UNIVERSITY OF SOUTHAMPTON

POSTPRANDIAL LIPID METABOLISM IN HEALTHY MEN AND IN PATIENTS WITH TYPE II DIABETES MELLITUS

BY

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"I believe the chief cause for premature development of arteriosclerosis in diabetes, save for advancing age, is excess fat: an excess of fat in the body; obesity, an excess of fat in the diet; and an excess of fat in the blood. With an excess of fat diabetes begins and from an excess of fat diabetes die; formerly of coma, recently of arteriosclerosis"

- E. P. Joslin (1927),
quoted by Paul Durrington
(Gotto & Pownall, 1999)

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ABSTRACT

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HUMAN NUTRITION

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POSTPRANDIAL LIPID METABOLISM IN HEALTHY MEN AND IN PATIENTS WITH TYPE II DIABETES MELLITUS

by Mohammad Arshad Humayun

Increasing age, obesity and type II diabetes are associated with disturbed postprandial lipid metabolism and increased risk for developing atherosclerosis. However, the exact mechanisms remains unclear partly due to problems in differentiating between exogenous and endogenous lipids in circulation.

The main purpose of this thesis was to use novel stable isotope tracer methodology for tracing the metabolism of exogenous lipids in healthy subjects and in those with type II diabetes. After an overnight fast, subjects ingested [1,1,1-^{13}C]tripalmitin within an emulsion and test meal (3.2 MJ; 90g carbohydrate; 36g lipid; 27g protein), followed by a second identical unlabelled meal 6 hours later. Blood and breath samples were collected and whole body CO_2 excretion was measured, by indirect calorimetry, before and at hourly intervals for 10 h following label administration. A chylomicron-rich fraction (CRF, Sf>400) was separated by discontinuous-gradient ultracentrifugation. The fatty acid profile of CRF triglycerides (CRF-TAG) and plasma non-esterified fatty acids (NEFA) and the ^{13}C-enrichment of palmitic acid (PA) methyl esters were determined by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). ^{13}C-enrichment of breath and stool was analysed by continuous flow isotope ratio mass spectrometry. Plasma TAG, NEFA and glucose concentrations were determined enzymically. Plasma insulin was determined by radio-immuno assay.

In the first study the validation of tracer methodology protocol was examined and repeatability of postprandial lipid metabolism was determined in healthy young men. The results of the validation study demonstrated precision in the lipid extraction procedures and in the measurement of enrichments and concentrations of ^{13}C palmitic acid by GC-C-IRMS system. The results of the repeatability study suggested that except for plasma glucose, the postprandial responses of plasma TAG, NEFA, ^{13}C palmitic acid in CRF-TAG and breath $^{13}CO_2$ were sufficiently repeatable to make it possible to explore potential differences between groups. Furthermore, ^{13}C excretion in stools was minimal in healthy subjects thereby eliminating the need to take faecal collections in subsequent studies.

In the subsequent studies the age-related changes and the effect of type II diabetes (normaltriglyceridaemic and hypertriglyceridaemic) on postprandial metabolism of lipids were examined. The combined results of these studies suggested that the presence of increasing fasting TAG resulted in a) decreased postprandial clearance of exogenous TAG from circulation b) decreased exogenous lipid oxidation and c) decreased appearance of exogenous fatty acids in NEFA fraction. In the absence of fasting hypertriglyceridaemia (NTG type II diabetics), diabetes or insulin resistance *per se* did not result in further decreased clearance of lipids from circulation and further decreased exogenous lipid oxidation as compared to controls. ^{13}C NEFA data of these studies suggested that the clearance of exogenous fatty acids from circulation increases with the increase in size of the mass of adipose tissue. Since hypertriglyceridaemia was associated with decreased clearance of exogenous TAG from circulation and decreased exogenous lipid oxidation, these findings supports the view that future diet and drug therapies should be targeted either to decrease the intake of dietary lipids to match the individual's metabolic competence or to increase the metabolic competence/capacity to handle dietary lipids by altering: a) the rate of exogenous TAG entry into circulation, b) the rate of endogenous TAG entry into circulation, c) the rate of TAG clearance from circulation d) rate of lipid oxidation. Tracing lipid metabolism using isotopic probes may provide a more direct way of assessing the contribution made by each of these components and determining the atherogenic risk.

To my loving Parents, Major Muhammad Humayun & Mst. Nawab Jan and my respected Teachers

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Bennoson, J., Humayun, M.A., Jones, A.E., Hounslow, A., Wootton, S.A. (1999). With-in individual variability in the gastrointestinal handling and metabolism of [1,1,1-^{13}C]tripalmitin. *Proc. Nutr. Soc.* **58**, 25A

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Humayun, A., Jones, A., Jones, J., Sandeman, D., Wootton, S.(1999). Defective postprandial lipid metabolism in hypertriglyceridaemic type II diabetes mellitus. *Atherosclerosis.* In press

Humayun, M.A., Jones, A.E., Bennoson, J., Wootton, A.A. (1999). Impaired postprandial lipid metabolism in middle-aged men. *Proc. Nutr. Soc.* In press.

