Investigation of Total HDL and HDL Subclass Kinetics Using Stable Isotope Techniques in Healthy Subjects

Ke Wang

Submitted for the Degree of Doctor of Philosophy from the University of Surrey

School of Biosciences and Medicine, Department of Nutritional Sciences Faculty of Health and Medical Sciences University of Surrey Guildford, Surrey GU2 7XH, U.K.

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Abstract

Background: HDLs are heterogeneous particles, and apoA-I is the major apolipoprotein in human HDL. CVD is a multifactorial condition, and various lipids and lipoproteins in the plasma are involved in the development of CVD. It has been suggested that HDLs have an inverse association with the risk of CVD. A high sugar intake (especially fructose and sucrose) was found to associate with a low HDL-C level and increased risk of CVD. However, the effect of dietary sugar on HDL kinetics is unclear. An insight of HDL subclass kinetics may provide a better understanding of the whole dynamic of HDL metabolism.

Methods: Two studies were undertaken: 1) A controlled, randomized crossover dietary intervention study was carried out in 6 overweight middle-aged men. Subjects underwent two 12-week dietary interventions with high and low non-milk extrinsic sugar diets. Total HDL kinetics was measured using a primed constant intravenous infusion of $[1-^{13}C]$ leucine for 10 hours. 2) A HDL subclass kinetic study was carried out in 6 healthy subjects (3 males and 3 females). An intravenous bolus injection of $[1-^{13}C]$ leucine was applied to measure HDL subclass kinetics. Blood samples were taken during a 10-hour study and the following 2 weeks. Total HDL, HDL$_2$ and HDL$_3$ were separated from the plasma by ultracentrifugation, and αHDL and preβHDL were isolated by agarose gel electrophoresis. ApoA-I in HDL fractions was separated by SDS-PAGE. After purification, hydrolysis and derivatization, the isotopic enrichment of apoA-I in HDL was measured by GC-MS and apoA-I fractional catabolic rate (FCR) and production rate (PR) was calculated for each subclass and total HDL.

Results: In the dietary intervention study, the FCR of total HDL apoA-I on the high and low sugar diet (0.20 ± 0.02 and 0.18 ± 0.02 pools/day) was similar, as was the PR (7.33 ± 0.66 and 6.05 ± 0.72 mg/kg/day respectively). In the HDL subclass study, the concentration of αHDL apoA-I (0.97 ± 0.05 g/L) was significantly higher than that of preβHDL apoA-I (0.15 ± 0.03 g/L) ($p<0.001$). The FCR of αHDL and preβHDL apoA-I was 0.10 ± 0.02 and 0.13 ± 0.04 pools/day, and the PR of αHDL and preβHDL apoA-I was 3.94 ± 0.73 and 0.67 ± 0.12 mg/kg/day respectively. The concentration of HDL$_2$ apoA-I (0.68 ± 0.04 g/L) was significantly higher than that of HDL$_3$ apoA-I (0.23 ± 0.06 g/L) ($p=0.002$). The FCR of HDL$_2$ and HDL$_3$ apoA-I was 0.15 ± 0.02 pools/day for both, and PR of HDL$_2$ and HDL$_3$ apoA-I was 1.35 ± 0.35
and 3.81 ± 0.51 mg/kg/day respectively. A significant difference was observed between αHDL and preβHDL apoA-I PR (p=0.010), and between HDL$_2$ and HDL$_3$ apoA-I PR (p=0.030) in the whole group. The concentration of HDL$_2$ apoA-I was higher in women (0.32 ± 0.08 g/L) than men (0.13 ± 0.02 g/L) though the difference was not significant. HDL$_2$ apoA-I PR was significantly higher in women than men (p=0.017).

**Conclusion:** The high and low sugar diet did not affect HDL metabolism in overweight men. The higher apoA-I concentration of αHDL and HDL$_3$ might be due to the higher apoA-I PR of αHDL and HDL$_3$ compared to preβHDL and HDL$_2$ respectively in healthy subjects. The higher level of HDL$_2$ apoA-I in female than male subjects might be due to the higher PR of HDL$_2$ apoA-I in women.
Declaration

This thesis and the work to which it refers are the results of my own efforts. Any ideas, data, images or text resulting from the work of others (whether published or unpublished) are fully identified as such within the work and attributed to their originator in the text, bibliography or in footnotes. This thesis has not been submitted in whole or in part for any other academic degree or professional qualification. I agree that the University of Surrey has the right to submit my work to the plagiarism detection service TurnitinUK for originality checks. Whether or not drafts have been so-assessed, the University reserves the right to require an electronic version of the final document (as submitted) for assessment as above. The thesis is available for library use on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement or consent.

Some data present in this thesis Chapter 3, including subject characteristics, lipid and lipoprotein profile, insulin and glucose, dietary intakes and lipases, were measured by Dr. Aryaty Ahmed and Dr. Najlaa Alsini.

Signed: Ke Wang  Data: 07/05/2015
Acknowledgement

This journey of PhD is a precious experience for me. I wish to take this opportunity to express my sincere gratitude to all those who contribute to this work and helped me to complete this thesis.

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## Contents

Abstract ........................................................................................................................................... 1
Declaration ....................................................................................................................................... 3
Acknowledgement ....................................................................................................................... 4
List of Figures ................................................................................................................................. 9
List of Tables ................................................................................................................................. 12
Abbreviations ............................................................................................................................... 14

### Chapter 1: Introduction .............................................................................................................. 17

1.1 Lipids and lipoproteins in the human body .............................................................................. 17
   1.1.1 Lipids .................................................................................................................................. 17
   1.1.2 Lipoproteins .................................................................................................................... 17
1.2 High-density lipoprotein .......................................................................................................... 23
   1.2.1 HDL structure and composition ...................................................................................... 23
   1.2.2 HDL subclasses ............................................................................................................. 24
   1.2.3 HDL apolipoproteins ..................................................................................................... 26
   1.2.4 HDL metabolism: Formation of HDL and Reverse Cholesterol Transport ............... 29
1.3 Cardiovascular disease ............................................................................................................. 36
   1.3.1 Definition and prevalence ............................................................................................. 36
   1.3.2 Cardio-metabolic risks ................................................................................................... 37
   1.3.3 Relationship between cardiovascular disease and HDL ............................................. 44
   1.3.4 Relationship between cardiovascular disease and diet ................................................. 50
1.4 Application of isotope techniques in lipoprotein metabolic studies ................................. 57
   1.4.1 Isotopes techniques ....................................................................................................... 57
   1.4.2 Detection of isotope enrichment by GC-MS ................................................................. 60
1.5 Aims ......................................................................................................................................... 62
1.6 Hypothesis .............................................................................................................................. 62

### Chapter 2: Methodology ............................................................................................................. 63

2.1 Clinical protocol ....................................................................................................................... 63
   2.1.1 The effect of a low and high extrinsic sugar diet on HDL kinetics ............................... 63
   2.1.2 Development of the study design for HDL subclass kinetic study (Pilot study) ......... 67
   2.1.3 HDL subclass kinetics study ....................................................................................... 68
2.2 Analytical methodology ......................................................................................................... 71
   2.2.1 Materials ....................................................................................................................... 71
   2.2.2 Summary of laboratory processes to measure leucine enrichment in total HDL, and HDL$_2$ and HDL$_3$ apoA-I ......................................................... 72
   2.2.3 Two-Dimensional Electrophoresis ................................................................................ 77
   2.2.4 Separation of apoA-I by SDS-PAGE ......................................................................... 81
2.2.5 Hydrolysis of apoA-I ................................................................. 83
2.2.6 Ion exchange chromatography ......................................................... 83
2.2.7 Derivatization ............................................................................... 84
2.2.8 Determination of leucine isotope enrichment by GC-MS ................. 85
2.2.9 Determination of α-KIC isotope enrichment ..................................... 87
2.2.10 Measurement of triglyceride, cholesterol and HDL concentration ....... 89
2.2.11 Measurement of glucose and insulin concentration, and HOMA......... 90
2.2.12 Determination of the lipase activity in plasma .................................. 91
2.2.13 Measurement of apoA-I concentration in plasma and HDL₂ and HDL₃ fractions ........................................................................... 91
2.2.14 Measurement of αHDL and preβ HDL apoA-I concentration: Western blotting ........................................................................... 91
2.2.15 Data analysis .................................................................................. 93

Chapter 3: The effect of a high and low non-milk extrinsic sugar diet on HDL kinetics ................................................................. 98

3.1 Introduction ...................................................................................... 98
3.2 Results ............................................................................................. 99
3.2.1 Subjects characteristics and lipid profile......................................... 99
3.2.2 Energy and macronutrient intakes of the high and low sugar diet ......... 100
3.2.3 The effect of the high and low sugar diets on body weight, body fat, plasma lipids and lipoproteins ......................................................... 102
3.2.4 The effect of the high and low sugar diets on blood glucose, insulin and insulin resistance ................................................................. 106
3.2.5 The effect of the high and low sugar diets on lipases ......................... 106
3.2.6 Isotopic enrichment, PR and FCR of total HDL apoA-I on the high and low sugar diets ........................................................................ 108
3.3 Discussion ....................................................................................... 111
3.3.1 Effects of high and low sugar diet on HDL-C and HDL kinetics......... 111
3.3.2 Effects of high and low sugar diet on body weight and body fat.......... 117
3.3.3 Effects of high and low sugar diet on plasma lipids, glucose and insulin 119
3.4 Comparison of subset results with those from whole cohort ............... 122
3.5 Limitations and further work ............................................................ 122

Chapter 4: Investigation of HDL subclass kinetics using a stable isotope technique ......................................................... 124

4.1 Introduction ...................................................................................... 124
4.2 Method summary ............................................................................. 124
4.3 Results ............................................................................................. 124
4.3.1 Optimising the laboratory method – SDS-PAGE ................................. 126
4.3.2 Pilot study – Development of the blood sampling protocol ................ 127
4.3.3 HDL subclass kinetic study ................................................................. 130

4.4 Discussion ........................................................................................................... 146

4.4.1 The metabolism of αHDL and preβHDL in healthy subjects ...................... 146

4.4.2 The metabolism of HDL\(_2\) and HDL\(_3\) in healthy subjects ...................... 152

Chapter 5: General discussion .................................................................................. 157

References: .............................................................................................................. 162

Appendices ............................................................................................................... 178
List of Figures

Figure 1: Exogenous and Endogenous pathways of lipoprotein metabolism .......... 20
Figure 2: The structure of spherical HDL ........................................................................ 23
Figure 3: Heterogeneity of human HDL ........................................................................ 24
Figure 4: The process of HDL maturation .................................................................. 30
Figure 5: The process of HDL remodelling ................................................................. 31
Figure 6: Factors contributing to global cardiovascular risk. ................................. 38
Figure 7: Biological activities of normal functional HDL ........................................... 47
Figure 8: Principles of gas chromatography-mass spectrometry ............................. 60
Figure 9: Study design of low and high sugar diet intervention study ....................... 66
Figure 10: Clinical study protocol and sampling time points ...................................... 70
Figure 11: Laboratory processes to separate and measure leucine enrichment in total HDL, HDL₂ and HDL₃ apoA-I ........................................................................... 72
Figure 12: Laboratory processes to separate and measure leucine enrichment in αHDL and preβHDL apoA-I .................................................................................. 77
Figure 13 A: Separation of αHDL and preβHDL by agarose gel electrophoresis with albumin as a standard ......................................................................................... 78
Figure 13 B: Separation of αHDL and preβHDL by agarose gel electrophoresis with apoA-I (from human plasma) as a standard .............................................................. 79
Figure 14: Separation of total HDL apoA-I on SDS-PAGE ........................................ 81
Figure 15: Separation of HDL₂ and HDL₃ apoA-I on SDS-PAGE. (A: HDL₂ apoA-I bands separation for five different time points; B: HDL₃ apoA-I bands separation for six different time points) .................................................. 82
Figure 16: Separation of αHDL and preβHDL apoA-I on SDS-PAGE. (A: αHDL apoA-I bands separation for five different time points; B: preβHDL apoA-I bands separation for five different time points) .................................................. 82
Figure 17: Chemical structure of leucine and the oxazolinone derivative ............... 85
Figure 18: Leucine standard curve (n=6). (Results are presented as mean ± SEM) .... 86
Figure 19: KIC standard curve (n=6). (Results are presented as mean ± SEM) ...... 89
Figure 20: Western blotting: Measurement of the percentage of αHDL and preβHDL ..................................................................................................................... 93
Figure 21: An example of the determination of FCR (pools/h) from the slope of a line of best fit ................................................................................................................ 95
Figure 22: The model structure for αHDL and preβHDL (k = rate constant) .......... 96
Figure 23: The model structure for HDL₂ and HDL₃ (m = rate constant) ............. 96
Figure 24: Correlations between plasma HDL-C and fraction HDL-C on the high and low sugar diet ................................................................. 104
Figure 25: Correlations between plasma TG and fraction HDL-TG on the high and low sugar diet ................................................................. 105
Figure 26: Correlation between plasma HDL-C and HDL apoa-I on the high and low sugar diet ........................................................................ 105
Figure 27: Correlation between HL and HDL fraction triglyceride on the high and low sugar diet ........................................................................ 107
Figure 28: Leucine enrichment of total HDL apoa-I on high and low sugar diets for each individual ................................................................. 108
Figure 29: Leucine enrichment of total HDL apoa-I on high and low sugar diets among all subjects (n=6) .............................................................. 109
Figure 30: Fractional clearance rate (FCR) and production rate (PR) of total HDL for individual subjects on the high and low sugar diet ............... 110
Figure 31: Optimizing the laboratory methods: SDS-PAGE ................................................................. 126
Figure 32: Leucine enrichment of αHDL and preβHDL apoa-I during 10h study and following 5 days in Pilot study 1 ......................................... 127
Figure 33: Leucine enrichment of αHDL and preβHDL apoa-I during 10h study and following 6 days in Pilot study 2 ......................................... 128
Figure 34: Leucine enrichment of αHDL and preβHDL apoa-I during 10h study and following 10 days in Pilot study 3 ......................................... 128
Figure 35: Leucine enrichment of HDL₂ and HDL₃ apoa-I during 10h study and following 6 days in Pilot study 2 ......................................... 129
Figure 36: Leucine enrichment of HDL₂ and HDL₃ apoa-I during 10h study and following 10 days in Pilot study 3 ......................................... 129
Figure 37: α-KIC enrichment during the first 10 hour in HDL subclass kinetic study of all subjects (n=6). (mean ± SEM) ........................................ 134
Figure 38: Leucine enrichment of αHDL & preβHDL apoa-I during the first 10 hours in the HDL kinetic study of all subjects (n=6). (mean ± SEM) ........ 135
Figure 39: Leucine enrichment of αHDL & preβHDL apoa-I during the first 10 hours in the HDL kinetic study of the females (n=3). (mean ± SEM).............. 135
Figure 40: Leucine enrichment of αHDL & preβHDL apoa-I during the first 10 hours in the HDL kinetic study of the males (n=3). (mean ± SEM) .............. 136
Figure 41: Leucine enrichment of αHDL & preβHDL apoa-I during the 14 day HDL kinetic study of all subjects (n=6) (mean ± SEM) ......................... 137
Figure 42: Leucine enrichment of αHDL & preβHDL apoa-I during the 14 day HDL kinetic study of the females (n=3) (mean ± SEM) ......................... 137
Figure 43: Leucine enrichment of αHDL & preβHDL apoA-I during the 14 day HDL kinetic study of the males (n=3) (mean ± SEM) ........................................ 138
Figure 44: Leucine enrichment of HDL₂ and HDL₃ apoA-I during the first 10 hours in the HDL kinetic study of all subjects (n=6). (mean ± SEM) ............... 139
Figure 45: Leucine enrichment of HDL₂ and HDL₃ apoA-I during the first 10 hours in the HDL kinetic study of the females (n=3). (mean ± SEM) .................. 139
Figure 46: Leucine enrichment of HDL₂ and HDL₃ apoA-I during the first 10 hours in the HDL kinetic study of the males (n=3). (mean ± SEM) .................. 140
Figure 47: Leucine enrichment of HDL₂ and HDL₃ apoA-I during the 14 day HDL kinetic study of all subjects (n=6) .................................................. 141
Figure 48: Leucine enrichment of HDL₂ and HDL₃ apoA-I during the 14 day HDL kinetic study of the females (n=3) ........................................ 141
Figure 49: Leucine enrichment of HDL₂ and HDL₃ apoA-I during the 14 days HDL kinetic study of the males (n=3) ........................................ 142
List of Tables

Table 1: Functions of the major apolipoproteins ......................................................... 19
Table 2: Classification and characteristics of lipoproteins ........................................ 20
Table 3: Functions of human plasma lipoproteins ...................................................... 22
Table 4: Criteria for clinical diagnosis of the metabolic syndrome ...................... 40
Table 5: Classification of dietary carbohydrates based on molecular size .... 53
Table 6: Preparation of leucine standard curve (n=6) ................................................. 86
Table 7: Preparation of KIC standard curve (n=6) .................................................... 88
Table 8: Subject characteristics at screening (n=6) .................................................... 99
Table 9: Intakes of energy and composition of macronutrients of the high and low sugar diet ........................................................................................................... 100
Table 10: Body weight, body fat and the concentrations of lipids and lipoproteins in plasma and total HDL fractions on the high and low sugar diets .... 103
Table 11: Glucose, insulin and insulin sensitivity on the high and low sugar diets . 106
Table 12: Total lipase, lipoprotein lipase and hepatic lipase on the high and low sugar diets ........................................................................................................... 107
Table 13: Fractional clearance rate (FCR) and production rate (PR) of total HDL on high sugar and low sugar diet ........................................................................................................... 110
Table 14: Summary of data on total HDL apoA-I kinetic parameters obtained with stable isotopes in the previous studies ................................................. 112
Table 15: The kinetics data of VLDL\(_1\)-TG and VLDL\(_1\)-apoB on the high and low sugar diet ........................................................................................................... 117
Table 16: Subject characteristics of HDL subclasses kinetic study (n=6).............. 130
Table 17: Lipid profile and apoA-I concentration of the subjects in HDL subclasses kinetic study ........................................................................................................... 133
Table 18: Fractional clearance rate and production rate of αHDL and preβHDL for all subjects ........................................................................................................... 143
Table 19: Comparison of fractional clearance rate and production rate of αHDL and preβHDL between female and male subjects ......................... 144
Table 20: Fractional clearance rate and production rate of HDL\(_2\) and HDL\(_3\) for all subjects ........................................................................................................... 145
Table 21: Comparison of fractional clearance rate and production rate of HDL\(_2\) and HDL\(_3\) between female and male subjects ......................... 145
Table 22: Summary of data on preβHDL and αHDL apoA-I kinetic parameters obtained with stable isotopes in the previous studies ......................... 147
Table 23: Recalculated results of APR of preβ_{1}HDL and αHDL in healthy subjects in the study by Chetiveaux et al., 2004 .................................................... 149

Table 24: Summary of data on HDL_{2} and HDL_{3} apoA-I kinetic parameters obtained with stable isotopes in the previous study by Li (2009) ......................... 153
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette transporter A1</td>
</tr>
<tr>
<td>AHA</td>
<td>The American Heart Association</td>
</tr>
<tr>
<td>αHDL</td>
<td>Alpha-migrating high density lipoprotein</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APR</td>
<td>Absolute production rate</td>
</tr>
<tr>
<td>APE</td>
<td>Atom percent excess</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>apoA-I</td>
<td>Apolipoprotein A-I</td>
</tr>
<tr>
<td>apoA-II</td>
<td>Apolipoprotein A-II</td>
</tr>
<tr>
<td>ATP III</td>
<td>Adult Treatment Panel III</td>
</tr>
<tr>
<td>AUC</td>
<td>The area under the curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesterol ester</td>
</tr>
<tr>
<td>CM</td>
<td>Chylomicron</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CHO</td>
<td>Carbohydrate</td>
</tr>
<tr>
<td>Chol</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>CI</td>
<td>Chemical ionization</td>
</tr>
<tr>
<td>CO</td>
<td>Cholesterol oxidase</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>2-DE</td>
<td>2-dimensional electrophoresis</td>
</tr>
<tr>
<td>DNL</td>
<td>de novo lipogenesis</td>
</tr>
<tr>
<td>DP</td>
<td>The degree of polymerization</td>
</tr>
<tr>
<td>EDHF</td>
<td>Endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>EI</td>
<td>Electron ionization</td>
</tr>
<tr>
<td>EL</td>
<td>Endothelial lipase</td>
</tr>
<tr>
<td>FC</td>
<td>Free cholesterol</td>
</tr>
<tr>
<td>FCR</td>
<td>Fractional clearance rate</td>
</tr>
<tr>
<td>FFM</td>
<td>Fat free mass</td>
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<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
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<tr>
<td>FSR</td>
<td>Fractional secretion rate</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography mass spectrometry</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GI</td>
<td>Glycaemic index</td>
</tr>
<tr>
<td>GL</td>
<td>Glycaemic load</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HDL-C</td>
<td>High density lipoprotein-cholesterol</td>
</tr>
<tr>
<td>HL</td>
<td>Hepatic lipase</td>
</tr>
<tr>
<td>HOMA</td>
<td>The homeostasis model assessment</td>
</tr>
<tr>
<td>HOMA2-IR</td>
<td>The homeostasis model assessment 2-Insulin resistance</td>
</tr>
<tr>
<td>HTGL</td>
<td>Hepatic triglyceride lipase</td>
</tr>
<tr>
<td>IDF</td>
<td>The International Diabetes Federation</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoproteins</td>
</tr>
<tr>
<td>IEC</td>
<td>Ion exchange chromatography</td>
</tr>
<tr>
<td>IHD</td>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>KIC</td>
<td>Ketoisocaproic acid</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin:cholesterol acyltransferase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MRS</td>
<td>The magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>MTBSTFA</td>
<td>N-Methyl-(tertbutyldimethylsilyl) trifluoroacetamide</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Nonalcoholic 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>NDNS</td>
<td>The National Diet and Nutrition Survey</td>
</tr>
<tr>
<td>NH₄OH</td>
<td>Ammonium hydroxide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline containing 0.05% Tween 20</td>
</tr>
<tr>
<td>PCI</td>
<td>Positive chemical ionization</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>PLTP</td>
<td>Phospholipid transfer protein</td>
</tr>
<tr>
<td>PON1</td>
<td>Paraoxonase 1</td>
</tr>
<tr>
<td>PR</td>
<td>Production rate</td>
</tr>
<tr>
<td>PreβHDL</td>
<td>Prebeta-migration high density lipoprotein</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>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SR-BI</td>
<td>Scavenger receptor B type I</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TFAA</td>
<td>Trifluoroacetic anhydride</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>VA-HIT</td>
<td>The Veterans Affairs HDL Intervention Trial</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>γHDL</td>
<td>Gamma high density lipoprotein</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Lipids and lipoproteins in the human body

1.1.1 Lipids

Lipids as a major source of energy can be utilized immediately by the body or stored as adipose tissue for later utilization when food intake is reduced. They perform a variety of essential functions including the maintenance of the structural and functional integrity of cell membranes, and are involved in hormone-like activities and cell signalling. The lipids circulating in the plasma are mainly fatty acids, triglycerides, cholesterol and phospholipids (Marshall, Bangert and Lapsley, 2012).

Due to the water insoluble property of lipids, they require specialized transport mechanisms associated with proteins. Free fatty acids are carried by albumin, and the other lipids circulate bound to complexes known as lipoproteins (Marshall, Bangert and Lapsley, 2012).

1.1.2 Lipoproteins

1.1.2.1 Lipoprotein structure

Lipoproteins are macromolecular complexes comprised of both lipids and proteins. They have a hydrophobic interior and a relatively hydrophilic external. A typical lipoprotein particle consists of a core of neutral lipid of triacylglycerol and cholesterol ester surrounded by a surface monolayer of polar lipid of phospholipid and free cholesterol. Phospholipid and cholesterol have amphipathic properties. Their hydrophobic (neutral) ends face the core and the hydrophilic (polar) ends face outward. These structures make the lipoprotein particles able to interact with the aqueous environment of the blood, and transport the water insoluble lipids from the intestine and the liver to the peripheral tissues, or from the peripheral tissues back to the liver (Frayn, 2010).
The components exposed on the outer surface of lipoproteins also include specific amphipathic proteins called apolipoproteins. Similarly, the hydrophobic domains of these proteins that attach to lipoprotein particles dip into its core, and the hydrophilic domains are exposed at the surface (Frayn, 2010). Apolipoproteins involved in lipoprotein metabolism are usually identified as five principle groups including: apolipoprotein A (apoA-I, apoA-II, ApoA-IV), apolipoprotein B (apoB-48, apoB-100), apolipoprotein C (apoC-I, apoC-II, apoC-III), apolipoprotein D and apolipoprotein E (Frayn, 2010). Apolipoproteins play the vital roles in determining the functions of the lipoproteins (Mann and Skeaff, 2007) (Table 1). They are indispensable for maintaining the structure and the solubility of the lipoproteins. Apolipoproteins are involved in the delivery and redistribution of lipids among specific cells and tissues. They play a role mediating the recognition between specific lipoproteins and cell surface lipoprotein receptors. Apolipoproteins also have function as cofactors for specific enzymes involved in lipid metabolism (Mahley et al., 1984).

<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-I</td>
<td>activates lecithin-cholesterol acyl transferase (LCAT); structural (in High density lipoprotein (HDL))</td>
</tr>
<tr>
<td>A-II</td>
<td>inhibits hepatic triglyceride lipase (HTGL) at high concentration; structural (in HDL)</td>
</tr>
<tr>
<td>B-100</td>
<td>structural (in very low density lipoprotein (VLDL) and low density lipoprotein (LDL)); receptor binding</td>
</tr>
<tr>
<td>B-48</td>
<td>structural (in chylomicrons)</td>
</tr>
<tr>
<td>C-II</td>
<td>activator of lipoprotein lipase (LPL)</td>
</tr>
<tr>
<td>C-III</td>
<td>inhibits LPL; inhibits clearance of chylomicrons and VLDL remnant particles</td>
</tr>
<tr>
<td>E</td>
<td>Binding to LDL and remnant receptors</td>
</tr>
</tbody>
</table>

Adapted from Marshall, Bangert and Lapsley (2012).
1.1.2.2 Classification and composition

Lipoproteins are a group of heterogeneous particles that differ in size, density, lipid and protein composition. They have been traditionally separated on the basis of either their electrophoretic mobility or flotation in an ultracentrifuge. The latter method has been used more commonly for classification (Frayn, 2010). Lipoproteins are divided into five major fractions based on their density, which include chylomicrons (CM), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) (Table 2). The density increases from CM through VLDL, IDL and LDL to HDL. HDL can be further divided into two subclasses, HDL₂ and HDL₃. Based on the content of lipid and protein, CMs and VLDL particles contain a relatively high amount of triglyceride, and are usually referred to as triglyceride-rich lipoproteins. IDL and LDL are cholesterol-rich particles, while HDL contains a high proportion of protein. According to their apolipoprotein (apo) composition, lipoproteins can be classified into two groups, apoA containing lipoproteins including HDL and apoB containing lipoproteins including CMs, VLDL, IDL and LDL.

Table 2: Classification and characteristics of lipoproteins

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Density (g/ml)</th>
<th>Mean diameter (nm)</th>
<th>Composition (weight%)</th>
<th>Major lipoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Protein</strong></td>
<td><strong>TG</strong></td>
</tr>
<tr>
<td>CM</td>
<td>&lt;0.95</td>
<td>500</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.95-1.006</td>
<td>43</td>
<td>5</td>
<td>65</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006-1.019</td>
<td>27</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019-1.063</td>
<td>22</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.210</td>
<td>8</td>
<td>55</td>
<td>5</td>
</tr>
</tbody>
</table>


Adapted from Mann and Skeaff (2007); Marshall, Bangert and Lapsley (2012).
1.1.2.3 General metabolic pathways of lipoproteins

There are two separate pathways in lipoprotein metabolism, the exogenous (dietary) pathway and the endogenous (hepatic) pathway (Figure 1). Both pathways similarly break down the large lipid-rich lipoproteins that are released from the intestine or liver, into smaller lipoproteins or remnants, which supply lipid to the peripheral tissue or liver (Davis and Wagganer, 2006).

Figure 1: Exogenous and Endogenous pathways of lipoprotein metabolism (Davis and Wagganer, 2006).

LPL: lipoprotein lipase; FFA: free fatty acids; LDL-R: low-density lipoprotein receptor; LRP: low-density lipoprotein receptor-related protein; HL: hepatic lipase.

1.1.2.3.1 Exogenous pathway

The major function of CMs is to transport the lipids which originated from the diet from the intestine to peripheral tissues and liver (Table 3). Therefore the metabolism of CMs is often considered as the exogenous pathway of lipoprotein metabolism (Davis and Wagganer, 2006). CMs are formed in the small intestine. After the absorption and re-esterification of the triacylglycerol and cholesterol in the cells of the intestine wall, CMs are secreted into the circulation via the lymphatics. The newly
synthesized CM particles have a core of triacylglycerol and cholesterol ester surrounded by a surface of unesterified cholesterol, phospholipid, and apoB 48, Al and AIV. CMs then obtain apoC-II, which is essential for the reaction between CMs and enzyme lipoprotein lipase (LPL). Triacylglycerol in CMs are hydrolyzed by LPL, and the particle becomes smaller. The free fatty acids are distributed to either the muscle or heart tissue for energy utilization or the adipose tissues for energy storage. CM remnants are catabolized by the liver.

1.1.2.3.2 Endogenous pathway

In contrast to the exogenous pathway, the endogenous pathway involves the transport of cholesterol and triacylglycerol released from the liver. This pathway consists of the metabolism of VLDL and LDL (Davis and Wagganer, 2006) (Table 3). VLDL particles are synthesized by the liver. The newly secreted VLDL is composed of triacylglycerol, cholesterol ester, apoB 100 and a small amount of apoE and C. Like CMs, triacylglycerol in VLDL particles are hydrolyzed by LPL and the free fatty acids are then distributed to the peripheral tissues. VLDL remnants are relatively cholesterol ester-rich particles known as IDL. IDLs are subsequently hydrolyzed by LPL and hepatic lipase (HL). They lose all surface components except the layer of phospholipids, free cholesterol and apoB 100. They have a cholesterol ester-rich core and become LDL particles. LDLS are the smaller and denser particles. ApoB 100 on the surface of LDL particles is recognized by the LDL-receptor. Cholesterol esters in LDL are hydrolyzed to free cholesterol, which is then delivered to the tissues.
### Table 3: Functions of human plasma lipoproteins

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td>Transport dietary lipids from intestine to peripheral tissue and liver</td>
</tr>
<tr>
<td>VLDL</td>
<td>Transport lipids from liver to peripheral tissues</td>
</tr>
<tr>
<td>IDL</td>
<td>Precursor of LDL</td>
</tr>
<tr>
<td>LDL</td>
<td>Transport cholesterol to peripheral tissues and liver</td>
</tr>
<tr>
<td>HDL</td>
<td>Removes cholesterol from tissues and transfer it to the liver or other lipoproteins</td>
</tr>
</tbody>
</table>

*Adapted from Mann and Skeaff (2007).*
1.2 High-density lipoprotein

1.2.1 HDL structure and composition

HDLs are the smallest (diameter 7.4-12 nm) and densest (1.063<d<1.21 g/ml) plasma lipoproteins (Barter, 2002). They comprise the largest amount of protein, approximately 50% and smallest amount of lipid compared with other lipoproteins (Barter and Rye, 2005; Davis and Wagganer, 2006). The mature form of HDL has a similar structure to the other plasma lipoproteins. They are mostly spherical particles with a hydrophobic core mainly consisting of cholesterol ester and a small quantity of triacylglycerol, and the outer surface contains unesterified cholesterol, phospholipids and apolipoproteins (Figure 2).

![Figure 2: The structure of spherical HDL (HDL forum, 2011)](http://www.hdlforum.org)

The predominant apolipoproteins in human HDL are apoA-I and apoA-II, which account for 70% and 20% of total HDL proteins respectively. There are also some minor apolipoproteins contained in HDL particles including apoA-IV, apoA-V, apoC-I, apoC-II, apoC-III, apoD, apoE, apoJ and apoL (Barter and Rye, 2005).
1.2.2 HDL subclasses

HDLs are a group of heterogeneous particles that vary in shape, size, density, apolipoprotein composition and electrophoretic mobility (Figure 3).

**Figure 3: Heterogeneity of human HDL (Barter, 2002)**

**Shape:** In normal plasma, there are most spherical HDLs. However, there is a minor population of HDLs, which are the discoidal particles. Discoidal HDLs are the nascent forms of HDL, and they comprise only surface constituents and exist as a molecular bi-layer of unesterified cholesterol and phospholipids surrounded by apolipoproteins (Barter and Rye, 2005). Discoidal HDLs normally have a very low concentration since they only exist transiently and convert into spherical HDLs rapidly (Barter and Rye, 2005).

**Size and density:** HDL can be isolated as two major subclasses based on density by ultracentrifugation: HDL$_2$ (1.063<d<1.125 g/ml) and HDL$_3$ (1.125<d<1.21 g/ml) (Barter, 2002). These two subclasses can be further separated into five distinct subfractions based on particle size by non-denaturing polyacrylamide gradient gel electrophoresis. Two distinct subfractions HDL$_{2b}$ (mean diameter 10.6 nm) and HDL$_{2a}$ (9.2 nm) are included in the HDL$_2$ density range, and the other three subfractions
HDL$_{3a}$ (8.4 nm), HDL$_{3b}$ (8.0 nm) and HDL$_{3c}$ (7.6 nm) are within the HDL$_3$ density range (Blanche et al., 1981).

