly before analysing any samples on the GC-MS. Therefore, a range of standards with different enrichment was prepared to determine the sensitivity, linearity, and accuracy of GC-MS. Both 1-¹²C-leucine and 1-¹³C-leucine were used for the preparation of standards. The standard curve was made with a constant ¹²C-leucine concentration, but increasing ¹³C-leucine concentrations in eight different concentrations. These were derivatized and analysed by the GC-MS. The theoretical ratio was plotted against observed (area ratio) AR from the GC-MS.

The preparation of the standard curve is shown in Table 3.5 which shows the concentrations, theoretical and observed ratios of tracer (¹²C) and tracee (¹³C) respectively. The standard
curve is demonstrated as the measured $^{13}\text{C}/^{12}\text{C}$ peak tracer: tracee ratio (TTR) plotted against the theoretical TTR. A linear relationship was indicated by a slope approximating to unity (0.9731) (Figure 3.6). It was essential to check that the range of TTRs in the standard curve was appropriate for the sample TTRs. Also, three sets of QCs was prepared and included in beginning and end of each assay which would imply that any subsequent sample run was accurately measured. The QCs for each assay were represented as QC$_1$ (low), QC$_2$ (middle), and QC$_3$ (high) with AR mean ± standard error of the mean (SEM), and the intra-assay Coefficient of Variations (CVs) were (0.62%, 0.74%, and 0.42%) respectively (n=6).

Table 3.5: Preparation of standards for TRL-apoB and HDL-apoA-I leucine samples

<table>
<thead>
<tr>
<th>standards</th>
<th>Tracer $^{13}\text{C}$ (µg)</th>
<th>Tracee $^{12}\text{C}$ (µg)</th>
<th>Theoretical TTR $^{13}\text{C}/^{12}\text{C}$</th>
<th>Observed ratio $^{13}\text{C}/^{12}\text{C}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.0015</td>
<td>0.00000</td>
<td>0.09110</td>
</tr>
<tr>
<td>2</td>
<td>0.000005</td>
<td>0.0015</td>
<td>0.00335</td>
<td>0.09514</td>
</tr>
<tr>
<td>3</td>
<td>0.000010</td>
<td>0.0015</td>
<td>0.00669</td>
<td>0.09662</td>
</tr>
<tr>
<td>4</td>
<td>0.000030</td>
<td>0.0015</td>
<td>0.02008</td>
<td>0.10975</td>
</tr>
<tr>
<td>5</td>
<td>0.000050</td>
<td>0.0015</td>
<td>0.03346</td>
<td>0.12291</td>
</tr>
<tr>
<td>6</td>
<td>0.000075</td>
<td>0.0015</td>
<td>0.05019</td>
<td>0.13929</td>
</tr>
<tr>
<td>7</td>
<td>0.000101</td>
<td>0.0015</td>
<td>0.06693</td>
<td>0.15750</td>
</tr>
<tr>
<td>8</td>
<td>0.000151</td>
<td>0.0015</td>
<td>0.10039</td>
<td>0.18800</td>
</tr>
</tbody>
</table>

$^{13}\text{C}$, levorotary labelled leucine; $^{12}\text{C}$, unlabeled leucine; TTR: tracer/tracee ratio.

Figure 3.6: Leucine standard curve for TRL-apoB and HDL-apoA-I fractions. Calibration graph showing the ratio of labelled ($L^{13}\text{C}_6$) to unlabelled Leucine ($^{12}\text{C}_6$). Results are mean ± SEM (n=6).
3.2.9.2 Measurement of $^{13}$C leucine isotope enrichment

Isotopic enrichment of apoB-100, apoB-48 and apoA-I were measured by the GC-MS (GC system, Agilent 5973C inert XL E/I/Cl network MSD, Agilent Technologies, Wokingham, Berkshire, UK) (Ackermans et al., 1998). The GC was equipped with a capillary column of 30 meters 0.25 mm inner diameter (J&W Scientific, Inc. CA, USA) in which helium was used as the carrier gas. The GC-MS was operated in a negative chemical ionisation (NCI) mode using methane as the reagent gas. The initial temperature of the GC oven was 50°C for one minute, and the ramp was 6°C/minute to 90°C, 30°C/minute to 280°C. Samples were loaded in the sequential order and injected with an Agilent 7683 autosampler.

The oxazolinone derivatives of $^{12}$C leucine and $^{1-13}$C leucine have a molecular mass of 209 and 210 respectively. Therefore, the AR of ions at m/z 210 (ion fragment of $^{1-13}$C Leucine), representing the tracer, and m/z 209 (ion fragment of $^{12}$C Leucine) representing the tracee, were calculated at each time point as TTR of 210/209. This specifies the concentration of enrichment after the initiation of tracer infusion at t=0. TTR at each time point after the start of tracer infusion was calculated by subtracting the background AR at t=0 multiplied by 100 (Dwyer et al., 2002) as: \[ \text{TTR} = \frac{\text{AR} \text{ 210/209 after infusion}}{\text{AR} \text{ 210/209 baseline (t=0)}}. \]

The calculation of TTR was used for the calculation of apoB-100, apoB-48 and apoA-I kinetics (Dwyer et al., 2002).

3.2.10 Determination of $\alpha$-ketoisocaproate ($\alpha$-KIC) isotope enrichment

Plasma samples obtained for the measurement of $\alpha$-KIC isotopic enrichment were kept at -80°C until analysis. Plasma samples were thawed, vortexed and then centrifuged at 2500 rpm (RCF of 1400 x g) for 10 minutes at 4°C to precipitate any proteins. One hundred µl of
the plasma was added into a small glass test tube containing 1 ml ethyl alcohol. Samples were vortexed and centrifuged at 2500 rpm (RCF of 1400 × g) for 10 minutes at 4°C. The supernatant was transferred to new vials by glass pipettes and dried under OFN at 50°C. The remainder was dissolved in a mixture of 200 μl deionized H₂O and 100 μl O-phenylenediamine (2%) dissolved in 4M HCl, and placed on the heating block at 90°C for 1 hour. Samples were left to cool down to room temperature. One ml of ethyl acetate was added to extract the keto-acid. The top layer containing the keto-acid was collected in another tube. The extraction process was repeated by adding another 1 ml ethyl acetate. The extracts were dried over sodium sulphate followed by evaporation under OFN at room temperature. The remainder was derivatized by 100 μl acetonitrile and 100 μl N-Methyl-N-(tertbutyldimethylsilyl) trifluoroacetamide (MTBSTFA) with 1% trimethylchlorosilane. Samples were placed on a heating block at 120°C for 45 minutes. The excess derivatizing reagent was removed by using OFN at room temperature. One hundred μl decane was added to the derivative 4-methyl-2-Oxpentanoic acid sodium salt, and the whole mixture was collected in GC vials.

The preparation of α-KIC standard curve is shown in Table 3.6 which shows the concentrations, theoretical and observed ratios of D1 tracer (¹²C) and D0 tracee (¹³C) respectively and Figure 3.7. The α-KIC derivative 4-methyl-2-Oxpentanoic acid sodium salt was analysed as D1 and D0. D1 represents the labelled 4-methyl-2-oxpentanoic-1-¹³C acid sodium salt tracer, and D0 was the unlabelled 4-methyl-2-oxpentanoic-1-¹²C acid sodium salt tracee. D1 and D0 have a molecular mass of 260 and 259 respectively. A-KIC isotopic enrichment (Figure 3.5) was measured by electron ionisation (EI) GC-MS by the selected ion monitoring of D0 fragments at m/z 259 (m) and D1 fragments m/z 260 (m+1). Chromatographic peaks were determined, and the TTR of α-KIC (D1 ¹³C/ D0 ¹²C) was
calculated. The α-KIC observed isotope TTR enrichment was plotted against the theoretical TTR. Also, three sets of QCs was prepared and included in beginning and end of each assay which would imply that any subsequent sample run was accurately measured. The QCs were represented as QC1 (low), QC2 (middle), and QC3 (high) with AR mean ± SEM with an intra-assay CVs of (0.67%, 0.66%, and 0.58%) respectively (n=6).

Table 3.6: Preparation of standards for TRL-apoB and HDL-apoA-I α-KIC samples

<table>
<thead>
<tr>
<th>Standards</th>
<th>D1 Tracer $^{13}$C (µg)</th>
<th>D0 Tracee $^{12}$C (µg)</th>
<th>Theoretical TTR $^{13}$C/$^{12}$C</th>
<th>Observed ratio $^{13}$C/$^{12}$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1.00</td>
<td>0</td>
<td>0.22716</td>
</tr>
<tr>
<td>2</td>
<td>0.1000</td>
<td>1.00</td>
<td>0.010513</td>
<td>0.24115</td>
</tr>
<tr>
<td>3</td>
<td>0.02500</td>
<td>1.00</td>
<td>0.026283</td>
<td>0.25756</td>
</tr>
<tr>
<td>4</td>
<td>0.05000</td>
<td>1.00</td>
<td>0.052566</td>
<td>0.28639</td>
</tr>
<tr>
<td>5</td>
<td>0.07500</td>
<td>1.00</td>
<td>0.078849</td>
<td>0.31540</td>
</tr>
<tr>
<td>6</td>
<td>0.10000</td>
<td>1.00</td>
<td>0.105132</td>
<td>0.34485</td>
</tr>
</tbody>
</table>

D1 $^{13}$C, labelled 4-methyl-2-oxpentanoic-1-$^{13}$C acid sodium salt; D0 $^{12}$C, unlabelled 4-methyl-2-oxpentanoic $^{12}$C acid sodium saline. TTR: tracer/tracee ratio.

Figure 3.7: α-KIC standard curve for TRL-apoB and HDL-apoA-I fractions. Calibration graph showing labelled (D1 $^{13}$C) to unlabelled α-KIC (D0 $^{12}$C) ratio. Data are mean ± SEM (n=6).

3.2.11 Measurement of TRL-apoB-100 and TRL-apoB48 by competitive Enzyme-linked immunosorbent assays (ELISA)

3.2.11.1 TRL-apoB-100 ELISA

This was an in-house assay used to measure TRL-apoB-100 concentration from the obtained TRL fraction. This quantitative sandwich enzyme immunoassay was prepared along with a set of standards and QCs to check the accuracy of the assay every time it was used. The main
The idea behind this assay was to capture the apoB-100 antigen in the sample by creating a sandwich between the primary antibody, a polyclonal anti apoB, and the secondary antibody, biotinylated monoclonal 4G3 antibody, specific to apoB-100. The excess of both antibodies along with the sample were washed off inside the well and nothing remained but the sandwich. Streptavidin was added to the wells as an enzyme to facilitate a colour producing reaction. Finally, a substrate was added in the end to produce a colour, and the colour intensity was measured by reading at 540nm in a plate reader (DYNEX Technologies, Opsys ELISA microplate reader, UK). A standard curve of the known standards concentration and optical densities (ODs), which is also known as absorbance, was used to calculate the concentration of the unknown sample concentration using its absorbance. The standards were prepared as eight standards with a gradual increase in concentration (S1-S8), and stored in -80°C to be used as slots for the assay as shown in Table 3.7 which shows the optical density (OD) and concentration of each standard prepared. In each assay, a freshly defrosted set of eight standards and QC were used. The plate reader software (DYNEX Technologies Revelation software version 4.25, UK) was used to calculate and create the best curve fitting for the standards based on the concentrations uploaded and measured ODs (Linear/Linear axes scaling by cubic regression was used as the standards curve fit for this assay). The standard curve was then used to calculate the TRL-apoB-100 concentrations based on their measured ODs (Figure 3.8). The QC used in this assay was an isolated in-house LDL fraction which was used as a positive QC for this assay to ensure that the assay was working properly.
Table 3.7: Preparation of standards for TRL-apoB-100 ELISA assay.

<table>
<thead>
<tr>
<th>Standards</th>
<th>OD (mean) n=4</th>
<th>Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.02025</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.13175</td>
<td>0.02</td>
</tr>
<tr>
<td>3</td>
<td>0.255</td>
<td>0.05</td>
</tr>
<tr>
<td>4</td>
<td>0.58625</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>0.85825</td>
<td>0.133</td>
</tr>
<tr>
<td>6</td>
<td>1.15475</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>1.816</td>
<td>0.4</td>
</tr>
<tr>
<td>8</td>
<td>2.259</td>
<td>1</td>
</tr>
</tbody>
</table>

OD; optical density.

Figure 3.8: ELISA standard curve for TRL-apoB-100 assay. Results are mean ± SEM (n=3)

3.2.11.2 Method of TRL-apoB-100 ELISA

Microplate wells are coated with primary antibody; sheep Anti-human apoB (IgG fraction) (Binding site, UK) in 100 µl coating phosphate buffer (PBS 1 pack, pH 7.5) 2-3 days before the assay, covered and stored at 4°C in a designated box. TRL-apoB-100 samples were diluted with dilution buffer A (phosphate buffer + 0.02% Thimerosal (VWR International LTD., UK) as 1:700, and the QC was diluted as to 1:35,000. On the day of the assay, the plate is allowed to warm up to the room temperature (R/T), washed three times with the washing buffer (0.02 % thiomersal and 0.1% Triton (Triton X-100, Merck Millipore, Germany)) in which each wash took 300µl/well. The plate was then blocked with the blocking buffer (Phosphate buffer + 1% bovine serum albumin (BSA) (Sigma, UK), 0.02 % thiomersal) at R/T for one hour and then washed three times as before. Standards and diluted samples were pipetted
(100µl) to the well and incubated at R/T for two hours. The plate was then washed three times followed by addition of secondary antibody B-4G3 (biotinylated monoclonal anti-apoB-100, prepared in-house) in 100µl diluting buffer B (Phosphate buffer, 0.01% thiomersal, 0.5% BSA) and incubated at R/T for two hours. Then the plate was washed three times as before, and a streptavidin-Alkaline Phosphatase dilutes at 1:30,000 in 100µl diluting buffer B and incubated either at R/T for one hour or overnight at 4°C. The plate was then washed three times as before, and a 100µl substrate solution (Phenolphthalein monophosphate di (Cyclohexylammonium) salt (Sigma, UK) was dissolved in 8mg/ml in substrate buffer (Diethanolamine, NaN₃, MgCl₂ (Sigma, UK), HCl in H₂O, pH 9.8) was added to the wells and incubated at R/T up to 45-60 minutes. Once a good intensity of pink colour was achieved in the most concentrated standard well, the reaction was stopped by addition of 200µl stop buffer (Glycine, EDTA, NaOH (Sigma, UK) at pH 10.4). The plate was then read immediately in the plate reader at 540nm, and TRL-apoB-100 concentrations were calculated in mg/ml by the plate reader revelation software.

3.2.11.3 TRL-apoB-48 ELISA assay

This assay was purchased commercially as an ELISA kit (Human apoB-48 ELISA Kit, AKHB48, Shibayagi Co., LTD. Japan, facilitated by Oxford Biosystems, UK). Therefore, all materials used for this assay such as buffers for dilution, washing, enzyme and substrate solution was obtained from the commercial kit, and only the QCs and microplate reader and its software were used for the calculation of TRL-apoB-48 concentrations. The assay performance characteristics such as the assay range, specificity, precision and reproducibility are shown in Table 3.8. The assay principle was that provided standards and samples were incubated in monoclonal antibody-coated wells to capture apoB-48. After an hour of incubation and proper washing, the biotin-conjugated anti-apoB-48 antibody was added and incubated for
one hour. After proper washing, horseradish peroxidase (HRP) conjugated avidin was added to react with a chromogenic substrate reagent (TMB) for 20 minutes, and the reaction was stopped by addition of acidic solution, and absorbance of yellow product was measured spectrophotometrically at 450nm using the plate reader used with the previous assay. The absorbance was proportional to aboB-48 concentration. The standards were freshly prepared on the assay day as eight standards with gradual increase in concentration (S1-S8) as seen in Table 3.9 which shows the optical density (OD) and concentration of each standard prepared. Plate reader software (DYNEX Technologies Revelation software version 4.25, UK) was used to calculate and create the best curve fitting for the standards based on the concentrations uploaded and measured ODs (Logarithmic/Logarithmic axes scaling by cubic spline was used as the standards curve fit for this assay). The standard curve was then used to calculate the TRL-apo-B-48 concentrations based on their measured ODs as seen in Figure 3.9. In terms of QC, positive and negative QCs was used in this assay; positive QCs were the lymph fractions which were full of chylomicrons-apoB-48 (batch 2, in-house prepared), and the negative QC was the LDL fraction (In-house prepared, used for the TRL-apoB-100 ELISA assay) to make sure that the assay was working properly.

<table>
<thead>
<tr>
<th>Performance characteristics</th>
<th>Value information*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay range</td>
<td>2.5 ng/ml - 160 ng/ml</td>
</tr>
<tr>
<td>Specificity</td>
<td>All antibodies in this kit were specific to human apoB-48 and had no crossreactivity with human apoB-100</td>
</tr>
<tr>
<td>Precision of the Assay</td>
<td>Assay of 3 samples with five replicates gave a Mean CV of 3.5%</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>Assay of 3 samples with five replicates repeated for three days gave a CV of 2.8-8.6%</td>
</tr>
</tbody>
</table>

*All values were obtained from the instruction manual included in the ELISA kit version 9 issued on December 3, 2012.
Table 3.9: Preparation of standards for TRL-apoB-48 ELISA assay

<table>
<thead>
<tr>
<th>Standards</th>
<th>OD (mean) n=4</th>
<th>Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1345</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.175</td>
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<td>3</td>
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</tr>
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</tr>
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<td>0.5085</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
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<td>40</td>
</tr>
<tr>
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</tr>
<tr>
<td>8</td>
<td>2.29</td>
<td>160</td>
</tr>
</tbody>
</table>

OD; optical density.

Figure 3.9: ELISA standard curve for TRL-apoB-48 assay. Results are mean ± SEM (n=3)

3.2.11.4 TRL-apoB-48 ELISA assay method

TRL-apoB-48 samples were diluted with assay dilution buffer as 1:250, and the QC s were diluted as 1:300. On the day of the assay, the plate is brought up to warm up to the R/T, washed four times with the assay washing buffer in which each wash took 300µl/well. Standards and diluted samples were pipetted (50µl) to the well, shaken and incubated at R/T for one hour. The plate was then washed four times as before followed by addition of 50µl biotin-conjugated anti-apoB-48 antibody assay solution, shaken and incubated at R/T for one hour. Then the plate was washed four times as previously and 50µl Peroxidase-conjugated streptavidin assay solution were added, shaken and incubated either at R/T for 30 minutes at R/T. The plate was then washed six times as before, and 50µl Chromogenic substrate assay reagent solution were added to the wells and incubated at R/T up to 20 minutes. The reaction was stopped by addition of 50µl of the reaction stopper assay buffer. The plate was
then read immediately in the plate reader at 450nm within a maximum period of 30-40 minutes, and TRL-apoB-48 concentrations were calculated in ng/ml by the microplate reader revelation software.

3.2.12 Measurement of TG, cholesterol and HDL-Cholesterol (HDL-C) concentration

Blood samples were collected in EDTA, lithium heparin, and plain tubes, and then they were separated by centrifugation at 2500 rpm (RCF of 1400 × g) for 10 minutes at 4°C to obtain either serum or plasma. The concentration of plasma TG, TRL-TG-F (TG concentration on CMs and VLDL fraction samples), plasma total cholesterol and total cholesterol fractions were measured.

The plasma TG and TRL-TG-F concentrations were determined by the ABX Mira analyser (Triacylglycerols CP, kit ref: A11A01640; Horiba ABX, France). The TG assay kit was used, and the measurement was based on an enzymatic photometric method. TGs in the sample underwent few enzymatic reactions to produce a colorimetric indicator “Quinoneimine” which was detected by a colorimetry spectrometry. The concentration of TGs was calculated by measuring the absorbance of quinoneimine at 500 nm wavelength. The QCs were represented as N (low) and P (high) with an intra-assay CVs of 1.98% and 2.23% respectively (n=3).

The plasma and fraction cholesterol concentrations were measured by the ABX Mira analyser using enzymatic cholesterol assay kits (kit ref: A11A01634; Horiba ABX, France) as prescribed previously to produce Quinoneimine. The concentration of cholesterol was calculated by measuring the absorbance of Quinoneimine at 500 nm wavelength. QCs were prepared and represented as N (low) and P (high) with an intra-assay CVs of 3.53 % and 1.45% respectively (n=3).
HDL-C concentrations in plasma and fractions were also measured by the ABX Mira analyser using HDL enzymatic kit (Horiba ABX, Northampton, UK). The method was based on accelerating the reaction of cholesterol oxidase (CO) with non-HDL unesterified cholesterol and dissolving HDL selectively using a particular detergent; ‘Accelerator Selective Detergent’ (kit ref: A11A01636; Horiba ABX, France). Two main enzymatic reagents were used; one was responsible for the solubilization of HDL specifically, and the other one was used to develop a colour for the quantitative determination of HDL-C which was measured at a wavelength of 600 nm. QCs were prepared with N (low) and P (high) with an intra-assay CVs of 8.48% and 7.35% respectively (n=3).

3.2.13 Measurement of apoA-I concentration in plasma and total HDL fractions

ApoA-I concentration in the HDL fraction was measured by an automatic ABX Mira analyser (Horiba ABX, France) using an immunoturbidimetric method. Two reagents from one kit (kit ref: A11A01687; Horiba ABX, France) were used for the assay; apoA-I specific reagent which is a fraction of purified immunoglobulins from rabbit antiserum and apoA-I immunogen from human HDL. ApoA-I reacts with its specific antibody and generates the immune complexes. The produced substance was subjected to a beam of light. The proportion of apoA-I concentration in the samples was represented by the intensity of the scattered light. The low and high-quality controls (QCs) with an inter-assay CVs of 7.531% and 3.173% respectively (n=3).

3.2.14 Measurement of glucose and insulin concentration

Plasma glucose concentrations were measured by an auto glucose analyser (YSI 2300 STAT Plus. Analytical @ Technologies, UK). The enzymatic reaction resulted in the production of
hydrogen peroxidase $\text{H}_2\text{O}_2$, which is used to determine the glucose concentration. Each sample was measured twice, and the average value was used.

Plasma insulin was measured using a double-antibody radioimmunoassay (RIA) called Millipore Human Insulin assay (Merck Millipore, MA, USA) using the double antibody/PEG technique (Desbuquois and Aurbach, 1971). This method was incubating the plasma sample with an antibody-bound tracer overnight. Then the sample was incubated with a second antibody for further 2 hours. The Labelled human insulin was determined by a radioactivity gamma counter which can distinguish the bound from the unbound tracer using the kit (Kit ref: HI-1AK; Merck Millipore, MA, USA). Also, a standard curve was prepared and included in each assay which would indicate that any subsequent sample run was accurately measured.