Jones, J., Humayun, A., Jones, A., Sandeman, D., Wootton, S. Postprandial lipids in patients with type II diabetes and hypertriglyceridaemia. 5th International Symposium on Multiple Risk Factors in Cardiovascular disease: Global Assessment and Intervention. *Venice, Italy, October 28-31, 1999.*

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Main abbreviations used

ANOVA	Analysis of variance
Apo	Apolipoprotein
AUC	Area under the curve
BMI	Body mass index
CHD	Coronary heart disease
CHO	carbohydrate
CF-IRMS	Continuous flow-isotope ratio mass spectrometry
CO_2	Carbon dioxide
CRF	Chylomicron-rich fraction
CVD	Cardiovascular disease
EE	Energy expenditure
Endo	Endogenous
ER	Endoplasmic reticulum
Exo	Exogenous
FAME	Fatty acid methyl esters
GC-C-IRMS	Gas chromatography-combustion-isotope ratio mass spectrometry
GI	Gastrointestinal
HDL	High density lipoprotein
HL	Hepatic lipase
HTG	Hypertriglyceridaemic
IRMS	Isotope ratio mass spectrometry
LDL	Low density lipoprotein
Lox	Lipid oxidation
LPL	Lipoprotein lipase
MUFA	Monounsaturated fatty acids
MTP	Microsomal triglyceride transfer protein
NEFA	Non esterified fatty acids
NTG	Normaltriglyceridaemic
PA	Palmitic acid
PDB	Pee Dee Belemnite
PUFA	Polyunsaturated fatty acid
RER	Rough endoplasmic reticulum

SE	Standard error
SFA	Saturated fatty acids
TAG	Triacylglycerol
TGRL	Triglyceride-rich lipoproteins
TEF	Thermic effect of food
VLDL	Very low density lipoprotein

CHAPTER 1

Postprandial Lipid Metabolism in Healthy Men and in Subjects with Type II Diabetes Mellitus

1.1 Background

On average, men eat about 100 g and women 75 g of fat each day, which corresponds to 40 % of food energy in both cases (Dept. of Health, 1994). Approximately 90-95% of this lipid is in the form of triacylglycerol (TAG). People do not always eat the same amount of lipid in a given meal and there are marked differences in the distribution of fat between meals *ie.* breakfast/lunch/dinner. Thus, a typical meal may contain between 30 and 50 g of lipid. It is estimated that by taking this amount of lipid, plasma TAG concentration will increase 10-fold after a meal (considering the circulating TAG to be approximately 3 g and taking a typical plasma TAG concentration of 1 mmol/l, Frayn, 1998). However, in "healthy" people the rise is usually considerably less than 2 fold. This suggest that in healthy individuals, the ingestion of meals results in a series of co-ordinated changes in lipid metabolism that tend both to suppress the entry of endogenous TAG (VLDL) in to the plasma and to increase the TAG disposal from the plasma (Frayn, 1993). On the other hand, if after a mixed meal there is increased synthesis and secretion of endogenous TAG and decreased clearance of total TAG from circulation (such as in insulin resistance state), an increased risk of atherogenesis will occur due to the accumulation of TAG-rich lipoproteins (TGRL) and their remnants. Several clinical studies have demonstrated a strong positive relationship between the progression and pathogenesis of atherosclerosis with the magnitude and duration of postprandial TAG (Groot *et al.,* 1991; Patsch *et al.,* 1993; Karpe *et al.,* 1994).

Prolonged circulation of TGRL and their remnants increases atherogenic risk in several ways. The most important of these is likely to be the process of neutral transfer of lipids by cholesteryl ester transfer protein (CETP) thus resulting in subsequent enrichment of low density lipoproteins (LDL) and high density lipoproteins (HDL) with TAG and

enrichment of TGRL and their remnants with cholesterol. The TAG in LDL and HDL particles is then hydrolysed by hepatic lipase (HL) thus resulting in small dense LDL and HDL particles, both of which are related to increased risk of cardiovascular disease (CVD).

Given important relationship between atherogenic risk and postprandial TAG concentrations, it is therefore of great interest to understand the factors that determine the lipaemic response. Studies have shown that several factors influence postprandial TAG concentrations such as the amount of fat in the test meal (Dubois *et al.,* 1998); age and, gender (Cohn *et al.* 1988); body weight (Lewis *et al.* 1990); type II diabetes (Lewis *et al.* 1991; Tan *et al.* 1995) and exercise (Cohen *et al.,* 1989; Weintrab *et al.,* 1989). Although the factors affecting postprandial TAG concentrations are mostly known, the exact mechanisms leading to postprandial lipaemia are still unclear partly because of methodological problems.