**Apolipoprotein composition:** HDLs can be divided into two predominant subpopulations on the basis of their two major apolipoproteins: apoA-I and apoA-II. One subpopulation involves HDL particles containing only apoA-I without apoA-II (A-I HDL), while another subpopulation comprise HDL particles consisting of both apoA-I and apoA-II (A-I/A-II HDL) (Cheung and Alberts, 1984). ApoA-I is distributed approximately equally between A-I HDLs and A-I/A-II HDLs in most human subjects, while almost all apoA-II exists in A-II/A-II HDLs (Cheung and Alberts, 1984; Bekaert et al., 1992). Most A-I/A-II HDLs are found in the HDL$_3$ density range, while A-I HDLs are the predominant components of both HDL$_2$ and HDL$_3$ (Cheung and Alberts, 1982). There is also a minor HDL subpopulation containing apoA-II but no apoA-I (A-II HDL), which had been separated from human plasma by using sequential immunoaffinity chromatography (Bekaert et al., 1992). Most of A-II HDL particles contain apoA-II as its sole apolipoprotein while a minority of A-II HDLs may also contain apoD and other minor apolipoproteins.

**Electrophoretic mobility:** HDL particles also vary in surface charge. HDL can have either prealpha (preα), alpha (α), prebeta (preβ) or gamma (γ) migration when separated by agarose gel electrophoresis (Asztalos et al., 1993; Huang et al., 1994). The α-migrating HDLs that exist as spherical particles account for the predominant proportion of HDL in plasma. As the mature forms of HDL, α-migrating HDLs comprise HDL$_2$ and HDL$_3$ subclasses, and also A-I HDL and A-I/A-II HDL subclasses (Rye and Barter, 2004). Preβ-migrating HDL exist as either lipid-poor apoA-I or discoidal particles consisting of two or three molecules of apoA-I, phospholipids and possibly a small quantity of unesterified cholesterol. The γ-migrating HDLs exist as
discoidal particles. They contain apoE and phospholipids but no apoA-I (Huang et al., 1994).

1.2.3 HDL apolipoproteins

1.2.3.1 Major apolipoproteins of HDL

1.2.3.1.1 Apolipoprotein A-I

Human apoA-I circulates in plasma predominantly as a component of HDL. There is also a small amount of apoA-I found in chylomicrons or their remnants, VLDL or their remnants, or LDL (Mahley et al., 1984). The concentration of plasma apoA-I is about 1.0-1.5 mg/ml. ApoA-I is mainly synthesized in two sites: the liver and small intestine. The intestinal apoA-I secreted into the circulation is associated with chylomicrons and then is rapidly transferred to HDL particles via lipoprotein lipase hydrolysis of chylomicrons (Mahley et al., 1984). The hepatic apoA-I secreted into the circulation is related to nascent HDL particles that contain little or no core of cholesterol ester.

Apolipoprotein A-I was one of the first apolipoproteins to be identified and characterized. Human apoA-I is synthesized initially as a 267-amino acid pre-pro-apoA-I (Brewer et al., 1983; Chau et al., 2006). This undergoes proteolytic processing to form the mature apoA-I, a single polypeptide of 243 amino acids with a molecular weight of 28.1 KD (Brewer et al., 1978; Mahley et al., 1984). Mature apoA-I is composed of a series of repeated 22- and 11-amino acid sequences in the primary structure, and amphipathic α-helices with hydrophobic and hydrophilic faces in the secondary structure (Frank and Marcel, 2000). The amphipathic properties of α-helices enables apoA-I to strongly bind with various lipids such as phospholipids and cholesterol via the hydrophobic faces, and interact with the aqueous phase through the hydrophilic faces (Davidson and Thompson, 2007; Frayn, 2010). Therefore, this property may play a crucial role in enhancing the stability of apoA-I particles,
enabling the interaction between apoA-I and the cell membrane, and enabling apoA-I to collect the cholesterol from the cells. ApoA-I has been found to be a self-associating system in aqueous medium, which may help to stabilize the lipid-free form of apoA-I (Frank and Marcel, 2000; Vitello and Scanu, 1976).

ApoA-I presents in various conformations with metabolic interconversion between forms (Ajees et al., 2006). It can exist in lipid free/poor, partially lipided, and fully lipidated states. A series of reactions are involved in the cycling of apoA-I between lipid-poor and lipid-bound forms (Barter, 2002).

It has been indicated that human apoA-I has a protective effect against cardiovascular disease (Saito et al., 2004). As the principle protein component of HDL, apoA-I plays a crucial role in the anti-atherogenic function of HDL. The protective action of apoA-I is attributed to its major functions in the reverse cholesterol transport pathway (see below) (Saito et al., 2004; Narayanaswami and Ryan, 2000). ApoA-I acts as an acceptor of collecting cholesterol from the cell membrane and transporting cholesterol to the liver for excretion. It also functions as a key activator for the plasma enzyme Lecithin:cholesterol acyltransferase (LCAT).

1.2.3.1.2 Apolipoprotein A-II
Human apoA-II occurs as a second primary protein component of HDL. It may be also associated, in a smaller quantity, with other lipoproteins, such as chylomicrons and VLDL (Blanco-Vaca et al., 2001). The plasma concentration of human apoA-II is around 0.3-0.35 mg/ml (Mahley et al., 1984). Human apoA-II is initially synthesized in the liver as a 100 amino acid precursor pre-pro-apoA-II (Tailleux et al., 2002). Pre-pro-apoA-II is then converted to pro-apoA-II by removal of an 18 amino acid sequence and released to the plasma. It is subsequently converted to the mature apoA-II with a molecular weight of 17.4 KD after removal of another 5 amino acid
sequence (Irshad and Dubey, 2005; Mahley et al., 1984). The majority of human apoA-II in the plasma appears as a dimer of two identical 77-amino acid polypeptide chains linked by a disulphide bond at residue 6 (Blanco-Vaca et al., 2001; Schaefer et al., 1982).

There are also other species present in plasma, the apoA-II/apoE dimer, the apoA-II/apoD dimer and the apoA-II monomer (Blanco-Vaca et al., 2001). Like apoA-I, apoA-II can bind well to lipid due to the amphipathic property of its secondary structure (Mahley et al., 1984; Saito et al., 2004). Each apoA-II monomer contains three long α-helices. ApoA-II has a higher affinity to lipoprotein surfaces (including HDL) than apoA-I, and this is possibly because apoA-II is more hydrophobic. Consistent with the higher binding affinity, apoA-II is known to displace apoA-I from HDL (Mahley et al., 1984; Eisenberg, 1984).

1.2.3.2 Minor apolipoproteins of HDL

1.2.3.2.1 Apolipoprotein E

Apolipoprotein E (apoE) was first recognized in human VLDL in the early 1970s (Shore and Shore, 1973). Human apoE in plasma is a single polypeptide consisting of 299 amino acids with a molecular weight of 34.2 KD (Zannis et al., 1984; Rye et al., 2006). It can be synthesized in many tissues such as the kidney and adrenal gland, however the predominant source of apoE in the plasma is probably the liver (Zannis et al., 1984; Frayn, 2010). ApoE not only exists in triglyceride-rich particles but also has been found in HDL particles. It has been discovered that apoE is present in various subclasses of HDL based on electrophoretic mobility, which includes γHDL, preβHDL and αHDL (Krimbou et al., 1997). As a constituent of subclasses of HDL, apoE has a function that affects cholesterol efflux from the cells (Mahley and Rall, 2000). In the reverse cholesterol transport pathway, apoE acts as
a receptor ligand and assists the delivery of cellular cholesterol to the liver. It has also been indicated that apoE involves the inhibition of platelet aggregation (Higashihara et al., 1991; Riddell et al., 1997).

1.2.3.2.2 Apolipoprotein M
Apolipoprotein M (apoM) is a recently identified human apolipoprotein. The majority of apoM in the plasma is associated with HDL, but there is also a minor proportion present in triglyceride-rich lipoprotein and LDL (Xu and Dahlback, 1999; Xu et al., 2004). ApoM is principally synthesized in the liver and kidney. It exists as a 188-amino acid residue long protein with the molecular weight of 26 KD (Xu and Dahlback, 1999). ApoM is indispensable in HDL metabolism. A study in mice deficient in apoM suggested that apoM play a crucial role in the formation of preβ HDL (Wolfrum et al., 2005). ApoM also has the ability to modulate cholesterol efflux from macrophages to HDL, which may contribute to the anti-atherogenic effect of HDL. Anti-oxidation and anti-inflammation effects of apoM also contribute to the anti-atherogenic effect of HDL (Christoffersen et al., 2008; Huang et al., 2007).

1.2.4 HDL metabolism: Formation of HDL and Reverse Cholesterol Transport
Excessive accumulation of cholesterol in the artery walls can result in the development of atherosclerosis. The metabolism of HDL has been indicated to have an inverse relationship with the risk of atherosclerotic cardiovascular disease. It involves a complex interaction of factors that modulate the synthesis, remodelling and catabolism of HDL. HDL is considered to play a key role in the reverse cholesterol transport (RCT) pathway, in which the excess cholesterol can be transported by HDL from peripheral tissues back to the liver and subsequently excreted in the bile (Lewis and Rader, 2005).
The formation of HDL begins with the secretion of lipid-poor apoA-I from the liver and intestine (Figure 4). Once released in plasma, apoA-I rapidly acquires free cholesterol and phospholipids from the liver via the receptor ATP-binding cassette transport A1 (ABCA1), and obtains cholesterol and phospholipids from chylomicrons and VLDL via lipoprotein lipase (LPL) to form the discoidal, prebeta-migrating nascent HDL particles. The nascent preβHDL particles acquire additional free cholesterol and phospholipids from peripheral tissues mediated by ABCA1 and generate the more cholesterol-rich preβHDL particles. The enzyme LCAT which is carried by HDL esterifies the free cholesterol to form cholesterol ester. Esterified cholesterol then moves into the centre of HDL particles as a hydrophobic core, altering the discoidal preβHDL to form small spherical, alpha-migrating HDL particles (HDL₃). Further esterification of free cholesterol by the reaction of LCAT leads to the conversion of HDL₃ to larger spherical alpha-migrating HDL₂. This process leads to the further changes in composition and size of HDL particles (Frayn, 2010; Lewis and Rader, 2005).

**Figure 4: The process of HDL maturation (Lewis and Rader, 2005)**

ABCA1: ATP-binding cassette A1; PL: phospholipids; FC: free cholesterol; LCAT: lecithin-cholesterol acyltransferase; CE: cholesterol ester; LPL: lipoprotein lipase.
The subsequent process of HDL remodelling can be achieved by either the direct or indirect pathway (Figure 5). In the direct pathway, cholesterol esters in spheroidal HDL particles interact with a specific receptor – the scavenger receptor B type I (SR-B1), and then are taken up by the liver for excretion in the bile. The indirect pathway involves the transport of cholesterol esters to the apoB-containing lipoproteins. By the action of cholesterol ester transfer protein (CETP) which is mainly bound to HDL, cholesterol ester in mature HDL particles exchange with triglyceride in chylomicron and VLDL. This exchange leads to cholesterol ester depletion and triglyceride enrichment in HDL particles. In addition, phospholipid transfer protein (PLTP) mediates the migration of phospholipid from triglyceride-rich lipoprotein to HDL. Hepatic lipase (HL) modification of triglyceride-rich HDL particles releases lipid-poor apoA-I and HDL remnants and leads to the reduction in HDL size. Lipid poor apoA-I can be degraded in the kidney (Frayn, 2010; Lewis and Rader, 2005).

**Figure 5: The process of HDL remodelling (Lewis and Rader, 2005)**

SR-B1: scavenger receptor class-B1; HL: hepatic lipase; CETP: cholesterol ester transfer protein; PLTP: phospholipase transfer protein; TG: triglyceride; CE: cholesterol ester; PL: phospholipids.
1.2.4.1 Factors involved in HDL metabolism

1.2.4.1.1 ATP-binding cassette transporter A1 (ABCA1)
ABCA1 is a cell membrane transport protein that uses ATP as an energy source to transport a wide variety of substrates across the membrane (Segrest et al., 2000). It largely occurs in the liver, macrophages, brain and various other tissues (Zannis et al., 2006). ABCA1 promotes the efflux of cholesterol and phospholipids from cell membranes to lipid-poor apoA-I in the extracellular region, which leads to the formation of discoidal apoA-I-containing HDL (Barter, 2002). Although several models have been proposed, the mechanism of ABCA1-mediated delivery of cholesterol to apoA-I remains elusive (Zannis et al., 2006). Patients with Tangier disease (caused by a mutation in ABCA1) have been reported to have a very small amount of plasma cholesterol and fail to synthesize the discoidal and spherical HDL, which leads to almost complete absence of HDL in plasma (Orso et al., 2000; Zannis et al., 2006). Therefore, ABCA1 is an important factor in lipidation of apoA-I and HDL formation in HDL metabolism.

1.2.4.1.2 Lecithin:cholesterol acyltransferase (LCAT)
LCAT is a 416-amino acid long protein that is released from the liver and circulates in the plasma. It is present in plasma in a lipid-free form or is bound the surface of HDL particles (Zannis et al., 2006; Barter, 2002). LCAT plays an important role in the conversion of nascent discoidal HDL to mature spherical HDL by delivery of cholesterol from peripheral tissues to HDL particles. LCAT facilitates the esterification of cholesterol to form cholesterol ester (Lewis and Rader, 2005). ApoA-I carried on HDL particles is the primary activator of LCAT (Frayn, 2010). Mutations in LCAT can cause classical LCAT deficiency. Classical LCAT deficiency can prevent the esterification of cholesterol in HDL and can lead to the accumulation of discoidal HDL particles in plasma (McIntyre, 1988; Zannis et al., 2006). Deficiency of LCAT is
associated with fish eye disease which is characterized by the impaired esterification of cholesterol in HDL but not in LDL and VLDL, and this can lead to a low level of HDL-cholesterol in plasma (McIntyre, 1988).

1.2.4.1.3 Scavenger receptor Class B type I (SR-BI)

SR-BI is a member of the scavenger receptor proteins. It is a 509-amino acid membrane glycoprotein with a molecular weight of 82KD (Wang and Briggs, 2004). SR-BI is mainly present in the liver and adrenal glands, but also can be found in other tissues and cells including the brain, the intestine, the testis, macrophages, endothelial cells and astrocytes (Zannis et al., 2006; Wang and Briggs, 2004). SR-BI can bind to various ligands including HDL, LDL and VLDL. As the first discovered HDL receptor, SR-BI has the function of facilitating the selective uptake of HDL cholesterol. SR-BI expression can increase cholesterol uptake mediated by HDL in culture cells (Ji et al., 1997). It has been found that deficiency of SR-BI in mice can lead to a great reduction in the clearance of HDL cholesterol from the circulation (Out et al., 2004). An inverse relationship has been shown between SR-BI expression and the levels of HDL cholesterol and apoA-I in studies using the SR-BI transgenic mice (Wang et al., 1998; Ueda et al., 1999). It has been suggested that increased SR-BI expression in the liver of SR-BI transgenic mice is associated with a decrease in HDL cholesterol and apoA-I levels (Wang et al., 1998; Uedua et al., 1999).

1.2.4.1.4 Cholesterol ester transfer protein (CETP)

CETP is a hydrophobic glycoprotein primarily bound to HDL (Barter, 2002). CETP is mainly present in the liver, the spleen and adipose tissue but is also present at low levels in the small intestine, adrenal gland, skeletal muscle, kidney and heart (Wang and Briggs, 2004). CETP promotes the redistribution of cholesterol ester and triglyceride between HDL and the apoB-containing lipoproteins (LDL, IDL, VLDL,
chylomicrons and remnants), and is thus important in reverse cholesterol transport. The net effect of its action is that the cholesterol esters in HDL produced by the LCAT reaction are transferred to triglyceride-rich lipoproteins, and triglycerides are transported in the reverse direction, from chylomicrons and VLDL to HDL (Rye et al., 1999). CETP mutations in humans are associated with increased HDL levels (Inazu et al., 1990). The level of CETP can be raised in dyslipidaemic state including type 2 diabetes mellitus and hypercholesterolaemia which are associated with low HDL concentrations in plasma (Bagdade et al., 1993).

1.2.4.1.5 Phospholipid transfer protein (PLTP)
PLTP is a plasma glycoprotein with a molecular weight of 81 KD (Wang and Briggs, 2004). PLTP activity is associated with larger HDL and moderates the transport of phospholipid from triglyceride-rich lipoproteins to HDL (Colhoun et al., 2002; Lewis and Rader, 2005). PLTP is able to promote extensive remodelling of HDL particles into larger HDL particles by particle combination, with the accompanying release of lipid-poor apoA-I (Huuskonen et al., 2001). In a study using human PLTP transgenic mice, the enhanced activity of PLTP was shown to be related to increased levels of apoA-I in preβ-migrating HDL (Jiang et al., 1996).

1.2.4.1.6 Hepatic lipase (HL)
HL is considered to be an endothelial enzyme, and a member of the triglyceride lipase family, that is involved in HDL metabolism (Wang and Briggs, 2004). HL is a 467-amino acid protein secreted from the liver. It has been shown that HL is bound to the surface of hepatocytes and hepatic endothelial cells. HL has the ability to hydrolyse a variety of lipids not only triglyceride and phospholipids but also cholesterol ester, monoglycerides and diglycerides (Wang and Briggs, 2004). It can hydrolyse the triglycerides and phospholipids of all lipoprotein fractions although the
preferred substrate is HDL triglyceride (Barter, 2002). A study that investigated HDL metabolism showed that HL can increase the uptake of HDL cholesterol ester by SR-BI (Lambert et al., 1999). Patients with HL deficiency have been found to have an increased level of HDL in plasma (Breckenridge et al., 1982).

1.2.4.1.7 Lipoprotein lipase (LPL)
LPL is the enzyme found predominantly in adipose tissue, skeletal muscle and heart muscle (Frayn, 2010; Wang and Briggs, 2004). It is a triglyceride lipase and it is located on the surface of endothelial cells in most tissues (Barter and Rye, 2005). The major activity of LPL is to promote the hydrolysis of triglyceride with a minor activity as a phospholipase (Lewis and Rader, 2005). LPL is the primary enzyme associated with the hydrolysis of triglycerides in chylomicrons and VLDL and the subsequent release of free fatty acids, in which the large triglyceride-rich particle is transformed into smaller triglyceride-depleted particles (Barter, 2002). This triglyceride hydrolysis process leads to the reduction of particle size as well as a redundancy of surface constituents such as phospholipids and apolipoproteins, which may be transported to HDL particles. Consequently, the hydrolysis can be a significant factor contributing to the plasma concentration of HDL cholesterol and HDL-associated apoA-I (Barter, 2002; Lewis and Rader, 2005).
1.3 Cardiovascular disease

1.3.1 Definition and prevalence

Cardiovascular disease (CVD) refers to a group of disorders occurring in the heart and the blood vessels that supply blood to the heart muscle, the brain and the peripheral regions of the body (World Health Organization (WHO), 2013a). Generally, it includes coronary heart disease (CHD), which is also known as coronary artery disease, ischaemic heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic arterial disease, congenital heart disease, and deep vein thrombosis and pulmonary embolism. Coronary heart disease (angina and myocardial infarction) and cerebrovascular disease (stroke) are the most prevalent forms of CVD. Myocardial infarction and stroke are primarily caused by a blockage that inhibits the blood flowing to the heart or brain. Stroke can also be caused by bleeding from the blood vessels in the brain (WHO, 2013a).

CVD is the major cause of death in both the developing and developed countries (WHO, 2013a). It has been estimated that 17.3 million people died from CVD in 2008, accounting for 30% of all global deaths. Approximately 7.3 million of these deaths were caused by CHD and about 6.2 million were due to stroke. Compared with high-income countries, low- and middle-income countries are more exposed to CVD risk factors such as tobacco use, unhealthy diet and physical inactivity. It has been reported that over 80% of CVD deaths occur in low- and middle-income countries, and it distributes almost equally in men and women. Moreover, death from CVD in low- and middle-income countries is in younger people, usually in their most productive years. WHO predicts that by the year 2030, the number of people who died from CVD, mainly CHO and stroke, will be increased to 23.3 million.
The death rate from CVD in the UK has been dropping since the early 1970s (British Heart Foundation, 2012). In the past ten years, the death rate has fallen by 44% among people aged under 75 years. Compared with a younger age group, the death rate from CHD has decreased more dramatically in those aged 55 and over in recent years (British Heart Foundation, 2012). From the 2000 to 2010, there was a decrease of 43% in the CHD death rate among men aged between 55 and 64 in the UK, compared to the decrease of 21% among men aged 35 to 44 years. For women, the death rate in those aged 55 to 64 declined by 52% while the rate in those aged between 35 and 44 years was barely changed. Although the mortality of CVD has been falling, CVD is still the most common cause of death in the UK, which accounted for approximately one in three of all deaths, almost 180,000 deaths in 2010 (British Heart Foundation, 2012).

1.3.2 Cardio-metabolic risks
Cardio-metabolic risk refers to a cluster of CVD risk factors which forms the diagnosis of the metabolic syndrome, the traditional risk factors and other unknown risk factors (Figure 6) (Despres and Lemieux, 2006).

CVD is a multifactorial condition, and there are various risk factors contributing to the likelihood of developing CVD. These risk factors are composed of the untreatable factors such as age and gender, and potentially modifiable risk factors including smoking, physical inactivity, hypertension, increased LDL cholesterol and a group of interrelated metabolic risk factors (British Heart Foundation, 2012; Cannon, 2007). The efforts of preventing and treating CVD risk factors during last several decades have significantly reduced the rates of CVD-related mortality. However, there are still a lot of patients who do not have appropriate control of their CVD risk factors although these factors have been recognized. The increased prevalence of obesity
and type 2 diabetes has been reported to be associated with the weakening improvements in CVD. Therefore, the focus has shifted to the assessment of cardio-metabolic risk, which may present the greatest potential origins of CVD risk (Cannon, 2007).

![Figure 6: Factors contributing to global cardiovascular risk (Despres and Lemieux, 2006).](image)

### 1.3.2.1 Metabolic syndrome

Metabolic syndrome is a cluster of metabolic disorders that are associated with an increased risk of CVD and type 2 diabetes mellitus (Alberti et al., 2009). The concept of metabolic syndrome was first described by Kylin, a Swedish physician, as a series of abnormalities characterized by hypertension, hyperglycaemia and gout in 1923 (Kylin, 1923; Eckel et al., 2005). Two decades later, in 1974, upper body adiposity (android or male-type obesity) had been suggested as the most common type of obesity related to metabolic abnormalities which occurred in patients with CVD and type 2 diabetes (Vague, 1974; Eckel et al., 2005). In 1988, Reaven introduced the term “Syndrome X” and postulated that insulin resistance could be the key underlying cause of hypertension, hyperlipidaemia, type 2 diabetes and CVD (Reaven, 1988;
Kahn et al., 2005). Following Reaven’s publication, various terms have arisen to describe the clustering of insulin resistance and related metabolic disorders which result in increased risk of CVD, such as syndrome X, insulin resistance syndrome and metabolic syndrome (Kahn et al., 2005).

Various criteria for clinical diagnosis of metabolic syndrome have been proposed by different organizations during the past decade. The first formalized definition of metabolic syndrome was introduced by a consultation group on the definition of diabetes for the WHO in 1998 (Alberti and Zimmet, 1998). This diagnostic criteria by WHO was built based on several markers of insulin resistance (impaired glucose tolerance or diabetes) combined with another two risk factors including obesity, hypertension, high plasma triglyceride level, reduced HDL cholesterol level or microalbuminuria. Another definition was proposed by the National Cholesterol Education Program Adult Treatment Panel III (ATP III) in 2002. ATP III criteria did not include insulin resistance but required the occurrence of 3 of the following 5 factors for diagnosis - abdominal obesity, elevated triglyceride, reduced HDL cholesterol, increased blood pressure and increased fasting glucose. The criteria of diagnosis for metabolic syndrome developed by the International Diabetes Federation (IDF) in 2005 had greater emphasis on abdominal obesity and particularly focused on waist circumference (Alberti et al., 2005). The rest of the criteria are essentially identical to the criteria provided by ATP III. More recently, in 2009, six major organizations tried to unify the criteria of metabolic syndrome. This included the International Diabetes Federation Task Force on Epidemiology and Prevention, National Heart, Lung and Blood Institute, American Heart Association, World Heart Federation, International Atherosclerosis Society, and International Association for the Study of Obesity. They presented a statement of common criteria for the clinical diagnosis for metabolic syndrome (Table 4) (Alberti et al., 2009). It was agreed that the risk related to waist measurement will differ in different populations.
Table 4: Criteria for clinical diagnosis of the metabolic syndrome

<table>
<thead>
<tr>
<th>Measure</th>
<th>Categorical Cut Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated waist circumference</td>
<td>Population- and country-specific definitions</td>
</tr>
<tr>
<td>Elevated triglycerides</td>
<td>≥150 mg/dL (1.7 mmol/L)</td>
</tr>
<tr>
<td>Reduced HDL-Cholesterol</td>
<td>&lt;40 mg/dL (1.0 mmol/L) in male; &lt;50 mg/dL (1.3 mmol/L) in females</td>
</tr>
<tr>
<td>Elevated blood pressure</td>
<td>Systolic ≥130 and/or diastolic ≥85 mm Hg</td>
</tr>
<tr>
<td>Elevated fasting glucose</td>
<td>≥100 mg/dL (5.6 mmol/L)</td>
</tr>
</tbody>
</table>

Adapted from Alberti et al. (2009).

The prevalence of metabolic syndrome has increased with the rise in glucose intolerance and type 2 diabetes worldwide (Ogbera, 2010). It has been demonstrated that the prevalence of metabolic syndrome in the general population is about 17% to 25%. Metabolic syndrome is a risk factor of CVD. Patients with metabolic syndrome have twice the likelihood of developing CVD during the next 5 to 10 years compared to those do not have metabolic syndrome (Alberti et al., 2009). Moreover, people with metabolic syndrome have a five times higher risk in developing type 2 diabetes mellitus.

1.3.2.2 Abdominal obesity

Obesity can be briefly described as the condition of abnormal or excessive fat accumulation in the body, which may be harmful for health (WHO, 2013b). It develops when energy intake exceeds energy expenditure for a period of time (Wilding, 2006). Obesity now is the most common nutritional disorder in the world, and it is associated with the increased intake of energy-dense foods that are high in fat and the increased tendency of physical inactivity. Obesity is often defined by body mass index (BMI), which is calculated as weight in kilograms divided by the square of height in meters (kg/m²) (Omari and Caterson, 2007). BMI is a simple and easy approach to assess overweight and obesity in both individual level and population
level. Adults with BMI $\geq 25$ kg/m$^2$ are classified as overweight, BMI between 30-34.9 kg/m$^2$ are categorized as obese class I, BMI from 35 to 39.9 kg/m$^2$ are obese class II, and BMI $\geq 40$ are obese class III with very high risk of comorbidities. Obesity is the dominant contributor to the metabolic disturbance related to lipid and glucose (Redinger, 2007). It has been indicated that obesity is the key factor in the pathophysiology of insulin resistance, diabetes mellitus, hypertension, dyslipidemia and atherosclerosis.

The cases of obesity have been nearly doubled in the world since 1980 (WHO, 2013b). In 2008, 35% of adults, aged 20 and over in the world (approximately 1.4 billion), were overweight or obese. Of these, there were over 200 million males and about 300 million females who were obese, in total accounting for about 11% of world’s adult population. Overweight and obesity have become the fifth leading risk of global death (WHO, 2013b). They contribute to 44% of diabetes burden, 23% of CHD burden and 7% to 41% of certain cancer burdens. Every year at least 2.8 million people die as the result of being overweight or obese. Thirty years ago, the major health issue of childhood in developing countries was focused on malnutrition. However today, attention has been drawn to the new pandemic of obesity and its accompanying noncommunicable diseases (Prentice, 2006). In 2011, more than 40 million children under five years old were overweight (WHO, 2013b). The prevalence of overweight and obesity in children living in low- and middle-income counties is higher, compared to high-income countries. There are over 30 million children in the developing countries that are overweight and 10 million in the developed countries.

The site of where the adipose tissue accumulates is known to be crucial for identifying the risk of obesity-related disease (Omari and Caterson, 2007). Abdominal obesity, a vital component of obesity, is particularly associated with the increase risk of CVD and type 2 diabetes (Westphal, 2008). Waist circumference, which has been
indicated to be a strong marker of obesity-related health risk, is used to assess abdominal obesity. The measurement >102 cm in Caucasian men and >88 cm in Caucasian women suggests the greater risk of developing metabolic disease (Omari and Caterson, 2007). It has been demonstrated that a combination of BMI and waist circumference would be a better predictor of health risk than using BMI alone (Janssen et al., 2004).

1.3.2.3 Insulin resistance

Insulin is a crucial metabolic hormone that stimulates glucose uptake in different organs, especially muscle and adipose tissue, and inhibits glucose production in the liver. It also inhibits lipolysis in adipose tissue (Greenfield and Campbell, 2004). Insulin resistance or a reduced biological response to insulin is known as a key component of metabolic syndrome, and is linked to type 2 diabetes and obesity. It has been suggested that insulin resistance is a strong predictor of the development of type 2 diabetes in adults (Greenfield and Campbell, 2004). Studies in obese children have also demonstrated this relationship (Lee et al., 2006). Insulin resistance is particularly associated with central obesity or abdominal obesity (Greenfield and Campbell, 2004). It has been suggested that the accumulation of fat in the abdominal area of the body can be a crucial determinant of whole-body insulin resistance. The increased level of FFAs, which can expand adipose mass, is commonly shown in obesity (Westphal, 2008). Visceral fat is less sensitive to the effect of insulin against lipolysis than subcutaneous fat, and the increased release of FFAs from visceral fat which goes directly to the liver can promote gluconeogenesis and hepatic insulin resistance.

Insulin resistance may be involved in the pathogenesis of nonalcoholic fatty liver disease (NAFLD) and most likely progression could be as follows: (i) Peripheral insulin resistance, which leads to the insufficient suppression of lipolysis and reactive
hyperinsulinemia; (ii) Increased fatty acid influx to the liver, which could lead to the increased level of hepatic triglycerides; (iii) Hepatic insulin resistance superimposed on peripheral resistance (Bugianesi et al., 2005). Insulin resistance has also been associated with an atherogenic plasma lipid profile (DeFronzo and Ferrannini, 1991). Insulin resistance can enhance the hepatic synthesis of VLDL and contribute to the increased plasma level of triglyceride. Resistance to the action of insulin on lipoprotein lipase can also cause hypertriglyceridaemia in patients with obesity and diabetes. It has been suggested that the latter process may facilitate the progressive elimination of lipid and apolipoproteins from VLDL particles and lead to the elevated production of IDL and LDL, which are both known to be atherogenic (DeFronzo and Ferrannini, 1991).

1.3.2.4 Fatty liver disease
The problem of fatty injury to the liver has arisen due to the globally increased prevalence of obesity (Preiss and Sattar, 2008). NAFLD can be defined as the accumulation of fat in the liver exceeding 5-10% by weight but in the absence of alcohol consumption (Hsiao et al., 2007). NAFLD is one of the most common liver disorders. It has been estimated that about 20-30% of the adult population in the developed countries are affected by NAFLD (Bellentani et al., 2010). Approximately 2-3% of the same population will have non-alcoholic steatohepatitis (NASH). The prevalence of NAFLD is also increasing in children. Paediatric NAFLD has increased from about 3% in 2000 age to 5% in 2010 (Bellentani et al., 2010).

There are several stages involved in the progression of NAFLD (Preiss and Sattar, 2008). Steatosis, which is the initial recognized stage, represents fat accumulation in liver tissue without inflammation. The subsequent stage in some cases is NASH, which is characterized by steatosis accompanying with hepatocellular injury, and inflammation with or without fibrosis. People who have developed NASH can be at
very high risk of CVD and progression to the severe stage of NAFLD, cirrhosis, which increases the risk of hepatocellular carcinoma. NAFLD is positively associated with obesity and BMI (Angulo, 2002). The prevalence of steatosis in obese individuals with BMI > 30kg/m² and BMI > 35 kg/m² is estimated at 65-75% and 85-90% respectively. As mentioned before, NAFLD is also associated with peripheral and hepatic insulin resistance. The content of liver fat is significantly increased in people with metabolic syndrome (Kotronen et al., 2007). In addition, the presence of severe fatty liver in the US population is greatly associated with the prevalence of hypertension, abnormal metabolism of glucose and triglyceride (Hsiao et al., 2007).

1.3.3 Relationship between cardiovascular disease and HDL

1.3.3.1 Pathogenesis of CVD

Atherosclerosis, which is caused by the development of fatty deposits in the arterial walls, is considered to be the most frequent cause underlying CVD (Frayn, 2010; Falk, 2006). Atherosclerosis is a chronic immunoinflammatory, fibroproliferative disease that affects the medium and large-sized arteries (Falk, 2006). In CHD, the most common form of CVD, atherosclerosis can cause the partial or complete blockage in one or more coronary arteries that provide blood to the muscular walls of the heart.

The development of the fatty deposits, which is known as atherosclerotic plaque, is a very complicated and slow progressive process, and it is related to various types of cells and lipids (Frayn, 2010; Singh et al., 2002). Accumulation of lipid (mainly cholesterol), infiltration of macrophages, proliferation of smooth muscles and connective tissue are involved in the development of atherosclerosis. Cholesterol from LDL particles is considered to be the principle lipid of the atherosclerotic plaque (Santos-Gallego et al., 2008). In brief, LDL particles from the circulation migrate into
the arterial intima following endothelial dysfunction. After undergoing an oxidation process, the oxidized LDLs become very toxic. As part of the mechanism of defense, the oxidized LDLs are taken up by macrophages. The macrophages engulf large quantities of lipid and become foam cells. Foam cells then accumulate in the arterial walls and lead to the first visible lesion in the atherosclerosis, which is called the fatty streak. Moreover, monocytes move into the intima as well. They exist in the subendothelial area distinguished from the macrophages and engulf further lipids. Advanced lesions, fibrous atheromas, may be formed via the migration and proliferation of smooth muscle cells and increasing synthesis of connective tissue (collagen fibrils). Therefore, excessive cholesterol accumulation in the arterial walls is the initial and pivotal phenomenon in the development of atherosclerosis (Santos-Gallego et al., 2008).