3.2.15 Anthropometrics

Body weight, body fat and BMI were measured using a body Composition Scale (Tanita Body Composition Analyzer BC-418MA) by applying electrical bio-impedance.

3.2.16 Magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) for the measurement of liver, muscle and whole body fat

Visceral and total fat mass was determined by MRI. Intrahepatocellular lipid (IHCL) and intramyocellular lipids (IMCL) were measured by $^1$H-magnetic resonance spectroscopy ($^1$H-MRS), and the spectra were acquired using a 1.5 T multinuclear system (Philips Medical Systems, Best, the Netherlands). All the measurements were performed in the Robert Steiner, MRI Unit at the Hammersmith Hospital in London, and participants were asked to fast for about 6 hours before they visit the unit. $^1$H-MRS has been used instead of a liver biopsy, ultrasound or CT as it safe to use (non-invasive), reliable and sensitive. It can detect a
low hepatic steatosis fat percentage down to less than 5% (Springer et al., 2010, Cowin et al., 2008, Dutour et al., 2016, Reeder et al., 2011).

3.2.17 HDL-apoA-I kinetics measurements

HDL-apoA-I kinetics (production rate (PR) and fractional clearance rate (FCR)) were calculated using the following variables;

a. ApoA-I TTR of each hourly time point throughout the study.

b. KIC TTR of each time points throughout the study.

c. Apo-A-I concentration from obtained HDL fraction of each time point.

d. Plasma volume (PV) of each participant at each visit which is determined by body weight (BW) and height.

The equations were applied as follows;

**Equation 1:** Fractional secretion rate (FSR) of HDL-apoA-I was calculated by linear regression as used in a previous study for the measurement of HDL apoA-I kinetics (Li et al., 2012).

\[ \text{FSR (pools/day)} = \left( \frac{\text{Slope of the apoA-I TTR time curve}}{\text{KIC}_{\text{TTR}}} \right) \times 24 \times 60. \]

**Equation 2:** In a steady state, the FSR is equal to the fractional catabolic rate (FCR). The production rate (PR) was calculated from the FSR and the pool size as follows;

\[ \text{PR (mg/kg/day)} = \text{FSR} \times \text{HDL apoA-I pool size} \]

**Equation 3:** ApoA-I pool size was calculated by the concentration of apoA-I and plasma volume (PV) divided by body weight (BW) as follows;

\[ \text{ApoA-I pool size (mg/kg)} = \text{HDL apoA-I concentration} \times \text{PV} / \text{BW} \]

**Equation 4:** Apo-Al PV was calculated (Pearson et al., 1995) as follows;

\[ \text{PV (ml)} = 1578 \times \text{Surface area m}^2 \text{ (SA)} \]

**Equation 5:** The surface area (SA) was calculated (Du Bois and Du Bois, 1989) as follows;

\[ \text{SA (m}^2) = \text{BW}^{0.425} \times \text{Height}^{0.725} \times 0.007184 \]
3.2.18 TRL-apoB-100 and B-48 kinetics

TRL-apoB kinetics was calculated using computer modelling software SAAM II (SAAM Institute, Seattle, WA, USA) in which a model developed by Dr Roman Hovorka (University of Cambridge, UK) was used to obtain the best-fit curves using TRL-apoB-100, TRL-apoB-48, and α-KIC concentrations (Figure 3.10 A and B). To calculate TRL-apoB kinetics, important values for each time point throughout the study were requested by the programme;

1. TTR of apoB-100 and apoB-48.
2. TTR of α-KIC.
3. ApoB-100 and apoB-48 concentration.
4. ApoB-100 and B-48 pool size which was calculated using the three equations used earlier to calculate apoA-I kinetics;

**Equation 1:** ApoB-100 and B-48 pool size were calculated from the concentration of apoB-100, apoB-48, and PV divided by BW as follows;

\[
\text{ApoB-100 pool size (mg/kg)} = \text{TRL-apoB-100 concentration} \times \frac{\text{PV}}{\text{BW}}
\]

\[
\text{ApoB-48 pool size (mg/kg)} = \text{TRL-apoB-48 concentration} \times \frac{\text{PV}}{\text{BW}}
\]

PV and SA were calculated using equations 4 and 5 which were used earlier to calculate apoA-I kinetics.
Figure 3.10: The compartmental model for measuring TRL-apoB-100 (A) and TRL-apoB-48 (B) kinetics using SAAM II model. A-KIC: α-ketoisocaproate; VLDL: very low-density lipoprotein; Apo: apolipoprotein; CM: chylomicron; K: rate of transfer of leucine.

3.2.19 Statistical analysis

All the statistics tests were performed using Graph Pad Prism Version 6.07, Microsoft Excel 2010 and SPSS 22 (SPSS Inc.; Chicago USA). The distributions of all data were examined by checking their skewness and kurtosis, and performing Kolmogorov-Smirnov, D'Agostino & Pearson omnibus and Shapiro-Wilk normality tests. All data were analysed using parametric tests.

3.2.19.1 Parametric tests used

- To determine whether postprandial TG concentrations were constant in a steady state over time Repeated Measures ANOVA was carried out.
- To compare repeated measurements after the two interventions in each study (exercise group vs. control group and post lixisenatide treatment vs. placebo) Paired-samples, two-tailed t-test was performed.
- To determine the differences (delta Δ values) and the effect of exercise within one group pre-intervention data values minus post-intervention data values were calculated.

- To determine whether the effects of exercise on post intervention data between the two groups are different (post-exercise versus post-control) in the NAFLD study (the end-point test), an unpaired sample Two-tailed t-test was performed.

- To determine whether the effects of exercise were different in the two groups in the NAFLD study, an unpaired sample Two-tailed t-test was performed on the Δ values. This test was applied to determine the effect of exercise between the two groups if the previous test failed to detect a significance difference due to an existing difference in the baseline measurements between the two groups after participant’s random distribution by coincidence.

- To test whether two curves were statistically different; Area under the curve (AUC) over time was calculated, and the slope of each curve was tested using Linear Regression.

- To test the difference between the baseline measurements of the NAFLD study vs. the lixisenatide study, unpaired samples Two-tailed t-test was performed.

- Testing for correlations between different variables was carried out by Pearson correlation analysis corrected by Bonferroni correction test to exclude false positive results.
Chapter 4: Development of a feeding and a laboratory protocol to measure postprandial TRL-apoB-100 and apoB-48 kinetics

4.1 Introduction

Atherogenic dyslipidaemia is one of the major characteristics of T2D and NAFLD which is strongly associated with CVD risk (Taskinen and Boren, 2015, Alcala-Diaz et al., 2014). Elevated Postprandial TRL concentrations are strongly related to atherogenic dyslipidaemia (Tushuizen et al., 2010). Therefore, many studies have looked at possible effects of elevated postprandial TRL on increasing the risk of CVD and CHD in patients with T2D and NAFLD via atherogenic dyslipidaemia (Tushuizen et al., 2010, Taskinen and Boren, 2015, Alcala-Diaz et al., 2014). Some studies have investigated possible mechanisms by measuring TRL-TG and TRL-apoB kinetics (Sun et al., 2013, Xiao et al., 2012). Feeding protocols were developed to establish a postprandial TG steady state in the plasma for a long duration such as 8 hours to study TRL kinetics under the influence of different dietary conditions and drugs (Sun et al., 2013, Xiao et al., 2012). These studies investigated TRL kinetics using protocols in which either the protein component (TRL-apoB) or the TG component (TRL-TG) of the lipoprotein was labelled using stable isotope technique (Sun et al., 2013, Xiao et al., 2012). Both studies used developed clinical and laboratory protocols to enable them to isolate and calculate TRL-apoB-100 and TRL-apoB-48 kinetics. Therefore, the current pilot study was conducted to validate the developed constant feeding, clinical and laboratory protocols to enable us to calculate fasting and postprandial TRL-apoB-100 and TRL-apoB-48 kinetics using a stable isotope trace-labelling technique to be able to use these protocols in other clinical trials.

4.2 Aims

The aims of the present study were a) to develop and validate a feeding and clinical protocol that could establish a postprandial TG steady state, and b) to develop and validate a and
laboratory protocol that could separate TRL-apoB-100 and apoB-48, and measure their kinetics using a stable isotope technique.

4.3 Methods

This pilot study was separated into two parts; 1) development of a feeding protocol to establish a postprandial TG steady state, 2) development of a laboratory protocol to measure TRL apoB-100 and apoB-48 using a validated feeding protocol from the first part. A total of 4 overweight healthy men with BMI 30-35 were recruited in the study (for detailed study recruitment see chapter 2 section 2.2.1). In the first part of the study, participants were given 12 hourly high-fat liquid meals for a duration of 13 hours. The meal composition was CHO 22%, fat 66.1%, and 11.9% protein with a total of 2280 Kcal (See Table 2.0 Chapter 2). Two protocols were tested to establish a postprandial TG steady state; A) using 12 hourly liquid meals, and 2) using 12 hourly liquid meals with bolus meal at the beginning of the study. Blood samples were taken hourly to measure plasma TG concentrations (Figure 2.1).

The second part of the study used a validated feeding protocol based on plasma TG measurements from the first part of the study to measure the kinetics of TRL-apoB-100 and apoB-48 using a stable isotope technique (Chapter 2, Figure 2.0). On the study day, 12 hourly liquid meals without a bolus meal were given, and an injection of $^{1-^{13}}$C leucine tracer of 1mg/kg followed by a constant infusion of 1mg/kg/min was given after 4 hours of the start of the study for a duration of 8 hours to measure TRL-apoB kinetics (Figure 2.2). Blood samples were taken hourly during the study. The TRL fraction was separated from plasma using ultracentrifugation, and ApoB-100 and apoB-48 in the fraction were isolated via SDS-PAGE (Chapter 3, Figure 3.0). After purification and derivatization, the leucine enrichment of apoB-100 and apoB-48 were finally determined by GC-MS (Chapter 3, Figure3.0). Plasma
metabolite concentration measurements throughout the study (see chapter 3 section 3.3 and 3.5).

4.4 Results

4.4.1 Subjects characteristics

Seven male subjects were screened, and four found suitable for the study. The characteristics of the subjects at screening in the PFS both parts are shown in Table 4.1. Three participants (PFS01, PFS02 and PFS05) were involved in part 1 to test the validity of the meal in establishing a postprandial TG steady state. The last participant PFS06 was involved in part 2 in which a feeding protocol was verified in part 1 then he was recruited for the second part to measure the TRL-apoB100 and apoB-48 kinetics.

| Table 4.1: Subject characteristics and fasting metabolite concentrations at screening. |
|-------------------------------------|--------------|--------------|--------------|--------------|------------------|
| Subject                            | PFS01        | PFS02        | PFS05        | PFS06        | Mean ± SEM (n=4) |
| Weight (kg)                        | 97.1         | 83.9         | 93.2         | 85.6         | 89.9 ± 2.7       |
| Height (m)                         | 1.8          | 1.7          | 1.7          | 1.7          | 1.7 ± 0.01       |
| BMI (kg/m²)                        | 30.6         | 29.0         | 31.0         | 28.7         | 29.8 ± 0.5       |
| Body fat (%)                       | 29.2         | 24.2         | 28.2         | 29.2         | 27.7 ± 1.0       |
| TG (mmol/L)                        | 1.3          | 1.1          | 2.2          | 2.0          | 1.7 ± 0.2        |
| Cholesterol (mmol/L)               | 4.8          | 6.2          | 4.3          | 5.2          | 5.1 ± 0.4        |
| Glucose (mmol/L)                   | 5.0          | 5.5          | 5.9          | 4.9          | 5.3 ± 0.2        |

Data are shown as Mean ± SEM. BMI: body mass index, TG: triacylglycerols.

4.4.2 Verification of postprandial TG steady state

4.4.2.1 TG concentrations in plasma and TRL fraction

The verification of the postprandial steady state reflected by TG concentrations is shown in Figure 4.0. Administration of 2 liquid meals at the beginning of the study, to act as a bolus, produced a rapid rise and fall in plasma TG concentration. On the contrary, without a bolus protocol resulted in a steady increase in plasma TG by 2.8 ± 0.1 mmol/L from basal, followed
by a plateau between 0 and 480 minutes (Figure 4.0). Repeated measures ANOVA was used to test if the TG concentration between 0 – 480 minutes was constant and found out no significant difference (P value = 0.6) over time in the three participants. This validates the feeding protocol in achieving a constant raised postprandial TG steady state which would enable an accurate stable isotope kinetic analysis.

The results from measuring postprandial TRL-TG (TG fraction of VLDL and CM particles) are shown in Figure 4.1. The use of a meal bolus feeding protocol produced an irregular change in TRL-TG with no notable pattern; this protocol would be unsuitable for isotopic tracer kinetic studies as TG steady state was not maintained. However, results from the protocol without a bolus gave a stable increase in TRL-TG by 1.8 ± 0.1 mmol/L from basal followed by a plateau between 0 and 480 minutes (Figure 4.1). Repeated measures ANOVA was used to test if the TRL-TG concentration between 0 – 480 minutes was constant and found out no significant difference (P value = 0.9) over time in the three participants. This validates the feeding protocol in achieving a constant raised postprandial TRL-TG steady state which would enable an accurate, stable isotope kinetic analysis.

![Graph depicting plasma TG concentrations throughout the study with or without the use of a meal bolus.](image)

Figure 4.0 Plasma TG concentrations throughout the study with or without the use of a meal bolus. Achieving a postprandial TG steady state without using a meal bolus was a better
approach as indicated. Bolus protocol n=1, without bolus protocol n=3. Plasma TG concentrations are increased by 2.8 ± 0.1 mmol/L from basal as indicated.

Figure 4.1: Plasma TRL-TG (TG concentrations measured in TRL fraction) concentration throughout the study with or without the use of a meal bolus. Achieving a postprandial TG steady state without using a meal bolus was a better approach as indicated. Bolus protocol n=1, without bolus protocol n=3. TRL-TG concentrations increased by 1.8 ± 0.1 mmol/L from basal as indicated.

4.4.2.2 Glucose and insulin concentrations in plasma and TRL fraction

Both plasma glucose and insulin concentrations in the bolus and non-bolus studies revealed a similar pattern shown for plasma TG and TRL-TG as shown in Figures 4.2 & 4.3 respectively. In the non-bolus meal protocol, plasma glucose concentrations were constant with a mean value of 5.5 ± 0.1 between 60 and 480 minutes (Figure 4.2), and plasma insulin concentrations were constant with a mean value of 124.0 ± 9.1 pmol/L between 0 and 480 minutes (Figure 4.3).
Figure 4.2: Plasma glucose concentrations. Plasma glucose concentration with and without the bolus protocol. Both methods achieved a steady state after 0-time point. Bolus protocol n=1, without bolus protocol n=3.

Figure 4.3: Plasma insulin concentrations. Plasma insulin concentration with and without the bolus protocol. The bolus method was more effective at achieving a steady state after 0 time point. With bolus protocol n=1, without bolus protocol n=3.

4.4.3 TRL-apo B-100 and apo-B-48 separation by SDS-PAGE

Fraction samples from subjects PFS01 and PFS02 were used to separate apolipoprotein B by SDS-PAGE after the precipitation process. Figure 4.4 shows the separated apo B-100 and apo B-48 bands from PFS01 samples. Sample order is as indicated in Figure 4.4 for the study time points from -240 – 480.

Figure 4.4: TRL-apo B-100 and apo B-48 separation using the SDS-PAGE method. TRL-TG-F samples were used for the separation of TRL-apo B-100 and apo B-48 using SDS-PAGE method.
TRL-apo B-100 and apo B-48 are indicated respectively by a black arrow. The control ladder which starts at 250 kDa is also indicated.

4.4.4 Characterisation of TRL-apo B-100 and apo B-48 by Western immunoblotting

Western immunoblotting was applied using antigen-antibody detection for identifying TRL-apo B-100 and apo B48 bands after they were separated by SDS-PAGE as shown in Figure 4.5 using PFS01 samples. This successfully identified the apolipoprotein B bands and shows complete separation which is important for band removal for further sample processing by IEC and GC-MS.

![Figure 4.5: TRL-apo B-100 and apo B-48 characterisation using western immunoblotting method. TRL-apo B-100 and B48 bands from the PFS01 study time points were identified and characterised using Primary AB 1D1-mouse and secondary AB AP-Anti mouse using SDS-PAGE and western immunoblotting methods respectively.]

4.4.5 TRL-apo B-100 and apo B-48 natural background enrichment (part 1)

The natural occurring enrichment profile of the $^{1-^{13}}$C leucine/${^{12}}$C leucine (as AR 210/209) in TRL-apo B-100 and apo B-48 is shown in Figure 4.6. This demonstrates that this method can be used to measure the TTR of TRL-apo B-100 and apo B-48.
Figure 4.6 AR 210/209 of the background enrichment of TRL-apo B-100 and B-48 from PFS02 samples in part 1. This figure illustrates the natural occurring background enrichment of AR 210/209 of apo B-100 and apo B48 throughout study time points between -280 – 480 minutes where the liquid meal was given hourly as indicated. AR 210/209 represent areas of ions at m/z 210 (ion fragment of 1\(^{-13}\)C Leucine) representing the tracer, and m/z 209 (ion fragment of \(^{12}\)C Leucine) representing the tracee.

4.4.6 TRL-apo B-100 and apo B-48 enrichment following the infusion of 1\(^{-13}\)C Leucine (part 2)

The enrichment profile of the incorporated 1\(^{-13}\)C Leucine tracer in the synthesis of TRL-apo B-100 and apo B-48 is shown in Figure 4.7. There was a marked increase in leucine enrichment in apo B-100 and apo B-48 from 0 minutes. The TTR did not plateau; this indicates that the incorporation of labelled and unlabelled apo B-100 in the liver and apo B-48 in the intestine has not completely equilibrated with the precursor pool. TRL-apo B-100 enrichment will continue increasing until it reaches a plateau which will reflect the precursor pool enrichment.
Discussion

The results from this study demonstrate successful development and verification of an hourly repeated feeding protocol. This protocol was validated by achieving a postprandial steady state of TRL. The TRL steady state was achieved after four hours, at this point, the $^{13}$C leucine labelled isotope infusion was given for accurate quantification of postprandial hepatic and intestinal TRL kinetics. Furthermore, all participants from the pilot studies accepted and tolerated the liquid meal which provided part of a recommended amount of a daily calorie allowance (2280Kcal of the 2500 Kcal). As seen in other repeated feeding studies shown in Table 1.2, different meal compositions of fat, proteins and carbohydrates have been used with the different amount of calories for each meal. As in the present study, most of the previous studies used meals with a large percentage of fat and meals were given every hour. A study conducted by Xiao et al. (2012) tried to achieve a postprandial steady state by administering a liquid feed via a radio-opaque polyvinyl feeding tube which had been placed through the nose into the duodenum under the guidance of a fluoroscope. The liquid formula contained 49% calories from fat, 38% from carbohydrates, and 13% from proteins. Also, the meal which was given at a rate of 40 mL/h for the first 2 hours and then
80 mL/h until the end of the study, did not achieve a good TG steady state to enable them to study TRL-apoB48 kinetics (Xiao et al., 2012) which was opposite results to the current study. Apo B-48 kinetics measurement may be difficult to measure due to the low concentrations of apo B-48 in the plasma compared with apo B-100 (Phillips et al., 1997). However, the current study was remarkably effective in developing and validating a method using isolation, precipitation and separation of apo B-100 and apo B-48 for accurate quantification of isotopic enrichment with $^{13}$C leucine.

The method used for the isolation of the TRL fraction by ultracentrifugation was similar to methods used in other studies such as Batista et al., 2004 and Xiao et al., 2012 which was originally developed by (Havel et al., 1955, Welty et al., 1997, Welty et al., 2004).

In this study, apo B-100 and B-48 were delipidated, precipitated and isolated from other apolipoproteins in the TRL fraction samples (CM and VLDL fractions) using Isopropanol. This method was validated by Egusa et al., 1983 and used for the isolation of apo B-100 and B-48. This method has also been used in other studies of apo B-100 and apo B-48 kinetics (Xiao et al., 2012, Cohn et al., 1988, Welty et al., 1997, Welty et al., 2004, Welty et al., 1999, Batista et al., 2004).

Similarly, separation of apo B-100 and B-48 by SDS-PAGE has been used previously (Cohn et al., 1988, Batista et al., 2004, Welty et al., 1997, Xiao et al., 2012).

In this study, isotopic enrichment of Apo B-100 and B-48 was measured using isolated purified free amino acids which were derivatized to the oxazolinone derivative. This derivative has a small molecular weight which is the advantage of reducing the contribution of naturally occurring isotopes. This method requires one single reaction step using cheap reagents for the derivatizing procedure. Also, the derivative has been shown to be suitable for Gas Chromatography-Mass Spectrometry (GC-MS), and can be detected by negative
chemical ionisation which allows determination of low concentrations of isotopic enrichment from small amounts of protein such as apo B-100 and apo B-48 (Dwyer et al., 2002).

1-¹³C Leucine tracer was successfully incorporated into the apo B-100 and B-48 which reflects the rate of VLDL assembly and synthesis in the liver, and CM assembly and synthesis in the intestine respectively. TRL-apo B-48 enrichment was lower than apo B-100 enrichment which could be due to dilution by the presence of leucine from the liquid meal which dilutes the precursor pool and causes a faster plateauing of the leucine enrichment. This indicates that the liquid meal and labelled isotope tracer protocols have been shown to be effective and are now validated for a further postprandial feeding study.

Although this study provided a unique and novel insight into the enrichment of the protein fractions of the TRL, this study did not investigate the lipid moiety of TRL. The kinetics of postprandial TRL lipid moiety can be investigated as seen in the study by Sun et al. (2013) in which a novel immunoaffinity method that completely separated hepatic and intestinal TRL lipid moiety (TG part) was used (Sun et al., 2013). Despite the effectiveness of this method, the separation of hepatic and intestinal lipoproteins using this method is a very time-consuming method. Also, this method is dependent on the availability of specific apoB-100 antibodies which are costly when compared with the SDS-PAGE method.

In conclusion, this study succeeded in developing a repeated feeding protocol that was used with a 1-¹³C leucine infusion to quantify the protein enrichment of hepatic VLDL-apo B-100 and intestinal CM-apo B-48 in postprandial TRL. The recruitment criteria in this study were selected to match the criteria in the lixisenatide study. Therefore, the feeding and laboratory protocols were used to measure TRL-apo B-100 and apo B-48 kinetics in the lixisenatide study (Chapter 6).
Chapter 5: The effect of exercise training on HDL kinetics in patients with non-alcoholic fatty liver disease (NAFLD).