Since increasing age, obesity and type II diabetes are associated with disturbed postprandial lipid metabolism and insulin resistance (both independently and together), hypertriglyceridaemia and its relationship with insulin resistance formed the basis of the central hypothesis of this thesis. The hypothesis states that with increasing age the levels of lipids in circulation also increase depending upon the individual's metabolic competence for clearing lipids from circulation and that this may be further modulated by types and amounts of lipids and CHO in diet and levels of physical activity. The levels of lipids in circulation will then determine the level of insulin resistance, the risk of developing obesity, type II diabetes and atherosclerosis (Figure 1.1). The concept of metabolic competence of clearing lipids from circulation is defined as an individual's efficiency of clearing lipids from circulation which depends upon one or more of the factors such as a) the mass and activity of enzymes such as lipoprotein lipase, hepatic lipase b) mass and activity of apo lipoproteins such as CII, CIII, apo E c) mass and activity of acylation stimulating protein d) mass and metabolic activity of tissues such as skeletal muscle and adipose tissue e) number and activity of receptors such as low density lipoprotein (LDL) receptor and LDL receptor related protein and f) size of organ such as liver. The Effect of these factors on lipid clearance and the role of lipids on insulin sensitivity are discussed in detail in the relevant sections of chapter 2.

The primary constraints in determining how secretion and clearance of TGRL from gut and liver are integrated in the postprandial state arise from difficulties in differentiating

between exogenous and endogenous lipids in circulation by traditional methodologies. Simply measuring postprandial TAG concentrations does not give the source of TAG in circulation (*ie.* gut or liver) and tells us little about the flux of ingested fatty acids through the different lipid pools of the body. For example to what extent do the increased postprandial TAG concentrations relate to the delivery of exogenous TAG in to the circulation, their removal from the circulation by skeletal muscle and adipose tissue or hepatic lipoprotein synthesis? Several studies have used either retinyl palmitate or apo B48 to serve as a marker for exogenous lipid metabolism. However, some authors have raised the concern that retinyl esters are not a specific marker of particles containing exogenous lipid (Cohn *et al.,* 1993; Lewis *et al.,* 1990). Retinyl esters from fat soluble preparations are criticised for delay in absorption as compared to triacylglycerol and apo B48 (Krasinski *et al.,* 1990; Jackson *et al.,* 1999) and transfer to other lipoproteins such as LDL and HDL (Krasinski *et al.,* 1990). Although apo B48 is exclusively attached to chylomicron particles and it is not transferred to other particles, the primary disadvantage for apo B48 is that it is the marker of particle carrying TAG but not the amount of TAG carried by these particles. Higher or lower concentrations of measured apo B48 and apo B100 may be misleading. As there is only one apo B per particle, it is possible that few TAG-rich particles from intestinal origin (*ie.* decreased apo B48 concentration) may contain more TAG than many TAG-poor particles produced by liver (*ie.* increased apo B100 concentration).

Since the mechanisms responsible for disturbed postprandial lipid metabolism in insulin resistant conditions (such as increased age, obesity and type II diabetes) are still unclear, the main objective of this thesis was to focus on the central part of the hypothesis. Namely, how exogenous lipid is metabolised in healthy individuals and those with various degrees of insulin resistance in a setting of controlled amount and type of lipid and CHO in test meal and level of physical activity. These studies shed more light into mechanisms responsible for postprandial hypertriglyceridaemia in these conditions by examining how the secretion and clearance of TGRL from gut and liver are integrated in the postprandial state especially in the insulin resistant conditions. In order to trace the exogenous lipid metabolism in the studies of this thesis, ^{13}C labelled tripalmitin was used in the test meal and traced through different pools of the body (for example, appearance of tracer in chylomicron rich fraction TAG, non-esterified fatty acids fraction and in breath; Figure 1.2). Furthermore, as the majority of the previous postprandial studies observed the differences in postprandial responses after an excessively large lipid load, it is not clear that the changes

observed in these studies are relevant to the ingestion of more typical amounts of lipid in normal daily life. Hence in contrast to many previous studies, a test meal was used which reflected the typical composition of lipid, CHO and protein present in the UK diet.

1.2 Objectives

- To determine the absorption of fatty acids as the proportion of the administered label excreted in stool (conducted only in repeatability study and for the reasons discussed in section 4.4.2.1 not conducted in other studies).
- To determine the processing of fatty acids by the enterocyte from the rate and time course of appearance of labelled fatty acid in CRF-TAG.
- To determine the extent to which fatty acids are released in to the plasma or are taken up by the tissues (after the hydrolysis of CRF-TAG by peripheral tissue lipoprotein lipase) by determining the rate and time course of appearance of labelled fatty acids in plasma NEFA pool.
- To determine the ultimate fate of labelled fatty acids in the form of oxidation (the proportion of administered dose of label recovered on the breath as $^{13}CO_2$ or retention in the body pool).
- To determine the effect of second unlabelled meal on the secretion of label as ingested in the previous meal.

In order to achieve these objectives, the following questions were addressed to this thesis:

a) What is the between-sample and within-sample variability in ^{13}C palmitic acid (PA) concentration in chylomicron rich fraction TAG (CRF-TAG)?
b) Is the postprandial metabolism of exogenous lipids repeatable after the ingestion of same types and amounts of lipid on two identical occasions in which strict adherence to experimental conditions are applied?
c) What is the effect of second unlabelled meal on the secretion of labelled TAG as ingested in the previous meal?
d) To what extent is exogenous lipid handling is influenced by insulin resistance as associated with increased age and type II diabetes?