### 1.3.3.2 Effect of HDL on CVD

HDLs are atheroprotective and have an inverse correlation with cardiovascular disease risk (Kontush and Chapman, 2006). Low concentration of HDL cholesterol has been revealed as an independent predictive risk factor for premature CHD. It has been suggested that the plasma concentration of HDL cholesterol could be a more powerful predictor of CHD compared with plasma total cholesterol or LDL cholesterol (Barter and Rye, 1996).

Numerous studies have investigated the relationship between HDL cholesterol and CHD. The Framingham Heart Study indicated that HDL cholesterol was significantly inversely associated with the incidence of CHD in both men and women aged 49-82, and it was the predominant potent lipid risk factor (Gordon et al., 1977). Since the results of the Framingham study were published, several other studies have also found a strong inverse association between HDL cholesterol and the risk of CHD (Gordon et al., 1989). It has been shown that a 1 mg/dL increase in HDL cholesterol
can lead to a 2% decrease of CHD risk in men and 3% in women, and it can also result in a significant reduction of CVD mortality rates, about 3.7% in men and 4.7% in women. The Helsinki Heart Study, which was a primary prevention trial in asymptomatic middle-aged men (40-55 years old) with dyslipidaemia found a reduction of 34% in the incidence of CHD after 5-year treatment of gemfibrozil, which is a member of fibrates used to increase the concentration of HDL cholesterol (Frick et al., 1987). In addition, this risk reduction was independently correlated to the 11% increase of plasma HDL cholesterol concentration and 11% decrease in LDL cholesterol (Frick et al., 1987). The Veterans Affairs HDL Intervention Trial (VA-HIT) also involved treatment with gemfibrozil (Boden, 2000 & Robins, 2001). After 1-year of gemfibrozil treatment in men with known CHD (with low HDL cholesterol and low LDL cholesterol levels), there was a significant increase in HDL cholesterol (6%) and a decrease in triglyceride (31%) and total cholesterol (4%). However, there was no change in LDL cholesterol. Moreover, the treatment of gemfibrozil also led to a notable reduction, about 22%, in incidence of death from CHD.

The protective effect of HDL against CVD is complex, and the understanding of the mechanism is still incomplete. The role of HDL in RCT promoting cholesterol efflux from cells has been most recognized to contribute to its protective effects. However, there are also many other properties of HDL which have been demonstrated to be antiatherogenic including inhibition of foam cell formation, inhibition of LDL oxidation, inhibition of endothelial cell dysfunction, anti-inflammatory, antiapoptotic activity, antithrombotic activity, anti-infectious and vasodilatory activity (Figure 7) (Kontush and Chapman, 2006).
Macrophages, RCT and inhibition of foam cell formation

The association between RCT and atherosclerosis was first reported by Ross and Glomset in 1973. After several decades of investigation, the relationship of RCT to atherosclerosis is still a hypothesis rather than an established fact (Cuchel and Rader, 2006). It has been clearly shown that the physiological process of RCT exists in all peripheral tissues. However in atherosclerotic lesions, the macrophage is the major cell that is overloaded with cholesterol (Cuchel and Rader, 2006). Macrophages are part of the body’s defence system and are involved in the immune response (Wang and Briggs, 2004). Macrophages also interact with T cells in the inflammatory response. These functions could contribute to the mechanism of the development of atherosclerosis associated with macrophages. As mentioned before,
Atherosclerosis originates by the accumulation and subsequent oxidation of LDL in the arterial intima. Macrophages take up the oxidized LDL and accumulate cholesterol, which leads to the formation of foam cells, the hallmark cell of atherosclerosis (Barter et al., 2004).

HDL-mediated cholesterol efflux from cholesterol-loaded macrophages is one of the well-established antiatherogenic functions of HDL (Brewer, 2004). HDL particles (both HDL$_2$ and HDL$_3$) can promote the removal of cholesterol from macrophages by interaction with the SR-BI receptor, by binding to the ABCA1 transporter, or by passive diffusion that involves exchange of free cholesterol between mature spherical αHDL and the cell membrane. After obtaining the excessive cellular cholesterol from the arterial macrophages, HDL delivers the excess cholesterol to the liver, where it is excreted as bile acids.

**Antioxidation**

HDL can reduce atherosclerosis by protecting LDL from oxidation (Brewer, 2004). The antioxidative function of HDLs is determined by the antioxidative properties of the apolipoproteins and enzymes they carry (Kontush et al., 2003). Binding and removing oxidative molecules could be one of the mechanisms. The major apolipoprotein of HDL, apoA-I, plays an important role in inhibiting LDL oxidation by removing LDL-derived oxidized phospholipids and LDL lipid hydroperoxides from LDL and/or from artery wall cells (Navab et al., 2001; Navab et al., 2004). HDLs are the principle carriers of plasma lipid hydroperoxides in animal models of atherosclerosis and in humans (Barter et al., 2004). They are also the carriers of antioxidant enzymes including paraoxonase and platelet-activating factor acetylhydrolase (Wang and Briggs, 2004). These enzymes can degrade the harmful oxidized phospholipids from LDL and inhibit the oxidation of LDL. The antioxidative effect of HDL subclasses is mainly correlated to the inactivation of LDL lipid
hydperoxides, and the heterogeneity of HDL results in a different contribution of distinct HDL subfractions in antiatherogenic activities (Kontush et al., 2003). It has been demonstrated that smaller and denser HDL₃ particles perform more efficient inhibition of LDL oxidation than larger and lighter HDL₂ particles. This could be due to their different cholesterol efflux capacity. HDL₃ was indicated to have higher capacity to accept oxidized lipids than HDL₂.

**Anti-inflammation**

An early stage of inflammatory process in atherogenesis is the adhesion of monocytes to endothelial cells that have been damaged or stimulated in a way to express adhesion proteins (Barter et al., 2004). These adhesion proteins are known to be expressed in the arteries that may develop atherosclerosis. The anti-inflammatory properties of HDL are demonstrated by the inhibition of the expression of adhesion proteins in endothelial cells, and reduction in the recruitment of monocytes into the artery wall in the early stage of atherosclerosis (Barter et al., 2004). It has been shown that smaller HDL₃ has a stronger ability to inhibit to adhesion protein expression in the endothelial cells compared with larger HDL₂ particles (Kontush et al., 2003).

**Inhibition of Endothelial Dysfunction**

The protective function of HDL against atherosclerosis also includes the inhibition of endothelial cell dysfunction (Wang and Briggs, 2004). HDL can facilitate the production and release of several atheroprotective factors in the endothelial cells such as nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF). The impairment of endothelium-dependent vasodilations, which is one the risk factors of atherosclerosis, can affect both the NO- and EDHF-mediated response. It has been shown that HDL can facilitate the normalization of endothelium-dependent vasodilation by enhancing the bioavailability of NO. This protective effect of HDL
associated with endothelium-dependent vasodilation can be partially attributed to the stimulation of expression of endothelial NO synthase by HDL (Wang and Briggs, 2004).

1.3.4 Relationship between cardiovascular disease and diet

It has been well established that there are a variety of lipids and lipoproteins in the plasma that are involved in the development of CVD (Posta et al., 2006). Dietary factors can either enhance or reduce the risk of CVD by altering the lipid and lipoprotein profile.

1.3.4.1 Influence of fat intake on CVD risk

Naturally occurring dietary lipids are derived from a variety of animal and plant sources including animal adipose tissue (the visible fat on meat, lard and suet), milk and products derived from it (cream, butter, cheese and yoghurt), vegetable seeds, nuts, oils and products from them, eggs, fish oil and plant leaves (Mann and Skeaff, 2007). Triglycerides account for approximately 95% of dietary lipids. Most foods contain a range of different fatty acids including saturated fatty acids, monounsaturated and polyunsaturated fatty acids (Mann and Skeaff, 2007). Trans fatty acids are less naturally occurring in dairy and meat products, but largely present in some margarines, commercially baked products and deep-fried foods as the result of human processing such as hydrogenation (Erkkila et al., 2008; Willett, 2006).

Numerous studies have investigated the relationship between dietary fat intake and CVD. In brief, trans fatty acids, saturated fatty acids and cholesterol can be inversely associated with CVD, whereas unsaturated fatty acids including monounsaturated fatty acids and polyunsaturated fatty acids may have favorable effects (Posta et al., 2006).
1.3.4.2 Influence of carbohydrate intake on CVD

Carbohydrates are an important dietary source of energy (Cummings and Mann, 2007). Carbohydrates are predominantly derived from cereals such as rich, wheat, maize, barley, rye, oats, millet and sorghum. They are also found in root crops like potatoes, sweet potatoes, cassava, yams and taro, sugar cane and beet, pulses, vegetables, fruits and milk products. Carbohydrates are mainly synthesized by plants from water and carbon dioxide by utilizing the sun’s energy and have a general formula \((\text{CH}_2\text{O})_n\). There is also a minority synthesized by animals such as lactose in milk. In humans, carbohydrates can be stored in muscle and liver as glycogen for energy. Carbohydrate-containing foods can supply about 40% to 80% total energy intake in the diet, which varies according to culture and economic status (Cummings and Mann, 2007). Carbohydrate-containing foods are also crucial sources of protein, vitamins and minerals. Carbohydrates are substances with diverse chemical and physiological properties. The primary classification of dietary carbohydrates is based on molecular size, which is characterized by the degree of polymerization (DP), the type of linkage (α or non-α), and the individual monomers (Table 5) (Cummings and Mann, 2007).
<table>
<thead>
<tr>
<th>Class (DP)</th>
<th>Subgroup</th>
<th>Principal components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugars (1-2)</td>
<td>Monosaccharides</td>
<td>Glucose, fructose, galactose</td>
</tr>
<tr>
<td></td>
<td>Disaccharides</td>
<td>Sucrose, lactose, maltose, trehalose</td>
</tr>
<tr>
<td></td>
<td>Polyols (sugar alcohols)</td>
<td>Sorbitol, mannitol, lactitol, xylitol, erythritol</td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td>Malto-oligosaccharides (α-glucans)</td>
<td>Maltodextrins</td>
</tr>
<tr>
<td>(3-9) (short-chain carbohydrates)</td>
<td>Non α-glucans oligosaccharides</td>
<td>Raffinose, stachyose, fructo- and galacto- oligosaccharides, polydextrose, inulin</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>Starch (α-glucans)</td>
<td>Amylose, amylopectin, modified starches</td>
</tr>
<tr>
<td>(≥10)</td>
<td>Non-starch Polysaccharides</td>
<td>Cellulose, hemicellulose, pectin, arabinoxylans, glucomannans, plant gums and mucilages</td>
</tr>
</tbody>
</table>

Adapted from Cummings and Mann (2007).

Monosaccharides and disaccharides are usually referred to as sugars. The major monosaccharides include glucose, fructose and galactose. Free glucose and fructose exist naturally in honey and fruits, and fructose is the sweetest among all carbohydrate-containing foods (Cummings and Mann, 2007). The major disaccharides include sucrose and lactose. Sucrose widely occurs in fruits, berries and vegetables and can be extracted from sugar cane or beet. Lactose is the principal sugar in the milk. Based on the physical location in the foods, sugar can also be subdivided into intrinsic and extrinsic sugars (Stephen et al., 1995). Intrinsic sugars refer to the sugar located inside the cell walls of plants, while extrinsic sugars refer to those located outside the cell walls and are added to the foods. Intrinsic sugars are found in fruits and vegetables as glucose, fructose and sucrose, and extrinsic sugars are found in processed foods like fruit juices. Lactose in milk does
not exist within the cell walls, however milk has important nutritional properties. Therefore, the term non-milk extrinsic sugar, which was firstly introduced in the UK, has been used to distinguish the group of sugars other than intrinsic and milk sugars that should be restricted in the diet (Stephen et al., 1995). Starch is the major constituent of carbohydrates and is composed of two different types of glucan polymers, amylose and amylopectin (Lafiandra et al., 2014). They occur in staple foods such as rice, bread, cereal and potatoes. Non-starch polysaccharides are considered as the carbohydrate component of dietary fibre. There are also some other terms have been used to describe carbohydrates, including complex carbohydrate, dietary fibre and glycaemic carbohydrate, which are based on their physiological properties with different effects on health.

The association between dietary carbohydrate intake and risk of CVD has been examined in a dietary survey among Korean adults. High consumption of carbohydrate, > 70% of energy, is associated with higher levels of BMI, blood pressure, fasting glucose, plasma triglyceride and LDL cholesterol in Korean women aged 20-69 years (Park et al., 2010). Moreover, high carbohydrate intake was significantly correlated with a low level of HDL cholesterol and type 2 diabetes in women. However in men, high carbohydrate intake was only inversely associated with total cholesterol in plasma. Glycaemic index (GI) is a measurement of glucose and insulin response after eating, which is related to the rate of digestion and absorption. Carbohydrates that are absorbed rapidly and induce a high postprandial glucose and insulin response are considered to be high GI carbohydrates. Glycaemic load (GL), which is calculated from GI and carbohydrate content, presents both quantity and quality of carbohydrate of a specific food. The relationship between GI, GL and risk of CVD has also been investigated. The results from a cohort prospective study has shown that the consumption of diets with high GL and high GI could increase the risk of CVD in middle-aged Dutch women, particularly in those
who were overweight (Beulens et al., 2007). It has also been found that higher dietary GL and GI were associated with a higher likelihood of developing obesity in Korean women but not in men (Youn et al., 2012).

Restriction of dietary carbohydrate has been found to induce a beneficial effect on reducing CVD risks by improving hepatic, intravascular and peripheral processing of lipoproteins, altering fatty acid composition and reducing inflammation (Volek et al., 2008). A six-week dietary carbohydrate restriction study was carried out in statin users (Ballard et al., 2013). At the end of the intervention, carbohydrate intake was reduced nearly 5 times compared to baseline, which accompanied with reduced energy intake. Body weight was reduced by 4% in 6 weeks, representing an average of 3.6 kg. Both systolic and diastolic blood pressure was decreased after 6-week carbohydrate restriction. The levels of serum triglyceride and insulin were also decreased. It was suggested that among people on statin therapy, 6 weeks dietary carbohydrate restriction could achieve additional improvements in metabolic and improved vascular health, such as increased insulin sensitivity and resistance vessel endothelial function, and decreased blood pressure and plasma triglycerides. Additionally, increased dietary fibre intake has been reported to have the beneficial effects on prevention of metabolic and cardiovascular diseases (Liu et al., 2002). It has been suggested that people who had higher dietary fibre intake by consuming three or more portions of whole grain cereals and their products per day had a lower risk of developing obesity, type 2 diabetes and CVD (Lafiandra et al., 2014).

Apart from its role as a source of energy, carbohydrate has an important function in regulating lipid metabolism (Volek et al., 2008). Dietary carbohydrate can affect insulin secretion and the availability of energy substrates like free fatty acids and glycogen. Dietary carbohydrate is usually digested and absorbed as glucose and fructose, and fructose is considered to be more lipogenic (Volek et al., 2008). Recent
data has suggested that fructose consumption is related to increased risk of CVD and type 2 diabetes including visceral obesity, insulin resistance and lipid dysfunction (Stanhope and Havel, 2010; Basciano et al., 2005). A high flux of fructose in the liver can cause the disturbance of glucose metabolism, and lead to a greatly increased synthesis of de novo lipogenesis (DNL) and TG by the enhanced flux of glycerol and acyl portions from fructose catabolism (Basciano et al., 2005). The effect of fructose on risk factors of CVD has been investigated by comparing the influence of consuming glucose, and fructose high fructose corn syrup (Stanhope et al., 2011). Men and women aged 18-40 years with BMI 18-35 kg/m² consumed usual al libitum diets along with 3 servings per day of glucose, fructose or high-fructose corn syrup sweetened beverages, which accounted for 25% of energy requirement. After 2 weeks intervention, the consumption of fructose and high-fructose corn syrup sweetened beverages increased postprandial triglycerides, LDL cholesterol and apolipoprotein B concentration. However, these effects were not found in the group who consumed glucose.

The dietary intervention study presented in this thesis was a part of a BBSRC funded trial, in which two iso-energetic diets with high and low non-milk extrinsic sugar content were designed and implemented in middle-aged Caucasian men who were at risk of metabolic syndrome with low or moderately increased liver fat. As mentioned, dietary carbohydrate intake can influence the secretion of insulin, DNL, and the plasma levels of triglyceride, LDL cholesterol and apoB, and is related to the risks of CVD. Moreover, it has been suggested that controlling the quality of dietary carbohydrate via reducing extrinsic sugar, such as fructose and sucrose, and increasing non-starch polysaccharides (dietary fibre), may have a beneficial improvement on CVD risks. Therefore, the whole trial aimed to determine the influence of the quality of dietary carbohydrate on lipoprotein metabolism including the formation of high and low risk lipoprotein phenotypes, VLDL kinetics (the
synthesis of VLDL-TG and VLDL apoB) and LDL kinetics (the production of small dense LDL). It also aimed to determine the effect of the quality of dietary carbohydrate on the sources of liver fat, whether VLDL-TG is derived from increased DNL and/or systemically derived NEFA (adipose tissue and peripheral lipolysis). The lipoprotein kinetics, DNL and systemic NEFA kinetics were measured by using stable isotope techniques. Furthermore, the whole trial also aimed to evaluate the effect of the high and low non-milk sugar diets on several cardio-metabolic risks including body weight, fasting plasma lipids, glucose and insulin. In the whole trial, it was hypothesized that compared to a low non-milk extrinsic sugar diet, a diet with high non-milk extrinsic sugar would increase plasma TG level and the production of LDL in subjects at risk of metabolic syndrome and a moderate increase in liver fat, and that the changes in plasma TG and lipoproteins would be due to increased VLDL-TG and/or VLDL apoB, and increased DNL and/or systemically derived NEFA. I was not involved in this BBSRC funded trial except for the analysis of the HDL samples. The study presented in this thesis was specifically focused on the effect of the high and low non-milk extrinsic sugar diets on total HDL kinetics which was measured by using a stable isotope technique in a small group of subjects (n=6) from the whole cohort (n=25). Some of the results presented in Chapter 3, including subject’s characteristics, lipid and lipoprotein profile, insulin and glucose, dietary intakes and lipase activities, were measured by Dr. Aryaty Ahmed and Dr. Najlaa Alsini.
1.4 Application of isotope techniques in lipoprotein metabolic studies

1.4.1 Isotopes techniques

Isotopes refer to the atoms of a chemical element with the same atomic number and similar chemical properties but different atomic weights and physical properties, such as $^{11}$C, $^{12}$C, $^{13}$C and $^{14}$C (Wong and Abrams, 2003). Some isotopes are radioactive (unstable), like $^{11}$C and $^{14}$C while others are non-radioactive (stable), like $^{13}$C and $^{12}$C (Sunehag and Haymond, 2003). In most cases, only the stable isotopes of the elements exist in natural environment (Wong and Abrams, 2003). The most widely used stable isotopes in metabolic tracer studies are $^2$H (hydrogen), $^{13}$C (carbon), $^{15}$N (nitrogen) and $^{18}$O (oxygen), and their natural abundances are 0.015%, 1.11%, 0.36% and 0.204% respectively (Chan et al., 2004; Sunehag and Haymond, 2003).

Lipoproteins are in a state of constant flux. Measurements of the plasma concentration of lipids and lipoproteins are traditionally applied to define the abnormalities of lipoprotein metabolism. However, lipoprotein metabolism is complex and abnormal plasma concentrations can be due to elevated or reduced production and/or catabolism rates of different lipoprotein particles. Therefore, static determinations of lipid and lipoprotein concentrations are unable to provide the information underlying the pathogenic mechanisms. The technique utilizing either radioactive or stable isotope tracers in kinetic studies is well established, and provides an insight for understanding lipoprotein metabolism and mechanisms of metabolic disorders in vivo (Chan et al., 2004).

In research that investigated lipoprotein kinetics in human, such as HDL, both exogenous and endogenous labelling methods have been used (Ooi et al., 2006). Radioactive isotopes have been commonly used for exogenous labelling, which are less used now, whereas endogenous labelling can be performed with either
radioactive isotopes or stable isotopes. Radioactive isotopes have the advantage that they can be easily detectable in blood, urine or faeces. However, they are a potential hazard due to the internal radiation exposure. Hence, radioactive isotopes are not suitable for use in the vulnerable groups, such as infants and pregnant women (Sandstrom, 1996).

The methodology of using stable isotopes as tracers to label amino acids has been established for more than two decades, and now it is widely used for endogenous labelling of proteins in lipoprotein metabolism studies (Chan et al., 2004). Compared to radioactive isotopes, the application of stable isotopes as endogenous tracers to investigate the metabolism of lipoprotein in vivo has several advantages. Firstly, it is safer and can be used in individuals of all ages, and is especially useful in studies among infants, pregnant and lactating women. Secondly, stable isotopes do not decay, so they can be detected in tissues and blood for longer periods of time. By using endogenous labelling, there are no worries about lipoprotein modification during the isolation and labelling process. Furthermore, it is possible to measure the production rate of several different proteins at the same time since all newly synthesized proteins will be labelled (Dwyer et al., 2002; Sandstrom, 1996).

The principle of the endogenous stable isotope labelling technique is to introduce the tracer into the biological system, such as the whole body or organ. The tracer is incorporated into a macromolecule and the ratio of tracer to tracee in the macromolecule is measured (Chan et al., 2004). Tracer refers to the labelled substance, such as stable isotopically labelled leucine, which is incorporated into the macromolecule under investigation. Tracee refers to the unlabelled substance. Since lipoproteins can be labeled in their lipid and/or protein components, stable isotopic labelled amino acids have been predominantly used as tracers to label apolipoproteins in studies of lipoprotein metabolism. The tracer can be introduced
into the circulation through intravenous infusion, bolus injection and oral administration. Ideally, a tracer needs: 1) to have properties identical to the tracee in terms of physical, chemical and biological characteristics; 2) to be quantitatively detected by the investigators; 3) does not disturb the system under study (Chan et al., 2004). L-[5,5,5\textsuperscript{2}H\textsubscript{3}] leucine, L-[1-\textsuperscript{13}C] leucine and [\textsuperscript{13}C\textsubscript{6}] phenylalanine are the stable isotopes that are most commonly used for endogenous labeling in the assessment of HDL apoA-I kinetics (Ooi et al., 2006; Rashid et al., 2006). When leucine is used as a tracer, the synthesis rate of protein can be determined if α-ketoisocaproate (KIC), which provides an estimate of the precursor pool, is measured.

Fisher et al., (1995) investigated the kinetics of apoA-I isolated from HDL by using three different clinical protocols: bolus intravenous injection of [\textsuperscript{3}H] leucine; bolus injection of [\textsuperscript{2}H] leucine; and primed dose followed by 12 hours constant intravenous infusion of [\textsuperscript{2}H] leucine. Both healthy subjects and subjects with familial hypercholesterolaemia were studied. Blood samples were taken over two weeks. Fisher et al. proposed that there were two apoA-I pools, a rapid turnover pool with a residence time of less than 1 day and a slow turnover pool which tracer enters after a delay of less than half day. The slowly turning-over pool contained the predominant mass of apoA-I and was the major determinant of the residence time of apoA-I. A larger quantity of apoA-I transported through the fast pathway. However, the mass of fast pool was relatively lower than the slow pool due to its rapid turnover. Fisher et al., (1995) suggested that apoA-I entered into both fast and slowly turning-over pools in plasma. Therefore, the kinetic data can be analysed by assuming either input pathways to each pools are separated or the fast pool is the precursor to the slow pool.
1.4.2 Detection of isotope enrichment by GC-MS

A GC-MS instrument consists of two components: the gas chromatograph (GC) and mass spectrometer (MS) (Sunehag and Haymond, 2003). The tracer: tracee ratios are measured by GC-MS. Mixtures of amino acids from a protein hydrolysate are isolated as individual amino acids by GC, which are subsequently identified and quantified by MS (Figure 8).

![Figure 8: Principles of gas chromatography-mass spectrometry (Sunehag and Haymond, 2003)](image)

To detect the isotopic enrichment by GC-MS, amino acids under analysis need to undergo a process called derivatization to produce complex molecules, which are more volatile (Sunehag and Haymond, 2003). The derivatized amino acids are vapourised at high temperature in the injector of the GC, and subsequently they are carried through the capillary column by the inert gas under pressure. The carrier gas can be helium, hydrogen, nitrogen or methane. The amino acids that need to be analysed are separated from other constituents of the sample in the column based on their volatility. The separation is achieved by the temperature-regulated interaction between the amino acids in the mobile phase (the carrier gas) and the stationary phase coating the inner surface of column.
After the separation, the samples move to the ion source in the MS, where they are ionized. Different ionization methods can be applied to identify and quantify the substance of interest, including electron ionization mode (EI) and chemical ionization mode (CI) (Sunehag and Haymond, 2003). In both modes, the electrons are released from the heated filament and adhere to the positive charged plate – the anode. In the EI mode, the ionization of the molecule is achieved by direct bombardment with electrons. In the process of EI, the molecule is broken into fragments when excess energy is applied. In the CI mode, different gases, such as methane, ammonia and isobutane, can be introduced to the ion source as the reagent gas. In the present study, the ionization of the molecule is achieved by reaction with methane ions. In the process of CI, methane molecules are hit by electrons and generate different methane ions. Methane ions react with sample molecules and ionize them. Since positive and negative ions can be produced in the reactions, CI mode is divided into two different types, positive chemical ionization (PCI) and negative chemical ionization (NCI). Compared with the EI mode, the CI mode is a lower energy process, and it can result in larger fragments of the analyte.

Following ionization, the ions that need to be analyzed move to the focusing lenses, which accelerate the ions for transporting to the mass analyzer. The ions are filtered by mass analyzer according to their mass. The quantity of an ion with specific mass is then detected and recorded by a computer (Sunehag and Haymond, 2003).

In this project, stable isotopic techniques will be used to measure HDL apoA-I kinetics. The most widely used method for this measurement, a primed continuous infusion will be used to investigate the effect of dietary sugar intake on total HDL apoA-I metabolism. Since HDL has a long half-life (5 days), measuring HDL apoA-I kinetics on a single day may not provide an accurate measurement. In addition, since HDLs are heterogenous particles, measuring total HDL apoA-I does not provide a
true measure of HDL metabolism. This project will attempt to address these two issues by measuring HDL subclass kinetics, using an intravenous injection of $^{13}$C leucine and measuring leucine enrichment over several days. An optimal sampling protocol will be developed and then this will be applied to measure preβHDL and αHDL kinetics, and HDL$_2$ and HDL$_3$ kinetics in a small group of subjects.

1.5 Aims
There are several aims of this project are:

1) To investigate the effect of a high sugar and low sugar diet on total HDL apoA-I metabolism in men at risk of metabolic syndrome.

2) To develop an optimal blood sampling protocol to measure HDL$_2$, HDL$_3$, preβHDL and αHDL flux in vivo in humans.

3) To measure apoA-I synthesis and clearance rates of HDL subclasses, including HDL$_2$, HDL$_3$, preβHDL and αHDL, in healthy men and women using stable isotope techniques.

1.6 Hypothesis

1) HDL cholesterol and apoA-I will be lower and HDL apoA-I FCR will be higher after a high sugar diet than a low sugar diet.

2) A two-week blood sampling protocol will be a feasible method to measure HDL$_2$, HDL$_3$, preβHDL and αHDL kinetics.

3) The FCR and PR of HDL$_2$ apoA-I will be higher than the FCR and PR of HDL$_3$ apoA-I.

4) The FCR of preβHDL apoA-I will be higher than the FCR of αHDL apoA-I, and the PR of preβHDL apoA-I will be lower than PR of αHDL apoA-I.
Chapter 2: Methodology

2.1 Clinical protocol

2.1.1 The effect of a low and high extrinsic sugar diet on HDL kinetics

2.1.1.1. Subjects

Ethical approval for the study was obtained from the Surrey NHS Research Ethics Committee, and University of Surrey Ethics Committee.

Caucasian men aged 40-65 with BMI 26 – 32 kg/m$^2$ were recruited in a dietary intervention study. Different approaches were applied in the recruitment. The major approach was through an existing Clinical Research Network with GP surgeries in the Surrey, which provided a valuable pre-screening tool including BMI, plasma lipids, blood glucose, blood pressure, current health, medication and socio-demographic status. The other approaches were through direct contact with local authorities, companies, schools and colleges by letters and email, distributing leaflets in the offices, shops and local residential areas, and displaying posters on local community notice boards. Men who expressed an interest were sent a participant information sheet (Appendix 1).

Subjects had two screening visits. At the first screening visit, subjects were asked to come to the CEDAR Centre, Royal Surrey County Hospital (RSCH) after an overnight fast. Subjects’ general health information including age, medical history, current medication, smoker/non smoker, were recorded. Weight, height and blood pressure were measured, and blood samples were taken to measure the haematology (haemoglobin, white blood cell count, platelet count), plasma triglyceride, total cholesterol and blood glucose. Subjects who had type 2 diabetes, renal disease, hepatic disease (except non-alcoholic fatty liver disease), unstable body weight in the past three months, and alcohol consumption more than 2
units/day were excluded. Subjects who were taking medication that lowered blood lipids, had any metal implants or claustrophobia were also excluded from the study. Participants who fulfilled the inclusion criteria then attended the second screening at Hammersmith Hospital in London for the magnetic resonance spectroscopy (MRS) examination to determine their percentage of liver fat. Subjects who had either low liver fat < 5% or high liver fat 7.5% - 30% were recruited.

2.1.1.2. Study protocol

The study was a controlled, randomized crossover trial with two 12 week dietary interventions, which were low and high sugar diets. The two diets had the same carbohydrate content in weight and total percentage energy but differed in the content of non-milk extrinsic sugars, high non-milk extrinsic sugar vs. low non-milk extrinsic sugar diet. The total carbohydrate content in the diets was set to 50%.

Before commencing the first dietary intervention, participants underwent a 4 week run-in diet. They were asked to maintain their habitual diet and level of physical activity. At the end of the 4 weeks, subjects came to the Cedar Centre for a fasting blood sample and subsequently they were allocated randomly to either the high or low non-milk extrinsic sugar diet for 12 weeks. Two-thirds of the participants' habitual total CHO intake was replaced by the study foods (≈ 180 g/day), and the remaining one-third of total CHO was derived from the subjects' habitual diet. Foods with sugar content ≥ 40% of total CHO per portion were classified as high sugar foods, while foods with ≤ 10% total CHO was classified as low sugar foods. Foods with sugar content between 10-40% of total CHO per portion were excluded from study food list.

Dietetic counseling was given to individuals before the run-in diet and at two weekly intervals during the study. Participants were asked to fill in 3-day diet diaries (2 on weekdays and 1 on weekend) at 0, 6 and 12 week. Some instructions for helping participants to fill in the diet diaries, such as reference food portion size photos,
examples of describing type and amount of food, and how to find information on food labels, were provided in the diet diary. An example of a completed diet diary was given as well, and participants were required to provide as much detail as possible about the foods they consumed. Subjects were asked to maintain their habitual level of physical activity throughout the study.

At the end of the first 12 week intervention, participants were asked to come to the Cedar Centre at 7:30 am after an overnight fast, for a 10 hour metabolic study. Participants needed to remain fasting during the study and only water was allowed. The stable isotope, L-[1-\textsuperscript{13}C] leucine (Cambridge Isotopes, 15mg/ml, \textsuperscript{13}C enrichment 99%) was injected intravenously as a priming dose (1 mg/kg) following by a constant infusion (1 mg/kg/h) for 10 hours with an IVAC 60 pump (IVAC, Hampshire, UK). Blood samples were taken at baseline and every hour up to 10 hours for measurement of apoA-I enrichment. The time points 0, 5 and 10 hour were used to measure the plasma lipid and apoA-I concentration. Blood samples for measuring α-KIC were taken at intervals during the study (0, 5, 15, 30, 60, 120, 180, 360, 480 and 600 min).

Subjects were placed on a 4 week wash-out period of their habitual diet before crossing over to the alternative diet for another 12 weeks. At the end of the second intervention, the metabolic study was repeated at the CEDAR Centre (Figure 9). Participants were also sent for magnetic resonance spectroscopy (MRS) at the Hammersmith Hospital to assess their percentage of liver fat at the end of each intervention.
MRS: magnetic resonance spectroscopy; Visit 1, screening visit; Visit 2, liver fat scan; Visit 3/5, start of study; Visit 4/6, kinetic study day

Figure 9: Study design of low and high sugar diet intervention study (Adapted from Ahmad (2012))
2.1.2 Development of the study design for HDL subclass kinetic study (Pilot study)

Pilot studies aimed to develop an optimal blood sampling protocol to measure HDL₂, HDL₃, preβHDL and αHDL metabolism in vivo were carried out.

Ethical approval for the study was obtained from the Surrey NHS Research Ethics Committee, and University of Surrey Ethics Committee. All aspects of the study were explained thoroughly to the subjects, and the subjects were given the opportunity to ask questions. Subjects were provided with detailed information in a participant information sheet (Appendix 2). Consent forms were signed by all the subjects before their participation.

Three healthy women aged 18-70 years were recruited. The subjects were asked to fill in a short questionnaire about their general health (Appendix 3). This questionnaire was for the purpose of confirming that the subjects had stable weight in the past three months and did not have a history of heart disease, diabetes, liver disease, kidney disease, hormone disorders, eating disorders, drug or alcohol abuse, and they did not have any food allergies or intolerances and were not taking any medication known to alter lipid and glucose metabolism, body weight or appetite. They participated in one clinical study that involved five or six visits to the CEDAR Centre, Royal Surrey County Hospital. The first visit lasted for 10 hours, and the other visits took about 15 minutes.

Subjects were required to be fasted overnight (approximately 10-12 hours) for each visit. At Visit 1 they remained in a fasting state during the 10 hour study while a moderate amount of water was allowed. Height and blood pressure were measured.
Weight and body fat were measured by a Tanita Body Composition Analyzer BC-418 MA (Tanita UK Ltd, Yiewsley).