5.1 Introduction

Patients with NAFLD are characterised by visceral obesity (Park et al., 2016, Yu et al., 2015, Yang and Chang, 2016, Cordeiro et al., 2015, Di Naso et al., 2015, Yoshimura et al., 2014, Fabbrini et al., 2010), IR (Delarue and Magnan, 2007, Samuel et al., 2010, Tang et al., 2011, Westphal, 2008), altered lipoprotein metabolism including hypertriglyceridaemia, reduced HDL-cholesterol concentrations and increased LDL-cholesterol concentrations (Corey and Cohen, 2000, Cohen and Fisher, 2013, Tessari et al., 2009, Katsiki et al., 2016) and hyperinsulinaemia (Giovannucci, 2007, Onyekwere et al., 2015). All these previous altered metabolic manifestations in patients with NAFLD are strongly associated with increasing the risk of CVD and CHD through atherogenic dyslipidaemia which is also called atherogenic lipoprotein phenotype (ALP) (Corey et al., 2014, Sdiri et al., 2013, Bhatia et al., 2012, Edens et al., 2009, Onyekwere et al., 2015). Exercise is one of the important treatment approaches in correcting and/or treating NAFLD (Neuman et al., 2015, Pinto et al., 2015). Various studies showed that physical exercise has strong effects in correcting reduced HDL-C concentrations in patients with metabolic syndrome of altered lipid metabolism such as T2D and NAFLD (Garcia Hermoso et al., 2014, Pattyn et al., 2013, Nuri et al., 2012, Hayashino et al., 2012, Iborra et al., 2008), and in healthy subjects (Gondim et al., 2015). Many clinical trials have been conducted to investigate the effect of exercise on liver fat content in patients with NAFLD (Cuthbertson et al., 2016, Hallsworth et al., 2011, Yoshimura et al., 2014, Sullivan et al., 2012, Johnson et al., 2009, van der Heijden et al., 2010). Similar studies conducted by Lehmann et al., 2001 and Kadoglou et al., 2012 investigated the effect of physical exercise on HDL-C and apoA-I concentrations in patients with T2D in relation with other lipolytic enzymes and inflammatory markers. Remarkably, the study conducted
by Thompson et al., 1997 is the only study which has investigated the effect of long-term physical exercise on HDL-apoA-I kinetics in healthy overweight patients. A significant increase of HDL-C concentration after exercise was reported in the studies conducted by Thompson et al., 1997, Lehmann et al., 2001 and Alam et al., 2004, but not in the studies by Kadoglou et al., 2012, Cuthbertson et al., 2016 and Sullivan et al., 2012. Also, a significant increase of apoA-I concentrations after exercise were reported in the studies by Thompson et al., 1997, Lehmann et al., 2001 but not in Kadoglou et al., 2012. The study by Thompson et al., 1997 showed a significant decrease in HDL-apoA-I FCR after exercise by 8%. Also, the study showed a significant increase in the HDL-apoA-I PR by 11%. To the extent of our knowledge, to date no clinical trials were conducted on patients with NAFLD to investigate the effect of exercise on HDL-C concentration, and HDL-apoA-I concentration and kinetics. Therefore, the current study was conducted to test whether a physical exercise programme (4-5 times weekly moderate-high intensity exercise, 20 minutes progressing to 60 minutes per session under 40-60% VO2MAX equivalent to 65-80% HRMAX) can cause direct/indirect effects on fasting lipoprotein metabolism in patients with NAFLD by calculating VLDL-apoB-100 and HDL-apoA-I kinetics using stable isotope trace-labelling technique.

5.2 Hypothesis

It is hypothesised that physical exercise will correct altered lipoprotein metabolism and reduce liver fat in patients with NAFLD by increasing hepatic and peripheral insulin sensitivity, reducing fasting hypertriglyceridaemia and increasing low HDL-C. This will be achieved by the exercise action on reducing the PR of VLDL-apoB-100 or increasing VLDL-apoB-100 FCR, and directly increasing the PR of HDL-apoA-I or indirectly reducing FCR of HDL-apoA-I. This will contribute to reducing CVD risk.
5.3 Aims

To conduct a clinical trial that will study the effects of physical exercise programme on HDL-apoA-I kinetics and concentration, fasting hypertriglyceridaemia, liver fat, IR and lipid profile in patience with NAFLD using stable isotope trace-labelling technique.

5.4 Methods

This study was designed with two groups; a control group and a supervised exercise group. Caucasian men aged 18 and over with BMI of 27-37 and diagnosed with NAFLD were recruited in the study (for detailed study recruitment see chapter 2 section 2.4.1). This study was a retrospective analysis of stored samples from the previous NAFLD study which was conducted two years ago. A total of 27 stored samples were used which was collected from the two groups; 12 stored samples from 12 subjects in the control group and 15 stored samples from 15 subjects in the supervised exercise group (chapter 2, Figure 2.7). The stored samples were collected hourly during the study day for 9 hours before and after the injection of the 1-¹³C leucine tracer (chapter 2, Figure 2.6) (for the detailed study protocol see chapter 2 section 2.4.4). The following measurements were performed before and after the four-month exercise intervention period (chapter 2, Figure 2.5); the measurement of HDL-C and HDL apoA-I concentration, and the calculation of HDL-apoA-I kinetics by following the verified laboratory protocol (for detailed laboratory protocol and methods see chapter 3). Other supporting results mentioned in this chapter was collected and analysed by another personnel in the laboratory. Pre and post exercise intervention measurements in both groups were compared to elucidate the exercise effect by observing any significant difference within each group and between the two groups by using certain statistical tests (for detailed statistical analysis tests used see chapter 3 section 3.2.19).
5.5  Results

5.5.1  Subjects characteristics

The main physical and physiological characteristics of the 27 male subjects recruited in the study are shown in Table 5.0. Age, height, body weight, waist circumference and BMI were similar in both groups. The BMI of 16 out of 27 subjects indicates that they were considered obese (NHSUK, 2015). The mean value of ALT was higher than AST in these patients with NAFLD. This is considered one of the ways to differentiate NAFLD from alcoholic liver disease (Sattar et al., 2014). High plasma-TG and total cholesterol concentrations indicate altered lipid metabolism in these patients which would be expected in patients with NAFLD. Notably, the mean systolic blood pressure (BP) in both groups was located in the range of the prehypertension category (120 – 139 mmHg) (WHO, 2015a, American Diabetes Association, 2016). The mean diastolic BP was in the normal range.

The main characteristics of participant’s pre and post the exercise intervention or control period in each group are shown in Table 5.1. Body weight, BMI, waist circumference and body fat percentage in the exercise group were consistently lower after the exercise period ($P \leq 0.001$, $\leq 0.001$, $\leq 0.005$ and 0.01, respectively). The systolic and diastolic BP were decreased significantly after the exercise intervention within the exercise group ($P= 0.01$ and 0.04 respectively). There were no significant differences between any of the pre and post measurements of the characteristics in the control group. The diastolic BP was decreased significantly ($P=0.05$) after exercise between the exercise and control group. The exercise effect observed between the two groups was calculated using pre-exercise minus post exercise ($\Delta$) measurements and the $P$ value was significant for all the above measurements (Table 5.1).
Table 5.0: Subject characteristics and fasting plasma measurements at screening visit

<table>
<thead>
<tr>
<th>Subjects characteristics</th>
<th>Exercise group (n=15)</th>
<th>Control group (n=12)</th>
<th>P value</th>
<th>Unpaired T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>52.4±2.5</td>
<td>52.8±3.2</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.8±2.0</td>
<td>1.8±3.0</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>101.2±3.4</td>
<td>102.3±6.7</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>109.3±2.4</td>
<td>110.0±4.3</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>31.5±1.0</td>
<td>31.7±1.1</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.8±0.3</td>
<td>1.6±0.2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Total CHOL (mmol/L)</td>
<td>5.6±0.2</td>
<td>5.5±0.2</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>IHCL (%)</td>
<td>27.4±6.8</td>
<td>19.7±4.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>38.4±3.9</td>
<td>29.0±2.2</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>52.6±7.1</td>
<td>40.9±4.4</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>γGT (IU/l)</td>
<td>55.1±13.7</td>
<td>37.0±4.9</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>133.4±5.3</td>
<td>131.3±5.1</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>83.4±2.8</td>
<td>82.7±2.7</td>
<td>0.9</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM. Data between the two groups were analysed by unpaired two-tailed t-test. P values ≤0.05 are in bold. BMI: body mass index; TG: triacylglycerols; IHCL: intrahepatocellular lipid; AST: aspartate transferase; ALT: alanine transferase; γGT: gamma-glutamyl transferase; CHOL: cholesterol; BP: blood pressure.

Table 5.1: Main subjects characteristics and fasting measurements pre and post the intervention period in the exercise and the control group.

<table>
<thead>
<tr>
<th></th>
<th>Pre-EX (n=15)</th>
<th>Post EX (n=15)</th>
<th>P value Paired T-test</th>
<th>Pre-Control (n=12)</th>
<th>Post Control (n=12)</th>
<th>P value Paired T-test</th>
<th>P value Post data between the two groups Unpaired T-test</th>
<th>Δ P value unpaired T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>101.3±2.6</td>
<td>97.3±12.2</td>
<td>≤0.001</td>
<td>102.3±6.1</td>
<td>102.9±6.4</td>
<td>0.3</td>
<td>0.5</td>
<td>≤0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>31.6±0.8</td>
<td>30.5±1.0</td>
<td>≤0.001</td>
<td>31.7±1.0</td>
<td>31.6±1.2</td>
<td>0.4</td>
<td>0.4</td>
<td>0.02</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>109.3±1.9</td>
<td>105.0±2.5</td>
<td>0.005</td>
<td>110.0±3.9</td>
<td>109.0±4.3</td>
<td>0.7</td>
<td>0.3</td>
<td>0.03</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>27.8±1.2</td>
<td>26.4±1.1</td>
<td>0.01</td>
<td>29.5±1.5</td>
<td>29.8±1.6</td>
<td>0.6</td>
<td>0.1</td>
<td>0.02</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>133.4±4.2</td>
<td>128.8±4.2</td>
<td>0.01</td>
<td>131.3±4.5</td>
<td>134.4±3.8</td>
<td>0.1</td>
<td>0.5</td>
<td>0.04</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>83.4±2.3</td>
<td>78.2±0.6</td>
<td>0.04</td>
<td>83.8±3.0</td>
<td>86.3±3.0</td>
<td>0.6</td>
<td>0.05</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM. Pre and post intervention data within the two groups were analysed by paired two-tailed t-test. Data after exercise intervention between the two groups were analysed by unpaired two-tailed t-test. Pre minus post exercise data (Δ) values between the two groups were analysed by unpaired two-tailed t-test. P values ≤0.05 are in bold. EX: exercise; BMI: body mass index; BP: blood pressure.
5.5.2 The effect of exercise on TG, cholesterol and total apoA-I concentrations

Table 5.2 shows the cholesterol, and total apoA-I measurements pre and post the exercise intervention for all participants in the exercise and control groups. LDL-CHOL was significantly decreased after the exercise period ($P=0.03$), and the cholesterol concentration in the obtained HDL fraction (F-HDL-C) was significantly increased after the exercise intervention ($P=0.03$) in the exercise group. Also, the cholesterol: HDL-cholesterol ratio was significantly reduced after the exercise intervention in the exercise group ($P=0.04$). There were no significant differences between pre and post exercise measurements for the participants in the control group. The exercise intervention did not cause any significant differences for any of the mentioned measurements between the two groups (Table 5.2).

<table>
<thead>
<tr>
<th></th>
<th>Pre EX (n=15)</th>
<th>Post EX (n=15)</th>
<th>$p$ value</th>
<th>Pre Control (n=12)</th>
<th>Post Control (n=12)</th>
<th>$p$ value</th>
<th>$P$ value</th>
<th>Δ</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Paired T-test</td>
<td></td>
<td></td>
<td>unpaired T-test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TG (mmol/L)</strong></td>
<td>2.0±0.2</td>
<td>1.8±0.2</td>
<td>0.3</td>
<td>1.6±0.2</td>
<td>1.9±0.2</td>
<td>0.3</td>
<td>0.8</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>FFA (mmol/L)</strong></td>
<td>0.4±0.1</td>
<td>0.4±0.1</td>
<td>0.4</td>
<td>0.5±0.1</td>
<td>0.5±0.1</td>
<td>0.6</td>
<td>0.1</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Total CHOL (mmol/l)</strong></td>
<td>5.0±0.2</td>
<td>4.7±0.2</td>
<td>0.1</td>
<td>5.1±0.2</td>
<td>5.1±0.2</td>
<td>1.0</td>
<td>0.2</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>LDL-CHOL (mmol/l)</strong></td>
<td>3.8±0.1</td>
<td>3.3±0.2</td>
<td><strong>0.03</strong></td>
<td>3.6±0.2</td>
<td>3.2±0.2</td>
<td>0.1</td>
<td>0.7</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>HDL-CHOL (mmol/L)</strong></td>
<td>1.0±0.1</td>
<td>1.0±0.1</td>
<td>0.3</td>
<td>1.1±0.1</td>
<td>1.1±0.1</td>
<td>0.9</td>
<td>0.5</td>
<td>0.6</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>F-HDL-C (mmol/L)</strong></td>
<td>0.5±0.1</td>
<td>0.6±0.1</td>
<td><strong>0.03</strong></td>
<td>0.6 ± 0.1</td>
<td>0.6±0.1</td>
<td>0.6</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>F-HDL-apoA-I (g/L)</strong></td>
<td>0.5±0.1</td>
<td>0.5±0.1</td>
<td>0.1</td>
<td>0.5±0.1</td>
<td>0.6±0.1</td>
<td>0.4</td>
<td>0.8</td>
<td>0.9</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>CHOL:HDL ratio</strong></td>
<td>5.2±0.4</td>
<td>4.8±0.4</td>
<td><strong>0.04</strong></td>
<td>5.1±0.1</td>
<td>4.9±0.4</td>
<td>0.6</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM. Pre and post intervention data within the two groups were analysed by paired two-tailed t-test. Data after exercise intervention between the two groups were analysed by unpaired two-tailed t-test. Pre minus post exercise data (Δ) values between the two groups were analysed by unpaired two-tailed t-test. P values ≤0.05 are in bold. EX: exercise; TG: triacylglycerols; FFA: free fatty acids; CHOL: cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; F-HDL-C: cholesterol in obtained HDL fraction; F-HDL-apoA-I: apolipoprotein A-I in obtained HDL fraction.
5.5.3 The effect of exercise on liver enzymes

Table 5.3 shows the plasma liver enzyme concentrations measured pre and post the exercise intervention period for all participants in the exercise and control groups. ALT, AST and γGT were significantly decreased after the exercise intervention in the exercise group (P= 0.01, 0.02, and 0.03 respectively). Also, ALT concentrations were lower after the intervention period with borderline significance in the control group (P=0.06). There were no significant differences caused by exercise between the two groups in all the liver transferase enzyme concentrations measured.

Table 5.3: Plasma liver enzymes concentrations pre and post the intervention period in the exercise and control group.

<table>
<thead>
<tr>
<th></th>
<th>Pre EX (n=15)</th>
<th>Post EX (n=15)</th>
<th>p value</th>
<th>Pre</th>
<th>Post</th>
<th>p value</th>
<th>Pre</th>
<th>Post</th>
<th>p value</th>
<th>Post data between the two groups</th>
<th>Δ</th>
<th>p value un-paired T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>51.1±5.3</td>
<td>36.8±5.2</td>
<td>0.01</td>
<td>40.9±6.2</td>
<td>31.1±4.7</td>
<td>0.06</td>
<td>40.9±6.2</td>
<td>31.1±4.7</td>
<td>0.06</td>
<td>0.5</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>36.9±3.2</td>
<td>29.4±3.5</td>
<td>0.02</td>
<td>29.0±2.5</td>
<td>26.3±1.84</td>
<td>0.24</td>
<td>29.0±2.5</td>
<td>26.3±1.84</td>
<td>0.24</td>
<td>0.5</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>ΓGT (U/L)</td>
<td>53.5±10.2</td>
<td>36.3±7.5</td>
<td>0.03</td>
<td>37.0±4.5</td>
<td>33.8±4.9</td>
<td>0.25</td>
<td>37.0±4.5</td>
<td>33.8±4.9</td>
<td>0.25</td>
<td>0.8</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM. Pre and post intervention data within the two groups were analysed by paired two-tailed t-test. Data after exercise intervention between the two groups were analysed by unpaired two-tailed t-test. Pre minus post exercise data (Δ) values between the two groups were analysed by unpaired two-tailed t-test. P values ≤0.05 are in bold. EX: exercise; ALT: alanine transferase; AST: aspartate transferase; γGT: gamma-glutamyl transferase.

5.5.4 The effect of exercise on physical fitness, glucose, insulin and insulin sensitivity

Table 5.4 shows the pre and post exercise intervention measurements of physical fitness (by VO2max physical fitness test), glucose, insulin and insulin sensitivity (as HOMA2) for participants in the exercise and control group. Physical fitness of the participants was significantly increased after exercise in the exercise group intervention (P≤0.001). Glucose and insulin concentrations were reduced significantly after exercise intervention in the exercise group (P= 0.005 and 0.007 respectively). Also, insulin sensitivity was increased...
significantly after the exercise intervention in the exercise group \((P = 0.006)\). There were no significant differences in the control group after the intervention period. Significant differences of the exercise effect between the two groups were detected for physical fitness, insulin and insulin sensitivity measurements \((\Delta P \leq 0.001, 0.02 and 0.02\) respectively).

Table 5.4: Pre and post intervention measurements of physical fitness, glucose and insulin sensitivity in participants in the exercise and control groups.

<table>
<thead>
<tr>
<th></th>
<th>Pre EX (n=15)</th>
<th>Post EX (n=15)</th>
<th>(p) value Paired T-test</th>
<th>Pre Control (n=12)</th>
<th>Post Control (n=12)</th>
<th>(p) value Unpaired T-test</th>
<th>(\Delta) (p) value Unpaired T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>(VO_{2\text{max}}) (ml kg(^{-1}) min(^{-1}))</td>
<td>25.5±1.1</td>
<td>33.0±1.5</td>
<td>\leq 0.001</td>
<td>23.3±1.0</td>
<td>23.8±1.3</td>
<td>0.5</td>
<td>\leq 0.001</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>6.0±0.2</td>
<td>5.8±0.2</td>
<td>0.005</td>
<td>5.9±0.2</td>
<td>5.6±0.1</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>183.0±17.0</td>
<td>138.0±16.0</td>
<td>0.007</td>
<td>164.0±17.0</td>
<td>170.0±17.0</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>HOMA2-IR (%)</td>
<td>3.45±0.3</td>
<td>2.61±0.3</td>
<td>0.006</td>
<td>3.1±0.3</td>
<td>3.2±0.3</td>
<td>0.8</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM. Pre and post intervention data within the two groups were analysed by paired two-tailed \(t\)-test. Data after exercise intervention between the two groups were analysed by unpaired two-tailed \(t\)-test. Pre minus post exercise data (\(\Delta\)) values between the two groups were analysed by unpaired two-tailed \(t\)-test. \(P\) values \(\leq 0.05\) are in bold. EX: exercise; \(VO_{2\text{max}}\): volume of maximum oxygen capacity; HOMA2-IR: The Homeostasis Model Assessment for IR percentage.

5.5.5 The effect of exercise on the body composition and fat measurements

The participants’ measurements of liver, muscle, visceral and body fat before and after the exercise intervention period are shown in Table 5.5. Exercise significantly reduced body fat measurements in the exercise group including IHCL, abdominal and subcutaneous fat, total visceral and subcutaneous fat and total body fat \((P \leq 0.001\) for all measurements). Exercise, however, did not significantly reduce the soleus and tibialis intramyocellular lipid percentage \((\text{IMCL}\ %)\) in the exercise group.

There were no significant differences in these measurements in the control group as shown in Table 5.5. There were significant differences between the two groups for all the measurements except IMCL as shown in Table 5.5.
Table 5.5: The pre and post intervention measurements of liver, muscle and body fat composition in the exercise and control groups.

<table>
<thead>
<tr>
<th></th>
<th>Pre EX</th>
<th>Post EX</th>
<th>$p$ value Paired T-test</th>
<th>Pre Control</th>
<th>Post Control</th>
<th>$p$ value Unpaired T-test</th>
<th>$\Delta$ $p$ value Un-paired T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHCL (%)</td>
<td>27.3±5.8</td>
<td>17.0±5.3</td>
<td>≤0.001</td>
<td>19.3±4.0</td>
<td>17.7±3.5</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>IMCL (Sol) (%)</td>
<td>21.7±3.8</td>
<td>19.0±3.5</td>
<td>0.1</td>
<td>19.2±1.8</td>
<td>20.5±2.6</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>IMCL (Tib. A) (%)</td>
<td>8.8±1.0</td>
<td>8.8±1.4</td>
<td>1.0</td>
<td>13.2±5.4</td>
<td>8.2±1.2</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Abdominal visceral Fat (kg)</td>
<td>5.7±0.4</td>
<td>4.7±0.4</td>
<td>≤0.001</td>
<td>5.7±0.6</td>
<td>5.4±0.6</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Abdominal Subcut. Fat (kg)</td>
<td>7.0±7.2</td>
<td>6.3±0.7</td>
<td>≤0.001</td>
<td>7.8±1.0</td>
<td>7.9±1.1</td>
<td>0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Total visceral fat (kg)</td>
<td>9.5±0.6</td>
<td>7.9±0.6</td>
<td>≤0.001</td>
<td>9.9±0.9</td>
<td>9.6±0.9</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Total Subcut. fat (kg)</td>
<td>22.1±1.7</td>
<td>19.5±1.6</td>
<td>≤0.001</td>
<td>24.9±2.4</td>
<td>24.9±2.6</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Total body fat (kg)</td>
<td>31.6±2.0</td>
<td>27.3±1.9</td>
<td>≤0.001</td>
<td>34.8±3.2</td>
<td>34.5±3.5</td>
<td>0.8</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM. Pre and post intervention data within the two groups were analysed by paired two-tailed t-test. Data after exercise intervention between the two groups were analysed by unpaired two-tailed t-test. Pre minus post exercise data (Δ) values between the two groups were analysed by unpaired two-tailed t-test. $P$ values ≤0.05 and are in bold. EX: exercise; IHCL: intrahepatocellular lipid; IMCL: intramyocellular lipid; Sol: soleus muscle; Tib. A: tibialis anterior muscle; Subcut: subcutaneous.

5.5.6 The effect of exercise on dietary intake using seven days diet diaries

The dietary intake of the participants in the exercise and control group pre and post the intervention is shown in Table 5.6. There were no significant differences in dietary intake in either the exercise or the control group. Also, there were no significant differences in measurements indicated between the two groups.
Table 5.6: The pre and post intervention dietary intake of participants in the exercise and control groups.