1.3 Thesis outline

This thesis describes the work carried out during the past four years which has helped in the development of standardised protocol for postprandial ^{13}C labelled TAG

studies and addressed questions relating to the general hypothesis. The thesis is composed of a introductory background to the research project (chapter 1); a review of literature describing (a) triacylglycerol digestion and absorption, (b) synthesis, secretion and metabolism of intestinal triglyceride-rich lipoproteins (TRL), (c) dietary and physiological factors affecting postprandial lipid metabolism and (d) insulin resistance, diabetic dyslipidaemia and risk of CHD, and e) stable isotopes tracer methodology (chapter 2). Description of methodology involved in the present research (chapter 3), with studies on the validation and standardisation of protocol for postprandial ^{13}C labelled TAG studies and repeatability of postprandial exogenous lipid metabolism (chapter 4). The two main experimental chapters that examined the lipaemic responses in young versus middle-aged men (chapter 5) and in subjects with type II diabetes (chapter 6). General discussion and summary (chapter 7); appendices (chapter 8) and references (chapter 9).

Figure 1.1 As the age increases levels of lipids in circulation also increases depending upon the individual's metabolic competence for clearing lipids from circulation, levels of physical activity and types and amounts of lipids and CHO in diet. The levels of lipids in circulation will then determine the level of insulin resistance, the risk of developing obesity, type II diabetes and atherosclerosis.

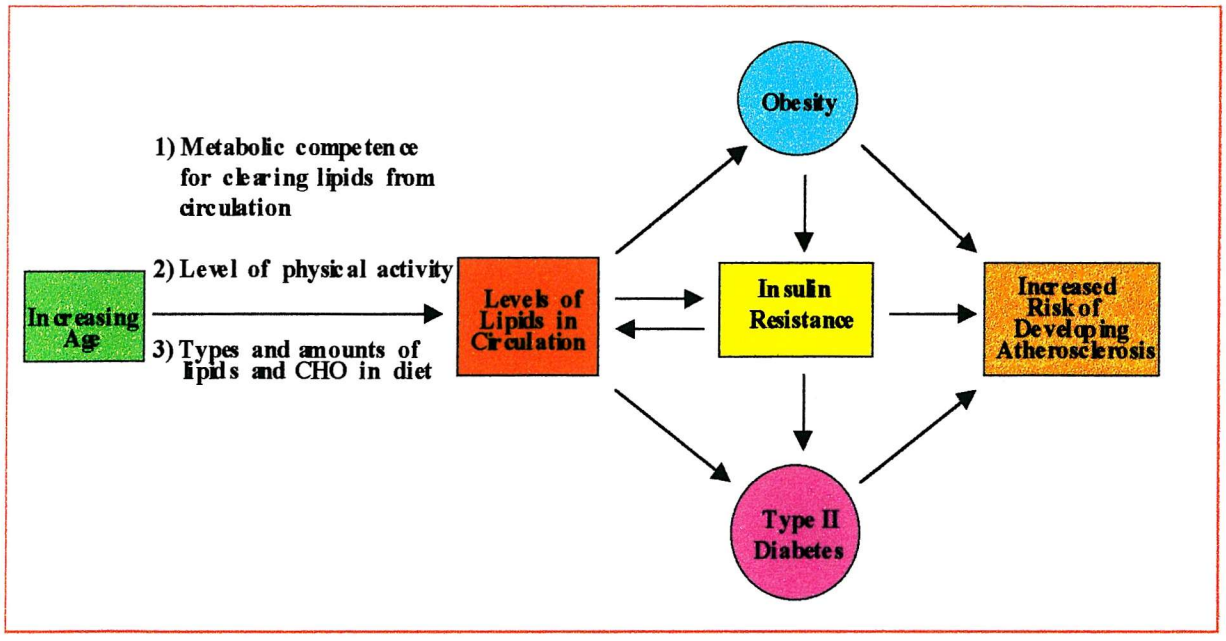
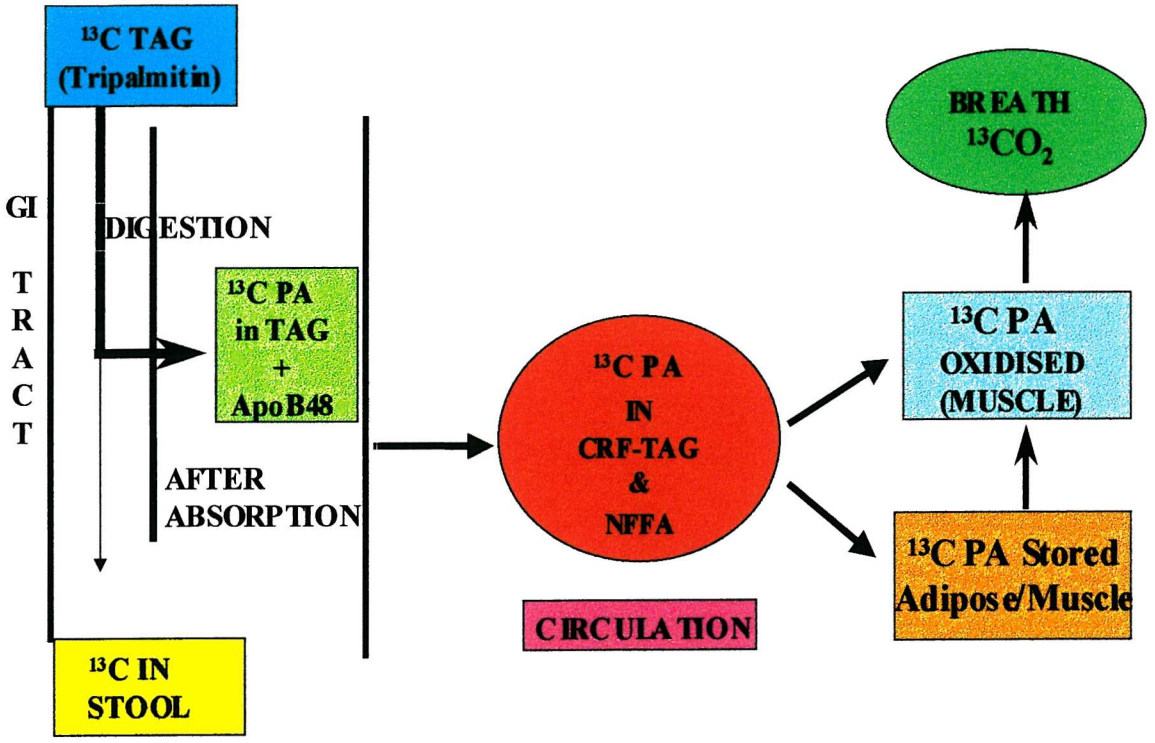