A flexible plastic tube was placed in the antecubital vein in one arm for blood sampling. After taking the baseline blood sample, L- [1-\textsuperscript{13}C] leucine (Cambridge Isotopes, 15mg/ml prepared by St Thomas' Hospital Pharmacy, \textsuperscript{13}C enrichment 99\%) was injected intravenously into the contralateral antecubital vein over 3 minutes as a bolus dose of 5mg/kg (Dwyer et al., 2002). Following the administration of leucine, blood samples were taken frequently in the first hours and then at intervals up to 600 minutes. This visit was identical in all the pilot studies.

A single blood sample was taken in the morning with the subject in a fasted state at the following times - 24, 48, 72 and 120 hours (Day1, Day2, Day3 and Day5) (Visit 2, 3, 4, 5) in the first pilot study for measurement of HDL kinetics. A single blood sample was taken at 24, 48, 72 and 144 hours (Day1, Day2, Day3 and Day6) (Visit 2, 3, 4, 5) in the second pilot study, and at 24, 48, 72, 144 and 240 hours (Day1, Day2, Day3, Day6 and Day10) (Visit 2, 3, 4, 5, 6) in the third pilot study.

2.1.3 HDL subclass kinetics study

2.1.3.1 Subjects

Ethical approval for the study was obtained from the Surrey NHS Research Ethics Committee and University of Surrey Ethics Committee. People who express an interest were sent a participant information sheet (Appendix 4). All aspects of the study were explained thoroughly to the subjects, and the subjects were given the opportunity to ask questions. A signed consent form was obtained from all subjects before their participation (Appendix 5).
Six healthy males and females aged 30-45 with a BMI of 18.5 – 25 kg/m² were recruited for this study. The participants were recruited by advertisement through the website of the University of Surrey (SurreyNet), local hospitals, public and private sector by either poster or email, and through GP Practices. Subjects were asked some questions related to their general health in a phone call. The questions were similar to those on the questionnaire that was used in the pilot study (Appendix 6). Height, weight, blood pressure, waist and hip circumference, and body fat were measured at a screening visit. Blood samples were taken to measure serum insulin, plasma glucose, triglyceride, total cholesterol and HDL cholesterol levels.

Subjects with cardiovascular or endocrine disease (such as unstable ischaemic heart disease), diabetes and diabetic complications, uncontrolled hypertension, hepatic and renal disorders, unstable weight in past three month, or taking any medication known to affect lipid metabolism, glucose metabolism, body weight or appetite, or having hormone therapy were excluded from the study.

2.1.3.2 Study protocol

The study involved 7 visits in total, and all the visits were in the CEDAR Centre, Royal Surrey County Hospital. Subjects were required to be fasted overnight (approximately 10-12 hours) before all the visits. On the first visit, weight, height, BMI and bioelectrical impedance analysis of body composition were measured, followed by a 10 hour study. The dose of L-Leucine [1-13C] leucine (15mg/ml, 13C enrichment 99%) was 8mg/kg and it was injected intravenously over 3 minutes as a bolus after taking a baseline blood sample. Blood samples were taken at 40mins and every hour (except 7 hour and 9 hour) up to 10 hours. Six more single fasting blood samples were taken in the morning during the next 2 weeks, Day1, Day3, Day7, Day9, Day10 and Day14 (Visit 2, 3, 4, 5, 6, 7) for further measurements of leucine enrichment (Figure 10). Blood samples at five time points (baseline, 10h, Day1, Day7, Day14)
were used to measure the concentration of plasma lipids and apoA-I. Blood samples for measuring α-ketoisocaproic acid (α-KIC) which provides an estimation of intracellular leucine enrichment were taken frequently in the first hour (0, 5, 10, 20, 30, 40, 60 min), and then at 1.5h, 2h, 2.5h, 3h, 4h, 5h, 6h, 8h and 10h. Blood samples were taken via sterile syringes and collected into the tubes containing different anticoagulants. Plasma for HDL subclasses separation was collected with EDTA tubes, and plasma for α-KIC was collected with tubes containing heparin and serum for insulin was collected in the tubes containing gel for serum.

Participants in both groups did not need to change their diets or other lifestyle behaviors. However, they were asked to avoid vigorous exercise in the 48 hours before the long study day (Visit 1).

Figure 10: Clinical study protocol and sampling time points
2.2 Analytical methodology

The laboratory procedures for measuring HDL subclass metabolism are according to the method developed by Li et al. (2012).

2.2.1 Materials

The stable isotope L-Leucine [1-\(^{13}\)C] (15mg/ml, \(^{13}\)C enrichment 99%) were 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, acetic acid, diethyl ether, acetonitrile (Far UV), toluene, ethyl acetate, ammonia solution S.G.0.88, (35%), and sodium dodecyl sulphate were obtained from Fisher Scientific UK Ltd (UK). Ethanol (100%) was obtained from Chemistry Store (University of Surrey, UK). Methanol (for HPLC) and Tricine (98%, for biochemistry) were purchased from Agros Organics (UK). Chemical reagents including 3-Methylbutanol, N,N,N’,N’-Tetramethylene-diamine, agarose (Type VI-A), glycine (for electrophoresis ≥99%), phosphate buffered saline (pH 7.4), bromphenol blue, trizma base (Primary standard and buffer ≥99.9%), sodium chloride, ammonium persulfate, trifluoroacetic acid, trifluoroacetic anhyfride, N-tert-butyldimethylsily-N-methyltrifluoroacetiamide (>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).
2.2.2 Summary of laboratory processes to measure leucine enrichment in total HDL, and HDL\(_2\) and HDL\(_3\) apoA-I

2.2.2.1 Separation of plasma

Plasma was separated from blood cells by using centrifugation at 3000 rpm for 10 minutes at 4°C. Plasma samples for the separation of HDL\(_2\) and HDL\(_3\) fractions were stored in a cold cabinet at 4°C overnight and the separation begun the following morning. Plasma samples for the separation of αHDL and preβ HDL, and measurements of α-KIC enrichment, lipids and HDL concentration, glucose and insulin levels, were stored at -80°C until used. Measurement of leucine enrichment in total HDL, HDL\(_2\) and HDL\(_3\) apoA-I required a series of laboratory processes, which are shown in Figure 11.

![Figure 11: Laboratory processes to separate and measure leucine enrichment in total HDL, HDL\(_2\) and HDL\(_3\) apoA-I](image)

- SDS-PAGE: sodium dodecyl sulphate – polyacrylamide gel electrophoresis
- IEC: ion exchange chromatography
- GC-MS: gas chromatography mass spectrometry
2.2.2.2 Removal of VLDL, IDL and LDL

2.2.2.2.1 Isolation of VLDL
Three ml plasma from each sample at the different time points was transferred into labeled ultraclear ultracentrifugation tubes (U/C) (Beckman Instrument, USA). Approximately 1.5 ml of saline containing 0.1% Na$_2$EDTA ($d =$ 1.006 g/ml) was added into each tube to make the total volume 4.5 ml. The volume was adjusted to the upper line on the tube shoulder. The tubes were capped, mixed well, and loaded in the pre-chilled type 50.4 Ti rotor. Samples were spun at 37000 rpm ($147425 \times g$) by an Optima LE-80K ultracentrifuge (Beckman Instrument, USA) for at least 16 hours at 4°C. After ultracentrifugation, approximately 1.2 ml of the top layer containing VLDL was cut by a centrifuge tube cutter (Beckman Instrument, USA) and removed.

2.2.2.2.2 Isolation of IDL and LDL
After removing VLDL, the remaining samples at the bottom of the cut U/C tubes were transferred into the new U/C tubes containing 0.5 ml 1.519 g/ml NaBr solution. The total volume was adjusted to the upper line on the tube shoulder by topping up with saline containing 0.1% Na$_2$EDTA ($d =$ 1.006 g/ml). The tubes were capped, mixed and loaded into the pre-chilled type 50.4 Ti rotor. Samples were spun at 37000 rpm for at least 20 hours at 4°C. After centrifugation, approximately 1.2 ml of the top layer containing IDL and LDL was cut and removed.

2.2.2.3 Separation of total HDL fraction
After cutting VLDL, IDL and LDL, the remaining samples at the bottom of the cut U/C tubes were transferred into the new U/C tubes containing 1.5 ml 1.504 g/ml NaBr solution. The density was adjusted to 1.21 g/ml to make a total volume of 4.5 ml. The final volume was adjusted to the upper line on the tube shoulder. The tubes were capped, mixed well and then loaded into a pre-chilled U/C rotor. The tubes were
placed to make the rotor balanced. The samples were ultracentrifuged at 40000 rpm (172301 × g) for at least 24 hours at 4°C.

After the spinning, the top layer that contained the total HDL fraction was cut by the centrifuge cutter. The cut part of the U/C tube and the well of the cutter were rinsed with saline containing 0.1% Na₂EDTA (d=1.006 g/ml). Both the top layer and the washing solutions were collected in a clean glass tube and stored at -20°C for further analysis. For the samples used to determine the concentration of total HDL fractions, the cut samples and the washing solution were transferred to a 2 ml mini-volumetric flask to make the total volume to 2 ml, and then collected into the clean glass tubes. Samples were kept at -20°C until used.

2.2.2.4 Separation of HDL₂ and HDL₃ fractions

2.2.2.4.1 Separation of HDL₂ fractions

After removal of IDL and LDL, the remaining samples at the bottom of the cut U/C tubes were transferred to new U/C tubes to which had been added 1.5 ml 1.249 g/ml NaBr density solution. The total volume was adjusted to the upper line on the tube shoulder. The tubes were capped, mixed, put into a pre-chilled 50.4 Ti rotor and spun at 40000 rpm (172301 × g) for at least 48 hours at 4°C. After ultracentrifugation the top layer (HDL₂ fraction), approximately 1.2 ml, was cut by the centrifuge cutter. The cut part of the U/C tube and the well of the cutter were washed with saline containing 0.1% Na₂EDTA (d= 1.006 g/ml). Both the cut samples and the washing solutions were collected in duplicate in two clean glass tubes and stored at -20°C until used.

For the samples used to measure the concentration of HDL₂ fractions, the top layer was collected into a 2 ml mini-volumetric flask (Sigma-Aldrich Ltd, UK) using a plastic pipette. The cut part of the U/C tube and the well of the cutter were washed with
saline containing 0.1% Na₂EDTA (d= 1.006 g/ml). The washing solution was transferred to the volumetric flask as well to make the total volume to 2 ml. The samples were then mixed well, placed into two new glass tubes and stored at -20°C until further analysis.

2.2.2.4.2 Separation of HDL₃ fractions
After removing the HDL₂ fraction, the remaining sample at the bottom of the cut U/C tube was transferred into a new U/C tube to which had been added 1.5 ml 1.38 g/ml NaBr density solution. The volume was adjusted to the upper line on the tube shoulder. The tubes were capped, mixed, and then loaded into the rotor, and spun at 40000 rpm (172301 × g) for at least 48 hours at 4°C. When the centrifugation had finished, the top layer containing the HDL₃ fraction was cut. The cut part of U/C tube and the well of the cutter were rinsed with saline containing 0.1% Na₂EDTA (d= 1.006 g/ml). The top layer and the washing solutions were collected in duplicate in new glass tubes. Samples were kept at -20°C for further analysis.

For those samples used to measure the concentration of the HDL₃ fraction, the top layer was transferred into a 2 ml mini-volumetric flask (Sigma-Aldrich Ltd, UK). After rinsing the cut part of the U/C tube and the well of the cutter with saline containing 0.1% Na₂EDTA (d= 1.006 g/ml), all the washing solutions were transferred to the volumetric flask to make the total volume to 2 ml. The samples were then mixed well, placed in two new glass tubes and stored at -20°C until further laboratory analysis.

2.2.2.5. Delipidation of total HDL, HDL₂ and HDL₃ fractions
Methanol and diethyl ether were precooled at 4°C for at least 30 minutes. Four hundred µl HDL fraction was added into 4 ml methanol. Four ml diethyl ether was then added into each sample and mixed vigorously. The samples were centrifuged at
4000 rpm (3684 × g) for 30 minutes at 1°C. The solvent was decanted gently. Another 4 ml diethyl ether was added into each sample and vortexed. Samples were centrifuged at 4000 rpm (3684 × g) for 20 minutes at 1°C. The diethyl ether was subsequently decanted and the tubes were left upside down to dry the sample at room temperature for about 30 minutes. Proteins were dissolved in 70 μl sample buffer (pH6.8) for SDS-PAGE. Samples were stored in the freezer at -20°C.
2.2.3 Two-Dimensional Electrophoresis

2.2.3.1 Summary of laboratory processes to measure leucine enrichment in preβHDL and αHDL apoA-I

Measurement of leucine enrichment of αHDL and preβHDL apoA-I required a series of steps as shown in Figure 12.

![Diagram](Image)

- SDS-PAGE: sodium dodecyl sulphate – polyacrylamide gel electrophoresis
- IEC: ion exchange chromatography
- GC-MS: gas chromatography mass spectrometry

Figure 12: Laboratory processes to separate and measure leucine enrichment in αHDL and preβHDL apoA-I

2.2.3.2 First dimension: Agarose gel electrophoresis

αHDL and preβHDL in plasma were separated by agarose gel electrophoresis (Figure 13A and 13B). Agarose gel at a concentration of 0.6% was prepared. Agarose powder (Sigma, UK) was dissolved in tris-tricine buffer (25mM, pH8.6) by heating in a microwave. The gel was left to cool down until the temperature was approximately 60°C and was then transferred into the flat-bed gel cassette. Two
hundred and sixty µl plasma was diluted with 65µl tris-tricine sample buffer (125mM, pH8.6) (4:1 v:v) (325 µl in total), and 300 µl of the mixture (equal to 240 µl plasma) was loaded onto the agarose gel. Albumin (bovine serum ≥96%) (Sigma, USA) and apoA-I (from human plasma, ≥85%, 3.25 mg protein/ml) were loaded to identify the position of the αHDL and preβ HDL bands. Albumin runs in the same position as αHDL while apoA-I is a marker for preβHDL. The gel was run in tris-tricine buffer at 170 V for 3 hours. The gel was subsequently stained with 0.125% coomassie blue R250 staining solution overnight. Excessive dye was removed by destaining with methanol/acetic acid/H₂O solution (5:1:3 v:v:v). αHDL and preβ HDL bands separated on agarose gel were excised and then dried on the freeze dryer (ModulyoD Freeze Dryer, Thermo Fisher Scientific Inc, Waltham, MA, USA) overnight.

Figure 13 A: Separation of αHDL and preβHDL by agarose gel electrophoresis with albumin as a standard
2.2.3.3 Extraction of proteins

Freeze dried gel was incubated in 3 ml 1% SDS solution for 2 hours at room temperature. The supernatant solution was collected into a 10 ml round bottom glass tube. The gel was repeatedly washed with 3 ml 0.2% SDS solution three times for αHDL and twice for preβ HDL. All the washing solution was transferred to the 10 ml glass tube and frozen in a freezer at -20°C overnight. Samples were dried on the freeze dryer and stored in the freezer at -20°C for further analysis.

2.2.3.4 Precipitation and delipidation

Ethanol and diethyl ether were precooled on ice for at least 30 minutes. Four ml ethanol was added into the HDL sample extracted from agarose gel and mixed well. The tubes were kept on ice for 1 hour to precipitate the proteins and then centrifuged at 4500 rpm (4662 × g) for 1 hour at 1°C. The blue supernatant containing coomassie blue was poured out gently. Four ml diethyl ether was added into each sample and then centrifuged at 4500 rpm (4662 × g) for another 1 hour. Diethyl ether was then
poured out gently. The tubes were left upside down to dry at room temperature for 1 hour for αHDL and 30 minutes for preβ HDL. Proteins were dissolved in 70 μl sample buffer (pH6.8) for SDS-PAGE and the samples were stored in the freezer at -20°C.
2.2.4 Separation of apoA-I by SDS-PAGE

ApoA-I in the HDL fractions was isolated by Sodium dodecyl sulphate – Polycrylamide gel electrophoresis (SDS-PAGE) (Figure 14, 15 and 16). Two layers of discontinuous SDS-PAG were prepared. The resolving gel (10%) was prepared using tris base-SDS buffer (tris base 0.75M, SDS 0.2%, pH8.8), and the stacking gel (4%) using tris base-SDS buffer (tris base 0.25M, SDS 0.2%, pH6.8). The stacking gel has the ability to enhance the resolution of protein separation and sharpen the sample bands on the resolving gel. Two gels were cast in a PROTEAN II gel cast (Bio-Rad, USA). Samples in sample buffer (containing SDS) were heated at 110°C for 5 minutes and subsequently cooled down on ice. SDS in the sample buffer can linearise the proteins after heating and give the protein a negative charge. Following loading the samples on SDS-PAG, the gel was run at 80v for 16 hours. Known molecular weight standards, prestained and unstained standards, and human apoA-I standard were loaded to identify the position of the apoA-I bands. Prestained standard is able to show the colours without staining, which was used to check whether the SDS-PAGE work normally or not after overnight running, while unstained standard will be visible only after the staining process.

![Figure 14: Separation of total HDL apoA-I on SDS-PAGE](image)
Figure 15: Separation of HDL$_2$ and HDL$_3$ apoA-I on SDS-PAGE. (A: HDL$_2$ apoA-I bands separation for five different time points; B: HDL$_3$ apoA-I bands separation for six different time points)

Figure 16: Separation of αHDL and preβHDL apoA-I on SDS-PAGE. (A: αHDL apoA-I bands separation for five different time points; B: preβHDL apoA-I bands separation for five different time points)
Gels were stained to develop the images by using a silver stain kit. The gel was firstly fixed in fixative enhancer solution (methanol, acetic acid, fixative enhancer concentration, H\textsubscript{2}O, 5:1:1:3 v:v:v) for 20 minutes. After washing the gel with nanopure H\textsubscript{2}O twice for 20 minutes in total, the silver staining solution was added to stain the gel for 10-20 minutes. Acetic acid (5\%) was added for 15 minutes to stop the staining reaction when the ideal image had appeared. Nanopure H\textsubscript{2}O was used to eliminate the excessive acetic acid. HDL apoA-I bands were excised for further analysis.

2.2.5 Hydrolysis of apoA-I

The apoA-I bands separated by SDS-PAGE were transferred to round bottom glass tubes. One ml 6M HCl was added to each sample, and the tubes were heated on a heating block at 120°C for 24 hours to hydrolyse apoA-I to amino acids.

2.2.6 Ion exchange chromatography

Amino acids from hydrolysed 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. In terms of cation exchange chromatography, which was used, positively charged amino acids in 6M HCl (at low pH) bind to the negatively charged solid support resin. To elute the amino acids, ammonium hydroxide (NH\textsubscript{4}OH) was added to increase the pH of the mobile phase, therefore the ionic interaction between amino acids and the resin were weakened.

AG50W – X8 cation exchange resin was weighed (1g/sample) and washed with nanopure H\textsubscript{2}O twice in the beaker. Following washing with 2M NH\textsubscript{4}OH (1:2 v:v) to
remove any possible contamination, resin was rinsed with nanopure H$_2$O until the pH of the eluted water was 7. The resin was then washed with 1M HCl (1:1 v:v). Nanopure H$_2$O was added to rinse the resin until the pH of the eluted water was 7, and the resin was ready for the columns to purify amino acids. Nanopure H$_2$O was added to resin (1:1 v:v) and stirred. Two ml of the mixture (containing 1g resin) was transferred to polypropylene columns (1 column/sample). Samples from hydrolysis were loaded to each column. Each hydrolysis tube was subsequently washed with 5 ml nanopure H$_2$O, and then the washing solution was transferred to the columns. The column was rinsed with 5 ml nanopure H$_2$O 4 times until the pH of the eluted water reached 7. Three ml 4M NH$_4$OH was added into each column and the eluted solution was collected in a new glass tube. Samples were frozen at -20°C overnight and then freeze dried on a freeze dryer.

2.2.7 Derivatization

Freeze dried samples containing purified amino acids need to be derivatized before the determination of $^{13}$C leucine enrichment by gas chromatography mass spectrometry (GC-MS). Leucine was converted into a volatile and thermally stable oxazolinone derivative (Figure 17). The oxazolinone derivative was analysed by negative ion chemical ionization GC-MS. Fifty μl of trifluoroacetic anhydride (TFAA) and 50 μl of trifluoroacetic acid (TFA) (1:1 v:v) was added separately to each sample. Samples were capped with Teflon lined caps and heated at 110°C for 5 minutes. Five hundred μl toluene and 1 ml nanopure H$_2$O were added to samples and mixed vigorously. Samples were then centrifuged at 2500 rpm (1439 × g) for 10 minutes at 4°C. The toluene layer at the top was removed and transferred to GC vials for GC-MS analysis.
2.2.8 Determination of leucine isotope enrichment by GC-MS

2.2.8.1 Leucine standard curve

It needed to be confirmed that the detector was functioning properly before analyzing any samples on the GC-MS. A range of standards with different enrichment was prepared to determine the sensitivity and linearity of GC-MS. L-$^{12}$C-leucine and L- [1-$^{13}$C] leucine were used for the preparation of standards. The standards were derivatized and analysed by GC-MS.

The preparation of leucine standard curve is shown in Table 6. The standard curve is demonstrated as the measured $^{13}$C/$^{12}$C peak area ratio plotted against the theoretical enrichment. A linear relationship ($R^2=0.999$) was indicated by a slope approximating to unity (1.0004) (Figure 18).
Table 6: Preparation of leucine standard curve (n=6)

<table>
<thead>
<tr>
<th>Concentration of $^{13}$C leucine (µg/µl)</th>
<th>Concentration of $^{12}$C leucine (µg/µl)</th>
<th>Theoretical $^{13}$C/$^{12}$C ratio</th>
<th>Observed $^{13}$C/$^{12}$C ratio (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>4.33</td>
<td>0.09159 ± 0.0008</td>
</tr>
<tr>
<td>2</td>
<td>0.004579</td>
<td>4.33</td>
<td>0.09248 ± 0.0008</td>
</tr>
<tr>
<td>3</td>
<td>0.009157</td>
<td>4.33</td>
<td>0.09402 ± 0.0008</td>
</tr>
<tr>
<td>4</td>
<td>0.018314</td>
<td>4.33</td>
<td>0.09577 ± 0.0008</td>
</tr>
<tr>
<td>5</td>
<td>0.027471</td>
<td>4.33</td>
<td>0.09807 ± 0.0007</td>
</tr>
<tr>
<td>6</td>
<td>0.036628</td>
<td>4.33</td>
<td>0.10017 ± 0.0007</td>
</tr>
<tr>
<td>7</td>
<td>0.045785</td>
<td>4.33</td>
<td>0.10240 ± 0.0009</td>
</tr>
</tbody>
</table>

$^{13}$C/$^{12}$C ratio: the concentration ratio of L-$^{12}$C-leucine and L-$[1^{-13}]$C leucine

Figure 18: Leucine standard curve (n=6). (Results are presented as mean ± SEM)

2.2.8.2 Measurement of $^{13}$C leucine isotope enrichment

The samples were analyzed by a GC-MS (7890A GC system, Agilent 5975C inert XL EI/Cl MSD, Agilent Technologies, Wokingham, Berkshire, UK). Samples were loaded in sequential order and injected with an Agilent 7683 autosampler. The GC was equipped with a capillary column with 30 meter 0.25 mm inner diameter, 0.25µm (J&W Scientific, Inc CA, USA) using helium as the carrier gas. The GC-MS was
operated in a negative chemical ionization (NCI) mode using methane as the reagent gas. The initial temperature of GC oven was 50°C for 1 minute and the ramp was 6°C/minute to 90°C, 30°C/minute to 280°C. The oxazolinone derivative of 12C leucine and 13C leucine have a molecular mass of 209 and 210 respectively, and the analysis of oxazolinone derivative of 12C leucine and 13C leucine would enable to measure apoA-I leucine enrichment.

2.2.9 Determination of α-KIC isotope enrichment

Plasma samples for measuring α-KIC isotope enrichment were kept at -80°C until analysis. After thawing, plasma was vortexed and then centrifuged at 2500 rpm (1439 × 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 (1439 × g) for 10 minutes at 4°C. The supernatant was transferred to new vials by glass pipettes and dried under oxygen free nitrogen (Nitroflow, Parker Filtration and separation, Parker Hannifin Ltd, Maidston, UK) at 50°C. The remainder was dissolved in a mixture of 200 μl H2O and 100 μl O-phenylenediamine (2% in 4M HCl), and put 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. The extraction process was repeated by adding another 1 ml ethyl acetate. The extracts were dried over sodium sulphate followed by evaporation under oxygen free nitrogen at room temperature. The remainder was derivatized by 100 μl acetonitrile and 100 μl N-Methyl-N-(tertbutyldimethylsilyl) trifluoroacetamide with 1% trimethylcholorsilane (MTBSTFA). Samples were put on a heating block at 120°C for 45 minutes. The excess derivatising reagent was removed by oxygen free nitrogen at
room temperature. One hundred µl decane was added to the derivative before loading to the GC-MS.

The preparation of KIC standard curve is shown in Table 7. The derivative of D1 and Do have a molecular mass of 259 and 260 respectively. Therefore, α-KIC isotope enrichment (Figure 19) was measured by El GC-MS by the selected ion monitoring of fragments at m/z 259 (m) and 260 (m+1). Chromatographic peaks were determined, and area ratio of α-KIC (13C/12C) was calculated. The α-KIC isotope enrichment was presented as APE.

Table 7: Preparation of KIC standard curve (n=6)

<table>
<thead>
<tr>
<th></th>
<th>Concentration of D1 (µg/µl)</th>
<th>Concentration of Do (µg/µl)</th>
<th>Theoretical 260/259 ratio</th>
<th>Observed 260/259 ratio (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>2.40</td>
<td>0</td>
<td>0.2259 ± 0.0009</td>
</tr>
<tr>
<td>2</td>
<td>0.15</td>
<td>2.40</td>
<td>0.0625</td>
<td>0.2900 ± 0.0012</td>
</tr>
<tr>
<td>3</td>
<td>0.30</td>
<td>2.40</td>
<td>0.1250</td>
<td>0.3571 ± 0.0073</td>
</tr>
<tr>
<td>4</td>
<td>0.45</td>
<td>2.40</td>
<td>0.1875</td>
<td>0.4178 ± 0.0060</td>
</tr>
<tr>
<td>5</td>
<td>0.90</td>
<td>2.40</td>
<td>0.3750</td>
<td>0.6019 ± 0.0042</td>
</tr>
<tr>
<td>6</td>
<td>1.20</td>
<td>2.40</td>
<td>0.5000</td>
<td>0.7213 ± 0.0123</td>
</tr>
<tr>
<td>7</td>
<td>1.60</td>
<td>2.40</td>
<td>0.6667</td>
<td>0.8870 ± 0.0097</td>
</tr>
<tr>
<td>8</td>
<td>1.80</td>
<td>2.40</td>
<td>0.7500</td>
<td>0.9682 ± 0.0108</td>
</tr>
</tbody>
</table>

259/260 ratio: the concentration ratio of D1 and Do, D1: 4-Methyl-2-Oxopentanoic-1-13C acid Sodium Salt; Do: 4-Methyl-2-Oxopentanoic acid Sodium Salt
2.2.10 Measurement of triglyceride, cholesterol and HDL concentration

The concentration of triglyceride (TG) in the plasma were determined by the ABX Mira analyzer (Horiba ABX, Northampton, UK). The TG assay kit was used and the measurement was based on the enzymatic photometric method. After the enzymatic reactions, the colorimetric indicator which was quinoneimine was detected by colorimetry. The N (low) and P (high) QCs were 1.23 and 2.29 mmol/L, and CVs were 1.98% and 2.23% respectively (n=3).

The concentration of cholesterol in the plasma was measured by the ABX Mira analyzer using an enzymatic colorimetric method. A total cholesterol assay kit (Horiba ABX, Northampton, UK) was needed for the determination. QCs were prepared with N (low) and P (high) concentration (2.28 and 4.81 mmol/L), and CVs were 3.53 % and 1.45% respectively (n=3).

Plasma HDL-Cholesterol was measured by the ABX Mira analyzer as well. The method is based on accelerating the reaction of cholesterol oxidase (CO) with non-HDL unesterified cholesterol and dissolving HDL selectively using specific detergent,
Accelerator Selective Detergent. Two reagents from Horiba ABX (Northampton, UK), which are subject to the enzyme reaction, the solubilization of HDL specifically and to develop color for the quantitative determination of HDL-C, were used. QCs were prepared with N (low) and P (high) concentration (0.83 and 1.82 mmol/L), and CVs were 8.48% and 7.35% respectively (n=3).

2.2.11 Measurement of glucose and insulin concentration, and HOMA

Plasma glucose concentration was measured by a glucose analyzer (YSI 2300 STAT Plus. Analytical @technologies, UK). Each sample was measured twice, and the average value was used.

Plasma insulin was measured by using a double antibody radioimmunoassay. Fifty μl plasma was incubated with 450 μl of the first antibody, guinea pig anti-insulin serum (GP 8) with tracer $^{125}$I-$A_{14}$-tyr-monoiodo-insulin (Amersham Pharmacia Biotech, UK), at room temperature overnight. One hundred μl of the second antibody, a solid phase sheep-anti-guinea pig serum antibody (Pharmacia Decanting Suspension, UK) was added to the sample and incubated for 2 hours. After adding 1 ml of H$_2$O, the samples were spun at 1500 rpm for 45 minutes at 4 °C. Then, the supernatant was decanted and the precipitate was counted on a gamma counter (Wallac Wizard 1470, UK).

The homeostasis model assessment (HOMA) was calculated from fasting glucose and insulin concentration by using the HOMA2 calculator, which was downloaded from the University of Oxford website: (http://www.dtu.ox.ac.uk/homacalculator/download.php).
2.2.12 Determination of the lipase activity in plasma

The activity of the total and hepatic lipase was analysed by a continuous fluorometric lipase test (Conflulip lipase test, Progen, Biotechnik Gubh, Heidleberg, Germany) via assessing the capacity of triglyceride hydrolysis in plasma samples. A lipase substrate, which is a triglyceride comprised of pyrene fluorescence, is included in the test kit. By adding the active lipase, the lipase substrate was hydrolysed and the intensity of pyrene fluorescence, which is related to lipase activity in the samples, was detected.

2.2.13 Measurement of apoA-I concentration in plasma and HDL$_2$ and HDL$_3$ fractions

Apolipoprotein A-I concentrations in plasma, HDL$_2$ and HDL$_3$ fractions were measured by an automatic ABX Mira analyzer (Horiba ABX, France) according to an immunoturbidimetric method. Reagents, ABX Pentra Apo AI (a fraction of purified immunoglobulins from rabbit antiserum) and immunogen (apoA-I from human HDL) were needed for the assay. ApoA-I reacts with its specific antibody and generates the immune complexes, scattering a beam of light through the sample. The proportion of apoA-I concentration in the samples is represented as the intensity of the scattered light. The low and high quality controls (QCs) were 1.137 and 2.424 mg/ml, and inter-assay coefficient of variations (CV) were 7.531% and 3.173% respectively (n=5).

2.2.14 Measurement of αHDL and preβ HDL apoA-I concentration: Western blotting

After separation of αHDL and preβ HDL on agarose gel electrophoresis, the agarose gel was covered with a 0.2 μm nitrocellulose membrane, filter paper and fibre pad to make a sandwich. The sandwich was then placed into a plastic cassette filled with
transfer buffer (pH8.3). The gel was on the negative polarity and the membrane was on the positive polarity. Following 24 hours electrophoretic transfer at a constant voltage (30V), the αHDL and preβ HDL bands were transferred to the membrane. The membrane was incubated in a blocking buffer containing 5% dried non-fat milk, 0.12% Tris base, 0.58% NaCl and 0.5% Tween 20 (pH7.5) for 1 hour at room temperature. After washing with 100 ml phosphate buffered saline containing 0.05% Tween 20 (PBST, pH7.4) three times, each time for 10 minutes, the membrane was incubated with the primary antibody (Goat polyclonal antibody to human apoA-I, Abcam, UK) at room temperature for 1 hour. The excess primary antibody was washed off with 100 ml PBST for 10 minutes three times, and the membrane was incubated with the secondary antibody (Donkey polyclonal antibody to goat IgG, H&L, Abcam, UK) at room temperature for 1 hour. The excess secondary antibody was washed off again with 100 ml PBST for 10 minutes three times. αHDL and preβ HDL bands were visualised on the membrane by staining with AP (alkaline phosphatase) staining reagent for 1 minute (Figure 20). The reaction was stopped by adding nanopure H₂O.

The membrane was subsequently put on the Calibrated Imaging Densitometer (Model GS-800, Bio-Rad, the United States) and scanned. The scanned image was created and analysed using Quantity One (Version 4.5.2, Bio-Rad Laboratories, the United States). The bands of αHDL and preβ HDL were selected as a rectangle shape on the image, and the volume of each band was quantified as the sum of the intensities of the pixels inside the volume boundary multiplied by the area of a single pixel (the area of the band) (mm²). The background intensity of each selected band was identified by Quantity One automatically, and the adjusted volume (Adj. Vol) (volume minus the background volume) was used to calculate the percentage of αHDL and preβ HDL in each sample. The formula is as follows:
\[ \alpha\text{HDL}\% = \frac{\text{Adj. Vol of } \alpha\text{HDL}}{\text{Adj. Vol of } \alpha\text{HDL} + \text{Adj. Vol of } \text{pre}\beta\text{HDL}} \]

\[ \text{pre}\beta\text{HDL}\% = 1 - \alpha\text{HDL}\% \]

The concentration of \( \alpha\text{HDL} \) and \( \text{pre}\beta \text{HDL} \) was calculated as:

\[ \alpha\text{HDL concentration} = \alpha\text{HDL} \% \times \text{total apoA-I} \]

\[ \text{pre}\beta\text{HDL concentration} = \text{pre}\beta\text{HDL} \% \times \text{total apoA-I} \]

![Western blotting: Measurement of the percentage of \( \alpha\text{HDL} \) and \( \text{pre}\beta\text{HDL} \)](image)

**Figure 20:** Western blotting: Measurement of the percentage of \( \alpha\text{HDL} \) and \( \text{pre}\beta\text{HDL} \)

### 2.2.15 Data analysis

After all the apoA-I samples had been run on the GC-MS, the area under the \( m/z \) 210 peak and \( m/z \) 209 peak were recorded, and the ratio of \( m/z \) 210/209 at each time point was calculated. The ratio was then converted to atom percent excess (APE), which is a measure of isotope enrichment taking into account the baseline isotopic enrichment.