<table>
<thead>
<tr>
<th></th>
<th>Pre EX (n=15)</th>
<th>Post EX (n=15)</th>
<th>p value Paired T-test</th>
<th>Pre Control (n=12)</th>
<th>Post Control (n=12)</th>
<th>p value Paired T-test</th>
<th>P value Post data between the two groups</th>
<th>Δ value p value unpaired T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (MJ/d)</td>
<td>9.9±0.8</td>
<td>10.2±0.7</td>
<td>NS</td>
<td>8.7±0.5</td>
<td>8.2±0.5</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>105.0±10.0</td>
<td>105.0±7.0</td>
<td>NS</td>
<td>89.0±8.0</td>
<td>83.0±7.0</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CHO (g/d)</td>
<td>279.0±23.0</td>
<td>272.0±24.0</td>
<td>NS</td>
<td>208.0±6.0</td>
<td>203.0±11.0</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sugars (g)</td>
<td>102.0±13.0</td>
<td>111.0±16.0</td>
<td>NS</td>
<td>76.0±6.0</td>
<td>74.0±6.0</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Fat (g/d)</td>
<td>87.0±9.0</td>
<td>96.0±9.0</td>
<td>NS</td>
<td>82.0±8.0</td>
<td>72.0±8.0</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Saturated fat (g/d)</td>
<td>33.0±4.0</td>
<td>32.0±4.0</td>
<td>NS</td>
<td>30.0±3.0</td>
<td>25.0±3.0</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Fibre (g/d)</td>
<td>29.0±6.0</td>
<td>37.0±13.0</td>
<td>NS</td>
<td>17.0±2.0</td>
<td>18.0±1.0</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sodium (g/d)</td>
<td>3.5±0.4</td>
<td>2.9±0.5</td>
<td>NS</td>
<td>2.9±0.3</td>
<td>2.7±0.2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM. Pre and post intervention data within the two groups were analysed by paired two-tailed t-test. Data after exercise intervention between the two groups were analysed by unpaired two-tailed t-test. Pre minus post exercise data (Δ) values between the two groups were analysed by unpaired two-tailed t-test. P values ≤0.05 are in bold. EX: exercise; CHO: carbohydrates; NS: non-significant.

5.5.7 The effect of exercise on leucine isotopic enrichment of HDL-apoA-I in the exercise and control group

The leucine isotopic enrichment of HDL-apoA-I in all the participants in the exercise group and the control group are shown in Figures 5.0 (A) and (B) respectively. Pre and post exercise intervention measurements of $^{13}$C leucine enrichment were calculated and expressed as TTRs. The mean area under the curve (AUC) of the leucine TTR enrichment was reduced with borderline significance (AUC=1.4±0.03xmin) after exercise when compared with the AUC of the pre-exercise TTR enrichment (AUC=1.8±0.03xmin) in the exercise group ($P=0.05$). The AUC of pre and post exercise leucine TTR enrichment in the control group was significantly difference (pre-exercise AUC= 1.7±0.03xmin and post-AUC= 1.5±0.03xmin, $P=0.09$). The slopes of pre and post-TTR enrichment in the exercise group were compared using Linear regression and showed a significant difference ($P=0.006$) (Figure 5.0 (A)). The slopes of pre
and post-TTR enrichment showed no significant difference in the control group ($P=0.5$) (Figure 5.0 (B)).

![Graphs showing leucine TTR enrichment of HDL-apoA-I in the exercise and control groups]

Figure 5.0: $^{13}$C Leucine TTR enrichment data are expressed as Mean ± SEM. Pre and post 16 weeks intervention measurements of $^{13}$C leucine TTR enrichment of HDL-apoA-I in the exercise group (A) and control group (B) across study time points.

### 5.5.8 The effect of exercise on HDL-apoA-I kinetics

Table 5.6 shows the HDL-apoA-I kinetics measurements pre and post exercise intervention in the participants of the exercise and control groups. HDL-apoA-I PS was increased significantly after the exercise intervention in the exercise group ($P=0.05$) as shown in Table 5.6. The kinetic measurements in the control group and between the two groups did not show significant differences.

<table>
<thead>
<tr>
<th>Pre EX</th>
<th>Post EX</th>
<th>p value</th>
<th>Pre</th>
<th>Post</th>
<th>p value</th>
<th>P value</th>
<th>Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=15)</td>
<td>(n=15)</td>
<td>Paired T-test</td>
<td>Control (n=12)</td>
<td>Control (n=12)</td>
<td>Paired T-test</td>
<td>Post data between the two groups Unpaired T-test</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------</td>
<td>--------</td>
<td>---------------</td>
<td>----------------</td>
<td>----------------</td>
<td>---------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>HDL-apoA-I PS (mg/kg)</td>
<td>17.4±0.8</td>
<td>18.9±0.75</td>
<td>0.05</td>
<td>17.9±2.0</td>
<td>19.3±2.0</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>HDL-apoA-I FCR (pools/day)</td>
<td>0.3±0.02</td>
<td>0.2±0.02</td>
<td>0.4</td>
<td>0.2±0.02</td>
<td>0.2±0.02</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>HDL-apoA-I PR (mg/kg/day)</td>
<td>4.4±0.3</td>
<td>4.5±0.3</td>
<td>1.0</td>
<td>3.3±0.5</td>
<td>3.5±0.5</td>
<td>0.6</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM. Pre and post intervention data within the two groups were analysed by paired two-tailed t-test. Data after exercise intervention between the two groups were analysed by unpaired two-tailed t-test. Pre minus post exercise data (Δ) values between the two groups were analysed by unpaired two-tailed t-test. P values ≤0.05 are in bold. EX: exercise; HDL: high-density lipoprotein; apoA-I: apolipoprotein A-I; PS: pool size; FCR: fractional catabolic rate; PR: production rate.

5.5.9 The effect of exercise on VLDL<sub>1</sub>-apoB-100 and VLDL<sub>2</sub>-apoB-100 kinetics.

Table 5.7 show that total VLDL<sub>1</sub>-apoB-100 PR was increased significantly after the exercise intervention in the exercise group (P = 0.004). VLDL<sub>1</sub>-apoB-100 FCR was increased significantly after the exercise intervention in the exercise group (P=0.02). Also, VLDL<sub>1</sub>-apoB-100 PR was increased significantly after the exercise intervention in the exercise group (P=0.003). VLDL<sub>2</sub>-apoB-100 PS was reduced significantly after the exercise intervention in the exercise group (P=0.02). The kinetics measurements in the control group did not show significant differences before and after the intervention period. However, the Δ values between the two groups showed significant differences in total VLDL-apoB-100 PR, VLDL<sub>1</sub>-apoB-100 FCR and VLDL<sub>1</sub>-apoB-100 PR (Δ P= 0.02, 0.006 and 0.01 respectively).
Table 5.7: VLDL<sub>1</sub>-apoB-100 and VLDL<sub>2</sub>-apoB-100 kinetics measurements before and after the intervention period in the exercise and control group.

<table>
<thead>
<tr>
<th></th>
<th>Pre EX (n=15)</th>
<th>Post EX (n=15)</th>
<th>P value Paired T-test</th>
<th>Pre Control (n=12)</th>
<th>Post Control (n=12)</th>
<th>P value Paired T-test</th>
<th>P value Post data between the two groups Unpaired T-test</th>
<th>Δ P value unpaired T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL&lt;sub&gt;1&lt;/sub&gt;-apoB-100 PS (mg/kg)</td>
<td>52.1±5.9</td>
<td>57.6±7.5</td>
<td>0.3</td>
<td>48.7±5.4</td>
<td>50.7±7.3</td>
<td>0.8</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>VLDL&lt;sub&gt;2&lt;/sub&gt;-apoB-100 PS (mg/kg)</td>
<td>36.5±3.8</td>
<td>28.1±3.1</td>
<td>0.05</td>
<td>32.9±4.6</td>
<td>32.7±3.3</td>
<td>0.9</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Total VLDL-apoB-100 PS (mg/kg)</td>
<td>88.6±8.1</td>
<td>85.6±8.2</td>
<td>0.6</td>
<td>80.7±7.9</td>
<td>83.4±9.9</td>
<td>0.8</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>VLDL&lt;sub&gt;1&lt;/sub&gt;-apoB-100 FCR (pools/day)</td>
<td>7.2±0.6</td>
<td>10.9±1.5</td>
<td>0.02</td>
<td>10.9±1.8</td>
<td>8.9±1.1</td>
<td>0.3</td>
<td>0.5</td>
<td>0.01</td>
</tr>
<tr>
<td>VLDL&lt;sub&gt;2&lt;/sub&gt;-apoB-100 FCR (pools/day)</td>
<td>12.3±1.3</td>
<td>11.8±1.3</td>
<td>0.7</td>
<td>16.9±3.0</td>
<td>12.9±1.8</td>
<td>0.1</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>VLDL&lt;sub&gt;1&lt;/sub&gt;-apoB-100 PR (mg/kg/day)</td>
<td>3.7±0.7</td>
<td>5.5±0.5</td>
<td>0.003</td>
<td>4.9±0.8</td>
<td>3.9±0.6</td>
<td>0.3</td>
<td>0.02</td>
<td>0.006</td>
</tr>
<tr>
<td>VLDL&lt;sub&gt;2&lt;/sub&gt;-apoB-100 PR (mg/kg/day)</td>
<td>4.1±0.4</td>
<td>3.2±0.4</td>
<td>0.2</td>
<td>4.9±1.0</td>
<td>3.9±0.6</td>
<td>0.3</td>
<td>0.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Total VLDL-apoB-100 PR (mg/kg/day)</td>
<td>4.2±0.7</td>
<td>6.0±0.5</td>
<td>0.004</td>
<td>5.7±0.9</td>
<td>4.9±0.5</td>
<td>0.5</td>
<td>0.6</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM. Pre and post intervention data within the two groups were analysed by paired two-tailed t-test. Data after exercise intervention between the two groups were analysed by unpaired two-tailed t-test. Pre minus post exercise data (Δ) values between the two groups were analysed by unpaired two-tailed t-test. P values ≤0.05 are in bold. EX: exercise; VLDL: very low density lipoprotein; apoB-100: apolipoprotein B-100; PS: pool size; FCR: fractional catabolic rate; PR: production rate.

5.5.10 Interrelationships between HDL-apoA-I and VLDL<sub>1</sub>-apoB-100, VLDL<sub>2</sub>-apoB-100 kinetics before and after the intervention.

Pearson correlation coefficient analysis was performed between the baseline HDL-apoA-I PS, FCR and PR and VLDL<sub>1</sub>-apoB-100 and VLDL<sub>2</sub>-apoB-100 kinetics and other variables after the intervention for all participants as shown in Table 5.8 in which there were analysed. HDL-apoA-I PS was directly correlated with VLDL<sub>1</sub>-apoB-100 FCR (r=0.42; P=0.03), F-HDL-C concentration (r=0.49; P=0.003) and F-HDL-apoA-I (r=0.99; P=<0.0001), and inversely correlated with, VLDL<sub>2</sub>-apoB-100 PS (r=-0.50; P= 0.01) and total VLDL-apoB-100 PS (r=-0.47; P=0.01). Also, HDL-apoA-I FCR was directly related with HDL-apoA-I PR (r=0.81; P=<0.001), ALT concentration (r=0.50; P=0.007) (Figure 5.1(C)), and AST concentration (r=0.44; P=0.02)
(Figure 5.1(D)), and inversely correlated with VLDL$_2$-apoB-100 FCR ($r$=-0.43; $P$=0.02) (Figure 5.1(A)), VLDL$_2$-apoB-100 PR ($r$=-0.47; $P$=0.01) (Figure 5.1(B)) and F-HDL-C concentration ($r$=-0.42; $P$=0.03). HDL-apoA-I PR inversely correlated with VLDL$_2$-apoB-100 PR ($r$=-0.45; $P$=0.02).

Table 5.8: Correlation between the exercise kinetic measurements of HDL-apoA-I, VLDL$_1$-apoB-100 and VLDL$_2$-apoB-100 at baseline (pre-exercise intervention) (n=27).

<table>
<thead>
<tr>
<th>Correlations</th>
<th>HDL-apoA-I PS (mg/kg)</th>
<th>HDL-apoA-I FCR (pools/day)</th>
<th>HDL-apoA-I PR (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL-apoA-I PS (mg/kg)</td>
<td>-0.037</td>
<td>-0.037</td>
<td>0.48**</td>
</tr>
<tr>
<td>HDL-apoA-I FCR (pools/day)</td>
<td>-0.42**</td>
<td>0.81***</td>
<td></td>
</tr>
<tr>
<td>HDL-apoA-I PR (mg/kg/day)</td>
<td>0.48**</td>
<td>0.12</td>
<td>-0.22</td>
</tr>
<tr>
<td>VLDL$_1$-apoB-100 FCR (pools/day)</td>
<td>-0.25</td>
<td>-0.22</td>
<td>-0.34</td>
</tr>
<tr>
<td>VLDL$_2$-apoB-100 FCR (pools/day)</td>
<td>0.47*</td>
<td>-0.20</td>
<td>0.034</td>
</tr>
<tr>
<td>Total VLDL-apoB-100 FCR (mg/kg)</td>
<td>0.16</td>
<td>0.29</td>
<td>-0.02</td>
</tr>
<tr>
<td>Total CHOL</td>
<td>-0.2</td>
<td>-0.47*</td>
<td>-0.42*</td>
</tr>
<tr>
<td>LDL-CHOL (mmol/L)</td>
<td>-0.05</td>
<td>0.17</td>
<td>0.03</td>
</tr>
<tr>
<td>Plasma TG (mmol/L)</td>
<td>-0.03</td>
<td>0.15</td>
<td>0.01</td>
</tr>
<tr>
<td>VLDL$_1$-apoB-100 FCR (pools/day)</td>
<td>-0.24</td>
<td>0.31</td>
<td>-0.09</td>
</tr>
<tr>
<td>Total VLDL-apoB-100 PR (mg/kg/day)</td>
<td>0.49**</td>
<td>-0.42*</td>
<td>-0.06</td>
</tr>
<tr>
<td>HDL-F-apo-A-I (g/L)</td>
<td>0.99***</td>
<td>-0.09</td>
<td>0.39</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>0.19</td>
<td>0.50**</td>
<td>0.33</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>0.01</td>
<td>0.44*</td>
<td>0.39</td>
</tr>
<tr>
<td>IHCL%</td>
<td>-0.05</td>
<td>0.36</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Data expressed and the $r$ values using two-tailed Pearson Correlation and corrected by Bonferroni correction test. $P$ values ≤ 0.05 were marked as; *$P$≤0.05. **$P$≤0.01 and ***$P$≤0.001. VLDL: very low density lipoprotein; LDL: low-density lipoprotein; HDL: high density lipoprotein; apo: apolipoprotein; PS: pool size; FCR: fractional catabolic rate; PR: production rate; CHOL: cholesterol; TG: triacylglycerols; F-HDL-C: cholesterol in obtained HDL fraction; F-HDL-apoA-I: apolipoprotein A-I in obtained HDL fraction; ALT: alanine transferase; AST: aspartate transferase; IHCL%: intrahepatocellular lipid percentage.
Figure 5.1: Baseline correlations analysis by Pearson correlation test and corrected by Bonferroni correction test in all participants (n=27) of A: baseline HDL-apoA-I FCR versus baseline VLDL2-apoB-100 FCR (r=-0.43; P=0.02), B: baseline HDL-apoA-I PS versus baseline VLDL2-apoB-100 PR (r=-0.47; P=0.01), C: baseline HDL-apoA-I FCR versus ALT (r=0.50; P=0.007), and D: baseline HDL-apoA-I FCR versus AST (r=0.44; P=0.02). VLDL: very low density lipoprotein; HDL: high density lipoprotein; apoB-100: apolipoprotein B-100; apoA-I: apolipoprotein A-I; PS: pool size; FCR: fractional catabolic rate; PR: production rate; TG: triacylglycerols; IHCL%: intra hepatocellular lipid percentage; ALT: alanine transferase; AST: aspartate transferase.
The relationship between the change of pre minus post exercise (delta (Δ)) in HDL-apoA-I PS, FCR and PR with Δ VLDL₁-apoB-100 and Δ VLDL₂-apoB-100 kinetics and other variables after the intervention for all participants was analysed as shown in Table 5.9. The Δ HDL-apoA-I PS was positively correlated with Δ HDL-apoA-I PR (r=0.40; P= 0.02), Δ VLDL₁-apoB-100 FCR (r= 0.41; P= 0.03) (Figure 5.2 (D)), and the Δ F-HDL-apoA-I (r= 0.98; P=<0.0001), and inversely correlated with Δ VLDL₁-apoB-100 PS (r=-0.43; P=0.03), Δ VLDL₂-apoB-100 PS (r=-0.41; P= 0.03) and Δ total VLDL-apoB-100 PS (r=-0.63; P=0.0004) as shown in Figure 5.2 (A, B and C) respectively. There was a positive correlation between HDL-apoA-I FCR and HDL-apoA-I PR (r=0.80; P=<0.001). HDL-apoA-I PR inversely correlated with total VLDL-apoB-100 PS (r=-0.45; P=0.02) (Figure 5.2 (E)), and directly correlated with F-HDL-apoA-I concentration (r=0.48; P=0.01).

Table 5.9: Interrelationships correlation between the Δ HDL-apoA-I measurements and Δ VLDL₁-apoB-100 and Δ VLDL₂-apoB-100 kinetic measurements (n=27).

<table>
<thead>
<tr>
<th>Correlations</th>
<th>Δ HDL-apoA-I PS (mg/kg)</th>
<th>Δ HDL-apoA-I FCR (pools/day)</th>
<th>Δ HDL-apoA-I PR (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ HDL-apoA-I PS (mg/kg)</td>
<td>-0.04</td>
<td>-0.04</td>
<td>0.40*</td>
</tr>
<tr>
<td>Δ HDL-apoA-I FCR (pools/day)</td>
<td>-0.04</td>
<td>-0.04</td>
<td>0.80***</td>
</tr>
<tr>
<td>Δ VLDL₁-apoB-100 PS (mg/kg)</td>
<td>0.40*</td>
<td>0.40*</td>
<td>0.80***</td>
</tr>
<tr>
<td>Δ VLDL₂-apoB-100 PS (mg/kg)</td>
<td>-0.43*</td>
<td>-0.43*</td>
<td>-0.21</td>
</tr>
<tr>
<td>Δ Total VLDL-apoB-100 PS (mg/kg)</td>
<td>-0.63***</td>
<td>-0.17</td>
<td>-0.45*</td>
</tr>
<tr>
<td>Δ VLDL₁-apoB-100 FCR (pools/day)</td>
<td>0.41*</td>
<td>0.41*</td>
<td>0.06</td>
</tr>
<tr>
<td>Δ VLDL₂-apoB-100 FCR (pools/day)</td>
<td>0.15</td>
<td>0.15</td>
<td>0.33</td>
</tr>
<tr>
<td>Δ VLDL₁-apoB-100 PR (mg/kg/day)</td>
<td>0.16</td>
<td>0.16</td>
<td>-0.04</td>
</tr>
<tr>
<td>Δ VLDL₂-apoB-100 PR (mg/kg/day)</td>
<td>-0.27</td>
<td>-0.27</td>
<td>-0.02</td>
</tr>
<tr>
<td>Δ Total VLDL-apoB-100 PR (mg/kg/day)</td>
<td>0.1</td>
<td>0.1</td>
<td>-0.04</td>
</tr>
<tr>
<td>Δ Total CHOL</td>
<td>-0.17</td>
<td>-0.17</td>
<td>0.15</td>
</tr>
<tr>
<td>Δ LDL-CHOL (mmol/L)</td>
<td>-0.03</td>
<td>-0.03</td>
<td>0.74</td>
</tr>
<tr>
<td>Δ Plasma TG (mmol/L)</td>
<td>-0.08</td>
<td>-0.08</td>
<td>-0.09</td>
</tr>
<tr>
<td>Δ F-HDL-C (mmol/L)</td>
<td>0.39</td>
<td>0.39</td>
<td>-0.05</td>
</tr>
<tr>
<td>Δ HDL-F-apo-A-I (g/L)</td>
<td>0.98***</td>
<td>0.15</td>
<td>0.5*</td>
</tr>
<tr>
<td>Δ IHCL (%)</td>
<td>-0.04</td>
<td>-0.04</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Data expressed and the r values using two-tailed Pearson Correlation corrected by Bonferroni correction test. P values ≤ 0.05 were marked as; *P≤0.05, **P≤0.01 and ***P≤0.001. Δ: Delta (the change); VLDL: very low density lipoprotein; LDL: low-density lipoprotein; HDL: high density lipoprotein; apo: apolipoprotein; PS: pool size; FCR: fractional catabolic rate; PR: production rate; CHOL: cholesterol; TG: triacylglycerols; F-HDL-C: cholesterol in obtained HDL fraction; F-HDL-apoA-I: apolipoprotein A-I in obtained HDL fraction; IHCL%: intrahepatocellular lipid percentage.
Figure 5.2: Delta correlations analysis by Pearson correlation test and corrected by Bonferroni correction test in all participants (n=27) of A: Δ HDL-apoA-I PS versus Δ VLDL-apoB-100 PS ($r=-0.43; P=0.03$), B: Δ HDL-apoA-I PS versus Δ VLDL₂-apoB-100 PS ($r=-0.41; P=0.03$), C: Δ HDL-apoA-I PS versus Δ total VLDL-apoB-100 PS ($r=-0.63; P=<0.001$), D: Δ HDL-apoA-I PS versus Δ VLDL₁-apoB-100 FCR ($r=0.41; P=0.03$), and E: Δ HDL-apoA-I PR versus Δ total VLDL-apoB-100 PS ($r=-0.45; P=0.02$). VLDL: very low density lipoprotein; HDL: high density lipoprotein; apoA-I: apolipoprotein A-I; apoB-100: apolipoprotein B-100; PS: pool size; FCR: fractional catabolic rate; PR: production rate.
5.6 Discussion

This study investigated the effect of a four-month moderate-intensity exercise intervention on HDL kinetics in patients with NAFLD. The current study in addition to other studies conducted by Thompson et al., 1997, Cuthbertson et al., 2016, Kadoglou et al., 2012, Lehmann et al., 2001, Sullivan et al., 2012 and Alam et al., 2004 in which participants were required to exercise at a range between 40-50% \( VO_{2\text{max}} \) (equivalent to 20-30% change in maximum heart rate (\( HR_{\text{max}} \)) (Lounana et al., 2007, Swain et al., 1994) in each study as shown in Table 5.10. The American College of Sports and Medicine (ACSM) (Garber et al., 2011) and the U.S. Office of Disease Prevention and Health Promotions (ODPHP) (ODPHP, 2016) both states that in order to gain health benefits, adults must perform a weekly 150-300 minutes of moderate-intensity physical activity equals to 5 days of 30-60 minute exercise as seen in the previous studies.