Figure 1.2 Schematic representation of ^{13}C labelled TAG studies.



PA, palmitic acid; CRF-TAG, chylomicron-rich fraction triacylglycerol, NEFA, non-esterified fatty acids

CHAPTER 2

Review of Literature

2.1 Introduction

The main objective of this chapter is to give background information about the processes and factors determining postprandial triacylglycerol handling (*ie.* the primary outcome variable of this thesis). This chapter is divided in to five sections. Section 2.2 gives a brief introduction about various classes of lipid and discusses lipid metabolism by focusing on triacylglycerol digestion and absorption, the assembly of chylomicrons (particles carrying exogenous lipids in circulation) in the enterocyte and their metabolism in the circulation over the postprandial period. The main objective of this section is to highlight the key metabolic processes and factors that can affect the synthesis, secretion and clearance of chylomicrons in the postprandial state. Section 2.3 concentrates on nutritional and physiological factors affecting postprandial lipid metabolism. The main objective of this section is to highlight the various nutritional and physiological factors affecting postprandial TAG metabolism. It also provides information on the difficulties to interpret results of individual studies and to compare results between studies due to methodological problems associated with the quantification of postprandial metabolism of exogenous lipids and/or lipoproteins and due to lack of standardised test meal for studying postprandial lipid metabolism. It also gives the opportunity for the reader to see that if controlled, these nutritional and metabolic factors would have minimal effect on primary outcome variable. Section 2.4 describes insulin resistance, the role of lipids in developing insulin resistance, diabetic dyslipidaemia and the associated abnormalities in lipoprotein composition and metabolism. It also covers a discussion on relationship of insulin resistance with hypertriglyceridaemia and cardiovascular disease risk in Type II diabetes. Section 2.5 deals with the basic concepts and instruments involved in the stable isotope tracer methodology. The final section gives summary from the review of the literature.

2.2 Lipids, triacylglycerol digestion and absorption and chylomicron assembly and metabolism

This section gives a brief introduction about various classes of lipids and discusses lipid metabolism by focusing on triacylglycerol digestion and absorption, and the assembly of chylomicrons in the enterocyte and their metabolism in the circulation over the postprandial period.

2.2.1 Dietary lipids

The role of lipids in biological systems goes well beyond the provision of energy. Understanding the various biological roles of lipids will facilitate a greater understanding of disease states, both directly and indirectly related to lipid metabolism.

Fatty acids and triacylglycerol

Fatty acids are a diverse group of molecules with many different functions in the body. They are found as a part of or are used as building blocks for other members of the lipid family such as triglycerides, phospholipids, and cholesterol. All fatty acids consists of a chain of carbon atoms, each attached to hydrogen atom with a carboxyl group (COOH) attached on one end of the chain. The hydrogen atom in COOH readily dissociates from the rest of the molecules, forming an H^+ (hydrogen ion), the hallmark of an acid. The other end of the chain consists of carbon atom and three hydrogen atoms, and is called the methyl end. Fatty acids with 2 to 36 carbon atoms have been found in higher plants and animals, but the commonest forms have carbon atoms between 14 to 22. A notation has been developed to denote the fatty acid chain length and number and position of any double bond in the chain. For example the palmitic acid, linoleic acid and α-linoleic are denoted by notions of 16:0, 18:2 n-6 and 18:2 n-3 respectively. Where the first number represents the number of carbon atoms, the number after the colon represents the number of double bonds and n-3 or n-6 represents the carbon atoms, numbered from the methyl end, at which the first double bond begins. Fatty acids are very weak acids compared to mineral acids like sulphuric acid and nitric acid. However, the COOH group is acidic enough for a high concentration of fatty acid to disrupt the biological working of the cell. It is for this reason fatty acids are bound as a part of larger complex molecules in body.