The APE for each time point was calculated by the following equation:

\[ \text{APE} (%) = \left[ \frac{(R_t - R_0)}{(R_t - R_0 + 1)} \right] \times 100 \]

*Equation 1*
Where \( R_t \) and \( R_0 \) represent the isotope area ratios of \( m+1/m \) (210/209) for sample at time \( t \), and baseline sample at time zero (before the injection of the isotope) respectively. The KIC APE was calculated using the isotope area ratio of \( m/z \) 260/259.

For the dietary intervention study, which used a primed bolus injection followed by a constant infusion of stable isotope (Chapter 3), the 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).

\[
\text{FSR (pools/day)} = \frac{(\text{Slope of the apoA-I APE time curve}/\text{KIC}_{\text{APE}})}{24} \quad \text{Equation 2}
\]

where plasma \( \alpha \)-ketoisocarproate (KIC\(_{\text{APE}}\)) is the measurement of the precursor pool enrichment of hepatic intracellular leucine. 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. ApoA-I pool size was calculated from the concentration of apoA-I and plasma volume (PV) divided by body weight. PV was calculated as shown below:

\[
\text{PR (mg/kg/day)} = \text{FSR} \times \text{HDL apoA-I pool size} \quad \text{Equation 3}
\]

\[
\text{ApoA-I pool size (mg/kg)} = \text{HDL apoA-I concentration} \times \text{PV} / \text{BW} \quad \text{Equation 4}
\]

For males, \( \text{PV (ml)} = 1578 \times S \quad \text{Equation 5} \)

For female, \( \text{PV (ml)} = 1395 \times S \quad \text{Equation 6} \)

Where \( S \) is surface area (m\(^2\))

\[
S (\text{m}^2) = \text{BW}^{0.425} \times \text{Height}^{0.725} \times 0.007184 \quad \text{Equation 7}
\]

where \( \text{BW} \) is body weight (kg), height (cm).

For the HDL subclasses study, which used a bolus injection of stable isotope, the apoA-I enrichment (APE) of HDL\(_2\), HDL\(_3\), \( \alpha \)HDL and pre\( \beta \)HDL for time points after the 10-hour study, which are Day1, Day3, Day7, Day9, Day10 and Day 14, was plotted
using a natural logarithmic scale. A line of best fit was fitted and the equation for this curve fit provided the slope of the curve. The slope is equal to FCR (pools/hour). An example is shown in Figure 21. The values of FCR in pools/hour were multiplied by 24 to express FCR in pools/day for HDL₂, HDL₃, αHDL and preβHDL. PR for HDL subclasses was then calculated by using Equation 3. ApoA-I pool size, plasma volume and surface area were calculated by using Equation 4, 5, 6 and 7 respectively. The area under the curve (AUC) of leucine enrichment data for αHDL, preβHDL, HDL₂ and HDL₃ during the first 10 hour study and the rest of days in the following two weeks was calculated automatically by SigmaPlot (Version 12.3, Systat Software Inc, USA).

![Figure 21](image_url)

**Figure 21: An example of the determination of FCR (pools/h) from the slope of a line of best fit**

It was hoped that a mathematical model developed by the Department of Mathematics, University of Surrey, could be used to determine HDL subclass kinetics. Two models were developed, one to describe αHDL and preβHDL, and one to describe HDL₂ and HDL₃. The model structures for αHDL and preβHDL, and for HDL₂ and HDL₃ shown in Figure 22 and Figure 23 respectively were based on models proposed previously (Fisher et al., 1995). The models were used to fit the leucine enrichment curve for αHDL, preβHDL, HDL₂ and HDL₃. However, the model
structures did not allow a good fit to all the data, suggesting the model structure needed to modification. Therefore, other model structures need to be explored in the future.

Figure 22: The model structure for αHDL and preβHDL (k = rate constant)

Figure 23: The model structure for HDL₂ and HDL₃ (m = rate constant)
Statistics analysis

The results are presented as mean ± SEM. Microsoft Excel 2003 was used to analyze data and create graphs and curves. Statistical analyses were performed by using Statistical Package for the Social Sciences (SPSS) version 22 (IBM Corporation, New York, USA). A paired two-tailed t-Test was used to analyse the differences in HDL subclass variables, and between the high and low sugar diet of all variables. An unpaired t-Test was used to compare HDL subclass kinetics between genders. The associations between variables were determined by calculating Pearson’s correlation. In the all comparisons, $P<0.05$ was considered statistically significant.
Chapter 3: The effect of a high and low non-milk extrinsic sugar diet on HDL kinetics

3.1 Introduction

This dietary intervention study was part of a BBSRC funded trial to investigate the effects of dietary sugar on lipoprotein kinetics. In this thesis, the association between dietary sugar intake and HDL metabolism is investigated. It also addresses the influence of dietary sugar consumption on several cardio-metabolic risks, such as body weight, fasting plasma lipids, glucose and insulin.

The study was a controlled, randomized crossover trial. Six Caucasian middle-aged men were recruited, and they underwent two 12-week dietary interventions with high and low sugar diets. The aim was for the macronutrient content of the two diets to be the same with the only difference being in the content of non-milk extrinsic sugars, high or low non-milk extrinsic sugar. The dietary intake of the subjects was measured by 3-day diet diaries. Anthropometrics, fasting plasma lipids and lipoproteins, glucose and insulin levels were measured at the end of the interventions. The assessment of HDL kinetics using a stable isotope technique was also measured at the end of each dietary intervention. L-[1-13C] leucine isotope was given intravenously as a priming dose of 1 mg/kg followed by a constant infusion (1 mg/kg/h) for 10 hours. Blood samples were taken at intervals during the study. The total HDL fraction was separated from plasma by using ultracentrifugation, and apoA-I in the fractions was isolated via SDS-PAGE. After the purification and derivatization, the leucine enrichment of apoA-I was finally determined by GC-MS (Chapter 2, section 2.2.8).
3.2 Results

3.2.1 Subjects characteristics and lipid profile

The characteristics of subjects at screening in the study are shown in Table 8. Six male subjects were aged between 49 to 65 years old. Subjects 01, 02, 03 and 05 were overweight, and subjects 04 and 06 were borderline obese. According to the categories defined by the American Heart Association (AHA, 2014), only subject 06 had a normal systolic blood pressure (less than 120 mmHg), while other subjects were at the prehypertension levels (120-139 mmHg). The diastolic blood pressure of subjects 03, 05 and 06 were in the normal range (less than 80 mmHg), and subjects 01 and 02 were in the prehypertension range (80-89 mmHg). The diastolic blood pressure of subject 04 was in high blood pressure stage 1 category (90-99 mmHg). In terms of liver fat, subjects 03 and 04 had low liver fat (<5.0% liver fat) while the other subjects had moderately enhanced liver fat.

Table 8: Subject characteristics at screening (n=6)

<table>
<thead>
<tr>
<th>Subject</th>
<th>01</th>
<th>02</th>
<th>03</th>
<th>04</th>
<th>05</th>
<th>06</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58</td>
<td>49</td>
<td>58</td>
<td>50</td>
<td>65</td>
<td>63</td>
<td>57.2 ± 2.7</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.78</td>
<td>1.75</td>
<td>1.83</td>
<td>1.75</td>
<td>1.66</td>
<td>1.77</td>
<td>1.76 ± 0.02</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>86.4</td>
<td>88.1</td>
<td>95.3</td>
<td>92.7</td>
<td>78.7</td>
<td>96.6</td>
<td>89.63 ± 2.72</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.3</td>
<td>28.9</td>
<td>28.3</td>
<td>30.3</td>
<td>28.6</td>
<td>30.8</td>
<td>29.03 ± 0.54</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>127</td>
<td>127</td>
<td>124</td>
<td>135</td>
<td>121</td>
<td>118</td>
<td>125.33 ± 2.40</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>80</td>
<td>87</td>
<td>76</td>
<td>91</td>
<td>75</td>
<td>76</td>
<td>80.83 ± 2.73</td>
</tr>
</tbody>
</table>

BP: blood pressure
3.2.2 Energy and macronutrient intakes of the high and low sugar diet

The mean of total energy and macronutrient intakes from the habitual, the high and low sugar diets are presented in Table 9. The dietary data of subject 02 was not available, so the mean ± SEM values were calculated from the other five subjects.

No significant differences were found in total energy, carbohydrate, protein, total fat and saturated fat intakes between the habitual and high sugar diet, habitual and low sugar diet. The differences in fibre intake between the habitual and high sugar diet was on the borderline of being significant \( (p = 0.051) \), whereas no significant difference was found between the habitual and low sugar diet. Sugar consumption \( (g/d) \) was significantly different between the habitual and low sugar diet \( (p = 0.006) \) but not between the habitual and high sugar diets. However, there was a significant difference in the percentage of energy intake from sugar between the habitual and high sugar diet, and the habitual and low sugar diet \( (p = 0.048 \) and \( p = 0.012 \) respectively).

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Habitual diet</th>
<th>High sugar diet</th>
<th>Low sugar diet</th>
<th>Paired T-test (Low vs. High)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy (MJ/d)</strong></td>
<td>10.3 ± 1.1</td>
<td>10.4 ± 1.2</td>
<td>11.2 ± 1.0</td>
<td>( p = 0.548 )</td>
</tr>
<tr>
<td><strong>CHO (g/d)</strong></td>
<td>270.7 ± 33.0</td>
<td>317.6 ± 26.3</td>
<td>278.7 ± 30.7</td>
<td>( p = 0.395 )</td>
</tr>
<tr>
<td>% Total energy</td>
<td>44.3 ± 2.3</td>
<td>53.5 ± 5.5</td>
<td>41.6 ± 2.8</td>
<td>( p = 0.084 )</td>
</tr>
<tr>
<td><strong>Sugar (g/d)</strong></td>
<td>115.3 ± 13.9</td>
<td>173.9 ± 14.0</td>
<td>60.2 ± 9.6*</td>
<td>( p = 0.007 )</td>
</tr>
<tr>
<td>% Total energy</td>
<td>19.4 ± 2.7</td>
<td>30.0 ± 4.5*</td>
<td>8.9 ± 1.1*</td>
<td>( p = 0.012 )</td>
</tr>
<tr>
<td><strong>Protein (g/d)</strong></td>
<td>88.6 ± 7.1</td>
<td>90.2 ± 13.1</td>
<td>116.0 ± 14.5</td>
<td>( p = 0.047 )</td>
</tr>
<tr>
<td>% Total energy</td>
<td>14.7 ± 0.8</td>
<td>14.4 ± 0.8</td>
<td>17.5 ± 1.5</td>
<td>( p = 0.076 )</td>
</tr>
<tr>
<td><strong>Fat (g/d)</strong></td>
<td>89.9 ± 12.7</td>
<td>75.8 ± 22.8</td>
<td>104.1 ± 14.9</td>
<td>( p = 0.173 )</td>
</tr>
<tr>
<td>% Total energy</td>
<td>32.7 ± 1.7</td>
<td>25.9 ± 5.7</td>
<td>34.5 ± 2.6</td>
<td>( p = 0.163 )</td>
</tr>
<tr>
<td><strong>Saturated fat (g/d)</strong></td>
<td>37.6 ± 4.8</td>
<td>33.1 ± 11.1</td>
<td>44.8 ± 9.6</td>
<td>( p = 0.211 )</td>
</tr>
<tr>
<td><strong>Fibre (g/d)</strong></td>
<td>27.7 ± 3.9</td>
<td>20.9 ± 2.6</td>
<td>28.2 ± 4.4</td>
<td>( p = 0.132 )</td>
</tr>
</tbody>
</table>

Data are presented in mean ± SEM (n=5). CHO: carbohydrate. * Significantly different from habitual diet \( p < 0.05 \).
There were no significant differences in the intakes of total energy, carbohydrate, total fat, saturated fat and fibre between the high sugar and low sugar diet. Although there was a statistically significant difference in the amount of protein consumption (g/d) \( (p = 0.047) \), the distribution of protein in total energy was not significantly different. The quantity of sugar intake and its percentage of total energy on the high and low sugar diet were both statistically different \( (p = 0.007 \text{ and } p = 0.012 \text{ respectively}) \). The mean intake of energy on both the high and low sugar diet in the present study was slightly higher compared with the mean energy intake in men aged 50-64 years old from the National Diet and Nutrition Survey (NDNS), which is 9.55 MJ (Hoare et al., 2004). Energy from carbohydrate in the high sugar diet was 6% higher and in the low sugar diet was almost 6% lower than the mean intake of middle-aged men from the NDNS.
3.2.3 The effect of the high and low sugar diets on body weight, body fat, plasma lipids and lipoproteins

The individual and the mean values of body weight, body fat, the concentration of lipids and lipoproteins in plasma and total HDL fractions on the high and low sugar diets are shown in Table 10. Body weight after the high sugar dietary intervention was higher than that after the low sugar dietary intervention, and this difference between the two diets was statistically significant \( (p = 0.020) \). The percentage of body fat was higher on the high sugar diet compared to the low sugar diet, and the difference between the two diets was also statistically significant \( (p = 0.014) \).

The fasting plasma triglyceride for subject 02, 03, 04, 05 was in the normal range \(<1.7 \text{ mmol/L}\) after the two dietary interventions, while the concentration of triglyceride for subject 01 and 06 was higher than the normal level on both diets. The fasting levels of plasma cholesterol for subject 01 to 05 was in the normal range \(<5 \text{ mmol/L}\) or just on the borderline after the two dietary interventions, whereas the cholesterol levels of subject 06 on both diets was above the normal range. Moreover, the values of plasma HDL-C for all subjects on the high sugar diet were below the normal level \( (>1.2 \text{ mmol/L}) \). Subject 02 and 04 had a borderline normal fasting HDL-C concentration on the low sugar diet, however the other subjects had a concentration below the normal range. Although the levels of fasting plasma triglyceride, cholesterol and HDL-C varied within individuals on the high and low sugar diets, there were no significant differences between the two diets. In addition, there were no significant differences in the levels of triglyceride, cholesterol and apoA-I in total HDL fractions between the diets.
Table 10: Body weight, body fat and the concentrations of lipids and lipoproteins in plasma and total HDL fractions on the high and low sugar diets

<table>
<thead>
<tr>
<th>CHOT</th>
<th>Diet</th>
<th>01</th>
<th>02</th>
<th>03</th>
<th>04</th>
<th>05</th>
<th>06</th>
<th>Mean ± SEM</th>
<th>Paired T-test</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>High sugar</td>
<td>89.8</td>
<td>89.1</td>
<td>91.8</td>
<td>92.5</td>
<td>76.6</td>
<td>98.1</td>
<td>89.65 ± 2.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low sugar</td>
<td>89.4</td>
<td>85.8</td>
<td>90.6</td>
<td>92.0</td>
<td>73.5</td>
<td>99.2</td>
<td>87.92 ± 3.20</td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td><strong>p = 0.020</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>High sugar</td>
<td>29.2</td>
<td>30.3</td>
<td>24.0</td>
<td>26.3</td>
<td>26.4</td>
<td>29.5</td>
<td>27.62 ± 0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low sugar</td>
<td>26.2</td>
<td>27.4</td>
<td>23.9</td>
<td>24.8</td>
<td>22.2</td>
<td>28.0</td>
<td>25.42 ± 0.90</td>
<td></td>
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<tr>
<td></td>
<td><strong>p = 0.014</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Plasma TG (mmol/L)</td>
<td>High sugar</td>
<td>1.75</td>
<td>1.67</td>
<td>1.58</td>
<td>1.52</td>
<td>1.24</td>
<td>3.69</td>
<td>1.91 ± 0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low sugar</td>
<td>2.74</td>
<td>1.64</td>
<td>1.64</td>
<td>0.93</td>
<td>1.07</td>
<td>3.12</td>
<td>1.86 ± 0.36</td>
<td></td>
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</tr>
<tr>
<td></td>
<td><strong>p = 0.835</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Plasma CHOL (mmol/L)</td>
<td>High sugar</td>
<td>4.15</td>
<td>4.33</td>
<td>4.26</td>
<td>4.61</td>
<td>4.02</td>
<td>7.12</td>
<td>4.75 ± 0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low sugar</td>
<td>5.02</td>
<td>4.15</td>
<td>3.90</td>
<td>4.34</td>
<td>3.70</td>
<td>6.33</td>
<td>4.57 ± 0.40</td>
<td></td>
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</tr>
<tr>
<td></td>
<td><strong>p = 0.474</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Plasma HDL-CHOL (mmol/L)</td>
<td>High sugar</td>
<td>1.15</td>
<td>1.16</td>
<td>0.79</td>
<td>1.12</td>
<td>0.86</td>
<td>1.10</td>
<td>1.03 ± 0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low sugar</td>
<td>1.19</td>
<td>1.23</td>
<td>0.62</td>
<td>1.25</td>
<td>0.87</td>
<td>1.01</td>
<td>1.03 ± 0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>p = 0.972</strong></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL fraction - TG (mmol/L)</td>
<td>High sugar</td>
<td>0.13</td>
<td>0.10</td>
<td>0.13</td>
<td>0.13</td>
<td>0.08</td>
<td>0.16</td>
<td>0.12 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low sugar</td>
<td>0.14</td>
<td>0.08</td>
<td>0.16</td>
<td>0.10</td>
<td>0.06</td>
<td>0.14</td>
<td>0.11 ± 0.02</td>
<td></td>
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</tr>
<tr>
<td></td>
<td><strong>p = 0.425</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HDL fraction-CHOL (mmol/L)</td>
<td>High sugar</td>
<td>1.18</td>
<td>1.26</td>
<td>0.81</td>
<td>1.00</td>
<td>0.72</td>
<td>1.03</td>
<td>1.00 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low sugar</td>
<td>1.24</td>
<td>1.06</td>
<td>1.06</td>
<td>1.09</td>
<td>0.61</td>
<td>0.83</td>
<td>0.98 ± 0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>p = 0.815</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HDL apoA-I (g/L)</td>
<td>High sugar</td>
<td>1.23</td>
<td>1.45</td>
<td>0.83</td>
<td>1.05</td>
<td>0.71</td>
<td>1.02</td>
<td>1.05 ± 0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low sugar</td>
<td>1.56</td>
<td>1.05</td>
<td>0.90</td>
<td>1.07</td>
<td>0.53</td>
<td>0.65</td>
<td>0.96 ± 0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>p = 0.477</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Plasma HDL-C was significantly correlated to fraction HDL-C on the high sugar diet ($r = 0.900, p = 0.007$) but not on the low sugar diet ($r = 0.407, p = 0.212$) (Figure 24). There was no correlation between body weight and body fat (%) on either the high or low sugar diets. There was also no relationship between body weight or body fat (%) and the level of plasma triglyceride and cholesterol. A positive correlation was observed between plasma concentration of triglyceride and triglyceride in the HDL fraction on the high sugar diet ($r = 0.753, p = 0.042$) (Figure 25) but not on the low sugar diet ($r = 0.620, p = 0.095$). A significant positive association was also observed between plasma HDL concentration and HDL apoA-I on the high sugar diet ($r = 0.845, p = 0.017$) (Figure 26), but this correlation was not found on the low sugar diet ($r = 0.529, p = 0.140$).

![Figure 24: Correlations between plasma HDL-C and fraction HDL-C on the high and low sugar diet](image-url)
Correlation between plasma TG and HDL fraction TG on the high sugar diet

\[ r = 0.753 \]

Correlation between plasma TG and HDL fraction TG on the low sugar diet

\[ r = 0.620 \]

\[ p = 0.095 \]

**Figure 25:** Correlations between plasma TG and fraction HDL-TG on the high and low sugar diet

Correlation between plasma HDL-C and HDL apoA-I on the high sugar diet

\[ r = 0.845 \]

\[ p = 0.017 \]

Correlation between plasma HDL-C and HDL apoA-I on the low sugar diet

\[ r = 0.529 \]

\[ p = 0.140 \]

**Figure 26:** Correlation between plasma HDL-C and HDL apoA-I on the high and low sugar diet
3.2.4 The effect of the high and low sugar diets on blood glucose, insulin and insulin resistance

The fasting plasma glucose, insulin and insulin sensitivity of the participants on the high and low sugar diets were measured, and the results are summarized in Table 11. There were no significant differences found in the levels of fasting glucose, insulin and HOMA2-IR between two diets.

Table 11: Glucose, insulin and insulin sensitivity on the high and low sugar diets

<table>
<thead>
<tr>
<th>CHOT</th>
<th>Diet</th>
<th>01</th>
<th>02</th>
<th>03</th>
<th>04</th>
<th>05</th>
<th>06</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>High sugar</td>
<td>4.85</td>
<td>5.19</td>
<td>4.80</td>
<td>4.34</td>
<td>4.90</td>
<td>5.49</td>
<td>4.93 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>Low sugar</td>
<td>5.48</td>
<td>4.30</td>
<td>4.47</td>
<td>4.87</td>
<td>5.83</td>
<td>5.50</td>
<td>5.08 ± 0.25</td>
</tr>
<tr>
<td>Paired</td>
<td>T-test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p = 0.620</td>
</tr>
<tr>
<td>Insulin</td>
<td>High sugar</td>
<td>11</td>
<td>8.6</td>
<td>18</td>
<td>24</td>
<td>21</td>
<td>27</td>
<td>18.27 ± 2.96</td>
</tr>
<tr>
<td></td>
<td>Low sugar</td>
<td>15</td>
<td>22</td>
<td>17</td>
<td>16</td>
<td>22</td>
<td>27</td>
<td>19.78 ± 1.84</td>
</tr>
<tr>
<td>Paired</td>
<td>T-test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p = 0.622</td>
</tr>
<tr>
<td>HOMA2-IR</td>
<td>High sugar</td>
<td>1.4</td>
<td>1.1</td>
<td>2.3</td>
<td>2.9</td>
<td>2.6</td>
<td>3.5</td>
<td>2.30 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>Low sugar</td>
<td>2.0</td>
<td>2.7</td>
<td>2.1</td>
<td>2.0</td>
<td>2.9</td>
<td>3.4</td>
<td>2.52 ± 0.24</td>
</tr>
<tr>
<td>Paired</td>
<td>T-test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p = 0.558</td>
</tr>
</tbody>
</table>

3.2.5 The effect of the high and low sugar diets on lipases

The total lipase, lipoprotein lipase (LPL) and hepatic lipase (HL) levels on the two dietary interventions are shown in Table 12. The total lipase levels were similar on the high and low sugar diet. The levels of LPL were also not statistically different between two diets. The HL level was significantly higher on the high sugar diet than that on the low sugar diet (p = 0.018). No correlation was observed between total lipase and HL. Moreover, there were no associations between HL and plasma or fraction HDL-C levels. A significant correlation was observed between HL and
plasma triglyceride on both the high and low sugar diet ($r = 0.915, p = 0.005$ and $r = 0.762, p = 0.039$). The association between HL and HDL fraction triglyceride was on the border of being significant ($r = 0.721, p = 0.053$) on the high sugar diet but not on the low sugar diet ($r = 0.638, p = 0.086$) (Figure 27).

Table 12: Total lipase, lipoprotein lipase and hepatic lipase on the high and low sugar diets

<table>
<thead>
<tr>
<th>CHOT</th>
<th>Diet</th>
<th>01</th>
<th>02</th>
<th>03</th>
<th>04</th>
<th>05</th>
<th>06</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipase (pmol/ml/min)</td>
<td>High sugar</td>
<td>6.25</td>
<td>2.38</td>
<td>1.99</td>
<td>4.86</td>
<td>2.35</td>
<td>4.01</td>
<td>3.64 ± 0.69</td>
</tr>
<tr>
<td></td>
<td>Low sugar</td>
<td>4.03</td>
<td>2.24</td>
<td>3.31</td>
<td>6.75</td>
<td>2.90</td>
<td>2.92</td>
<td>3.69 ± 0.66</td>
</tr>
<tr>
<td></td>
<td><strong>Paired T-test</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>p = 0.937</strong></td>
</tr>
<tr>
<td>Lipoprotein lipase (LPL) (pmol/ml/min)</td>
<td>High sugar</td>
<td>3.54</td>
<td>1.14</td>
<td>0.03</td>
<td>2.82</td>
<td>0.28</td>
<td>0.87</td>
<td>1.45 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>Low sugar</td>
<td>1.76</td>
<td>1.57</td>
<td>1.49</td>
<td>5.01</td>
<td>1.56</td>
<td>0.66</td>
<td>2.01 ± 0.62</td>
</tr>
<tr>
<td></td>
<td><strong>Paired T-test</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>p = 0.377</strong></td>
</tr>
<tr>
<td>Hepatic lipase (HL) (pmol/ml/min)</td>
<td>High sugar</td>
<td>2.71</td>
<td>1.23</td>
<td>1.96</td>
<td>2.04</td>
<td>2.07</td>
<td>4.88</td>
<td>2.48 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>Low sugar</td>
<td>2.27</td>
<td>0.67</td>
<td>1.82</td>
<td>1.74</td>
<td>1.34</td>
<td>3.58</td>
<td>1.90 ± 0.40</td>
</tr>
<tr>
<td></td>
<td><strong>Paired T-test</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>p = 0.018</strong></td>
</tr>
</tbody>
</table>

Figure 27: Correlation between HL and HDL fraction triglyceride on the high and low sugar diet.
3.2.6 Isotopic enrichment, PR and FCR of total HDL apoA-I on the high and low sugar diets

The leucine enrichment of HDL apoA-I during the 10 hour study after the high and low sugar diets expressed as APE for each subject is shown in Figure 28 and the mean APE is shown in Figure 29. No significant difference was found between the two diets.

Figure 28: Leucine enrichment of total HDL apoA-I on high and low sugar diets for each individual.
Table 13 and Figure 30 show the fractional clearance rate (FCR) and production rate (PR) of total HDL on the high sugar and low sugar diet. PR was lower in 5 out of the 6 subjects on the low sugar diet. There was no statistically significant difference in either FCR and PR between the two dietary interventions. In addition, no significant correlations were found between FCR and plasma or HDL fraction TG levels, and there was also no correlation between PR and the concentration of plasma or HDL fraction cholesterol.
Table 13: Fractional clearance rate (FCR) and production rate (PR) of total HDL on high sugar and low sugar diet

<table>
<thead>
<tr>
<th>Subjects (n=6)</th>
<th>ApoA-I pool size (mg/kg)</th>
<th>FCR (pools/day)</th>
<th>PR (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High sugar diet</td>
<td>Low sugar diet</td>
<td>High sugar diet</td>
</tr>
<tr>
<td>CHOT 01</td>
<td>45.0</td>
<td>57.2</td>
<td>0.17</td>
</tr>
<tr>
<td>CHOT 02</td>
<td>52.4</td>
<td>38.9</td>
<td>0.16</td>
</tr>
<tr>
<td>CHOT 03</td>
<td>30.6</td>
<td>33.4</td>
<td>0.24</td>
</tr>
<tr>
<td>CHOT 04</td>
<td>37.4</td>
<td>38.1</td>
<td>0.26</td>
</tr>
<tr>
<td>CHOT 05</td>
<td>26.9</td>
<td>20.5</td>
<td>0.21</td>
</tr>
<tr>
<td>CHOT 06</td>
<td>35.3</td>
<td>22.8</td>
<td>0.15</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>37.9 ± 3.8</td>
<td>35.2 ± 5.4</td>
<td>0.20 ± 0.02</td>
</tr>
</tbody>
</table>

Paired T-test:
- $p = 0.524$ for FCR
- $p = 0.533$ for PR
- $p = 0.115$

Figure 30: Fractional clearance rate (FCR) and production rate (PR) of total HDL for individual subjects on the high and low sugar diet.
3.3 Discussion

In this dietary intervention study, the diet with high non-milk extrinsic sugar induced higher body weight and body fat than the low non-milk extrinsic sugar diet among the middle-aged men. With respect to the other cardio-metabolic risk factors including plasma lipids (triglyceride and cholesterol) and lipoproteins (HDL), glucose, insulin and insulin resistance, beneficial effects of the low sugar diet compared to the high sugar diet were not observed. An association between plasma TG and HDL-TG, and between plasma HDL and HDL apoA-I was only found on the high sugar dietary intervention. Moreover, distinct effects of high and low sugar intakes on total HDL apoA-I FCR and PR was not shown.

3.3.1 Effects of high and low sugar diet on HDL-C and HDL kinetics

In two previous studies, HDL apoA-I FCR and PR were measured after 6-week consumption of a baseline average US diet (49% carbohydrate, 15% protein, 35% fat (14% saturated, 14% monounsaturated, 7% polyunsaturated) and 147mg of cholesterol/1000kcal) in men aged >40 or >50 years old (Velez-Carrasco et al., 2000; Tilly-Kiesi, 1997). Among healthy men aged >40 years (57±11 years) with HDL-C level of 0.97 ± 0.02 mmol/L, the mean values of HDL apoA-I FCR and PR were 0.225 ± 0.062 pools/day and 12.28 ± 3.64 mg/kg/day respectively (Velez-Carrasco et al., 2000). In another study, among men aged >50 years (58±12 years) who had high plasma cholesterol (5.90 ± 1.27 mmol/L) but normal triglyceride concentration (1.40 ± 0.36 mmol/L) and a HDL-C level of 1.14 ± 0.23 mmol/L, the mean levels of HDL apoA-I FCR and PR were 0.14 ± 0.04 pools/day and 8.5 ± 2.7 mg/kg/day respectively (Tilly-Kiesi et al., 1997). In the present study, the HDL apoA-I FCR levels for individual subjects on both the high and low sugar diet were similar to the data in the studies by Velez-Carrasco et al. (2000) and Tilly-Kiesi et al. (1997). The levels of HDL apoA-I PR for individuals in the present study were lower than the results in
those two previous studies on both diets, except subject 04 on the high sugar diet. The mean ages of the male subjects in the studies by Velez-Carrasco et al. (2000) and Tilly-Kiesi et al. (1997) were similar to the mean age in the present study. However, the kinetic measurements in the previous studies were carried out under constant feeding condition (Table 14), while the HDL apoA-I FCR and PR in the present study were measured under fasting state. This may account for the lower PR in the current study.

Table 14: Summary of data on total HDL apoA-I kinetic parameters obtained with stable isotopes in the previous studies

<table>
<thead>
<tr>
<th>Authors</th>
<th>Methodology</th>
<th>Subjects</th>
<th>Nutritional state</th>
<th>HDL apoA-I FCR (pools/d)</th>
<th>HDL apoA-I PR (mg/kg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Velez-Carrasco et al., 2000</td>
<td>15h primed-constant infusion of [5,5,5-2H3]L-Leucine</td>
<td>18 males aged &gt;40y (57 ± 11y)</td>
<td>constant-feeding</td>
<td>0.225 ± 0.062</td>
<td>12.28 ± 3.64</td>
</tr>
<tr>
<td>Tilly-Kiesi et al., 1997</td>
<td>15h primed-constant infusion of [5,5,5-2H3]L-Leucine</td>
<td>11 males aged &gt;50y (58 ± 12y)</td>
<td>constant-feeding</td>
<td>0.14 ± 0.04</td>
<td>8.5 ±2.7</td>
</tr>
<tr>
<td>Velez-Carrasco et al., 1999</td>
<td>15h primed-constant infusion of [5,5,5-2H3]L-Leucine</td>
<td>5 males aged &gt;40y (49 ± 12y)</td>
<td>constant-feeding</td>
<td>0.245 ± 0.029</td>
<td>14.67 ± 2.22</td>
</tr>
<tr>
<td>Frenais et al., 1997</td>
<td>14h primed-constant infusion of [5,5,5-2H3]L-Leucine</td>
<td>5 males aged 25 ± 3y</td>
<td>fasting</td>
<td>0.21 ± 0.06</td>
<td>12.0 ± 4.2</td>
</tr>
</tbody>
</table>

It has been suggested that various factors including female sex, exercise, alcohol consumption, low fat diet, high polyunsaturated fat diet, obesity, and smoking may alter plasma HDL levels (Tall, 1990). It has been found that a high carbohydrate diet (>57.4% of energy) is associated with a low concentration of HDL-C in men aged 25-64 years of the non-institutionalized population (Yang et al., 2002), while a carbohydrate-restricted diet (10-12% of energy) is associated with increased plasma
HDL-C concentration (by 12-13%) in overweight men aged 18-69 years (Wood et al., 2006; Volek et al., 2009). The proportion of total carbohydrate in the present study was about 53% and 41% of total energy on the high and low sugar diet respectively. This relatively moderate difference might not be able to achieve a remarkable difference in HDL-C concentration.

The relationship between the type of carbohydrate and HDL metabolism has been investigated previously. A prospective study indicated that energy from dietary sucrose was inversely associated with HDL-C in both cross-sectional (at baseline and year 7) and longitudinal analyses (from baseline to year 7) (Archer et al., 1998). Moreover, higher consumption of added sugars or soft drinks was also found to correlate with lower HDL-C levels (Welsh et al., 2010; Dhingra et al., 2007). It has been indicated that increased dietary carbohydrates, especially simple sugars and starches with high GI could lead to increased levels of small and dense HDL, which was considered to be positively related to atherogenic dyslipidemia (Siri and Krauss, 2005). Hence, although there was no difference in total HDL levels, changes in HDL subclasses might have occurred. Unfortunately, HDL subclasses were not measured in the present study.

Weight loss and decreased body fat induced by a carbohydrate-restricted diet with or without reduced energy intake has been shown to be associated with increased plasma HDL-C concentration (Wood et al., 2006; Volek et al., 2009; Dattilo and Kris-Etherton, 1992). Body weight was significantly higher in the high sugar diet than that in the low sugar diet, although there was considerable variation between individuals. This variance of body weight may account for the variance in HDL-C levels, which could be one of the possible explanations for the non-significant differences in HDL-C levels between two dietary interventions in the current study. Additionally, exercise can also increase the levels of HDL-C and apoA-I (Thompson et al., 1997). However,
subjects in the present study were asked to maintain their habitual physical activity, and the physical activity levels of the subjects during the two dietary interventions were not assessed.