There was no significant change in HDL-apoA-I kinetics after the exercise intervention in either group. Neither plasma HDL-C concentrations nor apoA-I fraction concentrations were significantly changed. However, HDL-C concentrations measured in the isolated HDL fraction (different samples from plasma) were increased significantly after exercise in the exercise group.

The only published study to date which has previously investigated the effect of exercise on HDL-apoA-I kinetics was by Thompson et al., 1997. This study with other studies which investigated the effect of exercise on HDL-apoA-I concentration and HDL-cholesterol but not kinetics (Kadoglou et al., 2012 and Lehmann et al., 2001) are shown in Table 5.10 which illustrates key information from each study (including the current study) such as the number of participants recruited, healthy or with a metabolic disease, period and type of exercise used. Sullivan et al., 2012 and Alam et al., 2004 investigated the effect of a similar exercise
programme on liver fat content, and they measured the concentrations of HDL-cholesterol after exercise (Table 5.10). Table 5.11 shows key information from some previous clinical studies that looked at the effect of different interventions on HDL-apoA-I kinetics (FCR and PR calculated before and after each intervention) in patients with altered lipid metabolism either healthy or with T2D.

Table 5.10: Comparison of the current study with previous studies which investigated the effect of exercise on HDL metabolism.

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of participants</th>
<th>Health and disease status</th>
<th>Period of exercise</th>
<th>Measurements of</th>
<th>Type of exercise programme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current study</td>
<td>27 males (12 control and 15 exercise)</td>
<td>NAFLD</td>
<td>16 weeks</td>
<td>HDL-apoA-I kinetics, apoA-I and HDL-C concentrations</td>
<td>4-5X weekly moderate-high intensity exercise, 20 minutes progressing to 60 minutes per session (40-60% VO2MAX) equivalent to 65-80% HRMAX. approved by ACSM guidelines</td>
</tr>
<tr>
<td>Thompson et al., 1997</td>
<td>17 males</td>
<td>Healthy overweight</td>
<td>12 Months</td>
<td>HDL-apoA-I kinetics, apoA-I and HDL-C concentrations</td>
<td>3 progressing to 5X weekly moderate-high intensity exercise, 30 minutes progressing to 45 minutes including warm-up and cooling down per session (60-80% 6-7 HRMAX). Own protocol.</td>
</tr>
<tr>
<td>Cuthbertson et al., 2016</td>
<td>39 males and 11 females</td>
<td>NAFLD</td>
<td>16 weeks</td>
<td>HDL-C concentrations</td>
<td>4-5X weekly moderate-high intensity exercise, 20 minutes progressing to 60 minutes per session (30-60% HRR) equivalent to 65-80% HRMAX. approved by ACSM guidelines</td>
</tr>
<tr>
<td>Kadoglou et al., 2012</td>
<td>12 males and 35 females</td>
<td>T2D</td>
<td>12 weeks</td>
<td>ApoA-I and HDL-C concentrations</td>
<td>3X weekly moderate-high intensity exercise, 60 minutes including warm-up and cooling down per session (60-80% HRMAX). Own protocol.</td>
</tr>
<tr>
<td>Lehmann et al., 2001</td>
<td>7 males and 3 post-menopausal Females</td>
<td>T2D</td>
<td>12 weeks</td>
<td>ApoA-I and HDL-C concentrations</td>
<td>Daily moderate-high intensity exercise aerobic exercise with 50-70% HRMAX. Own protocol.</td>
</tr>
<tr>
<td>Sullivan et al., 2012</td>
<td>18 males (6 control and 12 exercise)</td>
<td>NAFLD</td>
<td>16 weeks</td>
<td>HDL-C concentrations</td>
<td>5X weekly moderate-high intensity exercise, 30-60 minutes per session (45-55% VO2MAX) approved by ACSM guidelines</td>
</tr>
<tr>
<td>Alam et al., 2004</td>
<td>18 males</td>
<td>T2D</td>
<td>24 weeks</td>
<td>HDL-C concentrations</td>
<td>4X weekly moderate-high intensity exercise, (50-85% VO2MAX) approved by ACSM guidelines</td>
</tr>
</tbody>
</table>

NAFLD; non-alcoholic fatty liver disease, -X: how many times i.e. 5X: five times; VO2MAX: the volume of maximum oxygen capacity by physical fitness; ACSM: American College of Sports and Medicine; HRMAX: maximum heart rate during exercise; HRR: Heart rate reserve (maximal heart rate minus resting heart rate). HDL-C: high-density lipoprotein – cholesterol; apoA-I: apolipoprotein A-I.
Table 5.11: Previous studies which investigated the effect of different interventions on HDL-apoA-I kinetics.

<table>
<thead>
<tr>
<th>Study carried out by</th>
<th>Methodology</th>
<th>Subjects</th>
<th>Nutritional state</th>
<th>Intervention investigated</th>
<th>HDL-apoA-I FCR (pools/day) pre and post intervention</th>
<th>HDL-apoA-I PR (mg/kg/day) pre and post intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pang et al., 2014</td>
<td>96 hours primed-constant infusion of Leucine [(5,5,5-2H3)L-Leucine]</td>
<td>12 males with T2D aged &gt;18</td>
<td>Constant-feeding</td>
<td>Effect of niacin</td>
<td>Pre= 0.37±0.02 Post= 0.33±0.01</td>
<td>Pre= 21.72±0.2 Post= 20.93±0.2</td>
</tr>
<tr>
<td>Verges et al., 2009</td>
<td>16 hours primed-constant infusion of L-L-[1-13C] leucine</td>
<td>8 subjects (5 males and 3 females) with T2D aged &gt;40</td>
<td>Constant-feeding</td>
<td>Effect of 20mg Rosuvastatin</td>
<td>Pre= 0.32±0.02 Post= 0.25±0.02</td>
<td>Pre= 16.19±1.5 Post= 12.79±1.4</td>
</tr>
<tr>
<td>Frenais et al., 2001b</td>
<td>14 hours primed-constant infusion of Leucine [(5,5,5-2H3)L-Leucine]</td>
<td>5 females with T2D aged &gt; 36</td>
<td>Fasting</td>
<td>Role of LPL activity in patients with T2D</td>
<td>Patients with T2D = 0.27±0.04 Healthy control = 0.37±0.04</td>
<td>Patients with T2D = 12.1±1.3 Healthy control = 16.1±1.5</td>
</tr>
<tr>
<td>Frenais et al., 2001a</td>
<td>14 hours primed-constant infusion of Leucine [(5,5,5-2H3)L-Leucine]</td>
<td>14 females aged &gt; 40 (7 with T2D)</td>
<td>Fasting</td>
<td>Effect of omega-3 fatty acids</td>
<td>Pre= 0.32±0.02 Post= 0.23±0.01</td>
<td>Pre= 15.8±0.9 Post= 12.3±1.5</td>
</tr>
<tr>
<td>Frenais et al., 1997</td>
<td>14 hours primed-constant infusion of Leucine [(5,5,5-2H3)L-Leucine]</td>
<td>8 patients (7 females and 1 male) with T2D aged &gt; 40</td>
<td>Fasting</td>
<td>No intervention were investigated</td>
<td>0.39±0.1</td>
<td>13.6±1.8</td>
</tr>
<tr>
<td>Thompson et al., 1997</td>
<td>Administration of radioactive [125I]-HDL tracer before and after 12 months of exercise</td>
<td>17 overweight males aged &gt; 40</td>
<td>Constant-feeding</td>
<td>Effect of prolonged exercise training</td>
<td>Pre= 0.23±0.01 Post= 0.21±0.01</td>
<td>Pre= 9.0±0.1 Post= 10.1±0.1</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM. HDL: high-density lipoprotein; apoA-I: apolipoprotein A-I; FCR: fractional catabolic rate; PR: production rate.

5.6.1 Changes in TG, HDL-C, apoA-I concentrations and HDL-apoA-I kinetics and correlations with TG and IHCL

The current study is the first study to investigate the effect of exercise on HDL-apoA-I kinetics in patients with NAFLD. Plasma TG concentrations did not reduce significantly after exercise and plasma HDL-C concentrations not increase significantly after exercise in the exercise group. However, HDL-C concentrations in HDL fraction were significantly increased after exercise in the exercise group which was similar to the results found in the study by Thompson et al., 1997. Also, exercise did not cause a significant change in apoA-I concentrations in the exercise groups but increased the HDL-apoA-I PS in the exercise group without a significant difference between the exercise and the control group. Exercise also reduced HDL-apoA-I FCR insignificantly in the exercise group which it was opposite to the
results achieved in the study by Thompson et al., 1997. The HDL-apoA-I PR did not change in both groups after the exercise intervention which it was opposite to the results achieved in the study by Thompson et al., 1997. Also, a significant increase of apoA-I concentrations after exercise were reported in the studies by Thompson et al., 1997, Lehmann et al., 2001 but not in Kadoglou et al., 2012 which was similar to the results in the current study. Also, significant reduction of plasma TG concentrations after exercise were reported in the studies by Thompson et al., 1997, Lehmann et al., 2001 and Alam et al., 2004, but not in the studies by (Cuthbertson et al., 2016), Kadoglou et al., 2012 and Sullivan et al., 2012 which was not seen in the current study results. Reported significant increase in HDL-C and/or apoA-I concentrations after exercise is accompanied by a significant decrease in plasma TG which is seen in the studies by Thompson et al., 1997, Lehmann et al., 2001, Alam et al., 2004, Kadoglou et al., 2012 and Sullivan et al., 2012. HDL-apoA-I kinetics were reported to be significantly changed after 12 months exercise in healthy overweight males (Thompson et al., 1997) which was not seen in the current study results.

5.6.2 Changes in VLDL metabolism and kinetics

The study conducted by Sullivan et al., 2012 was the only study which has previously investigated the effect of exercise on the VLDL-apoB-100 secretion rate (SR), VLDL-TG SR kinetics and LDL-CHOL in patients with NAFLD. However, their results show no significant changes in VLDL-apoB-100 SR, VLDL-TG SR kinetics and LDL-CHOL after exercise. Alam et al., 2004 showed that a six-month exercise intervention reduced total VLDL-apoB absolute secretion rate (ASR) by 51.3% and reduced VLDL-apoB PS by 47.2% with no significant difference in VLDL-apoB FCR.
5.6.3 Interrelationship between VLDL$_1$, VLDL$_2$-apoB100 and HDL-apoA-I kinetics

The baseline correlations (pre-intervention) showed two important key associations; 1) baseline HDL-apo-A-I PS was inversely correlated with plasma TG concentration and 2) HDL-apoA-I FCR was borderline directly correlated with IHCL% (P=0.06). The study conducted by Verges et al., 2014 showed that HDL-apoA-I FCR was correlated liver fat content (IHCL) which was similar to our results. Our study data showed some significant correlations between Δ VLDL$_1$ & Δ VLDL$_2$-apoB-100 and Δ HDL-apoA-I Kinetics as shown in Table 5.8 (Figure 5.1). Similar results were observed in the study conducted by Welty et al., 2004 in which similar correlations were observed between HDL-apoA-I and VLDL and LDL apoB-100 and chylomicron (CM) apoB-48 kinetics. The study showed that HDL-apoA-I PS was directly correlated with HDL-apoA-I PR (r=0.65; P=<0.001) but not with HDL-apoA-I FCR (r=0.24; P=0.272). HDL-apoA-I PR was directly correlated with HDL-apoA-I FCR (r=0.81; P=<0.001). The study also showed that apoA-I PS and PR correlated inversely with LDL-apoB-100 PS but not VLDL-apoB-100 PS. Current correlations indicate strong evidence of a connection between liver fat accumulation, plasma TG concentrations, and VLDL and HDL production and clearance rates in patients with altered lipid metabolism. Also, any significant improvement in the previous parameters will lead to a significant improvement in the other ones with time.

5.6.4 Changes in IR and physical fitness

Our data demonstrate that exercise caused a significant reduction in fasting insulin and glucose concentrations by 24.5% and 4%, HOMA2-IR (IR assessment) by 24.3%, and an increase in VO$_{2\text{MAX}}$ (physical fitness) by 22.7% in the exercise group. Similar results were observed in the study conducted by van der Heijden et al., 2010 which showed that a 12-week exercise intervention caused a significant decrease in insulin concentrations by 16.5%.
and a significant increase in VO$_{2\text{MAX}}$ by 11%. Also, the study conducted by Alam et al., 2004 showed that exercise reduces fasting insulin and glucose concentrations by 27.6% and 19% respectively, and also exercise decreased the HOMA-IR by 35.6% and increased the VO$_{2\text{MAX}}$ by 14%. Also, a study by Devries et al., 2008 showed that exercise reduces the HOMA-IR by 9% and increases the VO$_{2\text{MAX}}$ by 11.7% in obese healthy males. Cuthbertson et al., 2016 also showed similar significant reductions in fasting plasma glucose, insulin and HOMA2-IR. However, their results indicated that exercise intervention improved peripheral IR but not hepatic IR based on peripheral and hepatic glucose production (HGP) in response to high and low insulin doses respectively. Also, they said that improved hepatic IR could not be achieved due to high accumulated liver fat in their participants. A significant reduction in hepatic IR was seen in previous studies such as the studies conducted by Lim et al., 2011 and Petersen et al., 2005. In both studies, the percentage of accumulated liver fat was lower than the percentage of liver fat in our participants (12.8 and 12% respectively compared to 27.3%). Therefore, weight loss intervention in both studies was able to clear almost all accumulated liver fat and resulted in the reversal of T2D in the study by Lim et al., 2011 and hepatic steatosis in the study by Petersen et al., 2005. As a result, an improvement in hepatic IR could not be achieved due to the high mean of liver fat content before the exercise in the exercise group 27.3%, and remained elevated even after exercise with a mean of 17.0%. Therefore, our data suggest that weekly physical exercise increases the physical fitness and improve peripheral insulin sensitivity in patients with NAFLD as the 16-week exercise intervention could not clear all accumulated liver fat as shown in (Cuthbertson et al., 2016, Bojesen-Moller et al., 2014).
5.6.5 Changes in weight loss, body and liver fat content

The significant reduction in body weight by 4%, BMI by 3.5% and body fat percentage by 5% in the exercise group is similar to the results observed in the study conducted by van der Heijden et al., 2010 which showed that a 12-week aerobic exercise intervention caused a significant reduction of body fat percentage by 2.6% in obese adolescents. Also, the study conducted by Alam et al., 2004 found a significant decline in body weight with 1.3%, BMI with 3.2% and body fat mass with a 6.5% after six-month exercise in patients with T2D. Sullivan et al., 2012 and Johnson et al., 2009 showed that a 16-week exercise intervention caused a reduction of with no significant changes in body weight, BMI and body fat percentage in NAFLD patients.

Also, our data demonstrate four-month moderate exercise activity caused a significant decrease in IHCL with 37.7%, abdominal visceral and subcutaneous fat by 17.5% and 10% respectively and total visceral, subcutaneous and body fat by 16%, 11.7% and 13.6% respectively. Sullivan et al., 2012 showed a significant reduction of IHCL by 10.3% after a 16-week exercise intervention. Cuthbertson et al., 2016 also showed a significant reduction in liver fat percentage by 4.7 % after exercise with an increased percentage of liver fat even after the exercise as seen in the current study. Therefore, hepatic IR could not be improved in the current study as shown in the study by Cuthbertson et al., 2016.

Also, the study carried out by Johnson et al., 2009 showed a significant decrease in hepatic triacylglycerol concentrations (HTGC) by 20.6% and visceral adipose tissue (VAT) area 12.5% after exercise. Also, the study conducted by van der Heijden et al., 2010 showed a significant reduction in the hepatic fat percentage by 30% and visceral fat area by 10% after exercise among the obese group.
Calorie restriction without exercise is considered as independent factors with a powerful impact on reducing weight and liver content in patients with metabolic syndrome of altered lipid metabolism such as NAFLD (Koot et al., 2011, Oza et al., 2009, Lazo et al., 2010, Promrat et al., 2010) and T2D (Lazo et al., 2010, Tamura et al., 2005, Schafer et al., 2007, Petersen et al., 2005). However, the current study suggests that moderate physical exercise without calorie restriction can be considered as an independent therapeutic approach to reduce weight and body and liver fat in NAFLD patients.

### 5.6.6 Changes in plasma liver enzymes

The study data demonstrated that a four-month exercise intervention significantly decreased plasma liver enzymes concentrations as shown by several previous exercise interventions in NAFLD (Sullivan et al., 2012, Khaoshbaten et al., 2013, Abd El-Kader et al., 2014). The studies by Johnson et al., 2009, van der Heijden et al., 2010, Cuthbertson et al., 2016 and Devries et al., 2008 showed reduction with no significance in ALT concentrations after exercise.

Baseline correlation showed a significant positive correlation between HDL-apoA-I FCR and liver enzymes ALT, AST and IHCL% \( (r=0.50, P=0.007, r=0.44, P=0.02 \) and \( r=0.36, P=0.05 \) respectively) \( \) (Figure 5.1 D, E and F respectively). This indicates that a decrease in HDL-C concentrations may be associated with increased liver enzymes and liver fat accumulation in patients with altered lipid metabolism, liver and cardiovascular disease due to the development of HDL-TG and SDLDL in the ALP (Chunming et al., 2015, Calanna et al., 2014, Siddiqui et al., 2013, Onat, 2011).

Our study data also showed a direct positive correlation between \( \Delta \) ALT and \( \Delta \gammaGT \) concentrations with \( \Delta \) IHCL% after exercise \( (r=0.48, P=0.01 \) and \( r=0.57, P=0.001 \) respectively). Similar results were observed in the study conducted by Sullivan et al., 2012 in
which ALT concentrations were directly associated with the IHCL% changes after exercise ($R^2=0.60$, $P≤0.001$) in patients with NAFLD. Also, in the study conducted by van der Heijden et al., 2010 showed that hepatic fat content was directly correlated with ALT concentrations before and after exercise (baseline: $R^2=0.56$, $P=≤0.05$; post: $R^2=0.67$, $P=≤0.01$) in patients with NAFLD.

Therefore, our data along with data from other studies suggest that regular moderate exercise activity reduces liver enzymes, especially ALT. The study conducted by Bi et al., 2014 showed that ALT, AST and GGT are strongly correlated with NAFLD disease progression and risk especially GGT. Therefore, studies investigated the physical activity association with liver enzymes in improving NAFLD conditions such as the recent study conducted by Loprinzi, 2016 which showed that reduced ALT and GGT concentrations with physical activity were strongly associated with reduced mortality in patients with NAFLD. Also, in the study conducted by Martins et al., 2015 showed that physical activity is related to central obesity and fatty liver markers such as ALT. As a result, all this evidence suggests that regular moderate physical activity improves NAFLD, as determined by fat liver content and liver enzymes (Omagari et al., 2011, Prati et al., 2002).

5.6.7 The overall effect of exercise on hypertriglyceridaemia and HDL metabolism

Cuthbertson et al., 2016 showed that physical exercise improves peripheral insulin sensitivity. This would be expected to increase the activity of LPL but not HL (Zhang et al., 2002). Increased LPL activity will lead to increased VLDL hydrolysis in peripheral tissues (VLDL$_1$ and VLDL$_2$) which increases VLDL clearance (Mead et al., 2002, Mead and Ramji, 2002, Lafontan and Langin, 2009).

Physical exercise increases skeletal muscle fat oxidation and blood flow which contributes to reducing circulating FFA (Hurren et al., 2011, Kiens, 2006). Reduced FFA release from the
adipose tissue due to increased insulin sensitivity (Cuthbertson et al., 2016) will also reduce circulating FFA. This will result in reduced hepatic FA uptake and reduced fat droplet accumulation in the liver (Cuthbertson et al., 2016, Gauthier et al., 2004, Alam et al., 2004).

Increased insulin sensitivity reduces the expression of MTP, which is a protein responsible for VLDL assembly and intracellular stabilisation of apoB within the hepatocyte (Gordon et al., 1996, Taghibiglou et al., 2000, White et al., 1998). However, instead of reduced VLDL-apoB-100 PR due to increased insulin sensitivity, VLDL₁ and total VLDL-apoB-100 PR was increased. This may be explained as exercise activity increases peripheral insulin sensitivity but not hepatic insulin sensitivity as shown in the study by Cuthbertson et al., 2016. Also, there is a evidence that physical exercise causes more TG enrichment VLDL₁ which increases VLDL₁ particle size and facilitates more clearance of accumulated intracellular TG and hence reduces liver fat content (Aadland et al., 2013, Al-Shayji et al., 2012, Herd et al., 2001, Ghafouri et al., 2015).

It has been reported that physical exercise reduced hepatic FFA accumulation by stimulation of hepatic FA oxidation via the activation of adenosine-monophosphate-activated protein kinase (AMPK) and SREBP-I to inhibit acetyl-coA carboxylase release which results in decreasing malonyl-CoA and results in increased mitochondrial FA oxidation (Carlson and Winder, 1999, Sim and Hardie, 1988, Kim et al., 1989). Increased FA oxidation and VLDL production will increase hepatic fat clearance (Boren et al., 2015). This however will lead to a decrease in plasma TG pool which will increase the exchange rate of TG and CE between HDL and the TG plasma pool via CETP which causes the formation of TG-enriched HDL (HDL-TG) as seen in Figure 5.2 (Frayn, 2010, Guerin et al., 2001, Barter, 2000). HDL-TG will be hydrolysed by LPL in the circulation and/or HL in the liver causing the rapid removal of HDL particles which will lead to a decrease in HDL-C concentrations and an increase in LDL-C and
In this study, HDL-apoA-I FCR was correlated with IHCL%, and HDL-apoA-I PS was correlated with plasma TG concentration which indicates that an increase in plasma TG, by increased VLDL production, will lead to a decrease in HDL-apoA-I concentration. This explanation fits with our study in which plasma TG did not decrease after four-month of exercise and hence HDL-C did not increase as seen in previous studies by Cuthbertson et al., 2016, Kadoglou et al., 2012 and Sullivan et al., 2012 (Figure 5.2).