Fatty acids are usually attached through their COOH group to alcohols (characterised by having OH group) through a reaction called esterification. Three

esterification reactions form a triacylglycerol molecule.

Fatty acids are used for energy production in most tissues such as muscles, liver, heart and energy storage as triglycerides in tissues such as adipose tissue, muscle, and liver (which mainly contains long chain fatty acids). Fatty acids are also found as phospholipids which are essential components of cell membranes and the surface layer of lipoproteins. Fatty acids may also be esterified to cholesterol to form cholesteryl esters, the principal storage and transport form of cholesterol. Certain fatty acids are the precursors of prostaglandins, intimately involved in the regulation of vascular events.

Fatty acids are released in to circulation after the hydrolysis of triglycerides in the lipoproteins by the activities of enzymes lipoprotein lipase and hepatic lipase or after the hydrolysis of TAG in adipose tissue by hormone sensitive lipase. In circulation fatty acids are transferred to large protein carrier molecules such as albumin and are called non-esterified fatty acids (NEFA) in this form.

Cholesterol

Cholesterol is a 20 carbon steroid with a hydroxyl group at position 3 and double bonds between carbons 5 and 6. Cholesterol occurs in the body as free alcohol or in an esterified form attached to a long chain fatty acid by an ester linkage. Cholesterol and cholesterol metabolites play a variety of essential roles in physiology. Cholesterol is essential for steroid hormones (adrenal and sex steroid hormones) synthesis and is a component of biological membranes (Bloch, 1991). The ratio of cholesterol:phospholipid within the membrane determines the extent of membrane fluidity and thus permeability of cells. Cholesterol is also precursor for bile acids. Cholesterol metabolites are active in the regulation of protein transcription integral to lipid transport (Goldstein & Brown, 1990). Cholesterol can be obtained from the diet or can be synthesised in the body. HMG-CoA reductase (3-hydroxy, 3-methylglutaryl-coenzyme A reductase) is the rate limiting enzyme in cholesterol synthesis. It controls the formation of mevalonic acid from acetoacetyl-CoA and is subject to feed back inhibition depending upon the final cholesterol concentrations.

Phospholipids

Phosphatidylcholine (lecithin) and spingomyelin are the two main phospholipids in plasma. Phospholipids have the ability to form a bridge between non polar hydrophobic lipids (TAG, cholesteryl esters) and water which is polar. This property of phospholipids stabilise lipids in the aqueous medium of plasma and thus occur as a major constituents of

lipoprotein particles. Phospholipids are also integral components of all cell membranes.

2.2.2 Digestion, absorption and metabolism of triacylglycerol

Triacylglycerol, which forms the bulk of the lipid in the diet must be digested, absorbed and processed in the enterocyte before they are released in the circulation and metabolised. The average British adult consumes 74 to 102 g TAG and 0.28 to 0.39 g of cholesterol (Department of Health, 1994). The most common fatty acids associated with dietary lipids are palmitic, stearic, oleic and linoleic acids (LCFA with 16 to18 carbons). Exogenous (dietary) cholesterol and phospholipids (predominantly lecithin) are quantitatively less important than endogenous cholesterol and lecithin secreted in bile during lipid absorption. In man, the estimated daily biliary secretion of phospholipids into the small intestine is 11 to 12 g, whereas dietary contribution is 1 to 2 g (Bogstorm, 1976).

Factors affecting lipid digestion and absorption

Digestion and absorption of TAG predominantly occur in the small intestine (Castelli, 1986) such as in duodenum and jejunum respectively. Two major variables such as chain length and degree of unsaturation of fatty acid profoundly affect the digestion, absorption, physiological transport and metabolism of dietary lipids (Hashim & Babayan, 1978). The intramolecular structure of dietary TAG has also implications for digestion and absorption. For example 1,2-dipalmitoyl, 3-butyroyl (a major TAG species of butterfat) digestibility is greatly enhanced because this structure gives fat that remains liquid at body temperature as compared to glyceryltripalmitate, which is liquid only above 65 ^{0}C. Moreover the position to which a fatty acid is esterified in TAG affects its absorption. For example, in rats fed synthetic TAG containing oleic and stearic acid, stearic acid was well absorbed when present at the *sn-2* position but not when present at *sn-1* and *sn-3* positions (Mattson *et al* 1979).

2.2.2.1 Triacyglycerol Digestion and Absorption in Stomach

Digestion

Lingual lipase (Van Dyke, 1989) and gastric lipase (Gargouri *et al.,* 1986) are responsible for dietary triacylglycerol hydrolysis in stomach. As much as 10 to 30% of total dietary TAG may be hydrolysed in stomach (Hamosh, 1984). Like pancreatic lipase, gastric lipase also cleaves ester bonds at the *sn-1* and *sn-3* positions of the TAG molecule. Both lipases are much more active on triacylglycerols with short chain fatty acids (SCFA) and medium chain fatty acids (MCFA) than on those with (LCFA) (Staggers *et al.,* 1981).