The rates of synthesis and/or catabolism of major HDL proteins determine plasma HDL concentration, and the variation of HDL and apoA-I between individuals is associated with differences in the PR and FCR of apoA-I (Tall, 1990). Blum et al. (1977) found that a reduced HDL concentration in normal subjects, which was induced by a low fat, high carbohydrate diet, resulted from the increased fractional catabolism of plasma apoA-I. The FCR of apoA-I has been considered to be more crucial than apoA-I PR in determining plasma HDL levels, and it was found to have a strong positive association with triglyceride levels (Brinton et al., 1991). In the present study, neither the concentration of HDL apoA-I or the PR and FCR of HDL apoA-I were significantly different between the high and low sugar diet. Although HDL apoA-I PR was not significantly different, surprisingly it was lower in 5 out of 6 subjects on the low sugar diet.

Tall (1990) suggested that apoA-I catabolism could be affected by alterations in the lipid composition in the HDL core or the HDL size. Increased level of triglyceride or triglyceride-rich lipoproteins result in increased exchange of cholesterol ester in HDL particles with triglyceride in triglyceride-rich lipoproteins, which may lead to an increased level of triglyceride-rich HDL. A positive correlation between HL and plasma triglyceride concentration was observed in the present study, which indicates that an increased level of plasma triglyceride may also relate to an increased level of HL. HL promotes the catabolism of triglyceride-rich HDL, which reduces HDL size and these smaller HDL particles may be more rapidly taken up by the liver. The reduction in HDL size also leads to a loss of apoA-I which may be cleared by the kidney (Tall, 1990). It has been suggested that the adverse relationship between
plasma triglyceride level and HDL-C (or apoA-I) might be partly due to the elevated exchange of cholesterol ester for triglyceride from HDL to triglyceride lipoproteins, which leads to the secondary reduction of apoA-I because of increased catabolism (Tall, 1990). Neither triglyceride nor cholesterol levels were significantly different on the high and low sugar dietary intervention in the present study, which might be explained by the non-significant change in FCR of total HDL as well as PR.

HL is the lipase that is important in reverse cholesterol transport, and its activity is inversely associated with HDL levels (Lambert et al., 1999). HL plays an important role in converting larger HDL particles, αHDL or HDL₂ and HDL₃, to smaller HDL remnants, preβHDL and lipid-poor apoA-I (Lewis and Rader, 2005). Therefore greater activity of HL can lead to an increased level preβHDL and decreased level of mature αHDL, the predominant form of HDL. The level of HL on the high sugar diet was significantly higher than that on the low sugar diet. This might suggest that high sugar diet can induce higher level of preβHDL and lower level of αHDL compared to the low sugar diet, even though the concentration of plasma HDL did not change. Unfortunately, only total HDL was measured in the present study.

LPL, CETP and PLTP, which are involved in the process of the formation of HDL and reverse cholesterol transport, have also been suggested to have effects on HDL levels and HDL apolipoprotein metabolism (Tall, 1990; Goldberg et al., 1990; Masson et al., 2009). It has been suggested that high consumption of fructose may reduce the activity of lipoprotein lipase at the adipocyte (Johnson et al., 2009) resulting in increased plasma triglyceride. LPL is also involved in the formation of nascent preβHDL. In the present study, LPL was not different on the two diets.

A recent study by Verges et al. (2014) has investigated the associations between the kinetics of VLDL subclasses and HDL catabolism among subjects with abdominal
obesity by using stable isotopic technique. They found that HDL apoA-I FCR was positively associated with VLDL\textsubscript{1}-triglycerides PR and VLDL\textsubscript{1}-apoB PR. Verges \textit{et al.} (2014) suggested that apoA-I FCR is independently correlated with the production and catabolism of VLDL\textsubscript{1}-TG. This study was part of BBSRC funded trial, which also measured VLDL triglyceride and apoB kinetics. The relationships between HDL apoA-I FCR and VLDL subclasses kinetics was therefore examined in the present study. The kinetics data of VLDL\textsubscript{1}-TG and VLDL\textsubscript{1}-apoB for the subjects in the dietary intervention study are shown in Table 15. However, no significant correlations were found between apoA-I FCR and VLDL\textsubscript{1}-triglycerides PR or FCR, and between apoA-I FCR and VLDL\textsubscript{1}-apoB PR or FCR on both the high and low sugar diet. The study population in the study by Verges \textit{et al.} (2014) and in the current study had the similar age. However, the subjects in this recent study were abdominal obese and had higher BMI compared to the present study. The study by Verges \textit{et al.} (2014) included a relatively larger sample size (n=62) with a mixture of male and female subjects. In the current study, only male subjects were involved and the sample size was small (n=6), which may not be powerful enough to induce a significant association between HDL catabolism and VLDL kinetics. Furthermore, the subjects in the current study were on the high and low sugar diet, which may influence the metabolism of HDL and VLDL. Therefore, these factors could lead to the difference in the results between the study by Verges \textit{et al.} (2014) and the present study.
Table 15: The kinetics data of VLDL\textsubscript{1}-TG and VLDL\textsubscript{1}-apoB on the high and low sugar diet

<table>
<thead>
<tr>
<th>Subject</th>
<th>VLDL\textsubscript{1}−TG FCR (pool/day)</th>
<th>VLDL\textsubscript{1}−TG PR (mg/kg/day)</th>
<th>VLDL\textsubscript{1}−apoB FCR (pool/day)</th>
<th>VLDL\textsubscript{1}−apoB PR (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High sugar</td>
<td>Low sugar</td>
<td>High sugar</td>
<td>Low sugar</td>
</tr>
<tr>
<td>CHOT 01</td>
<td>10.46</td>
<td>9.94</td>
<td>274.35</td>
<td>319.64</td>
</tr>
<tr>
<td>CHOT 02</td>
<td>8.79</td>
<td>9.79</td>
<td>234.86</td>
<td>284.60</td>
</tr>
<tr>
<td>CHOT 03</td>
<td>8.73</td>
<td>8.98</td>
<td>177.41</td>
<td>145.62</td>
</tr>
<tr>
<td>CHOT 04</td>
<td>10.03</td>
<td>9.94</td>
<td>201.59</td>
<td>133.12</td>
</tr>
<tr>
<td>CHOT 05</td>
<td>5.55</td>
<td>5.95</td>
<td>74.94</td>
<td>80.86</td>
</tr>
<tr>
<td>CHOT 06</td>
<td>5.03</td>
<td>4.83</td>
<td>207.44</td>
<td>187.65</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>8.10 ± 0.93</td>
<td>8.24 ± 0.92</td>
<td>195.10 ± 27.57</td>
<td>191.92 ± 37.80</td>
</tr>
</tbody>
</table>

* VLDL\textsubscript{1}-apoB data for subject CHOT 01 and 02 is not available.

3.3.2 Effects of high and low sugar diet on body weight and body fat

Although the study aimed to maintain body weight throughout the two dietary interventions, body weight on the high sugar diet was higher than that on the low sugar diet, and this difference occurred without any statistically significant difference in total energy intake. However, the dietary assessment methods may cause an underestimation of energy intake. It has been reported that the underreporting of energy intake occurred commonly in self-recorded diet diaries (Samaras et al., 1999; Bedard et al., 2004; Johansson et al., 2001). Many factors may explain the underreporting of energy intake, such as BMI, body fat, age, smoking habits, educational level and environment of living (Macdiarmid and Blundell, 1998; Novotny et al., 2003; Johansson et al., 2001). Moreover, the dietary assessment tool itself could also be a source of error (Bedard et al., 2004). For example, the reference portion size photos in the diet diary might be misused by the participants or not used at all.
The effects of dietary carbohydrate intake on body weight in previous studies are inconsistent. A meta-analysis of 30 randomized controlled trials has suggested that among the adults consuming *ad libitum* diets (with no strict control of food intake), a decreased dietary sugar intake was associated with reduced body weight and increased intake of sugars was correlated with increased body weight (Morenga *et al.*, 2013). This finding is consistent with the present study. However, some other randomized controlled trials found that an isocaloric diet with either a higher content of carbohydrate (54-55% energy contribution from carbohydrate) or a lower content of carbohydrate (15% or < 40% energy from carbohydrate), could achieve a reduction in body weight and body fat (Brehm *et al.*, 2003; Frisch *et al.*, 2009).

It has been suggested that the type of carbohydrate may be associated with a variation of body weight and body fat rather than the percentage of energy from carbohydrate (Ma *et al.*, 2005). Sugar intake, particularly fructose, has been considered to be a risk factor for obesity (Johnson *et al.*, 2007). Several systematic reviews and meta-analysis have examined the relationship between sugar-sweetened beverage, especially soft drinks, and body weight (Malik *et al.*, 2006; Vartanian *et al.*, 2007; Gibson, 2008). It found that higher consumption of sugar-sweetened beverages was positively associated with increased body weight in both short-term feeding trials and long-term follow-up prospective cohort studies (Malik *et al.*, 2006; Vartanian *et al.*, 2007). Gibson (2008) also reported that there was a significant correlation between sugar-sweetened soft drinks and adiposity. Consuming fructose-sweetened beverages for 10 weeks significantly increased visceral adipose volume (Stanhope *et al.*, 2009). It has been suggested that greater intake of sugar-sweetened beverages, especially carbonated soft drinks and fruit drinks containing a high quantity of added sugar, might be an predominate contribution to the prevalence of overweight and obesity (Malik *et al.*, 2006). Furthermore, it has been found that a high consumption of fruit juices such as apple
juice, which contained high fructose and sucrose, may also be associated with overweight and obesity.

It has been suggested that the potential mechanism of the promotional effect of sugar-sweetened beverages on weight gain is the low satiety of liquid carbohydrates, which may lead to the incomplete compensation of energy intake at subsequent meals (Malik et al., 2006). A crossover study found that the consumption of liquid carbohydrate (soda) promoted a higher energy intake and increased body weight during a 4 week study period compared with the isoenergetic solid carbohydrate (jelly beans) (DiMeglio and Mattes, 2000). Moreover, there were no differences in satiety or subsequent energy intake when people consumed isoenergetic beverages which contained different types of sugar, either fructose or sucrose (Monsivais et al., 2007; Soenen and Westerterp-Plantenga, 2007).

In the present study, carbonated soft drinks and fruit juices were included in the study foods in the high sugar diet but not in the low sugar diet, which could be one of the possible explanations of the significant difference in body weight on the high and low sugar diets. The carbonated soft drinks and fruit juices with low satiety may promote higher energy intake on the high sugar diet, inducing higher body weight than the low sugar diet.

3.3.3 Effects of high and low sugar diet on plasma lipids, glucose and insulin

A positive association between carbohydrate consumption and plasma triglyceride and cholesterol has been reported in a cross-sectional study (Leise et al., 2007). It has been shown that a high carbohydrate diet (>57.4% of energy in men and >59.1% in women) was associated with a higher triglyceride concentration compared with a low carbohydrate diet (<38.7% in men and <41% in women), though the total energy
intake was lower in the high carbohydrate diet (2467 ± 49 kcal in men and 1664 ± 32 kcal in women) than the low carbohydrate diet (2912 ± 54 kcal in men and 1998 ± 42 kcal in women) (Yang et al., 2002). A moderated carbohydrate restricted (26% of energy), low-saturated fat diet may achieve a decrease in the plasma level of triglyceride (Krauss et al., 2006). It was estimated that every 100g higher intake of carbohydrate could result in a 7-8 mg/dL increase in total cholesterol level in men and 13-17 mg/dL increase in triglyceride level in women (Leise et al., 2007). In contrast, another cross-sectional study by Nichols et al. (1976) did not detect any significant relationship between carbohydrate intake and plasma levels of triglyceride and cholesterol among people who had similar total energy intake. This finding was consistent with the results in the present study.

Two cross-sectional studies with large sample size (n>6000) have investigated the relationship between sugar intake and plasma triglyceride, and a strong positive association was reported (Welsh et al., 2010; Dhingra et al., 2007). It found that higher intake of added sugar is associated with increased triglyceride levels in US adults, and among middle-aged adults in the Framingham Heart Study. Individuals who consumed ≥1 soft drink per day had higher levels of triglyceride than those consuming <1 soft drink per day (Dhingra et al., 2007). The effect of different types of dietary sugars on plasma lipids has been investigated in the intervention studies, and the findings were variable. A short-term study found overfeeding of glucose in parallel with a hypercaloric diet for 7 days among 11 healthy young men (24.6 ± 0.6 years) had no effect on fasting plasma triglyceride (Sock et al., 2010), whereas 10-week consumption of glucose sweetened beverage (providing 25% of energy) in parallel with subjects’ usual ad libitum diet and an energy-balanced diet among 15 older adults (54 ± 3 years in men and 56 ± 2 years in women) induced about 10% increase in fasting plasma triglyceride concentration (Stanhope et al., 2009). Stanhope and Havel (2008) also suggested that consuming sucrose-sweetened beverages could
increase postprandial triglyceride levels. However, a single meal containing 75 g sucrose had no effect on postprandial triglyceride level (Brynes et al., 2002). Moreover, in an intermediate-term study, the effect of sucrose on fasting plasma triglyceride was not detected by feeding 70 g sucrose per day in parallel with subjects’ habitual diet for 30 days among 40 overweight or obese males and females (Yaghoobi et al., 2008).

It has been suggested that fructose, which is commonly found in added sugars, may mediate the adverse effects of carbohydrate on plasma lipids by increasing both postprandial and fasting triglyceride levels (Johnson et al., 2009). The findings from the feeding intervention studies suggested that consumption of either fructose or high fructose corn syrup-sweetened beverages (25-30% of energy) was associated with an increased 24-hour triglyceride area under the curve (Stanhope et al., 2011; Teff et al., 2009). However, an increase in fasting plasma triglyceride concentration was not found in another study, which involved the consumption of a fructose-sweetened beverage (25% of energy) for 10 weeks (Stanhope et al., 2009). It has been suggested that a high flux of fructose in the liver, which is the major organ that metabolizes this simple carbohydrate, can disturb glucose metabolism and glucose uptake and result in a notable increase in de novo lipogenesis in the liver, hepatic triglyceride synthesis and decrease in the clearance rate of peripheral triglyceride (Johnson et al., 2009; Basciano et al., 2005).

A remarkable association between soft drink consumption and type 2 diabetes has been reported (Vartanian et al., 2007). Sustained consumption of ≥1 soft drink per day was linked to impaired fasting glucose in a larger cross-sectional study (Dhingra et al., 2007). In feeding studies, consumption of fructose-sweetened beverages (25% of energy) for 10 weeks was correlated with raised levels of fasting plasma glucose and insulin and decreased insulin sensitivity (Stanhope et al., 2009).
In the present study, although the duration of dietary interventions were relatively long-term, the sample size was small and it may not be powerful enough to induce a significant effect of dietary sugars on plasma lipids, glucose, insulin and insulin sensitivity. Moreover, it has been suggested that it is only at an abnormally high amount (>20% of energy from sucrose and >5% of energy from fructose), that the consumption of fructose and sucrose may lead to the increase in plasma triglyceride concentration (Frayn and Kingman, 1995; Fried and Rao, 2003). Therefore, it might be possible that the quantity of fructose and sucrose consumed in the test diets in the present study was not large enough to promote an essential change in plasma triglyceride levels. Unfortunately, the respective amount of fructose and sucrose contained in the study foods was not assessed in the present study. The non-significant difference in the levels of plasma triglyceride might also be explained by the variability of fasting triglyceride response to dietary fructose within individuals (Stanhope et al., 2009).

3.4 Comparison of subset results with those from whole cohort
The results from 6 subjects that are presented in this chapter are just a small part of the whole dietary intervention study. In terms of the whole cohort (n=25), there was a significant difference in fasting plasma triglyceride level (1.79 vs. 1.48 mmol/L, high sugar vs. low sugar, p < 0.02). The concentration of HDL, glucose and insulin were not significantly different in the whole cohort.

3.5 Limitations and further work
The sample size in the present study was relatively small, and it may not be powerful enough to demonstrate any remarkable differences in plasma lipids, glucose and insulin levels, and HDL metabolism on the high and low sugar diet. It would be interesting to explore if the GI values in the high and low sugar diet had an impact not only on plasma glucose and insulin but also plasma lipid level and HDL
concentration. Only total HDL fractions were assessed, changes in HDL subclasses were not measured. Although total HDL apoA-I was not changed, there may have been changes in HDL subclasses. Moreover, the activities of some lipid transfer proteins involved in HDL metabolism, such as CETP and PLTP, could also provide valuable information.
Chapter 4: Investigation of HDL subclass kinetics using a stable isotope technique

4.1 Introduction
HDL has an atheroprotective effect, and it could be a more powerful predictor of CHD than plasma total cholesterol and LDL cholesterol (Kontush and Chapman, 2006; Barter and Rye, 1996). HDL is a heterogeneous particle and its metabolism is complex due to the bidirectional inter-conversion between HDL subclasses. Therefore, only measuring total HDL may not be able to provide enough information regarding the biological functions of individual HDL subclasses. In order to have a better understanding of HDL metabolism, an investigation of HDL subclass kinetics was performed in the present study.

4.2 Method summary
A pilot study was carried out firstly among three healthy women aged 18-70. Different durations of the study (up to 5, 6 and 10 days) were tested to develop an optimal blood sampling protocol for measurement of HDL metabolism. Kinetic studies using this optimal blood sampling protocol were carried out subsequently to measure HDL₂ and HDL₃, αHDL and preβHDL metabolism in healthy subjects.

Six Caucasian males (n=3) and females (n=3) aged 30-45 with BMI 18.5-25 kg/m² were recruited. Subjects were required to fast overnight before all the visits in the CEDAR Centre, Royal Surrey County Hospital. Anthropometrics were measured before the study, and HDL subclasses kinetics was assessed by using a stable isotope technique. L[1-13C] leucine isotope was injected intravenously as a bolus with dose of 8 mg/kg. Blood samples were taken at baseline and at intervals up to 10 hours. Six more single blood samples were collected in the morning in a fasted state during the following 2 weeks (Day1, 3, 7, 9, 10 and 14) for further measurement of
leucine enrichment. Blood samples for measuring the concentration of plasma lipids, HDL, apoA-I, glucose and insulin were taken at baseline, 10h, Day1, Day7 and Day14, and blood samples for measuring a-KIC were taken frequently during the first 10 hours at the study. HDL$_2$ and HDL$_3$ fractions were isolated from plasma via untracentrifugation, and αHDL and preβHDL fractions were isolated from plasma by agarose gel electrophoresis. After delipidation, apoA-I in the fractions was separated using SDS-PAGE. The samples were then purified and derivatized, and finally run on the GC-MS to determine the leucine enrichment of apoA-I (Chapter 2, section 2.2.8).
4.3 Results

4.3.1 Optimising the laboratory method – SDS-PAGE

All the laboratory procedures that were to be used were optimised before carrying out the HDL kinetic study. In the process of separation of HDL₃ apoA-I by using SDS-PAGE, the amount of sample that was loaded into each well on the gel was 50 μl in a previous study by Li (2009). However, it was found that by loading 50 μl sample, HDL₃ apoA-I might get contaminated with the samples in adjacent lanes since the size of the band was quite large (Figure 31A). This could be due to the overloading of the sample. Therefore, the amount of samples loaded on the gel was reduced to 25 μl and tested again. It has been found that HDL₃ apoA-I bands were reduced to a more optimal size, reducing the risk of contamination between samples (Figure 31B).

Moreover, the samples with different loading amount on the SDS-PAGE, 50 μl for HDL₂ and 25 μl for HDL₃, were assessed on the GC-MS. The isotopic area of ion 209 and 210 for both HDL₂ and HDL₃ samples had the same digital values, which indicated that reducing the loading amount to 25 μl for HDL₃ did not affect the measurement of leucine enrichment.

![Figure 31: Optimizing the laboratory methods: SDS-PAGE.](image)

A: 50 μl of sample was loaded for both HDL₂ and HDL₃ for separation of apoA-I bands; B: 50 μl of sample was loaded for HDL₂ and 25 μl was loaded for HDL₃ for separation of apoA-I.
4.3.2 Pilot study – Development of the blood sampling protocol

Because of the relatively slow turnover rate of apoA-I, which has a mean residence time of approximately 5 days (Schaefer et al., 1982), a series of pilot studies were undertaken of 1-2 week duration to determine an optimal protocol of blood sampling for measuring HDL subclass kinetics. Figure 32, 33, 34 show the αHDL and preβHDL apoA-I enrichment (APE) that was determined at timed intervals for 10 hours and over the following 5, 6 and 10 days in the first, second and third pilot study respectively. The isotopic enrichment values of the last time point in three pilot studies are shown in the figures. The values of the last time point in the third pilot study (Day 10) for both αHDL and preβHDL apoA-I enrichment (APE) were most close to the baseline value compared with the first and second study.

![Figure 32: Leucine enrichment of αHDL and preβHDL apoA-I during 10h study and following 5 days in Pilot study 1](image-url)
Figure 33: Leucine enrichment of αHDL and preβHDL apoA-I during 10h study and following 6 days in Pilot study 2

Figure 34: Leucine enrichment of αHDL and preβHDL apoA-I during 10h study and following 10 days in Pilot study 3

Isotopic enrichment of HDL$_2$ and HDL$_3$ apoA-I were not measured in pilot study 1. Figure 35 and 36 show the HDL$_2$ and HDL$_3$ apoA-I enrichment (APE) that was determined in a 10 hour study and over the next 6 and 10 days in pilot study 2 and 3 respectively. The values of isotopic enrichment (APE) at the last time point in the second and third pilot study are shown in the figures. The enrichment at last time point for HDL$_2$ and HDL$_3$ apoA-I in the second and third pilot study seemed to be
similar. The values at Day 10 for αHDL and preβHDL apoA-I enrichment in the third pilot study were most close to the baseline, and the duration of the study up to 10 days seemed to be the most optimal blood sampling protocol among three pilot studies. However, the enrichment of HDL₂ and HDL₃ apoA-I at Day 10 was not as close to the baseline as αHDL and preβHDL apoA-I. Therefore, the duration of the study was extended to 14 days in the final study design for a better measurement of HDL subclass kinetics.

![Figure 35: Leucine enrichment of HDL₂ and HDL₃ apoA-I during 10h study and following 6 days in Pilot study 2](image1.png)

![Figure 36: Leucine enrichment of HDL₂ and HDL₃ apoA-I during 10h study and following 10 days in Pilot study 3](image2.png)
4.3.3 HDL subclass kinetic study

4.3.3.1 Subjects characteristics and lipid profile

The characteristics of subjects are shown in Table 16. Three male and 3 female subjects were aged between 34 to 45 years old. The BMI of subjects 01, 02, 03 and 04 was in the normal range. Whereas subject 05 was borderline overweight, and subject 06 was overweight. All subjects had a normal fasting glucose level (3.9-5.5 mmol/L). The fasting insulin concentration of subject 01, 03, 04, 05 and 06 was in the normal range, while subject 02 had a borderline high fasting insulin concentration (fasting insulin level 5-15 mU/L is normal) (Wilcox, 2005).

Table 16: Subject characteristics of HDL subclasses kinetic study (n=6)

<table>
<thead>
<tr>
<th>Subjects</th>
<th>01</th>
<th>02</th>
<th>03</th>
<th>04</th>
<th>05</th>
<th>06</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>45</td>
<td>34</td>
<td>34</td>
<td>35</td>
<td>34</td>
<td>34</td>
<td>36 ± 1.81</td>
</tr>
<tr>
<td>Gender</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>-</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.72</td>
<td>1.67</td>
<td>1.66</td>
<td>1.79</td>
<td>1.79</td>
<td>1.76</td>
<td>1.73 ± 0.02</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.6</td>
<td>54.6</td>
<td>67.5</td>
<td>74.2</td>
<td>87.0</td>
<td>77.7</td>
<td>71.60 ± 4.46</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>25.7</td>
<td>15.6</td>
<td>24.1</td>
<td>13.7</td>
<td>17.4</td>
<td>15.0</td>
<td>18.58 ± 2.07</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>42.9</td>
<td>40.5</td>
<td>44.8</td>
<td>60.6</td>
<td>69.3</td>
<td>63.5</td>
<td>53.60 ± 5.02</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.2</td>
<td>19.6</td>
<td>24.5</td>
<td>23.2</td>
<td>25.1</td>
<td>27.2</td>
<td>23.80 ± 1.03</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.24</td>
<td>5.20</td>
<td>5.30</td>
<td>5.31</td>
<td>4.81</td>
<td>4.83</td>
<td>5.12 ± 0.09</td>
</tr>
<tr>
<td>Insulin (mU/L)</td>
<td>12.3</td>
<td>15.0</td>
<td>9.6</td>
<td>10.9</td>
<td>8.9</td>
<td>7.4</td>
<td>10.68 ± 1.10</td>
</tr>
<tr>
<td>HOMA2-IR</td>
<td>1.60</td>
<td>1.94</td>
<td>1.26</td>
<td>1.43</td>
<td>1.14</td>
<td>0.95</td>
<td>1.39 ± 0.14</td>
</tr>
</tbody>
</table>

F: female; M: male; FFM: fat free mass; HOMA-IR: The homeostasis model assessment 2 - insulin resistance
Table 17 presents the plasma lipids, HDL and apoA-I concentration of the six subjects. All subjects had a normal fasting plasma triglyceride (<1.7 mmol/L). The fasting plasma cholesterol level for female subjects 01 and 02, and male subjects 04 and 06 were in the normal range (<5 mmol/L), while female subject 03 and male 05 had slightly higher plasma cholesterol. All female subjects, 01, 02 and 03, had a normal plasma HDL-C level (1.2-2.0 mmol/L) (Barter, 1994). Male subjects 04 and 05 had a normal level of HDL-C (1.1-1.8 mmol/L), while the HDL-C concentration of subject 06 was below the normal range.

There were no correlations between body weight and plasma triglyceride, and between body weight and plasma cholesterol level. A significant positive association was found between plasma HDL-C concentration and plasma apoA-I level ($r = 0.954$, $p = 0.002$). There was no statistically significant difference found in triglyceride and cholesterol levels between HDL$_2$ and HDL$_3$ fractions. No correlations were observed between plasma triglyceride concentration and triglyceride level in HDL$_2$ and HDL$_3$ fractions. No correlation was found between plasma cholesterol and HDL$_2$ cholesterol ($r = -0.039$, $p = 0.470$), while a significant positive association was observed between plasma cholesterol concentration and cholesterol level in the HDL$_3$ fraction ($r = 0.839$, $p = 0.018$).

Plasma HDL-C concentration was significantly correlated with HDL$_2$ cholesterol ($r = 0.903$, $p = 0.007$) but not with HDL$_3$ cholesterol ($r = 0.483$, $p = 0.166$). A significant difference was observed between HDL$_2$ apoA-I and HDL$_3$ apoA-I ($p = 0.002$), and also between αHDL apoA-I and preβHDL apoA-I concentration ($p = 0.000$). Moreover, plasma HDL-C was significantly correlated with HDL$_2$ apoA-I and αHDL apoA-I ($r = 0.868$, $p = 0.012$ and $r = 0.986$, $p = 0.000$) but not with HDL$_3$ apoA-I and preβHDL apoA-I ($r = 0.134$, $p = 0.400$ and $r = 0.594$, $p = 0.107$ respectively). In addition, a significant correlation was found between plasma apoA-I concentration and HDL$_2$
apoA-I ($r = 0.900, p = 0.007$) but not HDL$_3$ apoA-I ($r = -0.039, p = 0.471$), whereas a significant positive association was observed between both plasma apoA-I and prebHDL apoA-I ($r = 0.787, p = 0.032$), and plasma apoA-I and aHDL apoA-I levels ($r = 0.953, p = 0.002$). In the whole group, the ratio of cholesterol to TG in HDL$_2$ and HDL$_3$ fractions were 7.00 and 7.13 respectively.

With respect to gender differences, there was no significant difference in plasma triglyceride and cholesterol levels. There was also no significant difference in plasma HDL-C between female and male subjects, whereas a significant difference was found in plasma apoA-I concentration ($p = 0.036$). Moreover, there were no statistically significant differences in triglyceride, cholesterol and apoA-I levels in HDL$_2$ and HDL$_3$ fractions. The only significant difference in HDL subclass variations was observed in aHDL apoA-I between females and males ($p = 0.041$) but not in prebHDL apoA-I.
Table 17: Lipid profile and apoA-I concentration of the subjects in HDL subclasses kinetic study

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Plasma-TG (mmol/L)</th>
<th>Plasma-CHOL (mmol/L)</th>
<th>Plasma HDL-CHOL (mmol/L)</th>
<th>HDL$_{2}$-TG (mmol/L)</th>
<th>HDL$_{3}$-TG (mmol/L)</th>
<th>HDL$_{2}$-CHOL (mmol/L)</th>
<th>HDL$_{3}$-CHOL (mmol/L)</th>
<th>Plasma apoA-I (g/L)</th>
<th>HDL$_{2}$ apoA-I (g/L)</th>
<th>HDL$_{3}$ apoA-I (g/L)</th>
<th>αHDL apoA-I (g/L)</th>
<th>preβHDL apoA-I (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>0.89</td>
<td>4.18</td>
<td>1.66</td>
<td>0.07</td>
<td>0.06</td>
<td>0.73</td>
<td>0.47</td>
<td>1.37</td>
<td>0.43</td>
<td>0.53</td>
<td>1.10</td>
<td>0.27</td>
</tr>
<tr>
<td>02</td>
<td>1.12</td>
<td>4.82</td>
<td>1.23</td>
<td>0.06</td>
<td>0.11</td>
<td>0.29</td>
<td>0.58</td>
<td>1.14</td>
<td>0.18</td>
<td>0.73</td>
<td>0.97</td>
<td>0.17</td>
</tr>
<tr>
<td>03</td>
<td>0.77</td>
<td>5.34</td>
<td>1.68</td>
<td>0.06</td>
<td>0.07</td>
<td>0.61</td>
<td>0.75</td>
<td>1.26</td>
<td>0.36</td>
<td>0.80</td>
<td>1.14</td>
<td>0.12</td>
</tr>
<tr>
<td>Female</td>
<td>0.93 ± 0.10</td>
<td>4.78 ± 0.33</td>
<td>1.52 ± 0.14</td>
<td>0.06 ± 0.00</td>
<td>0.08 ± 0.02</td>
<td>0.54 ± 0.13</td>
<td>0.60 ± 0.07</td>
<td>1.26 ± 0.08</td>
<td>0.32 ± 0.08</td>
<td>0.69 ± 0.08</td>
<td>1.07 ± 0.05</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>mean ± SEM</td>
<td>0.86 ± 0.19</td>
<td>4.70 ± 0.59</td>
<td>1.11 ± 0.11</td>
<td>0.06 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.29 ± 0.05</td>
<td>0.55 ± 0.05</td>
<td>1.01 ± 0.05</td>
<td>0.17 ± 0.05</td>
<td>0.71 ± 0.05</td>
<td>0.88 ± 0.05</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>04</td>
<td>1.07</td>
<td>4.28</td>
<td>1.12</td>
<td>0.07</td>
<td>0.09</td>
<td>0.38</td>
<td>0.55</td>
<td>1.01</td>
<td>0.17</td>
<td>0.71</td>
<td>0.88</td>
<td>0.13</td>
</tr>
<tr>
<td>05</td>
<td>0.49</td>
<td>5.85</td>
<td>1.30</td>
<td>0.06</td>
<td>0.09</td>
<td>0.29</td>
<td>0.64</td>
<td>1.07</td>
<td>0.10</td>
<td>0.75</td>
<td>0.94</td>
<td>0.13</td>
</tr>
<tr>
<td>06</td>
<td>1.03</td>
<td>3.95</td>
<td>0.91</td>
<td>0.05</td>
<td>0.07</td>
<td>0.21</td>
<td>0.45</td>
<td>0.89</td>
<td>0.12</td>
<td>0.58</td>
<td>0.80</td>
<td>0.09</td>
</tr>
<tr>
<td>Male</td>
<td>0.86 ± 0.19</td>
<td>4.70 ± 0.59</td>
<td>1.11 ± 0.11</td>
<td>0.06 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.29 ± 0.05</td>
<td>0.55 ± 0.05</td>
<td>1.01 ± 0.05</td>
<td>0.17 ± 0.05</td>
<td>0.71 ± 0.05</td>
<td>0.88 ± 0.05</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>mean ± SEM</td>
<td>0.90 ± 0.10</td>
<td>4.74 ± 0.30</td>
<td>1.32 ± 0.12</td>
<td>0.06 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.42 ± 0.05</td>
<td>0.57 ± 0.05</td>
<td>1.12 ± 0.07</td>
<td>0.23 ± 0.05</td>
<td>0.68 ± 0.07</td>
<td>0.97 ± 0.05</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>All subjects</td>
<td>0.90 ± 0.10</td>
<td>4.74 ± 0.30</td>
<td>1.32 ± 0.12</td>
<td>0.06 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.42 ± 0.05</td>
<td>0.57 ± 0.05</td>
<td>1.12 ± 0.07</td>
<td>0.23 ± 0.05</td>
<td>0.68 ± 0.07</td>
<td>0.97 ± 0.05</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>mean ± SEM</td>
<td>0.10 ± 0.01</td>
<td>0.30 ± 0.12</td>
<td>0.12 ± 0.00</td>
<td>0.01</td>
<td>0.01</td>
<td>0.08</td>
<td>0.05</td>
<td>0.07</td>
<td>0.06</td>
<td>0.04</td>
<td>0.05</td>
<td>0.03</td>
</tr>
</tbody>
</table>

TG: triglyceride; CHOL: cholesterol; HDL: high density lipoprotein; apoA-I: apolipoprotein A-I

* Significant difference (p < 0.05) between males and females
4.3.3.2 α-KIC enrichment during the first 10 hours in HDL subclass kinetic study

Figure 37 shows the mean α-KIC enrichment (APE) of 6 subjects in the HDL kinetic study. α-KIC enrichment peaked at 5 minutes and then decreased rapidly and returned to baseline values at the end of the first 10 hour of the study.