The study conducted by Thompson et al., 1997 and Alam et al., 2004 showed a decreased plasma TG and improved plasma HDL-C concentrations after twelve-month and six-month exercise intervention respectively. This indicated that in order to reach this stage, more exercise duration is needed to clear most of the hepatic fat up to an optimum point in which VLDL production is reduced. This will lead to a reduction in plasma TG pool which reduces the exchange rate of TG and CE between HDL and the TG plasma pool via CETP (Frayn, 2010, Guerin et al., 2001, Barter, 2000) which reduces the formation of HDL-TG and hence reduced HDL clearance rate. Corrected associated hypertriglyceridaemia in NAFLD via improving peripheral IR by exercise will increase HDL-CHOL and reduce LDL-CHOL which reduces the ALP risk in patients with altered lipid metabolism (Cuthbertson et al., 2016, Frayn, 2010) (Figure 5.2 (A)). The effect of exercise on correcting hypertriglyceridaemia and HDL-C is shown in Figure 5.2 (B) as discussed earlier.
Figure 5.2: (A) Increased exchange rate of TG and CE between HDL and the TG plasma pool via CETP causes the formation of HDL-TG and SD-LDL and reduces HDL-C due to hypertriglyceridaemia. (B) Exercise decreases plasma TG which decreases the exchange rate of TG and CE between HDL and TG plasma pool via CETP which reduces the formation of HDL-TG and SD-LDL and increases HDL-C. FFA: free fatty acids; TG: triacylglycerols; ApoB-100: apolipoprotein B-100; ApoB-48: apolipoprotein B-48; ApoA-I: apolipoprotein A-I; IHCL%: intrahepatocellular lipid percentage; VLDL: very low-density lipoprotein; HDL: high density lipoprotein; CETP: cholesteryl ester transfer protein; CE: cholesteryl ester; LPL: lipoprotein lipase; HL: hepatic lipase; SD-LDL: small dense low-density lipoprotein; HDL-C: high-density lipoprotein – cholesterol; FCR: fractional catabolic rate.
5.6.8 Study limitations and future work

Extending the exercise intervention from four to six months as in the study conducted by (Alam et al., 2004), could show a significant decrease in the plasma TG and an increase in HDL-C concentrations as explained earlier (Figure 5.2 (B)). Also, this study measured total HDL apo-A-I fraction only; changes in HDL subclasses were not measured. Although Total HDL-apoA-I kinetics (PR and FCR) did not change significantly after exercise, changes in HDL subclasses kinetics could have occurred. Measuring the activity concentrations of enzymes involved in VLDL and HDL metabolism such as PLTP, CETP and LPL could provide additional valuable information. In addition, the measurement of VLDL-TG kinetics by immunochromatography (Sun et al., 2013) will provide valuable information about the amount of TG exported in VLDL particles instead of particle number reflected by apoB kinetics measurement. This will also be helpful in measuring the VLDL particle size after exercise as seen in other previous studies which might also be correlated with liver fat content (Aadland et al., 2013, Al-Shayji et al., 2012, Herd et al., 2001, Ghafouri et al., 2015).

Hepatic fat oxidation through the measurement of certain enzymes such as AMPK, malonyl-CoA and acetyl-coA carboxylase using liver tissue biopsies (Lim et al., 2012) could be assessed in the future to investigate exercise effect on hepatic fat oxidation concentration (Carlson and Winder, 1999, Sim and Hardie, 1988, Kim et al., 1989, Hirschey and Verdin, 2010). The HDL-apoA-I kinetics measured (PR and FCR) as shown in Table 5.6 were lower than the kinetics measured in previous studies shown in Table 5.11. This could be due to three reasons;

1) The apoA-I concentration, HDL-apoA-I kinetics and HDL-C measurement were analysed using total HDL-fraction instead of blood plasma. This fraction was obtained by a three-step ultracentrifugation for at least 20 hours each. The shear forces generated by the
high-speed ultracentrifugation field may strip off the HDL-apoA-I protein and alter the HDL concentration measurements (Hafiane and Genest, 2015). Causing a low detection of HDL-apoA-I concentration.

2) The high salt density solution used during ultracentrifugation process may deplete HDL-associated apolipoproteins and alter their concentration during measurements (Davidsson et al., 2010).

3) The fraction samples were not used freshly as they were stored at -80°C for more than two years which might cause a reduction in HDL-C and HDL-apoA-I measurements (Nanjee and Miller, 1990, Warnick and Wood, 1995).

5.6.9 Conclusion

Our laboratory, exercise and study protocols were successful in detecting an effect of four-month moderate exercise activity on HDL kinetics, VLDL₁ and VLDL₂-apoB-100 kinetics, liver fat content, and altered lipid profile in patients with NAFLD. Novel investigation of HDL-apoA-I kinetics gave no significant differences for the total HDL-apoA-I FCR and PR before and after exercise. However, exercise increases the total HDL-apoA-I PS, which would increase the availability for more HDL particles. Moderate physical exercise corrected hyperinsulinaemia and increased peripheral insulin sensitivity which is associated with reducing liver fat content and improves overall lipoprotein kinetics in patients with NAFLD. Moderate exercise caused a decrease IHCL% which was explained by an increase in VLDL₁ and total VLDL-apoB-100 FCR and PS and increased VLDL₁-apoB-100 PR to eliminate accumulated liver fat content. All the demonstrated data provided substantial evidence that long-term moderate physical exercise activity is beneficial and recommended for the management for NAFLD without calorie restriction or lifestyle-induced weight loss.
Chapter 6: The effect of lixisenatide on postprandial TRL-apoB-100, TRL-apoB-48 and HDL-apoA-I kinetics

6.1 Introduction

Hyperglycaemia, IR (IR) and hypertriglyceridaemia in patients with T2D are associated with serious cardiovascular complications (Adiels et al., 2006, Sobenin et al., 1996, King et al., 2011, Matikainen and Taskinen, 2013, Pettersson et al., 2011). Postprandial hyperlipidaemia is considered one of the factors that increase the development of CVD in patients with T2D via increasing the extent of the atherogenic lipoprotein phenotype (ALP) (Boren et al., 2014, Taskinen and Boren, 2015, Manoria et al., 2013, Suryabhan et al., 2013), i.e. increased LDL-C and \( \text{s}_0 \)LDL, and reduced HDL-C concentrations (Stefanutti et al., 2014, Manoria et al., 2013, Schaefer et al., 2002, Taniguchi et al., 2000). Several studies investigated the effect of glucagon-like peptide -1 (GLP-1) receptor agonists on postprandial hyperlipidaemia and triacylglycerol-rich lipoprotein (TRL) metabolism (Voukali et al., 2014, Hermansen et al., 2013, Chiquette et al., 2012). Hermansen et al., (2013) conducted a randomized, double-blind, placebo-controlled, cross-over clinical trial and found out that a 3 week treatment with once daily 1.8mg liraglutide reduced hypertriglyceridaemia and apoB-48 concentration versus placebo in patients with T2D. Bunck et al., (2010) illustrated that a 51-week treatment with twice daily 10µg exenatide period caused a reduction in postprandial hypertriglyceridaemia and apoB-48 concentrations but not TRL-apoB-100 concentrations, and a significant increase in serum HDL-C concentrations in patients with T2D. Another study showed similar results in which a 3-week treatment with once daily 10µg exenatide period also reduced postprandial increase of TG and apoB-48 concentrations (Schwartz et al., 2010). However, Xiao et al., (2012) was the only study to date which investigated the effect of GLP-1 receptor agonist (exenatide) on postprandial TRL-apoB-100 and TRL-apoB-48 kinetics in healthy obese men and found that exenatide inhibited the production of TRL-apoB-48 which
was independent of reduced gastric emptying. Xiao et al., (2012) also showed that 4 to 6-week treatment with once daily 10µg exenatide caused a significant reduction in apoB-48 concentrations, but not TRL-apoB-100 concentrations, which was explained by a significant decrease in TRL-apoB-48 PR, but not FCR, and found no significant changes in TRL-apoB-100 concentrations and kinetics. To date, there are no published studies on the effect of GLP-1 receptor agonist on postprandial TRL-apoB-100, TRL-apoB-48 and HDL-apoA-I kinetics in patients with T2D. Lixisenatide, also known as Lyxumia, is a GLP-1 receptor agonist which is been licensed to be used as a treatment for uncontrolled hyperglycaemia associated with patients T2D along with metformin (EMA, 2013). Therefore, this study was conducted to test whether a 4 week lixisenatide treatment (once daily (10µg for 14 days followed by an increase to 20µg for 14 days) versus placebo can cause direct/indirect effects on postprandial lipoprotein metabolism in patients with T2D by calculating TRL-apoB-48, TRL-apoB-100 and HDL-apoA-I kinetics using stable isotope trace-labelling technique.

6.2 Hypothesis

Lixisenatide treatment for patients with T2D will correct altered lipoprotein metabolism by increasing hepatic and peripheral insulin sensitivity and reducing postprandial hypertriglyceridaemia. This will be accomplished by reducing plasma postprandial TG concentration by decreasing CM-apoB-48 PR as a result of indirect and direct effects of lixisenatide on intestinal enterocytes, and will also reduce VLDL-apoB-100 PR from the liver as a consequence of an improvement in insulin sensitivity. The latter will indirectly increase the PR of HDL-apoA-I. These effects will contribute to reducing CVD risk.

6.3 Aims

To conduct a clinical trial that will study the effects of a 4-week treatment with lixisenatide (a GLP-1 receptor agonist) versus placebo on postprandial lipoprotein metabolism,
hypertriglyceridaemia, IR and lipid profile in patients with T2D using a constant feeding protocol and a stable isotope trace-labelling technique.

6.4 Methods

This study was a double-blind crossover study of a treatment with subcutaneous injections of once daily 10-20μg lixisenatide versus placebo for the duration of 12 weeks including the washout period. Six Caucasian men aged 40-65 with BMI 27-37 kg/m2 diagnosed with T2D were recruited in the study (for detailed study recruitment see chapter 2 section 2.3.1) (chapter 2, Figure 2.3). Samples from 4 out of 6 patients were used to measure HDL-C, HDL-apoA-I concentration and HDL-apoA-I kinetics (chapter 2, Figure 2.3). Subjects were assigned blindly and randomly to the two treatment options; starting lixisenatide treatment for four weeks followed by a four-week washout period versus another four weeks of placebo or vice versa (chapter 2, Figure 2.4). Blood samples were collected hourly during the study before and after the injection of the 1-¹³C leucine tracer for 12 hours (Figure 2.2) (for detailed study protocol see chapter 2 section 2.3.3). The following measurements were performed at the end of each treatment period at visits 4 and 8 (chapter 2, Figure 2.4); plasma glucose, plasma lipid concentrations, TRL-apoB-100, TRL-apoB-48 and HDL-apoA-I concentrations and kinetics by following the verified laboratory protocol (for detailed laboratory methods and protocol of measurement see chapter 3). Other supporting results mentioned in this chapter such as insulin concentration were collected and analysed by other personnel in the laboratory. Data of lixisenatide treatment versus placebo were compared and analysed by certain statistical tests to elucidate the effect of lixisenatide (for detailed statistical analysis tests used see chapter 3 section 3.2.19).
6.5 Results

6.5.1 Subject characteristics

The main physical and physiological characteristics of the six male subjects recruited in the study are shown in Table 6.0. Age, height, body weight, BMI and body fat percentage were similar in both groups. The BMI of 3 out of 6 subjects indicates that they were considered obese (NHSUK, 2015). Elevated fasting glucose, HbA1c, TG and total cholesterol concentrations show hyperglycaemia and altered lipid metabolism in these patients respectively which would be expected in patients with T2D. Notably, the mean systolic blood pressure (BP) in both groups was located in the range of the prehypertension category (120 – 139 mmHg) (WHO, 2015a, American Diabetes Association, 2016), while the mean diastolic BP was in the normal range. The main characteristics of the participants after lixisenatide treatment intervention versus placebo are shown in Table 6.1. Body weights, BMI, waist circumference and body fat percentage, were not significantly changed after lixisenatide treatment versus placebo. This indicates that lixisenatide treatment did not cause a significant effect on weight loss in the participants of this study, and all the following results occurred independently of weight loss. Plasma liver enzymes; alanine transaminase (ALT) and aspartate transaminase (AST) were not significantly changed after lixisenatide treatment versus placebo. Systolic and diastolic BP were not significantly changed after lixisenatide treatment versus placebo.
Table 6.0: Subject characteristics and fasting plasma measurements at the screening visit.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Baseline measurements (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58.8±2.2</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>173.2±2.7</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>91.2±4.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30.4±1.2</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>7.9±0.7</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>57.5±3.5</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>Total CHOL (mmol/L)</td>
<td>3.5±0.4</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>28.2±1.6</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>126.5±8.9</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>78.7±2.2</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM. Data between the two groups were analysed by paired two-tailed t-test. P values ≤0.05 are in bold. BMI: body mass index; TG: triacylglycerol; HbA1c: haemoglobin A1C; CHOL: cholesterol; BP: blood pressure.

Table 6.1: Main subjects characteristics after lixisenatide treatment versus period.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Lixisenatide (n=6)</th>
<th>Placebo (n=6)</th>
<th>P value Paired T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>91.8±4.0</td>
<td>91.0±4.3</td>
<td>0.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.5±1.1</td>
<td>29.3±1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>27.5±1.3</td>
<td>26.8±1.6</td>
<td>0.7</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>30.5±6.5</td>
<td>34.7±10.4</td>
<td>0.8</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>44.2±8.6</td>
<td>46.7±11.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>126.3±6.6</td>
<td>128.6±7.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>81.8±2.6</td>
<td>80.3±3.6</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM. Data after lixisenatide and placebo were compared between each other and analysed by paired two-tailed t-test. P values ≤0.05 are in bold. BMI: body mass index; BP: blood pressure.

6.5.2 The effect of lixisenatide treatment on glucose and insulin concentrations and IR

Concentrations of fasting and postprandial plasma glucose, insulin and IR calculated by the Homeostasis Model Assessment 2 (HOMA2-IR) after lixisenatide treatment versus placebo are shown in Figure 6.0 A, B and C respectively. Figure 6.0 (A) shows the mean concentrations of plasma glucose after lixisenatide treatment versus placebo for the six participants. Fasting glucose concentrations with a mean concentration of 7.6±0.6 at -240 minutes mmol/L were
significantly lower after the lixisenatide treatment ($P=0.05$) than placebo (8.3 ±1.0 mmol/L). Postprandial glucose concentrations with a mean concentration of 8.2±1.1 mmol/L between 0 – 480 minutes after the lixisenatide treatment were significantly lower ($P=0.001$) than placebo (9.2±1.1 mmol/L) (Figure 6.0 (A)). Fasting insulin concentrations with a mean concentration of 157.5±28.2 pmol/L at -240 minutes were not significantly different after the lixisenatide treatment ($P=0.33$) versus placebo (123.5±27.5 pmol/L) (Figure 6.0(B)). Postprandial insulin concentrations with a mean concentration of 264.8±52.5 pmol/L between 0 – 480 minutes after lixisenatide treatment were significantly higher ($P=<0.001$) than placebo (221.2±46.3 pmol/L) (Figure 6.0 (B)). The mean IR value of 2.3±0.3 after lixisenatide versus placebo was not significantly different (2.8±0.4) (Figure 6.0 (C)).
Figure 6.0: Plasma fasting glucose (A) and insulin (B) concentrations and HOMA2-IR (C) after lixisenatide treatment versus placebo (n=6). Data expressed as mean ± SEM. Fasting concentration at -240 minutes after lixisenatide and placebo treatments were analysed by paired two-tailed t-test. The mean of postprandial concentrations between 0 – 480 minutes after lixisenatid and placebo treatments were analysed by paired two-tailed t-test. *P≤0.05, **P<0.01 and ***P<0.001. HOMA2-IR: The Homeostasis Model Assessment 2 for IR calculation.
6.5.3 The effect of lixisenatide treatment on plasma TG, total cholesterol and FFA concentrations

Plasma TG, total cholesterol and FFA concentrations after lixisenatide treatment versus placebo for the six participants are shown in Figure 6.1 (A, B and C respectively). A repeated measure ANOVA test was used to verify if the TG concentrations between 0 – 480 minutes were constant and in a steady state. TG concentrations were not significantly different after lixisenatide treatment (\( P=0.4 \)) or placebo (\( P=0.5 \)) over time in the six participants. This validates the feeding protocol in achieving a constant raised postprandial TG steady state which is necessary for the analysis of stable isotope kinetics. Fasting TG concentrations with a mean concentration of 1.5±0.1 mmol/L at -240 minutes were not significantly different (\( P=0.6 \)) between the lixisenatide treatment and placebo (1.6±0.2 mmol/L) (Figure 6.1 (A)). Postprandial TG concentration with a mean concentration of 2.8±0.3 mmol/L between 0 – 480 minutes after lixisenatide treatment was significantly lower (\( P=0.002 \)) than placebo (3.3±0.3 mmol/L) (Figure 6.1 (A)). Fasting total cholesterol concentrations with a mean value of 3.7±0.4 mmol/L at -240 minutes after the lixisenatide treatment was not significantly different (\( P=0.09 \)) from placebo (4.2±0.5 mmol/L) (Figure 6.1(B)). Postprandial total cholesterol concentration with a mean concentration of 3.8±0.4 mmol/L between 0 – 480 minutes after the lixisenatide treatment was significantly lower (\( P=0.02 \)) than placebo (4.1±0.5 mmol/L) (Figure 6.1 (B)). Fasting FFA concentrations with a mean concentration of 0.7±0.1 mmol/L at -240 minutes after the lixisenatide treatment was not significantly different (\( P=0.5 \)) from placebo (0.7±0.1 mmol/L) (Figure 6.1 (C)). Postprandial FFA concentration with a mean concentration of 0.5±0.1 mmol/L between 0 – 480 minutes after the lixisenatide treatment was significantly lower (\( P=0.05 \)) than placebo (0.6±0.03 mmol/L) (Figure 6.1 (C)).
Figure 6.1: Plasma fasting TG (A), cholesterol (B) and FFA (C) concentrations after lixisenatide treatment versus placebo (n=6). Data expressed as mean ± SEM. Fasting concentrations at -240 minutes after lixisenatide and placebo treatments were analysed by paired two-tailed t-test. The mean of postprandial concentrations between 0 – 480 minutes after lixisenatide and placebo treatments were analysed by paired two-tailed t-test. *P≤0.05, **P<0.01 and ***P<0.001. TG: triacylglycerol; FFA: free fatty acids.
6.5.4 The effect of Lixisenatide treatment on TRL-TG and TRL-cholesterol concentrations.

The TRL-TG and TRL-cholesterol concentrations after lixisenatide treatment versus placebo for the six participants are shown in Figure 6.2 (A and B respectively). Fasting TRL-TG concentrations with a mean concentration of 1.0±0.1 mmol/L at -240 minutes after the lixisenatide treatment were not significantly different (\(P=0.9\)) from placebo (0.9±0.2 mmol/L) (Figure 6.2 (A)). Postprandial TRL-TG concentrations with a mean concentration of 2.3±0.2 mmol/L between 0 – 480 minutes after the lixisenatide treatment were significantly lower (\(P=0.05\)) than placebo (2.6±0.30 mmol/L) (Figure 6.2 (A)).

Fasting TRL-cholesterol concentrations with a mean concentration of 0.3±0.02 mmol/L at -240 minutes after the lixisenatide treatment were not significantly different (\(P=0.6\)) versus placebo (0.3±0.1 mmol/L) as shown in Figure 6.2 (B). Postprandial TRL-cholesterol concentrations with a mean concentration of 0.4±0.1 mmol/L between 0 – 480 minutes after the lixisenatide treatment were significantly lower (\(P=0.04\)) than placebo (0.5±0.1 mmol/L) (Figure 6.2 (B)).
Figure 6.2: TRL-TG (A) and TRL-cholesterol (B) concentrations after lixisenatide treatment versus placebo (n=6). Data expressed as mean ± SEM. Fasting concentrations at -240 minutes after lixisenatide and placebo treatments were analysed by paired two-tailed t-test. The mean of postprandial concentrations between 0 – 480 minutes after lixisenatide and placebo treatments were analysed by paired two-tailed t-test. *P≤0.05. TRL: triacylglycerol rich-lipoprotein; TG: triacylglycerol.
6.5.5  The effect of Lixisenatide treatment on fasting plasma HDL-C, F-HDL-C and F-HDL-apoA-I concentrations.

Figure 6.3 (A and B) shows the cholesterol and apoA-I concentrations in the HDL fraction (F-HDL-C) and (F-HDL-apoA-I) respectively after lixisenatide treatment versus placebo for the six participants. Fasting F-HDL-C concentration with a mean concentration of 0.57±0.1 g/L at -240 minutes after lixisenatide treatment was not significantly different (P=0.4) versus placebo (0.63±0.1 g/L) as shown in Figure 6.3 (A). Postprandial F-HDL-C concentration with a mean concentration of 0.6±0.1 g/L between 0 – 480 minutes after the lixisenatide treatment was lower, but not statistically significant (P=0.07) versus placebo (0.5±0.1 g/L) (Figure 6.3 (A)).

Fasting plasma HDL-C concentration with a mean concentration of 1.1±0.06 g/L at -240 minutes after lixisenatide treatment was not significantly different (P=0.4) versus placebo (1.1±0.1 g/L) as shown in Figure 6.3 (B).

Fasting F-HDL-apoA-I concentration with a mean concentration of 0.5±0.03 g/L at -240 minutes after the lixisenatide treatment was not significantly different (P=0.6) versus placebo (0.5±0.1 g/L) as shown in Figure 6.3 (C). Postprandial F-HDL-apoA-I concentrations with a mean concentration of 0.5±0.03 g/L between 0 – 480 minutes after the lixisenatide treatment was not changed significantly (P=0.9) versus placebo (0.5±0.04 g/L) (Figure 6.3 (C)).
Figure 6.3: F-HDL-C (A) plasma HDL-C (B) and F-HDL-apoA-I (C) concentrations after lixisenatide treatment versus placebo (n=4). Data expressed as mean ± SEM. Fasting concentrations at -240 minutes after lixisenatide and placebo treatments were analysed by paired two-tailed t-test. The mean of postprandial concentrations between 0 – 480 minutes after lixisenatide and placebo treatments were analysed by paired two-tailed t-test. HDL: high density lipoprotein; CHOL: cholesterol; apoA-I: apolipoprotein A-I; F-HDL-CHOL: cholesterol concentrations in HDL fraction; F-HDL-apoA-I: apolipoprotein A-I concentrations in HDL fraction.
6.5.6 The effect of lixisenatide treatment on TRL-apoB-100 and TRL-apoB-48 concentrations

The measurements of TRL-apoB-100 and TRL-apoB-48 concentrations after lixisenatide treatment versus placebo are shown in Figure 6.4 A and B respectively. The fasting mean TRL-apoB-100 concentration of 26.8±2.6 mg/L at -240 minutes after lixisenatide treatment was not significantly different ($P=0.4$) versus placebo (24.5±3.6 mg/L) (Figure 6.4 (A)). Postprandial TRL-apoB-100 concentration with a mean concentration of 28.8±4.3 mg/L were between 0 – 480 minutes after lixisenatide treatment was not significantly different ($P=0.1$) from placebo (31.6±5.3 mg/L) Figure 6.4 (A).