However gastric lipase differs from pancreatic lipase in several ways. Firstly, in case of gastric lipase, the fatty acids at position *sn-3* are preferentially hydrolysed as compared to the fatty acids at the position *sn-1* by a ratio of 2:1 (Paltauf *et al.,* 1974) regardless of what fatty acid is present. Secondly, the digestive products of gastric lipase are diacylglycerols (DAG) and fatty acids, whereas most of the pancreatic lipase are 2-monoacylglycerol (MAG) and fatty acids.

The partial digestion of dietary lipids in the stomach facilitates duodenal-jejunal hydrolysis in many ways (Carey et al, 1983; Bernback *et al.,* 1989). Firstly, LCFA dissolved within lipid droplets become partially ionised and promote fine emulsification in the duodenum (Roy *et al.,* 1979). Secondly, small amounts of partially ionised LCFA increase the binding of colipase to the emulsion interface (Patton *et al.,* 1978). As a result, promoting the binding of colipase to pancreatic lipase. Thirdly, DAG produced by gastric hydrolysis may be preferentially located at the emulsion water interface, hence are hydrolysed faster by pancreatic lipase (Richardson and Nelson, 1967). Finally, gastric hydrolysis is continued in the upper small intestine at pH 5-7 because it is not affected by bile salts (Dutta *et al.,* 1982).

Absorption

The preferential release of SCFA and MCFA in stomach allows the fatty acids to be rapidly absorbed in the gastric mucosa (Bugaut, 1987). There is no evidence of any carrier-mediated transport mechanisms for SCFA and MCFA in the gastric epithelial membrane. Because of their molecular size, water solubility and shorter chain length, SCFA should be more readily absorbed in the stomach than MCFA. The SCFA serve as a preferred energy source for gastric mucosa. After absorption, any SCFA not utilised by the gastric mucosa are transported to cystol without being esterified. They then enter the portal vein and are transported to the liver bound to albumin (Aw & Grigor, 1980).

In the stomach, the fate of released fatty acids differs depending upon their chain length and position in the TAG molecule. For example, significant hydrolysis and absorption of butterfat TAG is expected to occur in the stomach, due to its unique content of short- and medium-chain fatty acids and their preferential location at the *sn-3* position. Whereas hydrolysis of LCFA in other dietary lipids is likely to occur to a much lesser extent since they require emulsification prior to hydrolysis.

The products remaining in the stomach after hydrolysis of triacylglycerol

by lingual and gastric lipases principally LCFA, LCFA containing triacylglycerol, diacylglycerols and monoacylglycerols contained in emulsion droplets are propelled through the pylorus into the duodenum.

2.2.2.2 Triacyglycerol digestion, absorption and metabolism in small intestine

Dietary TAG that enter the duodenum are emulsified in the intestinal lumen (especially in the jejunum) by interaction with bile salts. The bile is synthesised in the liver and released in to the intestine via the gall bladder and bile duct when required. The active constituents in the bile are complex salts of cholesterol that emulsifies lipid in much the same way as soap does, and thereby make them accessible to enzymes that operate best in aqueous rather than a fatty environment.

The TAG in emulsion droplets is then hydrolysed by pancreatic lipase (Van Dyke, 1989). Intestinal digestion and absorption of lipid appears to be calcium dependent because pancreatic lipase requires calcium as an enzyme cofactor (Patton, 1981). Colipase (secreted by the pancreas), absorbed to the surface of the bile-TAG droplet, mediates the interaction of emulsified TAG with pancreatic lipase (Brindly, 1991). Pancreatic lipase, like lingual lipase, specifically hydrolyses the ester bonds at position *sn-1* and *sn-3* of triacylglycerol regardless of fatty acid chain length. However, hydrolysis appears to be faster with triacylglycerol containing SCFA. Only less than 5% of the lipid ingested remain in the form of di- and triacylglycerols. The acyl chain on *sn-2* position of dietary triacylglycerol is relatively resistant to the lipolytic action of pancreatic lipase, so that approximately 75% of the fatty acids at *sn-2* position remain intact as monoacylglycerols (Mattson & Volpenhein, 1964). Hydrolysis of *sn-2* monoacylglycerol occurs only after isomerization of fatty acids to *sn-1* position but this is unusual so that most of the *sn-2* located fatty acid are absorbed in their monoacylglycerol form.

During lipolysis, the released free fatty acids diffuse away from the lipid emulsion droplet. The LCFA and 2-monoacylglycerols become solubilized in the bile salt micelles and the remaining SCFA and MCFA partition into the aqueous phase depending upon their water solubility. Under alkaline or slightly acidic conditions, calcium ions react with released LCFA causing the release of protons and formation of insoluble calcium soaps (Benzonana & Desnuelle, 1968). These soaps may comprise from 5 to 50% of faecal lipid (Bliss *et al.*, 1972).

Absorption

The smaller molecular weight fatty acids can diffuse rapidly from the aqueous phase of the lumen, whereas the high molecular weight fatty acids diffuse slowly and the large mixed bile acid micelles diffuse much more slowly. No evidence has been found for carrier proteins participating in either active or facilitated transport. The intestinal absorbability of LCFA such as stearic acid is similar to that of palmitic acid and both saturated fatty acids appear to be absorbed almost as well as oleic acid (16:1) (Bonanome & Grundy, 1989).