Figure 37: α-KIC enrichment during the first 10 hour in HDL subclass kinetic study of all subjects (n=6). (mean ± SEM)
4.3.3.3 Isotopic enrichment of preβHDL and αHDL

The mean leucine enrichment of αHDL & preβHDL apoA-I (APE) for all subjects during the first 10 hour of the study is shown in Figure 38 and the data for females and males is shown in Figure 39 and Figure 40. The enrichments for each subject are shown in Appendix 7. Among all subjects, the leucine enrichment of αHDL & preβHDL apoA-I was increased similarly in the first 6 hours while the enrichment of preβHDL apoA-I started to decrease at 8 hours and the enrichment of αHDL apoA-I started to decrease at 10 hours.

![Figure 38](image_url)

*Figure 38: Leucine enrichment of αHDL & preβHDL apoA-I during the first 10 hours in the HDL kinetic study of all subjects (n=6). (mean ± SEM)*

![Figure 39](image_url)

*Figure 39: Leucine enrichment of αHDL & preβHDL apoA-I during the first 10 hours in the HDL kinetic study of the females (n=3). (mean ± SEM)*
The mean leucine enrichment of αHDL & preβHDL apoA-I (APE) for all subjects during the whole 14 day study is shown in Figure 41 and the data for the female and male subjects is shown separately in Figure 42 and Figure 43. The enrichments for each subject are shown in Appendix 7. Leucine enrichment for both αHDL & preβHDL apoA-I continued to decrease after Day 1 and the values on the last day (Day 14) were close to the baseline level.

There was no significant difference in the area under the curve (AUC) of leucine enrichment between αHDL & preβHDL apoA-I during the first 10 hours of the study and the following 14 days among all 6 subjects and within the female and male group. There was also no statistically significant difference in the AUC of αHDL & preβHDL apoA-I leucine enrichment between female and male subjects.
**Figure 41:** Leucine enrichment of αHDL & preβHDL apoA-I during the 14 day HDL kinetic study of all subjects (n=6) (mean ± SEM)

**Figure 42:** Leucine enrichment of αHDL & preβHDL apoA-I during the 14 day HDL kinetic study of the females (n=3) (mean ± SEM)
Figure 43: Leucine enrichment of αHDL & preβHDL apoA-I during the 14 day HDL kinetic study of the males (n=3) (mean ± SEM)
4.3.3.4 Isotopic enrichment of HDL$_2$ and HDL$_3$

Figure 44 shows the leucine enrichment of HDL$_2$ and HDL$_3$ apoA-I (APE) during the first 10 hours of the study for all subjects, and the results for female and male subjects are shown in Figure 45 and Figure 46 respectively. The enrichment in each subject is shown in Appendix 7.

Figure 44: Leucine enrichment of HDL$_2$ and HDL$_3$ apoA-I during the first 10 hours in the HDL kinetic study of all subjects (n=6). (mean ± SEM)

Figure 45: Leucine enrichment of HDL$_2$ and HDL$_3$ apoA-I during the first 10 hours in the HDL kinetic study of the females (n=3). (mean ± SEM)
The mean leucine enrichment of HDL$_2$ and HDL$_3$ apoA-I (APE) for all subjects during the 14 day study is shown in Figure 47, and the data for the female and male subjects is shown separately in Figure 48 and Figure 49. The enrichment in each subject is shown in Appendix 7. Compared to HDL$_2$ apoA-I, leucine enrichment of HDL$_3$ apoA-I increased more rapidly, and the values of HDL$_3$ apoA-I enrichment were higher during the first 10 hours of the study and the next 14 days.

No significant difference was observed in the AUC of leucine enrichment between HDL$_2$ and HDL$_3$ apoA-I during the first 10 hours of the study and next two weeks among all subjects and within female and male group. However, a statistically significant difference was found in the AUC of HDL$_3$ apoA-I leucine enrichment during the first 10 hours between female and male subjects ($p = 0.024$) but not in the AUC of HDL$_2$ apoA-I leucine enrichment ($p = 0.593$).
Figure 47: Leucine enrichment of HDL$_2$ and HDL$_3$ apoA-I during the 14 day HDL kinetic study of all subjects (n=6) (mean ± SEM)

Figure 48: Leucine enrichment of HDL$_2$ and HDL$_3$ apoA-I during the 14 day HDL kinetic study of the females (n=3) (mean ± SEM)
Figure 49: Leucine enrichment of HDL₂ and HDL₃ apoA-I during the 14 days HDL kinetic study of the males (n=3) (mean ± SEM)
4.3.3.5 Fractional clearance rate and production rate of HDL subclasses

Table 18 shows the fractional clearance rate (FCR) and production rate (PR) of αHDL and preβHDL for all subjects, and Table 19 shows the comparison of the FCR and PR (in both mg/kg BW/day and mg/kg FFM/day) of αHDL and preβHDL between female and male subjects. There was a statistically significant difference was found between αHDL and preβHDL PR for all subjects \((p = 0.010)\) but no significant difference between αHDL and preβHDL FCR \((p = 0.546)\). Moreover, no significant differences were observed in αHDL and preβHDL FCR and PR between female and male subjects. There was also no significant difference in αHDL and preβHDL FCR within female and male groups. The αHDL PR and preβHDL PR in mg/kg FFM/day was on the borderline of being significant different within female group \((p = 0.050)\) but not within male group \((p = 0.093)\). In addition, there was no association between αHDL and preβHDL apoA-I concentration and FCR or PR of αHDL and preβHDL.

Table 18: Fractional clearance rate and production rate of αHDL and preβHDL for all subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>FCR (pools/day)</th>
<th>PR (mg/kg BW/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>αHDL</td>
<td>preβHDL</td>
</tr>
<tr>
<td>01</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>02</td>
<td>0.17</td>
<td>0.03</td>
</tr>
<tr>
<td>03</td>
<td>0.11</td>
<td>0.10</td>
</tr>
<tr>
<td>04</td>
<td>0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>05</td>
<td>0.13</td>
<td>0.19</td>
</tr>
<tr>
<td>06</td>
<td>0.08</td>
<td>0.29</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>0.10 ± 0.02</td>
<td>0.13 ± 0.04</td>
</tr>
<tr>
<td>Paired T-test</td>
<td>(p = 0.546)</td>
<td>(p = 0.010)</td>
</tr>
</tbody>
</table>

BW: body weight
Table 19: Comparison of fractional clearance rate and production rate of αHDL and preβHDL between female and male subjects

<table>
<thead>
<tr>
<th>Subjects (Mean ± SEM)</th>
<th>FCR (pools/day)</th>
<th>PR (mg/kg BW/day)</th>
<th>PR (mg/kg FFM/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>αHDL</td>
<td>preβHDL</td>
<td>αHDL</td>
</tr>
<tr>
<td>Females (n=3)</td>
<td>0.12 ± 0.03</td>
<td>0.07 ± 0.02</td>
<td>4.89 ± 1.07</td>
</tr>
<tr>
<td>Males (n=3)</td>
<td>0.09 ± 0.02</td>
<td>0.19 ± 0.05</td>
<td>3.00 ± 0.77</td>
</tr>
<tr>
<td>Independent Samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p = 0.399</td>
<td>p = 0.130</td>
<td>p = 0.226</td>
<td>p = 0.229</td>
</tr>
</tbody>
</table>

* p = 0.05, the differences between αHDL and preβHDL PR within female group
BW: body weight; FFM: free fat mass

Table 20 shows the fractional clearance rate (FCR) and production rate (PR) of HDL₂ and HDL₃ for all subjects, and Table 21 shows the comparison of FCR and PR (in both mg/kg BW/day and mg/kg FFM/day) of HDL₂ and HDL₃ between female and male subjects. There was a significant difference between HDL₂ and HDL₃ PR for all subjects (p = 0.030) but not between HDL₂ and HDL₃ FCR (p = 0.878). There was a significant difference in HDL₂ PR in both mg/kg BW/day and mg/kg FFM/day between females and males (p = 0.017 and p = 0.028). However, there was no significant difference in HDL₂ FCR, HDL₃ FCR and HDL₃ PR between female and male subjects. Moreover, within the male group a significant difference was observed between HDL₂ and HDL₃ FCR (p = 0.023), and between HDL₂ and HDL₃ PR (p = 0.023 for mg/kg BW/day and p = 0.026 for mg/kg FFM/day).
Table 20: Fractional clearance rate and production rate of HDL\(_2\) and HDL\(_3\) for all subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>FCR (pools/day)</th>
<th>PR (mg/kg BW/day)</th>
<th>PR (mg/kg FFM/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDL(_2)</td>
<td>HDL(_3)</td>
<td>HDL(_2)</td>
</tr>
<tr>
<td>01</td>
<td>0.15</td>
<td>0.14</td>
<td>2.40</td>
</tr>
<tr>
<td>02</td>
<td>0.21</td>
<td>0.10</td>
<td>1.56</td>
</tr>
<tr>
<td>03</td>
<td>0.18</td>
<td>0.10</td>
<td>2.29</td>
</tr>
<tr>
<td>04</td>
<td>0.09</td>
<td>0.13</td>
<td>0.62</td>
</tr>
<tr>
<td>05</td>
<td>0.14</td>
<td>0.21</td>
<td>0.54</td>
</tr>
<tr>
<td>06</td>
<td>0.14</td>
<td>0.20</td>
<td>0.67</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>0.15 ± 0.02</td>
<td>0.15 ± 0.02</td>
<td>1.35 ± 0.35</td>
</tr>
</tbody>
</table>

Paired T-test
- \(p = 0.878\)
- \(p = 0.030\)

BW: body weight

Table 21: Comparison of fractional clearance rate and production rate of HDL\(_2\) and HDL\(_3\) between female and male subjects

<table>
<thead>
<tr>
<th>Subjects (Mean ± SEM)</th>
<th>FCR (pools/day)</th>
<th>PR (mg/kg BW/day)</th>
<th>PR (mg/kg FFM/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDL(_2)</td>
<td>HDL(_3)</td>
<td>HDL(_2)</td>
</tr>
<tr>
<td>Females (n=3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.18 ± 0.02</td>
<td>0.11 ± 0.01</td>
<td>2.08 ± 0.26</td>
<td>2.86 ± 0.08</td>
</tr>
<tr>
<td>Male (n=3)</td>
<td>0.13 ± 0.02</td>
<td>0.18 ± 0.03*</td>
<td>0.61 ± 0.04</td>
</tr>
<tr>
<td>Independent Samples T-test</td>
<td>(p = 0.078)</td>
<td>(p = 0.090)</td>
<td>(p = 0.017)</td>
</tr>
</tbody>
</table>

* \(p < 0.05\), the differences between HDL\(_2\) and HDL\(_3\) FCR or PR within male group

BW: body weight; FFM: free fat mass

The mathematical model developed in the present project did not allow a good fit to all the leucine enrichment data of αHDL, preβHDL, HDL\(_2\) and HDL\(_3\). Therefore, the kinetic data calculated from the modelling are not present in this thesis.
4.4 Discussion

This study is the first study to measure preβHDL, αHDL, HDL₂ and HDL₃ over a time period which captures the whole dynamics of HDL metabolism. Although this was a small study, with 3 men and 3 women, a significant difference in HDL₂ kinetics was observed between the genders.

4.4.1 The metabolism of αHDL and preβHDL in healthy subjects

In the present study, a bolus injection of $^{13}$C leucine stable isotope was used to measure the kinetics of αHDL and preβHDL apoA-I, and a two-dimensional electrophoresis technique was applied to separate αHDL and preβHDL fractions from plasma, and apoA-I from these two fractions. The measurement of αHDL and preβHDL apoA-I kinetics used the same laboratory methods described in a previous study by Li et al. (2012). The FCR and PR of αHDL and preβHDL apoA-I in this previous study are shown in Table 22, and these values were higher than the results in the current study. Moreover, in the study by Li et al. (2012), the FCR of preβHDL apoA-I was significantly higher than FCR of αHDL apoA-I. In the present study, though the FCR of preβHDL apoA-I was higher than FCR of αHDL apoA-I, the difference was not significant. In the current study, the PR of preβHDL apoA-I was significantly lower than PR of αHDL apoA-I, as shown in the previous study by Li et al. (2012).

In the study by Li et al. (2012), the study population (2 men and 4 women) was similar to the current study, whereas the clinical protocol was different. In this previous study, a precursor product technique in which $^{13}$C leucine stable isotope was given as a priming dose followed by a constant infusion for 9 hours was used, and FSR of preβHDL and αHDL apoA-I were calculated from the precursor pool enrichment of hepatic intracellular
leucine, which is α-KIC, and the slope of the apoA-I enrichment curve for 9 hours. Theoretically, FSR is equal to FCR in a steady state. However, in the current study, FCR of preβHDL and αHDL apoA-I was directly estimated from the slope of the apoA-I enrichment (APE) decay curve (2-14 days). Hence, the differences in the results between the present study and the previous study Li et al. (2012) may due to the stable isotope labelling method for kinetic analysis, the duration of the clinical protocol, and the calculation method for FCR.

<table>
<thead>
<tr>
<th>Study</th>
<th>FCR (pools/day)</th>
<th>PR (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>preβHDL*</td>
<td>αHDL</td>
</tr>
<tr>
<td>Li et al. (2012) –</td>
<td>0.204 ± 0.017</td>
<td>0.170 ± 0.016</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>(0.124 – 0.237)</td>
<td>(0.107 – 0.209)</td>
</tr>
<tr>
<td>Chetiveaux et al. (2004) – Healthy subjects</td>
<td>0.263 ± 0.085</td>
<td>0.112 ± 0.026</td>
</tr>
<tr>
<td></td>
<td>(0.142 – 0.367)</td>
<td>(0.076 – 0.121)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chetiveaux et al. (2006) – T2D subjects</td>
<td>0.448 ± 0.210</td>
<td>0.249 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>(0.286 – 0.850)</td>
<td>(0.230 – 0.259)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*For Chetiveaux et al. (2004 & 2006) preβ1HDL was measured rather than preβHDL.
T2D: Type 2 diabetes

It has been found that preβHDL can be divided into preβ1HDL and preβ2HDL subclasses based on particle size (Asztalos et al., 2004). PreβHDL is present in low concentrations in plasma, which leads to technical difficulties in separating it and assessing its kinetics. Very few studies have investigated the kinetics of preβHDL, and most studies working on preβHDL and αHDL kinetics were reported by Chetiveaux et al. (2002, 2004, 2006). An infusion of [5,5,5-\text{H}_3] leucine for 14 hours was used to examine apoA-I-HDL kinetics.
in a fasting state, and preβ₁HDL and αHDL were isolated by fast protein liquid chromatography (FPLC). The kinetics of preβ₁HDL and αHDL was investigated in both healthy and type II diabetes patients. Chetiveaux et al. (2004) reported that in healthy male subjects (n=6), the FCR of preβ₁HDL and αHDL apoA-I was 0.263 ± 0.085 and 0.112 ± 0.026 pools/day (Table 22), which is similar to the previous study by Li et al., (2012) but higher than the current study. The absolute production rate (APR) of preβ₁HDL and αHDL apoA-I in the study by Chetiveaux et al. (2004) was 7.65 ± 2.75 and 396.75 mg/kg/day, which is also higher than the current study.

Only total preβHDL was separated in the current study rather than preβ₁HDL, and the kinetic data in the study by Chetiveaux et al. (2004) was obtained from a multi-compartmental model using computer software for simulation, analysis and modelling, SAAM II. Therefore, these factors may cause the difference in the results in the present study and in Chetiveaux' study. Chetiveaux et al. (2002) reported that using FPLC method to separate preβ₁HDL and αHDL could lead to the dilution of samples. Li et al. (2012) also suggested that compared to the two-dimensional electrophoresis technique which separates preβHDL and αHDL directly from plasma, using FPLC may result in overlap between HDL subclasses which may occur when samples were processed in the buffers during the isolation. These disadvantages of the FPLC method may cause errors in the kinetic results. Moreover, the different methods used for stable isotope labelling, as well as the different duration of the study may also lead to the different results.

In the study by Chetiveaux et al. (2004, 2006), the APR was estimated as the product of FPR and pool size of apoA-I in HDL subclasses, which is similar to the calculation method of PR used in the current study. Furthermore, in Chetiveaux’ study, the pool size of preβ₁HDL and αHDL apoA-I in healthy subjects (7.72 ± 2.86 and 56.13 ± 6.13 mg/kg
respectively) was similar to the current study (8.1 ± 1.7 and 51.3 ± 4.4 mg/kg for preβHDL and αHDL apoA-I respectively). Ooi et al. (2006) reviewed 29 published articles which measured the kinetics of plasma total HDL apoA-I in healthy subjects using both radioactive and stable isotopes. They reported that the mean PR of total HDL apoA-I assessed by stable isotopes was 12.1 ± 0.67 mg/kg. In the study by Chetiveaux et al. (2004, 2006), the APR of preβ1HDL and αHDL apoA-I is much higher than PR of total HDL apoA-I reported by Ooi et al. (2006). The possible explanation for this huge difference in PR could be the calculation error. Based on the equation 3 (Chapter2, section 2.2.15), the PR in healthy subjects was recalculated using the data of FCR and apoA-I pool size reported by Chetiveaux et al. (2004), and the results are shown in Table 23. The recalculated PR of preβ1HDL and αHDL seems to be much closer to the data reported by Ooi et al. (2006) and Li et al., (2012).

Table 23: Recalculated results of APR of preβ1HDL and αHDL in healthy subjects in the study by Chetiveaux et al., 2004

<table>
<thead>
<tr>
<th>Subjects (n=6)</th>
<th>preβ1HDL (mg/kg/day)</th>
<th>αHDL (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>2.51</td>
<td>4.98</td>
</tr>
<tr>
<td>S2</td>
<td>2.71</td>
<td>4.32</td>
</tr>
<tr>
<td>S3</td>
<td>2.65</td>
<td>8.88</td>
</tr>
<tr>
<td>S4</td>
<td>0.88</td>
<td>6.49</td>
</tr>
<tr>
<td>S5</td>
<td>0.77</td>
<td>6.09</td>
</tr>
<tr>
<td>S6</td>
<td>NI</td>
<td>6.77</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>1.9 ± 0.99</td>
<td>6.25 ± 1.59</td>
</tr>
</tbody>
</table>

APR: absolute production rate; NI: not identifiable

In the present study, the PR of preβHDL apoA-I was lower than the PR of αHDL apoA-I, which was not expected since preβHDL is considered to be the precursor pool of αHDL. However, it has been suggested that preβHDL is a mixture of nascent HDL, recycled lipid-poor apoA-I and the remnants of large HDL particles (Rye and Barter, 2004; Lewis
and Rader, 2005; Barrans et al., 1994). Li et al. (2012) suggested that as the precursor of αHDL, the enrichment of preβHDL apoA-I could be diluted by the recycling of apoA-I, which would lead to an erroneously low production rate.

It has been reported that both pre- and post-menopausal women have higher levels of HDL-C and apoA-I than age-matched men (Collins, 2008; Schaefer et al., 1982; Velez-Carrasco et al., 2000), as shown in the current study. Schaefer et al. (1982) investigated apoA-I metabolism in young healthy men and women aged 22 ± 2 and 21 ± 2 years respectively, and they suggested that the higher plasma apoA-I concentration in females might due to the higher apoA-I synthesis rate compared to male subjects. The higher production rate of apoA-I in women could be due to hormonal effects. It has been found that estrogen administration mainly increases the levels of HDL that contain apoA-I without apoA-II rather than HDL particles which contained both apoA-I and apoA-II (Walsh et al., 1991). Another study which investigated apoA-I metabolism in older subjects (11 postmenopausal women and 11 men aged >50 years) also showed a higher secretion rate of total apoA-I in females compared with males (Tilly-Kiesi et al., 1997). However, in the study by Velez-Carrasco et al. (2000) which examined the apoA-I kinetics in 14 postmenopausal women and 18 men aged >40 years, it found that the secretion rate of apoA-I was similar between men and women while gender differences was shown in apoA-I FCR. The study population and study protocol was similar in the study by Tilly-Kiesi et al. (1997) and by Velez-Carrasco et al. (2000), and the calculation method of apoA-I SR, which was as FCR (pools per day) × apolipoprotein pool size (mg)/body weight (kg), was the same in these two studies. However, the apoA-I FSR, which is equal to apoA-I FCR under the steady state, was estimated by fitting tracer/trace ratios to a monoexponential function using the SAAM II program in the study by Velez-Carrasco et al. (2000), and was calculated by dividing the rate of appearance
of deuterated leucine in HDL subclass apoA-I by the VLDL apoB-100 plateau enrichment (assuming to be the precursor pool enrichment) in the study by Tilly-Kiesi et al. (1997). Therefore, this difference in analysed methods of kinetic data may cause the difference in the results of apoA-I FCR and SR between the two studies. A study by Brinton et al. (1994) which involved 57 subjects with a wide range of age and HDL-C levels also found no difference in apoA-I secretion rate between men and women, but a lower apoA-I FCR in females than males.

The PR of apoA-I in the studies discussed above was all expressed in terms of body weight. It has been known that women usually have higher levels of body fat than men, and apoA-I is synthesised from the intestine and liver but not from adipose tissues. Hence, expressing the PR of apoA-I by body weight, which includes body fat, might affect the accuracy of assessing the gender differences. In the present study, apoA-I PR was presented in both body weight and FFM to investigate the difference between the two genders. However, no differences were observed between males and females by expressing the apoA-I PR in either body weight or FFM. The non-significant difference in both FCR and PR of αHDL apoA-I between the two genders in the current study, which was also showed in the study by Li et al. (2012), might be due to the small sample size.

For future work, a larger sample size is needed to assess the difference in αHDL and preβHDL metabolism between men and women, and also in the whole group. For the calculation of kinetic parameters, modelling using multi-compartmental analysis may improve the understanding of HDL metabolism, since it can provide not only the FCR and PR but also the conversion rate between the HDL subclasses.
4.4.2 The metabolism of HDL₂ and HDL₃ in healthy subjects

A bolus injection of ¹³C leucine stable isotope was used to measure the kinetics of HDL₂ and HDL₃ apoA-I in the present study. Ultracentrifugation and SDS-PAGE technique were utilized to separate HDL₂ and HDL₃ fractions from plasma, and apoA-I in these two fractions. A previous study measured HDL₂ and HDL₃ kinetics in six healthy subjects using a constant infusion of ¹³C leucine stable isotope (Li, 2009), and the laboratory method used in this study was the same as the present study. In this previous study, the FCR of HDL₂ and HDL₃ apoA-I was 0.182 ± 0.017 and 0.155 ± 0.013 pools/day, and the PR of HDL₂ and HDL₃ apoA-I was 3.174 ± 0.688 and 3.861 ± 0.740 mg/kg/day (Table 24). Except HDL₂ apoA-I PR, which is higher than the present study, the FCR of HDL₂ and HDL₃ apoA-I and PR of HDL₃ apoA-I were remarkably similar between Li’s study and the current study. Additionally, in Li’s study, the FCR of HDL₂ apoA-I was significantly higher than FCR of HDL₃ apoA-I, and PR of HDL₂ and HDL₃ apoA-I was similar. However, in the present study, FCR of HDL₂ apoA-I and FCR of HDL₃ apoA-I are similar, and PR of HDL₂ apoA-I was significantly lower than PR of HDL₃ apoA-I.

The study population in Li’s study was similar to the present study (aged 36 ±1.81 vs. 37.67 ± 3.4 years, BMI 23.80 ± 1.03 vs. 23.85 ± 1.04 kg/m²), with 4 females and 2 males. In the current study, the number of men and women was equal, and the PR of HDL₂ apoA-I in men was much lower than that in women, which would lead to a lower value of mean PR of HDL₂ apoA-I for the whole group. Furthermore, as mentioned in the previous section 4.4.1, the differences between the results in the present study and Li’s study (2009) may also be caused by the different method of stable isotope labelling for kinetic measurement, the different duration of blood sampling protocol and the different calculation method for FCR.
Two previous studies have investigated the kinetics of HDL$_2$ and HDL$_3$ in healthy subjects, patients with type 2 diabetes and postmenopausal women (Frenais et al., 1997; Walsh et al., 1994). However, both of these studies showed no significant difference in HDL$_2$ and HDL$_3$ apoA-I metabolism. It is known that HDL$_2$ and HDL$_3$ can be interconverted between each other, and the similarity of HDL$_2$ and HDL$_3$ apoA-I kinetics may indicate that there is a rapid cycle of apoA-I between HDL particles or a fast interconversion rate between these subclasses (Frenais et al., 1997; Walsh et al., 1994).

In the study by Frenais et al. (1997), HDL$_2$ and HDL$_3$ were separated by gradient ultracentrifugation. By using this gradient ultracentrifugation method, lipoproteins in a sample are separated based on different density in a single spin. Therefore, this method may cause difficulties in avoiding the disturbance of different fractions when removing them, and in identifying the density ranges for each fraction without any visible marker, which may lead to a mixture of HDL$_2$ and HDL$_3$ (Li, 2009). However, in the present study, the isolation of HDL$_2$ and HDL$_3$ fractions was achieved by sequential ultracentrifugation.
for each fraction, and each lipoprotein fraction was removed after each ultracentrifugation step, which reduced the possibility of mixing of fractions. In the study by Walsh et al. (1994), although sequential ultracentrifugation was also used to separate HDL₂ and HDL₃, the plasma was stored at -80 °C before sequential ultracentrifugation, and freezing and thawing affect the integrity of HDL₂ and HDL₃. The differences in the results between the current study and previous studies may be also due to the differences in subject characteristics, the duration of clinical study protocol and the small sample size.

Hasselwander et al. (1999) investigated the composition of total HDL among 10 healthy subjects (5 female and 5 male), and found that that HDL particles contain higher proportion of cholesterol (0.59 ± 0.13 mmol/g HDL protein) and lower amount of triglyceride (0.05 ± 0.03 mmol/g HDL protein). In terms of HDL subclasses, it has been shown that both HDL₂ and HDL₃ comprise higher percentage of cholesterol esters and lower percentage of triglyceride among healthy subjects (Shuhei et al., 2010). These results match the data in the present study. Moreover, Shuhei et al. (2010) also reported that the ratio of CE/TG in HDL₂ and HDL₃ fractions were 6.00 and 7.07 respectively, which is similar to the cholesterol to TG ratio in HDL₂ and HDL₃ fractions in the current study (7.00 and 7.13 respectively).

According to the particle size, HDL₂ can be further divided into HDL₂b and HDL₂a, and HDL₃ can be divided into HDL₃a, HDL₃b and HDL₃c (Barter, 2002). It has been suggested that HDL₂ is a more variable subfraction and reflects the alteration in total HDL levels (Bakogiani et al., 2001). Schaefer et al. (1982) suggested the level of total HDL is significantly associated with the level of HDL₂b and HDL₂a. In the present study, the total HDL-C concentration was strongly associated with HDL₂ cholesterol and HDL₂ apoA-I
levels, which matches the findings in the previous studies. Moreover, PR of HDL$_2$ was significantly higher in women than men, which could be a possible explanation of a higher total HDL concentration in females compared to males.

Pathological conditions like CHD and type 2 diabetes have been found to be associated with a decreased level of HDL$_2$ cholesterol and increased HDL$_3$ cholesterol concentration (Bakogianni et al., 2001). Shuhei et al. (2010) investigated the distribution of HDL subclasses in 36 low-HDL-C subjects (12 of them had CHD) and 41 healthy subjects with normal HDL-C levels, and they found that the proportion of HDL$_{2b}$ was remarkably lower, while the amount of HDL$_{3a}$ and HDL$_{3b}$ were significantly higher in subjects with low HDL-C compared to the healthy subjects. Moreover, it has been suggested that the protective effect of total HDL against CHD is primarily performed by the HDL$_2$ fraction (Bakogianni et al., 2001). However, an inconsistent finding arose in the study by Kontush et al. (2003). It has been known that oxidation of LDL is a key stage in inflammation and atherogenesis, and HDL has the effect on protecting LDL from oxidation. Kontush et al. (2003) examined the capacity of HDL subclasses to protect the oxidation of LDL, and reported that the antioxidative activity of HDL enhanced with the increment of the density of subclass particles: HDL$_{2b}$<HDL$_{2a}$<HDL$_{3a}$<HDL$_{3b}$<HDL$_{3c}$. They suggested that the small and dense HDL$_3$ particles had a higher capacity to protect LDL against oxidation than HDL$_2$ particles, which implies that HDL$_3$ has a stronger protective effect against CHD.

A mathematical model has been used commonly to analyse the kinetic data in the previous lipoprotein kinetic studies. It can provide not only the FCR and PR, but also the interconversion rates. A mathematical model was developed using the data from the present HDL subclass study. However, this model was unable to fit all leucine
enrichment curves for αHDL, preβHDL, HDL₂ and HDL₃ very well, especially for the decaying phase of the curve. In the future, it may be possible to develop a model which provides a better description of the data.
Chapter 5: General discussion

It is well established that HDL plays a key role in the reverse cholesterol transport (RCT) pathway, which transports excessive cholesterol from the peripheral tissues back to the liver, and there is considerable evidence that it has a protective effect against the risk of atherosclerotic cardiovascular disease. This thesis has focused on the effect of dietary sugar intake on total HDL kinetics in overweight men at risk of metabolic syndrome. It has also investigated the kinetics of HDL subclasses, αHDL and preβHDL, HDL₂ and HDL₃, in healthy males and females.

The stable isotopic technique has been applied to investigate both total HDL and HDL subclass kinetics, whereas the clinical protocols were different. The administration of a primed constant infusion of stable isotope for a relative short study period (10 hours) was used to examine total HDL kinetics. A bolus injection of stable isotope was used for assessing the kinetics of HDL subclasses, and the study duration was longer (up to 14 days). ApoA-I as the major apolipoprotein of HDL has a relatively slow turnover rate with a mean residence time of about 5 days, and the stable isotope enrichment of apoA-I was measured to determine the HDL kinetic parameters. Therefore, the study duration of up to 14 days seems to be a more appropriate protocol to measure the kinetics of HDL compared to the study with short duration. However, compared to a primed constant infusion technique, a bolus injection of stable isotope would be a less feasible clinical protocol for patients’ convenience when the study duration is up to two weeks.

αHDL, which contains both HDL₂ and HDL₃, is the mature form of the HDL particle and should account for the major proportion of total HDL in the plasma. Therefore, the PR of αHDL was expected to be similar to the total PR of HDL₂ and HDL₃. However, the PR of
αHDL apoA-I in the HDL subclass study (3.94 ± 0.73 mg/kg/day) was comparatively lower than the total PR of HDL₂ and HDL₃ (1.35 ± 0.35 and 3.81 ± 0.51 mg/kg/day respectively). A possible explanation for the low PR of αHDL apoA-I in the HDL subclass study could be that there might be some other HDL subclasses beside αHDL and preβHDL. Asztalos et al. (2004) measured the values of HDL subspecies in plasma in 1277 CHD-free male subjects from the Framingham Offspring Study. They separated HDL subclasses based on different charge by using agarose gel electrophoresis, which was the same technique used in the present study. However, in addition to αHDL and preβHDL, Asztalos et al. (2004) also isolated one more apoA-I-containing HDL subclass, which is pre αHDL. Even though the level of pre αHDL was much lower than αHDL, it was slight higher than the level of preβHDL. Moreover, Asztalos et al. (2004) found that the level of pre αHDL was significantly lower in subjects with CHD compared to CHD-free subjects.

The total PR of HDL₂ and HDL₃ (1.35 ± 0.35 and 3.81 ± 0.51 mg/kg/day respectively) in the subclass study was expected to be similar to total HDL PR in the diet study (7.33 ± 0.66 and 6.05 ± 0.72 mg/kg/day for the high and low sugar diet respectively). However it was also lower. This may be a consequence of the different subject characteristics. Frenais et al. (1997) compared the HDL apoA-I FCR and PR between healthy subjects and subjects with type 2 diabetes (with poor metabolic control). They found that subjects with type 2 diabetes who had a lower plasma concentration of apoA-I and HDL-C, had a higher apoA-I FCR and PR than healthy subjects. The participants in the dietary intervention study were at metabolic risk and had a lower level of HDL apoA-I and HDL-C compared to the healthy subjects in the HDL subclass study.
Numerous previous studies have examined the relationship between HDL cholesterol and cardiovascular risk, and a strong inverse association was found. Hence, increasing plasma HDL cholesterol levels via lifestyle modification or pharmacological intervention was considered to be a therapeutic target for reducing the risk of CVD. However, some recently genetic studies may challenge this concept (Haase et al., 2011; Haase et al., 2012; Voight et al., 2012). The study by Haase et al. (2011) has examined the effect of mutations in apoA-I on HDL cholesterol and risk of ischemic heart disease (IHD) and myocardial infarction (MI). They suggested that mutation in apoA-I could predict an increased risk of IHD and MI in the general population independent of plasma HDL cholesterol levels. The other two recent genetic studies have investigated whether the inverse association between HDL cholesterol and the risk of IHD and MI is a causal relation (Haase et al., 2012; Voight et al., 2012). Both of these studies found that genetically reduced HDL cholesterol did not associated with an increased risk of IHD or MI.