The fasting TRL-apoB-48 concentration with a mean concentration of 2.3±0.7 mg/L at -240 minutes after lixisenatide treatment was not significantly different ($P=0.7$) from placebo (2.0±0.4 mg/L) (Figure 6.4 (B)). Postprandial TRL-apoB-100 concentration with a mean concentration of 9.2±2.4 mg/L between 0 – 480 minutes after lixisenatide treatment was not significantly different ($P=0.3$) from placebo (9.8±2.7 mg/L) (Figure 6.4 (B)).
Figure 6.4: Fasting and postprandial concentrations of TRL-apoB-100 (A) and TRL-apoB-48 (B) (n=6). Data expressed as mean ± SEM. Fasting concentrations at -240 minutes after lixisenatide and placebo treatments were analysed by paired two-tailed t-test. The mean of postprandial concentrations between 0 – 480 minutes after lixisenatide and placebo treatments were analysed by paired two-tailed t-test. TRL: triacylglycerol rich-lipoprotein; apoB-100: apolipoprotein B-10; apoB-48: apolipoprotein B-48.
6.5.7 The effect of lixisenatide treatment on leucine isotopic enrichment of TRL-apoB-100, TRL-apoB-48 and HDL-apoA-I versus placebo

The $^{13}$C leucine isotopic enrichment of TRL-apoB-100, TRL-apoB-48 and HDL-apoA-I after lixisenatide treatment versus placebo are shown in Figures 6.5 (A, B and C respectively). Post lixisenatide treatment versus placebo treatment measurements of $^{13}$C leucine enrichment was calculated and expressed as TTRs. The area under the curve (AUC) of the leucine TTR enrichment of TRLapoB-100 was not significantly different (AUC=10.9±0.2xmin) ($P=0.8$) after lixisenatide treatment versus placebo (AUC=10.0±0.2xmin) as shown in Figure 6.5 (A).

The mean AUC of the leucine TTR enrichment of TRLapoB-48 not significantly different (AUC=4.4±0.1xmin) ($P=0.8$) after lixisenatide treatment versus placebo (AUC=4.0±0.1xmin) as shown in Figure 6.5 (B).

Figure 6.5 (C) shows the mean AUC of the leucine TTR enrichment of HDL-apoA-I which was not significantly different (AUC=1.0±0.02xmin) ($P=0.8$) after lixisenatide treatment versus placebo (AUC=1.1±0.03xmin).
Figure 6.5: $^{13}$C Leucine TTR enrichment of TRL-apoB-100 (A), TRL-apoB-48 (B) and HDL-apoA-I (C) across study time points. Data expressed as mean ± SEM. The mean of postprandial TTR enrichment measurements between 0 – 480 minutes after lixisenatide and placebo treatments were analysed by AUC.
6.5.8 The effect of lixisenatide treatment on TRL-apoB-100, TRL-apoB-48 and HDL-apoA-I kinetics

TRL-apoB-100 fractional catabolic rate (FCR), pool size (PS) and production rate (PR) were calculated after lixisenatide treatment and placebo treatment (Figure 6.6 A, B and C respectively). TRL-apoB-100 FCR was increased significantly \((P=0.01)\) after lixisenatide treatment \((6.3\pm0.4)\) pools/day versus placebo \((4.1\pm0.6)\) pools/day as shown in Figure 6.6 (A). TRL-apoB-100 PS was not significantly different \((P=0.1)\) after lixisenatide treatment versus placebo as shown in Figure 6.6 (B). TRL-apoB-100 PR was higher, but not statistically significant \((P=0.06)\) after lixisenatide treatment versus placebo as shown in Figure 6.6 (C).

TRL-apoB-48 FCR, PS and PR were calculated after lixisenatide treatment and placebo and are shown in Figure 6.7 A, B and C respectively. TRL-apoB-48 FCR, PS and PR were not significantly different after lixisenatide treatment versus placebo.

The HDL-apoA-I kinetics; FCR, PS and PR were calculated in 4 out of 6 participants after lixisenatide treatment and placebo and are shown in Figure 6.8 A, B and C respectively. HDL-apoA-I FCR, PS and PR were not significantly different after lixisenatide treatment versus placebo.
Figure 6.6: TRL-apoB-100 kinetics after lixisenatide treatment versus placebo (n=6). Data expressed as mean ± SEM. The mean of postprandial measurements between 0 – 480 minutes after lixisenatide and placebo treatments were analysed by paired two-tailed t-test. The P values <0.05 are marked as *P≤0.05.
Figure 6.7: TRL-apoB-48 kinetics after lixisenatide treatment versus placebo (n=6). Data expressed as mean ± SEM. The mean of postprandial measurements between 0 – 480 minutes after lixisenatide and placebo treatments were analysed by paired two-tailed t-test.
Figure 6.8: HDL-apoA-I kinetics after lixisenatide treatment versus placebo (n=4). Data expressed as mean ± SEM. The mean of postprandial measurements between 0 – 480 minutes after lixisenatide and placebo treatments were analysed by paired two-tailed t-test.
6.6 Discussion

This study was a randomised cross-over clinical trial, which investigated the effect of a 4-week lixisenatide treatment intervention versus placebo treatment on TRL-apoB-100, TRL-apoB-48 and HDL-apoA-I kinetics in patients with T2D. The study showed that TRL-apoB-100 FCR was significantly increased after lixisenatide treatment versus placebo, and TRL-apoB-100 PS and PR did not change significantly which resulted in a decrease in plasma TG concentration. TRL-apoB-48 and HDL-apoA-I FCR, PS and PR were not changed significantly after lixisenatide treatment versus placebo. Plasma and fractional concentrations of TG, cholesterol, plasma FFA and glucose were significantly reduced after lixisenatide treatment versus placebo.

Previous studies conducted by Hermansen et al., 2013, Xiao et al., 2012, Bunck et al., 2010 and Schwartz et al., 2010 investigated the effect of GLP-1 receptor agonists on postprandially elevated plasma lipid profiles, especially TG (Table 6.2). There are no previous clinical studies on the effects of GLP-1 receptor agonists on postprandial TRL-apoB-100 and TRL-apoB-48 and HDL-apoA-I kinetics in patients with T2D.

The only published study which has investigated the effect of a GLP-1 receptor agonist on postprandial TRL-apoB-100 and TRL-apoB-48 kinetics was conducted by Xiao et al., 2012 in healthy subjects as shown in Table 6.2 which included key information from each study (including the current study) such as the number of participants recruited, period of treatment, healthy or with a metabolic disease, clinical protocol and GLP-1 receptor agonist dose used. Other studies carried out by Hermansen et al., 2013, Bunck et al., 2010 and Schwartz et al., 2010 investigated the effect of GLP-1 receptor agonists on postprandial plasma or serum lipid profile, glucose and insulin concentrations but not TRL-apoB-100, TRL-
apoB-48 or HDL-apoA-I kinetics. Table 6.3 and 5.11 (see chapter 5) show key information from previous clinical studies that investigated the effect of different interventions on TRL-apoB-100 and TRL-apoB-48 and HDL-apoA-I kinetics (FCR and PR calculated before and after each intervention) respectively in patients with altered lipid metabolism either healthy or with T2D.

Table 6.2: Comparison of the current study with previous studies which investigated the effect of GLP-1 receptor agonists on postprandial lipid metabolism.

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of participants</th>
<th>Health and disease status</th>
<th>Period of treatment</th>
<th>Measurements</th>
<th>Study protocol and GLP-1 receptor agonist used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current study</td>
<td>6 males</td>
<td>T2D</td>
<td>Four weeks of treatment with four weeks washout period</td>
<td>Plasma and TRL fraction lipid profile and TRL-apoB-100 and TRL-apoB-48 kinetics</td>
<td>1X daily (10µg for 14 days followed by an increase to 20µg for 14 days) lixisenatide treatment versus placebo.</td>
</tr>
<tr>
<td>Hermansen et al., 2013</td>
<td>20 subjects (11 males and nine females)</td>
<td>T2D</td>
<td>Three weeks of treatment with 3-9 weeks washout period</td>
<td>Plasma lipid profile and plasma apoB-48 concentrations</td>
<td>1X daily gradually increased dose (0.6 to 1.8 mg) of liraglutide versus placebo</td>
</tr>
<tr>
<td>Xiao et al., 2012</td>
<td>15 males</td>
<td>Healthy obese</td>
<td>Four-six weeks</td>
<td>Plasma and TRL fraction lipid profile and TRL-apoB-100 and TRL-apoB-48 kinetics</td>
<td>1X daily 10µg exenatide treatment versus placebo</td>
</tr>
<tr>
<td>Bunck et al., 2010</td>
<td>30 males</td>
<td>T2D</td>
<td>Fifty one week</td>
<td>Plasma lipid profile and plasma apoB-48 concentrations</td>
<td>2X daily 5µg exenatide progressing to 10µg and if needed to 20µg versus placebo</td>
</tr>
<tr>
<td>(Schwartz et al., 2010b)</td>
<td>35 subjects (31 males and four females)</td>
<td>Impaired glucose tolerance or recent-onset T2D</td>
<td>Three weeks</td>
<td>Plasma lipid profile and plasma apoB-48 concentrations</td>
<td>1X daily 10µg exenatide treatment versus placebo</td>
</tr>
</tbody>
</table>

TRL- triacylglycerol-rich lipoprotein; -X: how many times i.e. 5X: five times; T2D: type 2 diabetes; TRL: triacylglycerol-rich lipoprotein; apo: apolipoprotein.
### Table 6.3: Previous studies which investigated the effect of different interventions on apoB-100 and apoB-48 kinetics.

<table>
<thead>
<tr>
<th>Study carried out by</th>
<th>Methodology</th>
<th>Subjects</th>
<th>Nutritional state</th>
<th>Intervention investigated</th>
<th>Apo B-100 FCR (pools/day) for each intervention</th>
<th>Apo B-100 PR (mg/kg/day) for each intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xiao et al., 2012</td>
<td>10 hours primed-constant infusion of D3-Leucine ([5,5,5-2H3]L-Leucine)</td>
<td>15 healthy obese males</td>
<td>Constant-feeding</td>
<td>Effect of exenatide on TRL-apoB-100 and TRL-apoB-48 kinetics</td>
<td>placebo TRL-apoB-100 = 3.83±0.4</td>
<td>Placebo TRL-apoB-100 = 9.88±1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Exenatide TRL-apoB-100 = 3.03±0.2</td>
<td>Exenatide TRL-apoB-100 = 7.84±0.7</td>
<td></td>
</tr>
<tr>
<td>Duez et al., 2008a</td>
<td>12 hours primed-constant infusion of D3-Leucine ([5,5,5-2H3]L-Leucine)</td>
<td>12 healthy obese males</td>
<td>Constant-feeding</td>
<td>Effect of short-term acute elevation of plasma FFA</td>
<td>Controls VLDL-apoB-100 = 5.1±0.1</td>
<td>Controls VLDL-apoB-100 = 20.7±0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>After acute elevation of FFA VLDL-apoB-100 = 5.0±0.1</td>
<td>After acute elevation of FFA VLDL-apoB-100 = 27.9±1.2</td>
<td></td>
</tr>
<tr>
<td>Hogue et al., 2007b</td>
<td>12 hours primed-constant infusion of D3-Leucine ([5,5,5-2H3]L-Leucine)</td>
<td>24 males (11 males with T2D versus 13 healthy males as control)</td>
<td>Constant-feeding</td>
<td>TRL-apoB-100 and TRL-apoB-48 kinetics in patient with T2D against healthy controls</td>
<td>Controls VLDL-apoB-100 = 9.1±3.4</td>
<td>Controls VLDL-apoB-100 = 32.1±7.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T2D VLDL-apoB-100 = 4.1±0.4</td>
<td>T2D VLDL-apoB-100 = 41.1±4.8</td>
<td></td>
</tr>
<tr>
<td>Welty et al., 2004</td>
<td>15 hours primed-constant infusion of D3-Leucine ([5,5,5-2H3]L-Leucine)</td>
<td>23 healthy subjects (17 males and 6 females)</td>
<td>Constant-feeding</td>
<td>No interventions</td>
<td>VLDL-apoB-100 = 6.1±0.7</td>
<td>VLDL-apoB-100 = 15.0±1.3</td>
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<table>
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<tr>
<th>Study carried out by</th>
<th>Methodology</th>
<th>Subjects</th>
<th>Nutritional state</th>
<th>Intervention investigated</th>
<th>Apo B-100 FCR (pools/day) for each intervention</th>
<th>Apo B-100 PR (mg/kg/day) for each intervention</th>
</tr>
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<tr>
<td>Xiao et al., 2012</td>
<td>10 hours primed-constant infusion of D3-Leucine ([5,5,5-2H3]L-Leucine)</td>
<td>15 healthy obese males</td>
<td>Constant-feeding</td>
<td>Effect of exenatide</td>
<td>placebo TRL-apoB-48 = 1.5±0.2</td>
<td>Placebo TRL-apoB-48 = 0.12±0.02</td>
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<td>Exenatide TRL-apoB-48 = 1.36±0.2</td>
<td>Exenatide TRL-apoB-48 = 0.08±0.02</td>
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<td>Duez et al., 2008a</td>
<td>12 hours primed-constant infusion of D3-Leucine ([5,5,5-2H3]L-Leucine)</td>
<td>12 healthy obese males</td>
<td>Constant-feeding</td>
<td>Effect of short-term acute elevation of plasma FFA</td>
<td>Controls TRL-apoB-48 = 4.2±0.09</td>
<td>Controls TRL-apoB-48 = 3.5±0.2</td>
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<td>After acute elevation of FFA TRL-apoB-48 = 4.8±0.2</td>
<td>After acute elevation of FFA TRL-apoB-48 = 5.9±0.3</td>
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<td>Hogue et al., 2007b</td>
<td>12 hours primed-constant infusion of D3-Leucine ([5,5,5-2H3]L-Leucine)</td>
<td>24 males (11 males with T2D versus 13 healthy males as control)</td>
<td>Constant-feeding</td>
<td>TRL-apoB-100 and TRL-apoB-48 kinetics in patient with T2D against healthy controls</td>
<td>Controls TRL-apoB-48 = 7.8±0.6</td>
<td>Controls TRL-apoB-48 = 3.0±0.5</td>
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<td>T2D TRL-apoB-48 = 5.8±0.5</td>
<td>T2D TRL-apoB-48 = 11.0±0.6</td>
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<tr>
<td>Welty et al., 2004</td>
<td>15 hours primed-constant infusion of D3-Leucine ([5,5,5-2H3]L-Leucine)</td>
<td>23 healthy subjects (17 males and 6 females)</td>
<td>Constant-feeding</td>
<td>No interventions</td>
<td>TRL-apoB-48 = 4.3±0.4</td>
<td>TRL-apoB-48 = 0.94±0.2</td>
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Data are expressed as Mean ± SEM. TRL: triacylglycerol-rich lipoprotein; VLDL: very low-density lipoprotein; apoB-100: apolipoprotein B-100, apoB-48: apolipoprotein B-48; FCR: fractional catabolic rate; PR: production rate.
6.6.1 Changes in fasting and postprandial lipid profile, TRL-apoB-100, TRL-apoB-48 and HDL-apoA-I kinetics

A 3-week liraglutide treatment period caused no significant change in fasting TG or FFA concentrations versus placebo in the study of T2D by Hermansen et al., 2013 which was similar to the results in the current study. In contrast, the study carried out by Bunck et al., 2010 showed a significant reduction in fasting plasma TG after a 51-week exenatide treatment versus placebo. A significant reduction in postprandial plasma TG was observed after a 3-week liraglutide treatment period (Hermansen et al., 2013) and after a 3-week exenatide treatment period in patients with T2D (Schwartz et al., 2010) which was also similar to the results in the current study. However, the study conducted by Xiao et al., 2012 in healthy obese subjects did not show a significant decrease in postprandial plasma TG or TRL-TG after a 4 to 6-week exenatide treatment period versus placebo contrary to the current study results. A significant overall prolonged reduction of postprandial FFA was detected after a 51-week exenatide treatment period (Bunck et al., 2010) and a 3-week exenatide treatment period (Schwartz et al., 2010) versus placebo which was similar to the current study. There was no significant reduction in plasma FFA after a 3-week liraglutide treatment period (Hermansen et al., 2013) and after a 4-week exenatide treatment period (Xiao et al., 2012) versus placebo. No significant changes in plasma and TRL total cholesterol were found after a 4 to 6-week exenatide treatment period (Xiao et al., 2012) and a 51-week exenatide treatment period (Bunck et al., 2010) versus placebo. However, in the study carried out by Schwartz et al., 2010 plasma remnant lipoprotein cholesterol (RLP-C) was significantly decreased after a 3-week exenatide treatment period versus placebo.
No significant changes in apoB-100 concentrations were observed after a 4 to 6-week exenatide treatment period (Xiao et al., 2012) and a 51-week exenatide treatment period (Bunck et al., 2010) versus placebo which is similarly seen in the current study. On the other hand, significant reductions in apoB-48 concentrations were detected after a 3-week liraglutide treatment period (Hermansen et al., 2013), after a 4 to 6-week exenatide treatment period (Xiao et al., 2012), after a 3-week exenatide treatment period (Schwartz et al., 2010) and after a 51-week exenatide treatment period (Bunck et al., 2010) versus placebo contrary to the finding in the current study. These previous studies might have had different effects due to the use of different GLP-1 analogues in each study with different durations of treatment.

Xiao et al., 2012 measured the postprandial TRL-apoB-100 kinetics after a 4 to 6-week exenatide treatment in healthy obese subjects and showed that TRL-apoB-100 FCR and PR did not significantly change against placebo. On the contrary, the current study showed a significant increase in TRL-apoB-100 FCR after a 4-week lixisenatide treatment versus placebo. A possible mechanism for this is that lixisenatide significantly increased postprandial insulin concentrations in patients with T2D (Wilkins et al., 2014, Patel et al., 2014, Xiao et al., 2012) (Figure 6.0). Increased insulin secretion increases uptake of TG by the adipose tissue via increased LPL activity in the adipose tissues (Fried et al., 1993, Taskinen, 1987, Romon et al., 1983, Tan et al., 2006). An increased TRL-apoB-100 FCR contributes to decreasing the postprandial TG plasma pool.

The study showed that TRL-apoB-100 PR is increased with borderline significance (P=0.06) after lixisenatide treatment versus placebo. This could be due to an increase in liver de novo lipogenesis (DNL) due to increased insulin concentrations (Dimitriadis et al., 2011, Patel et
This would increase the availability of TG for VLDL production despite the fact that insulin would be expected to reduce apoB-100 PR.

Xiao et al., 2012 demonstrated that TRL-apoB-48 PR was significantly decreased, but not FCR, after 4 to 6-week exenatide treatment which was not shown in the current study.

Serum HDL-C concentrations were significantly increased after a 51-week exenatide treatment period versus placebo (Bunck et al., 2010) but this was not seen in the current study in either plasma or fraction HDL-C concentrations. The study by Bunck et al., 2010 also showed no significant change in plasma apoA-I concentrations after treatment period with exenatide which is similar to the results seen in the current study. The reason for the different findings may be that the current study was not sufficiently powered to detect a significant increase in HDL-C (n=4).

The current study is the only study which has investigated the effect of lixisenatide treatment on HDL-apoA-I kinetics against placebo in patients with T2D. This novel investigation showed no significant changes in HDL-apoA-I PS, FCR and PR after four-week lixisenatide treatment against placebo.

### 6.6.2 Changes in plasma glucose, insulin and IR

Fasting plasma glucose concentrations were significantly decreased after a 3-week liraglutide treatment period versus placebo (Hermansen et al., 2013) which is similar to the results in the current study. Postprandial glucose was significantly decreased after a 3-week liraglutide treatment period (Hermansen et al., 2013), after a 4 to 6-week exenatide treatment period (Xiao et al., 2012), after a 51-week exenatide treatment period (Bunck et al., 2010) and after a 3-week exenatide treatment period (Schwartz et al., 2010) versus placebo which is similar to the results of the current study.
Hermansen et al., 2013 showed that fasting insulin concentrations were significantly increased after a 3-week liraglutide treatment period versus placebo which was not found in the current study. Postprandial insulin concentrations were not significantly changed after a 4 to 6-week exenatide treatment period (Xiao et al., 2012) and after a 51-week exenatide treatment period (Bunck et al., 2010) versus placebo which differs from the current study. Postprandial insulin concentrations were significantly increased after a 3-week liraglutide treatment versus placebo (Hermansen et al., 2013) which is similar to the current results, but significantly decreased after a 3-week exenatide treatment period versus placebo (Schwartz et al., 2010) which is opposite to the finding in the current study.

The current study showed that IR, measured by HOMA2-IR, was not significantly different after a 4-week treatment period versus placebo. Reduced IR in patients with altered glucose metabolism was observed in previous studies after treatment with GLP-1 receptor agonists (Rizzo et al., 2009, Nystrom, 2008).

6.6.3 GLP-1 receptor agonist administration, dosage and the meal given in this study

Lixisenatide was administered daily in the present study with an increase after two weeks from the start of the treatment period for the duration of 4 weeks. A similar method of administration was seen in the studies conducted by Hermansen et al., 2013 and Bunck et al., 2010 for three weeks and 51-weeks respectively. Xiao et al., 2012 and Schwartz et al., 2010 used a fixed dose of exenatide and administered for similar durations of 3-4 weeks. Administration of gradual dosing of GLP-1 receptor agonists is advised to be effective in reducing side effects which occur during the initial treatment period (Prasad-Reddy and Isaacs, 2015).
In this study, the feeding protocol used to produce a postprandial TG steady state was an hourly fat-rich liquid meal (Carbohydrates (CHO) 22%, total fat 66.1%, and protein 11.9% with a total of 2280 Kcal) (See Table 2.0 Chapter 2). The studies conducted by Hermansen et al., 2013, Bunck et al., 2010 and Schwartz et al., 2010 used fat-rich solid test meals during the clinical trial to measure the effect of a GLP-1-receptor agonist on apoB-48 concentrations. Xiao et al., 2012 was the only study which used a similar hourly feeding protocol with CHO 38%, total fat 49%, and protein 13% to increase plasma TG concentrations to calculate TRL-apoB-100 and TRL-apoB-48 kinetics, but they did not achieve a steady state in their study as shown by changes in TRL-apoB-48 concentrations. They stated that they might use non-steady state modelling in the future to reanalyse their kinetics. In the latter study, both TRL-apoB-100 and TRL-apoB-48 kinetics were calculated using a one pool model based on the assumption that plasma TG was in a steady state. This indicates that their results could be questionable and needs reanalysis as they stated (Xiao et al., 2012). In contrast, the study design and the meal given in the current study were successful in achieving raised plasma TG and TRL-apoB-48 steady states.

It is important to note, that in the clinical protocol for the current study the lixisenatide was administered on the study day 30 minutes prior to giving the liquid meal in order to achieve a raised postprandial TG steady state under the effect of lixisenatide after 4 hours of administration, at which point the stable isotope infusion for the kinetic study was started. Lixisenatide is a once daily treatment with a longer half-life and action duration (EMA, 2013) that exenatide (EMA, 2011). Duration of action of lixisenatide was expected to be for more than 10 hours enabling the measurements of TRL kinetics between 0 – 480 minutes (EMA, 2013). On the contrary, the study carried out by Xiao et al., 2012 used a clinical protocol in which exenatide, with a shorter half-life of 2 hours (EMA, 2011), and the stable isotope was
injected at the same time, which is the reason for not achieving a steady state as they stated.

6.6.4 Overall effects of lixisenatide on altered lipoprotein metabolism

Many studies showed that GLP-1 receptor agonists cause a reduction in postprandial hypertriglyceridaemia by reducing TRL-apoB-48 concentrations (Hermansen et al., 2013, Xiao et al., 2012, Bunck et al., 2010, Schwartz et al., 2010). The mechanisms for this may be a reduction in gastric emptying which is a recognised effect of GLP-1 receptor agonists (Becker et al., 2015, Lorenz et al., 2013). Another possible mechanism is a reduction in activity of microsomal transfer protein (MTP) which is increased in patients or animal models with altered lipid metabolism and IR (Gutierrez-Repiso et al., 2015, Brahm and Hegele, 2015, Adelis and Lewis, 2008). Reducing gastric emptying plays a significant role in decreasing the lipid substrate needed for chylomicron (CM) formation in the enterocytes which causes a reduction in CM production rate (Becker et al., 2015, Lorenz et al., 2013). A decrease in MTP activity in the enterocytes will also result in a reduction in the assembly of CM particles which causes a decrease in CM production and chylomicron remnant (CMR) concentrations (Gutierrez-Repiso et al., 2015, Brahm and Hegele, 2015, Carpentier et al., 2002, Xiao et al., 2012, Adelis and Lewis, 2008). A decrease in TRL-apoB-48 PR was not seen in the current study which may be due to the sample size of (n=6) being too small to detect the hypothesised differences.

There is evidence that GLP-1 may decrease hepatic DNL via AMPK in DPP-4 deficient rats (Ben-Shlomo et al., 2011). Also, a GLP-1 receptor agonist has been shown to reduce MTP activity in hepatocytes which reduces TRL-apoB-100 production in hamsters (Carpentier et al., 2002). It was anticipated that TRL-apoB-100 PR would be reduced due to reduced hepatic
and adipose tissue DNL, decreased circulating FFA, increased hepatic fat oxidation and decreased CMR concentrations (Parlevliet et al., 2009b, Patel et al., 2014). However, the current study results showed that TRL-apoB-100 PR was increased with borderline significance after lixisenatide treatment which was not anticipated. A possible explanation is that acute suppression of hepatic VLDL production by insulin was ineffective due to elevated liver fat and hepatic IR in patients with the metabolic syndrome and altered lipid metabolism (Adiels et al., 2008, Adiels et al., 2007). Therefore, increased insulin secretion may not suppress VLDL production in patients with elevated liver fat and/or hepatic IR. The liver fat percentage was not measured in the current study, however, elevated body fat % and abnormal ALT and AST concentrations suggest the presence of high liver fat percentage in the participants of the current study (Sanal, 2015, Calanna et al., 2014, Hsiao et al., 2007, Clain and Lefkowitch, 1987). Another possible explanation could be that an increase in TRL-apoB-100 PR may also be due to an increase in hepatic DNL in response to increased insulin secretion (Dimitriadis et al., 2011, Patel et al., 2014) despite the fact that GLP-1 may decrease hepatic DNL in a DPP-4 deficient rat-model (Ben-Shlomo et al., 2011). Therefore, both explanations are debatable and the reason behind the increase in TRL-apoB-100 PR after GLP-1 receptor agonist treatment requires further investigation.

6.6.5 Study limitations and future work

The sample size for this study was estimated to be 18 based on the study power calculation of TRL-apoB-48 PR (see Chapter 2 section 2.3.3). Only six patients were recruited in this study which made the study underpowered to detect a 25% significant reduction of TRL-apoB-48 PR after lixisenatide treatment with a power of 80% at the 5% concentration. However n=6 was estimated to detect a 40% significant reduction in TRL-apoB-48 PR and
TRL-apoB-100 kinetics after lixisenatide treatment with a power of 80% at the 5% concentration.

The sample size for this study was estimated to be 14 based on another study power calculation for HDL-apoA-I FCR (see Chapter 2 section 2.3.3). Only four patients were recruited in this study which made the study underpowered to detect a 20% significant reduction of HDL-apoA-I FCR after exercise intervention with a power of 80% at the 5% concentration. However n=4 was estimated to detect a 35% significant reduction in HDL-apoA-I FCR after exercise intervention with a power of 80% at the 5% concentration.

The limited number of recruited patients was due to;

1. Restricted selection criteria of type 2 diabetic patients with uncontrolled glucose concentrations taking only metformin for the maintaining their glucose and elevated TG concentrations.

2. The lack of funds and financial support and time for the recruitment procedure. The recruitment process lasted for two years which was considered a long time in relation to a 4-year PhD programme.

Therefore, recruiting further participants as indicated earlier in each study power calculation for TRL-apoB-48 PR and HDL-apoA-I FCR will enhance the power to find statistical differences in TRL-apoB-48 PR (further n=9 are needed) and HDL-apoA-I FCR (further n=11 are needed) kinetics.

It was not possible to measure TRL-apoB-100 and TRL-apob-48 concentrations using a simple technique such as the measurement of HDL-apoA-I by MIRA COBAS auto analysers as they are chemically and structurally similar to each other. Therefore, two separate sensitive and
specific competitive ELISA methods were used for TRL-apoB-100 and TRL-apoB-48 which was financially expensive and time-consuming.

Measuring the activity and concentrations of enzymes involved in VLDL and HDL metabolism such as CETP, HL and LPL could provide additional valuable information.

The measurement of intrahepatic cellular lipid (IHCL) by magnetic resonance spectroscopy (MRS) could indicate whether the participants had a high percentage of liver fat, which might change after lixisenatide treatment (Armstrong et al., 2016a, Armstrong et al., 2016b, Li et al., 2015, Blaslov et al., 2014, Olaywi et al., 2013, Fruci et al., 2013, Sathyanarayana et al., 2011) and might explain the increase in TRL-apoB-100 PR after lixisenatide treatment.

Estimation of gastric emptying using paracetamol after lixisenatide treatment could provide more information about the magnitude of the effect of lixisenatide on increasing the lipid substrate for CM formation in the enterocytes (Becker et al., 2015, Lorenz et al., 2013).

Also, the measurement of VLDL-TG and CM-TG kinetics by immunochromatography (Sun et al., 2013) will provide valuable information about the amount of TG exported in VLDL particles instead of particle number reflected by apoB kinetics measurement. This will also be helpful in measuring the VLDL particle size after lixisenatide. The concentration of hepatic fat oxidation through the analysis of certain enzymes such as AMPK, malonyl-CoA and acetyl-coA carboxylase (Lim et al., 2012) could be assessed in the future to investigate lixisenatide effect on hepatic fat oxidation concentration using a T2D animal model (Ben-Shlomo et al., 2011, Svegliati-Baroni et al., 2011, Patel et al., 2014).

TRL-apoB-100 and TRL-apoB-48 FCR and PR were similar to those reported by Xiao et al., 2012 but were lower than other studies as seen in Table 6.3. This is due to the different methods used in measuring the two most important factors which contribute in the TRL-
apoB-100 and TRL-apoB-48 kinetic calculations; 1) the method of measuring apoB-100 and apoB-48 concentrations, and 2) using a measurement of precursor pool such as $^{13}\text{C}$ α-KIC measurements as an indicator of the Leucine precursor pool. The current study used a more robust method for measuring TRL-apoB-100 and TRL-apoB-48 concentrations using a highly sensitive and specific competitive ELISA. Xiao et al., 2012 used commercial ELISA kits with high sensitivity and specificity to determine TRL-apoB-100 and TRL-apoB-48 concentration which was similar to the method used in the current study especially the kit used for determining TRL-apoB-48 concentration which was from the same company (Shibayagi Co Ltd, Shibukawa, Gunma, Japan) (Xiao et al., 2012). Other studies such as the study conducted by Hogue et al., 2007 used a less specific and sensitive method in which they directly calculated the concentration using the density of the electrophoresis band for apoB-48 and apoB-100 using western immunoblotting. Hogue et al., 2007 and Xiao et al., 2012 extrapolated that the TTRs of TRL-apoB-100 and TRL-apoB-48 to plateau without measuring α-KIC which is a different method to estimate the precursor pool from the one used in the current study. In the lixisenatide study, a continuous feeding protocol was developed to achieve a relatively steady state of plasma TG concentration for the calculation of TRL kinetics. Feeding a high fat, low carbohydrate meal hourly for the duration of 12 hours may affect the physiology of digestion and absorption, and therefore lipoprotein metabolism kinetics may differ from that following a single large meal. Therefore, this could interfere with TRL kinetic calculations.

HDL-apoA-I kinetics were lower than the kinetics measured in previous studies. This has been justified previously (see Chapter 5 Table 5.11 and discussion under study limitations section).
6.7 Conclusion

The laboratory and clinical protocols were successful in measuring the effect of four-weeks of lixisenatide treatment versus placebo on TRL-apoB-100, TRL-apoB-48 and HDL-apoA-I kinetics and lipid profile in patients with T2D. TRL-apoB-100 FCR increased significantly after lixisenatide while TRL-apoB-48 and HDL-apoA-I FCR and PR did not change significantly after lixisenatide treatment versus placebo. Lixisenatide treatment decreased postprandial plasma TG, cholesterol, FFA and glucose concentrations but did not affect plasma and fractional HDL-C. Lixisenatide caused a reduction in postprandial plasma TG due to increased removal of VLDL particles. This may be due to significantly increased insulin production. Increased insulin concentrations after lixisenatide treatment did not significantly reduce TRL-apoB-100 PR which might be due to the presence of high liver fat percentage and increased hepatic IR, and might be due to an increase in hepatic DNL by the action of increased insulin after lixisenatide treatment. An increase in sample size is needed to investigate the effect of lixisenatide on TRL-apoB-48 and HDL-apoA-I kinetics. All the presented data provided substantial evidence that long-term lixisenatide treatment can improve glycemic control and correct altered lipid metabolism. Further investigations are needed to investigate the effects on lixisenatide on liver fat and hepatic DNL as there is a growing evidence which suggest the potential use of GLP-I receptor agonists in the treatment of patients with high liver fat percentage such as patients with NAFLD.
Chapter 7: General discussion

7.1 Altered lipid metabolism in patients with T2D and NAFLD

This project investigated the effect of exercise on HDL-apoA-I kinetics in NAFLD and the effect of lixisenatide on postprandial TRL-apoB-100, TRL-apoB-48 and HDL-apoA-I kinetics in patients with T2D. Both NAFLD and T2D are strongly associated with altered lipid metabolism, IR and visceral obesity (Noureddin and Rinella, 2015, Saponaro et al., 2015). About 30-80% of patients with T2D are also diagnosed with NAFLD (Richard and Lingvay, 2011, Tolman and Dalpiaz, 2007). Therefore, there is a strong association between NAFLD and T2D and cross-interaction may worsen the pathogenicity and progression of each disease (Noureddin and Rinella, 2015, Saponaro et al., 2015, Fruci et al., 2013). The fasting baseline measurements of the participants in both studies were analysed by unpaired T-test to investigate if there were any major differences in the plasma lipid profile, glucose, insulin, IR and liver enzymes as shown in Table 7.0. The results indicate that there were no significant differences for all the measurements except fasting glucose concentration which was significantly higher ($P=0.001$) in patients with T2D, and fasting insulin concentration which was significantly higher ($P=0.05$) in patients with NAFLD. One of the exclusion criteria in the NAFLD study was patients with T2D. The clear difference between the two groups is that the patients with NAFLD had functional insulin secretion which maintains normal glucose metabolism (Sattar et al., 2014, Preiss and Sattar, 2008).
Table 7.0: Baseline measurements for the participants in the NAFLD (n=27) and Lixisenatide (n=6) studies.

<table>
<thead>
<tr>
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<th>NAFLD study</th>
<th>Lixisenatide Study</th>
<th>Un-Paired T-test P value</th>
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<td>Baseline measurements (n=27)</td>
<td>Baseline measurements (n=6)</td>
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<tr>
<td>Age (years)</td>
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<tr>
<td>Height (cm)</td>
<td>179.1±1.6</td>
<td>173.2±2.7</td>
<td>0.1</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>101.7±3.0</td>
<td>89.7±3.3</td>
<td>0.09</td>
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<td>BMI (kg/m2)</td>
<td>31.6±0.6</td>
<td>29.3±1.2</td>
<td>0.1</td>
</tr>
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<td>Body fat (%)</td>
<td>28.6±0.9</td>
<td>26.8±1.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>139.9±3.0</td>
<td>128.6±7.1</td>
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<td>Diastolic BP (mm Hg)</td>
<td>89.4±2.8</td>
<td>80.3±3.6</td>
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<td>Fasting glucose (mmol/L)</td>
<td>5.9±0.1</td>
<td>7.6±0.7</td>
<td>0.001</td>
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<tr>
<td>Fasting TG (mmol/L)</td>
<td>1.8±0.2</td>
<td>1.5±0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Fasting Total CHOL (mmol/L)</td>
<td>5.0±0.1</td>
<td>4.0±0.4</td>
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<td>ALT (IU/l)</td>
<td>46.6±4.1</td>
<td>46.7±11.8</td>
<td>0.9</td>
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<tr>
<td>AST (IU/l)</td>
<td>33.4±2.2</td>
<td>34.7±10.4</td>
<td>0.8</td>
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<td>Serum fasting HDL-C (g/L)</td>
<td>1.0±0.1</td>
<td>1.1±0.1</td>
<td>0.9</td>
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<td>F-HDL-C (g/L)</td>
<td>0.6±0.1</td>
<td>0.5±0.1</td>
<td>0.3</td>
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<td>Insulin (pmol/L)</td>
<td>174.7±11.9</td>
<td>132.7±22.2</td>
<td>0.05</td>
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<tr>
<td>HOMA2-IR (%)</td>
<td>3.3±0.2</td>
<td>2.8±0.4</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM. Data between the two groups were analysed by unpaired two-tailed t-test. P values ≤0.05 are in bold. BMI: body mass index; BP: blood pressure TG: triacylglycerols; CHOL: cholesterol; ALT: alanine transferase; AST: aspartate transferase; HDL-C: high-density lipoprotein cholesterol; F-HDL-C: fractional high-density lipoprotein cholesterol; HOMA2-IR: The Homeostasis Model Assessment for IR percentage.

7.2 Exercise and lixisenatide for the treatment of altered lipoprotein metabolism via plasma insulin and IR

Exercise significantly increased VLDL1-apoB-100 FCR via decreasing IR. Increased insulin sensitivity after exercise may have increased LPL activity in the muscle tissues which increases the hydrolysis of VLDL particle. This may eventually correct fasting hypertriglyceridaemia which was not however seen in this study, and a longer exercise intervention period might be needed to observe a difference (Figure 7.0). Exercise also significantly reduced percentage liver fat and plasma liver enzymes which indicated that exercise improved the overall liver function and condition in patients with NAFLD.
A four-week treatment period with Lixisenatide, a GLP-1 receptor agonist, versus placebo was shown to lower postprandial plasma hypertriglyceridaemia, hyperglycaemia and hypercholesterolaemia in patients with T2D which may have been due to the direct GLP-1 receptor agonist action on β cells which caused a postprandial increase in plasma insulin concentrations (Figure 7.0). Reduced fasting and postprandial plasma hypertriglyceridaemia may have been due to the significant increase in postprandial insulin concentrations and TRL-apoB-100 FCR, reduced circulating FFA due to increased FFA uptake by adipose tissue and reduced lipolysis in adipose tissue. Lixisenatide, however, did not cause a significant change in TRL-apoB-48 kinetics.

It appears that both exercise and lixisenatide exerted their effects on altered lipoprotein metabolism mainly via either increasing insulin concentration or increasing insulin sensitivity.

7.3 Limitations of the project

The laboratory work was time-consuming for both studies. It took at least five weeks to analyse samples from each study for a single subject. The analysis protocol was complicated with multiple methodologies, and errors could be generated at any single step which required repetition if needed. Visible results were seen at the last two steps; bands on the gel after the SDS-PAGE step and the ion peaks generated at the GC-MS step. Therefore, any errors made in all the previous steps including delipidation, hydrolysis, IEC, freeze drying and derivatization were not detectable. Thus the project needed to be cautiously designed to minimise errors as possible, and the work to be carried out by a skilled experimenter.
Figure 7.0: Exercise and lixisenatide as combined treatment for altered lipoprotein metabolism can be achieved by;

- Lixisenatide treatment which increases postprandial insulin concentration.
- Regular exercise which decreases fasting insulin concentration and increases peripheral insulin sensitivity.

Corrected hypertriglyceridaemia will result in reduced exchange of TG and CE between TRL and HDL-C via CETP which reduces the risk of developing ALP which is associated with T2D and NAFLD.

Two studies were conducted to investigate potential effects on altered lipoprotein metabolism by;

Exercise effects on fasting hypertriglyceridaemia
- Decreased insulin concentration.
- Increased insulin sensitivity.
- Increased VLDL-apoB-100 FCR.
- Increased VLDL-apoB-100 PR.
- Decreased IHCL%.
- Fasting plasma TG, FFA and cholesterol were not significantly changed. A longer exercise intervention period might be needed to correct Fasting hypertriglyceridaemia.

Lixisenatide treatment effects on postprandial hypertriglyceridaemia
- Increased postprandial insulin concentration.
- IR was not significantly decreased.
- Increased VLDL-apoB-100 FCR.
- Increased VLDL-apoB-100 PR.
- Postprandial hypertriglyceridaemia and hyperglycaemia were corrected.

Figure 7.0: Exercise and lixisenatide as combined treatment for altered lipoprotein metabolism. IR: IR; VLDL: very low-density lipoprotein; apo: apolipoprotein; FCR: fractional catabolic rate; PR: production rate; IHCL%: intrahepatocellular lipid percentage; TG: triacylglycerol; FFA: free fatty acid; CE: cholesterol ester; TRL: triacylglycerol-rich lipoprotein; HDL-C: high-density lipoprotein cholesterol; CETP: cholesterol ester transfer protein; ALP: atherogenic lipoprotein phenotype; T2D: type 2 diabetes; NAFLD: non-alcoholic fatty liver disease.
7.4 Combined treatment of exercise and GLP-1 receptor agonist for the treatment of dyslipidaemia associated with NAFLD and T2D

The final results of both studies suggest two possible approaches which can be used in correcting hypertriglyceridaemia: 1. Increasing postprandial insulin secretion by lixisenatide treatment to correct postprandial hypertriglyceridaemia in patients with T2D and altered lipoprotein metabolism. 2. Regular exercise to increase peripheral insulin sensitivity and correct fasting hypertriglyceridaemia in patients with NAFLD and altered lipoprotein metabolism. Therefore, a potential combination treatment approach of exercise and lixisenatide is shown in Figure 7.1 in which both treatment increases VLDL-apoB-100 FCR by both decreasing insulin concentration and resistance in the fasting state by the exercise effects or by increasing insulin concentrations by the lixisenatide treatment effect. As a result, it is been suggested that a combined treatment will be more efficient than using one treatment approach to reduce fasting and postprandial dyslipidaemia associated with NAFLD and T2D in fasting and postprandial states, and it could be considered as a new treatment approach as shown in Figure 7.1. Also, the combined treatment might reduce liver fat percentage and improve liver function.

Recent studies indicate that exercise does not increase plasma GLP-1 in patients with altered lipid metabolism (Bailey et al., 2015, Nyhoff et al., 2015, Eshghi et al., 2013, Ueda et al., 2009, Martins et al., 2007). Therefore, it is suggested to conduct a clinical study investigating the effect of combined approaches of exercise and lixisenatide on postprandial hypertriglyceridaemia associated with altered lipid metabolism in diagnosed patients with T2D with an IHCL% above 5% to observe secondary effects on lipoprotein metabolism and liver fat accumulation and clearance. This may lead to a reduction in the TG plasma pool which will reduce the exchange rate between TRL and HDL via CETP (Sheridan, 2016,
This would lead to a decrease in the formation of \( s_0 \)LDL and increase HDL-C reducing CVD risk (Barter, 2000, Frayn, 2010).

**Figure 7.1:** Potential combination of exercise and lixisenatide treatments as a new treatment approach for dyslipidaemia associated with NAFLD and T2D. NAFLD: Non-alcoholic fatty liver disease; T2D: type 2 diabetes; VO\(_{2\text{MAX}}\): measurement of blood oxygen capacity during physical exercise; VLDL: very low-density lipoprotein; HDL: high-density lipoprotein; TRL: triglycerides-rich lipoprotein; \( s_0 \)LDL: small dense low-density lipoprotein; HDL-C: high-density lipoprotein – cholesterol; apoB-100: apolipoprotein B-100; FCR: fractional catabolic rate; LPL: lipoprotein lipase; TG: triglycerides; CETP: cholesterol ester transfer protein; CVD: cardiovascular disease; CHD: coronary heart disease.
7.5 Conclusion

Exercise and lixisenatide treatment increased VLDL-apoB-100 FCR. This may contribute to a correction of hypertriglyceridaemia. Both treatments increased VLDL-apoB-100 PR which may have been due to increased liver fat or increased hepatic DNL as both increases the availability of TG to export in VLDL which may contribute to increased plasma TG. Correcting hypertriglyceridaemia may result in increased HDL-C by reducing the rate of exchange of TG and CE between HDL and TRL. However, exercise did not cause a significant increase in HDL-C due uncorrected hypertriglyceridaemia in the NAFLD study. The failure to detect a decrease in HDL-C in the lixisenatide study despite the fact that lixisenatide treatment decreased postprandial plasma TG may be due to the small sample size.

The measurements of TRL-apoB-100, TRL-apoB-48 and HDL-apoA-I FCR and PR using stable isotope technique gives a better understanding of the possible metabolic mechanisms underlying hypertriglyceridaemia and low HDL-C concentration in patients with altered lipoprotein metabolism. Understanding these mechanisms provides information for new potential treatment approaches which can be used to correct hypertriglyceridaemia and low HDL-C concentration which will reduce the risk of CHD and CVD.
References


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