The findings from pharmacological intervention studies, which examined the clinical effects of HDL-C raising therapy, have been inconsistent. Fibrates are recommended for the treatment of people with low HDL-C or high triglyceride levels. In the Helsinki Heart Study, the Veterans Affairs High-density Lipoprotein Cholesterol Intervention Trial (VA-HIT), and the Bezafibrate Infarction Prevention study, the administration of fibrates in subjects with low HDL-C or high TG and cholesterol levels, or with CHD, induced a moderate increase in HDL-C after a long-term follow-up, and this increase was positively associated with a modest to significant reduction in the incidence and/or mortality of CHD (Frick et al., 1987; Rubins et al., 1999; Robins et al., 2001; the BIP Study Group, 2000). However, in some other studies, the beneficial effect of fibrates on CHD was only showed in patients with reduced HDL-C and increased TG but not in the subjects without
dyslipidaemia (Keech et al., 2005; Group et al., 2010; Scott et al., 2009). Niacin has been considered to be the most effective pharmacological method for raising low HDL-C levels (Rader, 2006). A number of previous studies have investigated the effects of niacin, which was used as a mono-therapy or in a combination dyslipidaemic therapy, on cardiovascular outcomes after a long-term follow-up (Canner et al., 1986; Carlson et al., 1988; Brown et al., 1990; Brown et al., 1998; Brown et al., 2001). They found that niacin was associated with a moderate to significant reduction in CHD mortality and nonfatal MI. Some studies showed the increased level of HDL-C was in parallel with the reduction in CHD events (Brown et al., 1990; Brown et al., 1998; Brown et al., 2001). However, not all of the previous studies measured the HDL-C levels. Therefore, the current data may not be sufficient to estimate the effect of the alteration in HDL-C levels on the reduction of CHD after a long-term treatment. CETP inhibition has also been considered to be a possible therapeutic target for HDL-raising therapy. In a trial by Barter et al. (2007), although the CETP inhibitor torcetrapid increased HDL-C levels dramatically (about 72%) in subjects with a high risk of CHD, it was also associated with an increased incidence and mortality of CHD. Barter et al. (2007) stated that the reason for this finding was uncertain, and they suggested that increasing the level of HDL-C might not be adequate for achieving a protection against CVD.

Based on these genetic and pharmacologic intervention studies, there is some doubt as to whether HDL cholesterol is a reasonable therapeutic target. A consensus statement from the National Lipids Association was published last year, which assessed the current status of HDL-C as a therapeutic target and evaluated the raised question regarding to the role of HDL-C in preventing or contributing to atherosclerotic disease (Toth et al., 2013). They concluded that HDL cholesterol is not a therapeutic target at the present time even though a lot of investigations have suggested that low HDL-C is associated
with an increased risk of CVD and HDL may play various roles in protecting against atherogenesis. Toth et al. (2013), however, still emphasised that “rigorous research into the biology and clinical significant of low HDL-C should continue. The development of novel drugs designed to modulate the serum levels and functionality of HDL particles should also continue”. They also suggested that research exploring the therapeutic effect of modulating HDL structure and function needs to be continued. This consensus statement by Toth et al. (2013) focused on the plasma concentration of HDL-C. However, as discussed in this thesis HDL is a heterogeneous mix of subclasses and measurement of these subclasses and their functionality rather than total HDL-C may be a more relevant measurement. Understanding the kinetics of these HDL subclasses may also provide more valuable information about the role of HDL in reducing atherosclerosis.
References:


165


Orso, E., Broccardo, C., Kaminski, W. E., Bottcher, A., Liebisch, G., Drobnik, W., Gotz, A., Chambenoit, O., Diederich, W., Langmann, T., Spruss, T., Luciani, M. F., Rothe, G.,


Appendix 1: Participant information sheet for dietary intervention study

Participant Information sheet

Study Title: “The Effect of Dietary Carbohydrate on Lipids.”

You are being invited to take part in a study investigating how dietary carbohydrate affects fat metabolism. Before you decide if you wish to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with your friends, relatives and anyone involved with your care. Please ask us if there is anything that is not clear or if you would like more information.

Why have I been chosen?
In this study we are looking for men, aged between 40-65, who are overweight but do not have diabetes. We want participants to be weight stable and not taking any other medication that may affect metabolism.

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

What would happen to me if I take part?
If you are interested in taking part in this study, you would come to the Cedar Centre at the Royal Surrey County Hospital in the morning, without having had breakfast, for an initial chat about the project, when we would measure your height, weight, take your blood pressure and take a small blood sample to measure your glucose, insulin, blood chemistry and fat levels. After this you will be provided with a snack and drink. This is a screening visit (VISIT 1) to make sure you do not have diabetes or any other disorder that may affect you taking part in the study. We also ask you to fill in two short questionnaires about your general health and diet. We would normally inform you of your results within 2 weeks of this visit.
If following this screening visit, you are found to fulfil the recruitment criteria and you are still happy to take part we will ask you to visit the Hammersmith Hospital in London for a second screening visit (VISIT 2) to measure the amount of fat in your liver. We are recruiting subjects with very low or moderate levels of liver fat.

If you fulfil these criteria we will ask you to undertake a dietary intervention for a period of 32 weeks. This will comprise a 4 week diet based on the average UK diet followed by a 12 week diet with either high or low sugar foods, followed by a 4 week diet based on the average UK diet and finally a 12 week diet with either high or low sugar foods (depending on which diet you followed for the first 12 weeks). Half the men in the study will have the high sugar foods first and the low sugar diet second and the other half will have the low sugar diet first and the high sugar diet second. The nutritionist will give you guidance and advice on how to follow this diet at the Cedar Centre before you start, and on how to complete a 3-day diet diary (VISIT 3). Diet diaries will be completed at the end of the run-in diet, and at 6 and 12 weeks during each 12 week dietary period. We will ask you to meet the nutritionist every 2 weeks, who will also contact you by telephone during the weeks you do not meet.

At the end of the first 4 week diet we will ask you to visit the Cedar Centre at the Royal Surrey County Hospital for a blood test to measure your blood fat levels (VISIT 4). You will then be allocated to one of the two diets. At week 11 of the diet you will be asked to visit Hammersmith Hospital for another MRI scan (VISIT 5). This visit is identical to visit 2. At the end of the first 12 week diet we will ask you to visit the Cedar Centre on 2 successive days for a series of tests (VISITS 6 and 7). At the end of the second 4 week wash-out we will ask you to visit the Cedar Centre for a blood test to measure fat levels (VISIT 8) to start the next diet. At the end of the second 12 week diet at week 11 you will be asked to visit Hammersmith Hospital for another MRI scan (VISIT 9). This visit is identical to visit 2 we will ask you to visit the Cedar Centre on 2 successive days for a series of tests (VISITS 10 and 11). These tests will be identical to the tests done at visits 6 and 7.

**VISITS 2, 4 and 7**

You will be asked to go to the Hammersmith Hospital, London after a 10 hour fast to have an MRI (magnetic resonance imaging) scan. MRI is a body scanning technique, which is used in hospitals worldwide to create images of the inside of
the body. MRI has been used safely for several decades and has no known side-effects. Each scan is directed to the specific requirements of the research study in which you are taking part. **The MRI scan is purely for the detection of liver fat and is not for the purposes of a health check.**

You will be given an appointment for magnetic resonance scans at the Robert Steiner MRI unit and asked to come fasted (nothing to eat or drink for 10 hours before your scan) however you may drink some water and take any medication you would normally do.

As the MRI scanner uses high magnetic field we have to make sure you do not have any metallic object in or on your body to ensure your safety. Before starting the scanning we will ask you to complete a short checklist. This will ensure that you do not have any magnetic implant (such as a cardiac pacemaker) which will stop us performing the scan. These scans are safe and do not involve any radioactivity or X rays.

You will be in the MR scanner for up to 1 hour. None of the magnetic resonance imaging techniques uses ionising radiation or intravenous contrast agents and may be performed during a single imaging session. You lie flat in the scanner and are automatically moved through the scanner. Whole body anatomical MR scanning will be performed to determine total and regional fat volumes, and magnetic resonance spectroscopy (MRS) performed to measure lipid content in internal organs, such as liver (IHCL) and muscles (IMCL), such as soleus and tibialis.

You may hear a knocking noise during part of the procedure, and you will experience no discomfort. We will give you an alarm bell to sound at any time if you are upset or worried during the examination and if the procedure does not suit you for any reason it can be stopped at any time.

**ANTHROPOMETRY**

Baseline anthropometric measurements including height, weight, waist and hip circumference, skinfold thickness, blood pressure, body-volume, and bioelectrical impedance analysis of percentage body fat. The bioelectrical impedance is a painless, safe procedure which involves either lying on a couch with sticky pads placed on a hand and foot, or standing on a metal platform for 1 minute so that the body’s electrical resistance can be measured.
What if anything abnormal is discovered?

MRI is a powerful, diagnostic body scanning technique used in hospitals worldwide to create images of the inside of the body. MRI has been used safely for several decades and has no known side-effects. These scans cannot be viewed as a health screening procedure. However, very rarely, unexpected information can be detected which may warrant further investigation. In this event, a report will be sent to your GP, who will arrange further tests and coordinate your further care. The doctor involved in the research study will also be informed.

VISIT 3
You will be asked to come to the Royal Surrey County Hospital in the morning having fasted since 10 pm the previous day. At this visit we will take a blood sample to measure fat levels. After the sample is taken you will be provided with a snack and drink. You will then meet with the nutritionist who will explain about the 12 week diet we would like you to be on. She will supply you with the food you will need for this period. If you do not attend by car, we can arrange for the food to be delivered to your home. This visit will take about 30-45 minutes.

VISIT 4
Hammersmith Hospital MRI scan. This visit is identical to visit 2.

VISIT 5
This visit will take place at the end of the 12 week diet with high or low sugar content. We ask you to attend the Cedar Centre at the Royal Surrey County Hospital. A blood sample will be taken and you will be given 2 small bottles of water containing labelled water and one large bottle of water containing labelled water. You will be asked to drink one small bottle of the labelled water at 7pm after your evening meal and the other small bottle at 10pm the same night. After 7pm, if you need to drink we will ask you to drink only the water in the large bottle without any other food. This visit will take about 20 minutes. We will provide you with a standard ready-meal for your evening meal (of your choice) to take home.

VISIT 6
The next day you will be asked to come to the Cedar Centre at the Royal Surrey County Hospital at 7.30am having fasted since 8pm the previous day. We will
provide you with a standard ready-meal for your evening meal to eat at home the day before the study. We ask you not to undertake any vigorous activity for 2 days before this visit and not to drink alcohol the day before.

We will put 3 fine plastic tubes into your veins. Two will be placed in a vein in each elbow and one in a wrist vein. The tubes are made of a flexible plastic and so once placed should not cause any discomfort and will not impede your arm movements. After taking a baseline blood sample, we will inject a labelled molecule of glycerol (which is a non-harmful fat that our body normally produces) to measure how much fat your liver is making. We will also start pumping (infusing) 2 solutions at a slow rate into one of the tubes in your elbow for 10 hours. This contains a labelled molecule of palmitic acid (another non-harmful fat that our body normally produces) bound to a blood protein albumin which can be used to measure how much fat your body is burning. This solution also contains a labelled amino acid which measures the number of particles of fat that your liver makes. At frequent time intervals during the 10 hour study blood samples will be taken from the vein in your elbow and the vein in your wrist. While we are taking blood from your wrist we will ask you to place your hand inside a “hot-box” for 20 minutes before we take a blood sample. This box is made of Perspex and heats the blood in your wrist making the blood similar to blood found in arteries. It will make your hand hot but this is not uncomfortable. You will not feel the blood samples being taken. At the end of the study we will measure the activity of 2 enzymes which break down fat in your blood. A small amount of a substance called heparin will be injected which releases the enzymes then a blood sample will be taken 15 minutes later. The total amount of blood we will take in this test is 11 tablespoons of blood (220mls).

During the study you will be free to write/read or watch DVDs within the unit. Drinking water will be made available throughout the study although food will only be provided at the end of the study. Although you will be connected to an infusion pump, that pump can be moved so there will be no limit to going to the toilet etc. At the end of the study day you will be provided with a meal of your choice with tea or coffee. The whole procedure (from arrival to going home) would take approximately 11 hours.

VISIT 7
This is before you start your second diet and identical to visit 4.
VISIT 8
Hammersmith Hospital MRI scan. This visit is identical to visit 2.

VISITS 9 and 10
These are at the end of your second diet and identical to visits 6 and 7.

Expenses
All travelling expenses will be reimbursed and there will be an inconvenience allowance of £200 payable upon completion of the study.

What do I have to do?
You will need to have fasted overnight (for 12 hours) for some of the visits. This means having no food or drink, other than water, after your evening meal the day before your visit. In addition, we will ask you to eat a standard ready-meal, which we will provide for you to eat at home, for your evening meal the night before visit 6 and visit 9. We also ask you to refrain from vigorous exercise for 2 days before these visits and to avoid alcohol.

What are the possible disadvantages and risks of taking part?
The risks of having a cannula in a vein are those associated with any blood sample. These are slight pain, possible bruising and, in principle, a local infection. We will try and avoid these problems.
In visit 5 and 8 one of the labelled molecules is bound to albumin. As this is a blood protein there is a small risk of allergic reaction. Albumin is a blood protein and is purified from human blood. As such there is a very small risk of transmission of infectious diseases. To minimise this, the albumin is purified from blood donors who have been screened for pathogenic viruses and the albumin is heat treated to inactivate viruses.
In visit 5 and 8 we will administer a small amount of heparin which thins the blood. You will be advised to avoid caffeine for 4 hours after this. You may have excessive bruising if you hit yourself hard within 4 hours of the procedure.

What are the possible benefits of taking part?
You will be helping medical science to understand how different types of carbohydrate affect fat metabolism. This will help formulate appropriate dietary advice for men at risk of metabolic syndrome.
**What if something goes wrong?**
If you are harmed by taking part in this research project then you may have grounds for legal action. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal University complaints mechanisms are available to you.

**Complaints**
Any complaint or concerns about any aspects of the way you have been dealt with during the course of the study will be addressed. Please contact Prof. Bruce Griffin, Principal Investigator on **01483 689724**.

**Would my taking part in this study be kept confidential?**
All information which is collected about you during the course of this research would be kept strictly confidential. Any information about you which leaves the hospital or University would have your name and address removed so that you cannot be recognised from it. With your permission, your GP will be notified of your participation in this study.

**What would happen to the results of the research study?**
The results of this study will be published in the scientific press and you will not be identifiable in any way. It is our usual practice to send copies of any resultant publications to research participants.

**Who is organising the funding and research?**
This research is being funded by the Biotechnology and Biological Sciences Research Council, a government funded research body.

**Who reviewed this study?**
The study was reviewed by the Surrey Research Ethics Committee.

**Contact for further information?**
Cheryl Isherwood (01483 688642) c.isherwood@surrey.ac.uk
Prof Bruce Griffin (01483 689724) b.griffin@surrey.ac.uk
Dr John Wright (01483 571122 ext 4844) john.wright@surrey.ac.uk
Appendix 2: Participant information sheet for pilot study

**Participant Information Sheet**

**Study Title:**

“Development of a study design to measure HDL metabolism”

You are being invited to take part in a study to develop an optimal study design to measure HDL metabolism. We want to determine an optimal blood sampling protocol and whether it is necessary for subjects to be fasted. Before you decide if you wish to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with your friends, relatives and anyone involved with your care. Please ask us if there is anything that is not clear or if you would like more information.

**Why have I been chosen?**

In this study we are looking for healthy women age 18-70y.

**Do I have to take part?**

If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw at any time and without giving a reason. You will undertake 1 study. In this study you will need to be fasted on all the study visits.

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

Each study will involve 5-6 visits to the Cedar Centre at the Royal Surrey County Hospital in the morning. One visit will take up to 10.5 hours and the other visits will take about 15 minutes.

If you are interested in taking part in this study, we will ask you to fill in a short questionnaire about your general health.
VISIT 1: CEDAR
Measurement of HDL metabolism (approximately 10.5 hours)

You are will be invited to come to the CEDAR Centre at 8am on a Monday, without having had breakfast for a metabolic study to measure HDL (good cholesterol) metabolism. Height, weight, waist and hip circumference, and body fat will be measured. A fine plastic tube will be placed in a vein in the crease of one elbow. The tube is made of flexible plastic and should not cause any discomfort and will not impede your arm movement once placed. After taking a blood sample via this tube, we will inject a labelled molecule of an amino acid (a non-harmful building block of proteins that our body normally produces) into a vein in your other arm to measure how much good cholesterol your liver is making. Small blood samples (each will be approximately 2 teaspoons of blood) will be taken at hourly intervals for 10 hours via the plastic tube. During the study you will be only given water to drink. You will be able to read, watch DVDs or listen to music. At the end of the study the tube will be removed, we will give you a drink and a meal (of your choice) and you will be free to go home.

VISIT 2, 3, 4, 5: CEDAR
Continued measurement of HDL metabolism (approximately 15 min)

The next morning and 4 other mornings during the next two weeks we will ask you to come to the CEDAR Centre in the morning, without having had breakfast for a single blood sample (2 teaspoons). We will give you a drink and snack afterwards.

Expenses
All travelling expenses will be reimbursed.

What do I have to do?
You will need to have fasted overnight (for 12 hours) for all visits. This means having no food or drink, other than water, after your evening meal at 8 pm the day before your visit.

What are the possible disadvantages and risks of taking part?
The risks of having a cannula in a vein are those associated with any blood sample. These are minor discomfort, possible bruising and, in principle, a local infection. We will try and avoid these problems.
What are the possible benefits of taking part?
You will be helping medical science. Once a study design has been optimised we will use this to understand more about good cholesterol metabolism. This could help in developing treatments which raise good cholesterol levels.

What if something goes wrong?
If you are harmed by taking part in this research project then you may have grounds for legal action. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal University complaints mechanisms are available to you.

Complaints
Any complaint or concerns about any aspects of the way you have been dealt with during the course of the study will be addressed. Please contact Prof. Margot Umpleby, Principal Investigator on 01483 688579.

Would my taking part in this study be kept confidential?
All information which is collected about you during the course of this research would be kept strictly confidential. Any information about you which leaves the hospital or University would have your name and address removed so that you cannot be recognised from it. With your permission, your GP will be notified of your participation in this study.

What would happen to the results of the research study?
The results of this study will be used to optimise a study design which can be used to understand more about good cholesterol metabolism.

Who is organising the funding and research?
This research is being funded and organised by the University of Surrey.

Who reviewed this study?
This research has been reviewed by two human ethics committees (Surrey Research Ethics Committee and University of Surrey Ethics Committee).

Contact for further information?
Ke Wang (01483 688639)
k.wang@surrey.ac.uk
Prof Margot Umpleby (01483 688579)
m.umpleby@surrey.ac.uk

Dr Fariba Shojae-Moradie (01483 688644)

f.shojae-moradie@surrey.ac.uk
Appendix 3: Participant screening sheet for pilot study

Development of a study design to measure HDL metabolism

Participant screening sheet

GENERAL INFORMATION

Name:
Address:

Contact telephone numbers:
Best time to call / on what number?

Email address:

Next of kin contact name:

Next of kin telephone number:

GP Name:

GP Address:

Date of birth:
Age:

Occupation & town:

Date informed consent obtained:

Dates of any holidays booked
Would you like to be sent information on other studies carried out at The University of Surrey that you may be suitable for?  

Yes          No

VISIT 1. SCREENING VISIT

Date:  

Weight: .................  Height: .................  BMI: .................... (kg/m²)  

Waist circumference: ........................................


<table>
<thead>
<tr>
<th></th>
<th>YES</th>
<th>NO</th>
</tr>
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<tbody>
<tr>
<td>Has been weight stable for the past three months (max var. 2.5 kg)</td>
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<tr>
<td>Prior/present history of Type 1 or Type 2 diabetes; self and/or family</td>
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<tr>
<td>Prior/present history of hormone disorders</td>
<td></td>
<td></td>
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<tr>
<td>Prior/present history of liver disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior/present history of kidney disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior/present history of heart disease, Angina or Stroke</td>
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<tr>
<td>Prior/present history of eating disorders, inc anorexia or bulimia nervosa</td>
<td></td>
<td></td>
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<tr>
<td>Prior/present history of drug or alcohol abuse within the last 2 years</td>
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<tr>
<td>Average consumption of alcoholic drinks a day? What kind?</td>
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<tr>
<td>Any previous surgical procedure to aid weight loss</td>
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<td></td>
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<tr>
<td>Any known food allergies or intolerances</td>
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<tr>
<td>If yes please specify</td>
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<tr>
<td>Medications known to alter lipid metabolism or body weight</td>
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<tr>
<td>Beta-blockers</td>
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<tr>
<td>Statins</td>
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<tr>
<td>Fibrates</td>
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<tr>
<td>Metformin</td>
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</table>


Non-smoker / Smoker: .................................................................

Amount of Exercise/week ......................................................

Sedentary/non sedentary occupation........................................

**Blood samples analyses:**

**Haematology:**
- Haemoglobin.................................................................
- White blood cell count.............................................
- Platelet count............................................................

**Clinical chemistry:**
- Triglyceride..............................................................
- Total cholesterol ...................................................
- Blood glucose .........................................................
- Insulin .................................................................

Suitable to participate in study:

a) Yes ............ No ............ Maybe ............

Pease give further details:

...........................................................................................................

..................................................
Appendix 4: Participant information sheet for HDL subclass kinetic study

Participant Information Sheet

Study Title:
“Effect of gender differences on HDL subclass kinetics”

You are being invited to take part in a study to develop an optimal study design to measure HDL metabolism. We want to determine an optimal blood sampling protocol and whether it is necessary for subjects to be fasted. Before you decide if you wish to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with your friends, relatives and anyone involved with your care. Please ask us if there is anything that is not clear or if you would like more information.

Why have I been chosen?
In this study we are looking for healthy women and men age 18-70y.

Do I have to take part?
If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw at any time and without giving a reason. You will undertake 1 study. In this study you will need to be fasted on all the study visits.

What would happen to me if I take part?
Each study will involve 8 visits to the Cedar Centre at the Royal Surrey County Hospital in the morning. One visit will take up to 11 hours and the other visits will take about 15 minutes.

If you are interested in taking part in this study, we will ask you to fill in a short questionnaire about your general health.

Visit 1: Screening (approximately 1 hour)
If you are interested in taking part in this study, you would come to the Cedar Centre at the Royal Surrey County Hospital in the morning, without having had
breakfast. Someone from the research team will ask you some questions about your health. The research nurse will measure your height, weight, take your blood pressure and take a blood sample (3 teaspoons) to measure your blood glucose and check you are not anaemic. After this you will be provided with a snack and drink.

This is a screening visit to make sure you do not have diabetes or any other disorder that may affect you taking part in the study. We would normally inform you of your results within 2 weeks of this visit.

**VISIT 2: CEDAR**

**Measurement of HDL metabolism (approximately 11 hours)**

If you are healthy we will invite you to come to the CEDAR Centre at 8am on a Monday, without having had breakfast for a metabolic study to measure HDL (good cholesterol) metabolism. Height, weight, waist and hip circumference, and body fat will be measured. A fine plastic tube will be placed in a vein in the crease of one elbow. The tube is made of flexible plastic and should not cause any discomfort and will not impede your arm movement once placed. After taking a blood sample via this tube, we will inject a labelled molecule of an amino acid (a non-harmful building block of proteins that our body normally produces) into a vein in your other arm to measure how much good cholesterol your liver is making. Small blood samples (each will be approximately 2 teaspoons of blood) will be taken at hourly intervals for 10 hours via the plastic tube. During the study you will be only given water to drink. You will be able to read, watch DVDs or listen to music. At the end of the study the tube will be removed, we will give you a drink and a meal (of your choice) and you will be free to go home.

**VISIT 3, 4, 5, 6, 7 & 8: CEDAR**

**Continued measurement of HDL metabolism (approximately 15 min)**

The next morning and 5 other mornings during the next two weeks we will ask you to come to the CEDAR Centre in the morning, without having had breakfast for a single blood sample (2 teaspoons). We will give you a drink and snack afterwards.
Expenses
All travelling expenses will be reimbursed. A payment of £200 will be made on completion of the last visit.

What do I have to do?
You will need to have fasted overnight (for 12 hours) for all visits. This means having no food or drink, other than water, after your evening meal at 8 pm the day before your visit.

What are the possible disadvantages and risks of taking part?
The risks of having a cannula in a vein are those associated with any blood sample. These are minor discomfort, possible bruising and, in principle, a local infection. We will try and avoid these problems.

What are the possible benefits of taking part?
You will be helping medical science. Once a study design has been optimised we will use this to understand more about good cholesterol metabolism. This could help in developing treatments which raise good cholesterol levels.

What if something goes wrong?
If you are harmed by taking part in this research project then you may have grounds for legal action. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal University complaints mechanisms are available to you.

Complaints
Any complaint or concerns about any aspects of the way you have been dealt with during the course of the study will be addressed. Please contact Prof. Margot Umpleby, Principal Investigator on 01483 688579.

Would my taking part in this study be kept confidential?
All information which is collected about you during the course of this research would be kept strictly confidential. Any information about you which leaves the hospital or University would have your name and address removed so that you cannot be recognised from it. With your permission, your GP will be notified of your participation in this study.
What would happen to the results of the research study?
The results of this study will be used to optimise a study design which can be used to understand more about good cholesterol metabolism.

Who is organising the funding and research?
This research is being funded and organised by the University of Surrey.

Who reviewed this study?
This research has been reviewed by two human ethics committees (Surrey Research Ethics Committee and University of Surrey Ethics Committee).

List of visits: Design of study for HDL metabolism

<table>
<thead>
<tr>
<th>Visit</th>
<th>Approximate Week</th>
<th>Test approx. time</th>
<th>Fasted</th>
<th>Place</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visit 1</td>
<td>Screening</td>
<td>1hour</td>
<td>Required</td>
<td>CEDAR</td>
<td></td>
</tr>
<tr>
<td>Visit 2</td>
<td>Week 1</td>
<td>11 hours</td>
<td>Required</td>
<td>CEDAR</td>
<td>HDL metabolism study</td>
</tr>
<tr>
<td>Visit 3</td>
<td>Week 1: 1 days from Visit 2</td>
<td>15 min</td>
<td>Required</td>
<td>CEDAR</td>
<td>Single blood test</td>
</tr>
<tr>
<td>Visit 4</td>
<td>Week 1: 4 days from Visit 2</td>
<td>15 min</td>
<td>Required</td>
<td>CEDAR</td>
<td>Single blood test</td>
</tr>
<tr>
<td>Visit 5</td>
<td>Week 2: 7 days from Visit 2</td>
<td>15 min</td>
<td>Required</td>
<td>CEDAR</td>
<td>Single blood test</td>
</tr>
<tr>
<td>Visit 6</td>
<td>Week 2: 9 days from Visit 2</td>
<td>15 mins</td>
<td>Required</td>
<td>CEDAR</td>
<td>Single blood test</td>
</tr>
<tr>
<td>Visit 7</td>
<td>Week 2: 11 days from Visit 2</td>
<td>15 min</td>
<td>Required</td>
<td>CEDAR</td>
<td>Single blood test</td>
</tr>
<tr>
<td>Visit 8</td>
<td>Week 3: 14 day from Visit 2</td>
<td>15 min</td>
<td>Required</td>
<td>CEDAR</td>
<td>Single blood test</td>
</tr>
</tbody>
</table>

Contact for further information?
Ke Wang (01483 688639)
k.wang@surrey.ac.uk

Prof Margot Umpleby (01483 688579)
m.umpleby@surrey.ac.uk

Dr Fariba Shojae-Moradie (01483 688644)
f.shojae-moradie@surrey.ac.uk
Appendix 5: Study consent form for HDL subclass kinetic study

Centre Number:  
Study Number:  
Patient Identification Number for this trial:

CONSENT FORM

Title of project: Effect of gender differences on good cholesterol

Name of Researcher: Dr Fariba Shojae-Moradie/ Dr. John Wright

Please initial box

I confirm that I have read and understand the Information Sheet dated January 20\textsuperscript{th} 2010 (Version 2) for the above study and have had the opportunity to ask questions. ☐

I consent to my personal data, as outlined in the accompanying information sheet, being used for the research project detailed in the information sheet, and agree that data collected may be shared with other researchers or interested parties. I understand that all personal data relating to volunteers is held and processed in the strictest confidence, and in accordance with the Data Protection Act (1998). ☐

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

I agree to take part in the above study ☐

Name of participant __________________________ Date __________________________ Signature __________________________

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

Researcher __________________________ Date __________________________ Signature __________________________
Appendix 6: Participant screening sheet for HDL subclass kinetic study

Effect of gender differences on HDL subclass kinetics

Participant screening sheet

GENERAL INFORMATION

Name:
Address:

Contact telephone numbers:
Best time to call / on what number?
Email address:
Next of kin contact name:
Next of kin telephone number:
GP Name:
GP Address:

Date of birth:
Age:
Occupation & town:
Date informed consent obtained:
Dates of any holidays booked
Would you like to be sent information on other studies carried out at The University of Surrey that you may be suitable for?

Yes  No

VISIT 1. SCREENING VISIT

Date:……………..

Weight: ……………… Height: ………………… BMI: ………………. (kg/m²)

Waist circumference …………………………….

Blood pressure:   R1……………………. R2 ……………………… R3……………….. ave

………………………….

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Has been weight stable for the past three months (max var. 2.5 kg)</td>
<td></td>
</tr>
<tr>
<td>Prior/present history of Type 1 or Type 2 diabetes; self and/or family</td>
<td></td>
</tr>
<tr>
<td>Prior/present history of hormone disorders</td>
<td></td>
</tr>
<tr>
<td>Prior/present history of liver disease</td>
<td></td>
</tr>
<tr>
<td>Prior/present history of kidney disease</td>
<td></td>
</tr>
<tr>
<td>Prior/present history of heart disease, Angina or Stroke</td>
<td></td>
</tr>
<tr>
<td>Prior/present history of eating disorders, inc anorexia or bulimia nervosa</td>
<td></td>
</tr>
<tr>
<td>Prior/present history of drug or alcohol abuse within the last 2 years</td>
<td></td>
</tr>
<tr>
<td>Average consumption of alcoholic drinks a day? What kind?</td>
<td></td>
</tr>
<tr>
<td>Any previous surgical procedure to aid weight loss</td>
<td></td>
</tr>
<tr>
<td>Any known food allergies or intolerances</td>
<td></td>
</tr>
<tr>
<td>If yes please specify</td>
<td></td>
</tr>
</tbody>
</table>

Medications known to alter lipid metabolism or body weight

Beta-blockers

Statins

Fibrates

Metformin

Any other medication / supplements
Non-smoker / Smoker: .................................................................

Amount of Exercise/week .........................................................

Sedentary/non sedentary occupation...........................................

**Blood samples analyses:**

Haematology:
- Haemoglobin.........................................................
- White blood cell count.................................
- Platelet count..................................................

Clinical chemistry:
- Triglyceride..................................................
- Total cholesterol .................................
- Blood glucose ..........................................
- Insulin ...........................................

Suitable to participate in study:

b) Yes .............  No .............  Maybe ..........

Please give further details:

...............................................................................

If yes please specify

.............................................................................

 .............................................................................

 .............................................................................
Appendix 7: HDL subclass kinetic data for individual subject in Chapter 4

1. The isotopic enrichment of leucine in preβHDL and αHDL apoA-I

Figure A1(a): The isotopic enrichment of leucine in preβHDL and αHDL apoA-I during the first 10 hour study for subject F01

Figure A1(b): The isotopic enrichment of leucine in preβHDL and αHDL apoA-I during 14 days for subject F01
Figure A2(a): The isotopic enrichment of leucine in preβHDL and αHDL apoA-I during the first 10 hour study for subject F02.

Figure A2(b): The isotopic enrichment of leucine in preβHDL and αHDL apoA-I during 14 days for subject F02.
Figure A3(a): The isotopic enrichment of leucine in preβHDL and αHDL apoA-I during the first 10 hour study for subject F03

Figure A3(b): The isotopic enrichment of leucine in preβHDL and αHDL apoA-I during 14 days for subject F03
Figure A4(a): The isotopic enrichment of leucine in preβHDL and αHDL apoA-I during the first 10 hour study for subject M01

Figure A4(b): The isotopic enrichment of leucine in preβHDL and αHDL apoA-I during 14 days for subject M01
Figure A5(a): The isotopic enrichment of leucine in preβHDL and αHDL apoA-I during the first 10 hour study for subject M02

Figure A5(b): The isotopic enrichment of leucine in preβHDL and αHDL apoA-I during 14 days for subject M02
Figure A6(a): The isotopic enrichment of leucine in preβHDL and αHDL apoA-I during the first 10 hour study for subject M03

Figure A6(b): The isotopic enrichment of leucine in preβHDL and αHDL apoA-I during 14 days for subject M03
2. The isotopic enrichment of leucine in HDL$_2$ and HDL$_3$ apoA-I

Figure A7(a): The isotopic enrichment of leucine in HDL$_2$ and HDL$_3$ apoA-I during the first 10 hour study for subject F01

Figure A7(b): The isotopic enrichment of leucine in HDL$_2$ and HDL$_3$ apoA-I during 14 days for subject F01
Figure A8(a): The isotopic enrichment of leucine in HDL$_2$ and HDL$_3$ apoA-I during the first 10 hour study for subject F02

Figure A8(b): The isotopic enrichment of leucine in HDL$_2$ and HDL$_3$ apoA-I during 14 days for subject F02
Figure A9(a): The isotopic enrichment of leucine in HDL$_2$ and HDL$_3$ apoA-I during the first 10 hour study for subject F03

Figure A9(b): The isotopic enrichment of leucine in HDL$_2$ and HDL$_3$ apoA-I during 14 days for subject F03
Figure A10(a): The isotopic enrichment of leucine in HDL$_2$ and HDL$_3$ apoA-I during the first 10 hour study for subject M01

Figure A10(b): The isotopic enrichment of leucine in HDL$_2$ and HDL$_3$ apoA-I during 14 days for subject M01
Figure A11(a): The isotopic enrichment of leucine in HDL$_2$ and HDL$_3$ apoA-I during the first 10 hour study for subject M02

Figure A11(b): The isotopic enrichment of leucine in HDL$_2$ and HDL$_3$ apoA-I during 14 days for subject M02
Figure A12(a): The isotopic enrichment of leucine in HDL$_2$ and HDL$_3$ apoA-I during the first 10 hour study for subject M03

Figure A12(b): The isotopic enrichment of leucine in HDL$_2$ and HDL$_3$ apoA-I during 14 days for subject M03