2.2.3 Triacyglycerol resynthesis in enterocytes

The absorbed products of lipid digestion (free fatty acids, monoacylglycerols, and lysophospholipids) are then utilised for the synthesis of lipids in enterocytes (Johnston, 1977; Small, 1991). Since the absorbed fatty acids are potentially toxic for the enterocytes, they are rapidly converted to the essentially bio-inert triacylglycerol. Thus after crossing the enterocyte membrane, the fatty acids are rapidly transported to smooth endoplasmic reticulum (SER) where the triacylglycerol resynthesis takes place. The cytosolic transport of the fatty acids to SER is facilitated by an intracellular fatty acid binding protein (FABP) (Ockner & Manning, 1974; Ockner & Manning, 1976), which has a high affinity for LCFA. FABP thus also prevents accumulation of potentially toxic unbound fatty acids in cell (Ockner & Manning, 1974). Re-esterification of fatty acids and 2-monoacylglycerols also creates a gradient that helps to drive continued absorption (Tso & Fujimoto, 1991). It has also been proposed that FABP might be involved in directing fatty acids to 2-monoacylglycerol pathway (Lowe *et al.*, 1987).

In the SER, triacylglycerols are resynthesised from coenzyme A (CoA) activated MCFA, LCFA, and 2-monoacylglycerols (Tso & Balint, 1986). This process of triacylglycerol resynthesis is mediated by fatty acyl-colligases, monoacylglycerol acyltransferase, and diacylglycerol acyltransferase which are located in a complex on the cytoplasmic face of SER (Grigor & Bell, 1982). Fatty acids with more than 12 carbon atoms are re-esterified to triacylglycerols (Pfeffer *et al.*, 1977). Whereas SCFA and MCFA (containing less than 10-12 carbon atoms) pass from the mucosal cells directly without re-esterification and, bound to albumin, they are transported as nonesterified fatty acids to liver and other tissues. Readily soluble free glycerol from lipid hydrolysis is also

transported by portal blood. Roughly 75% of LCFA originally at *sn-2* position in triacylglycerols are maintained there (Akesson *et al.,* 1978). The fatty acids re-esterified to *sn-1* and *sn-3* positions, in contrast, have been randomised during digestion, absorption, and intracellular resynthesis.

Newly synthesised TAG from mucosal SER, which are the major pool of enterocyte TAG (Mansbach & Parthasarathy, 1982) are then packaged in to chylomicrons for transport in to the circulation. As the acylglycerol transferases involved in the TAG resynthesis are present on the cytoplasmic side of SER, the TAG formed there must cross the lipid bilayer to the microsomal lumen for packaging into chylomicrons. The exact mechanism of this transference is not known but may be facilitated by the TAG transfer protein (Hamilton & Havel, 1993).

2.2.4 Chylomicrons

Chylomicrons are triglyceride-rich lipoprotein particles (water-soluble macromolecules), which are synthesised exclusively by the small intestine. Their function is to transport absorbed dietary lipids and lipid-soluble vitamins and bile derived lipids from gut in to the circulation during the postprandial state. Chylomicrons are spherical particles which are assembled in the endoplasmic reticulum (ER)-golgi region of the enterocyte. Structurally, bulk of neutral lipids like triacylglycerols along with some cholesteryl esters forms the apolar oily inner core of chylomicron, while the protein, phospholipid, and free cholesterol forms a polar surface coat around the triacylglycerol core. A small amount of free fatty acids are also found in the surface coat.

Physical characteristics

Chylomicrons are large lipoprotein particles ranging from 100 to 1000 nm diameters, with a density of less than 0.95 g/ml and flotation rates (Sf) >400. The size of the chylomicron depends on the lipid content of meal ingested. During lipid absorption, instead of increasing the number of chylomicron, the enterocyte expands the size of the chylomicron particle (Hayashi *et al.,* 1990), thus conserving surface components. Studies in the rabbit (Redgrave & Dunne, 1975) have shown that when lymph TAG is greater than 1.5 to 3.0 mg/ml, most TAG is transported in larger chylomicron-sized particles, while below this concentration smaller chylomicrons predominate in lymph. The fatty acid composition of the meal also determines chylomicron size, with polyunsaturated fatty acids (PUFA) producing larger chylomicrons than meals containing saturated fatty acid

(Levy *et al.*, 1991).

2.2.4.1 Chylomicron lipid composition

Triacylglycerol

The major lipid in chylomicrons is TAG (86-92%), whilst other lipids are present in small quantities such as cholesteryl ester (0.8-1.4%), free cholesterol (0.8-1.6%), phospholipid (6-8%) and protein 1-2% of the mass (Glickman, 1976; Scanu & Landsberger, 1980). Triacylglycerol composition largely reflects dietary fatty acids (Redgrave & Dunne, 1975; Levy *et al.*, 1991).

Phospholipids

Phospholipid, largely derived from dietary and biliary phospholipid, are hydrolysed in the intestine, absorbed passively by enterocytes as lysophospholipids, reacylated on SER membrane and transported into SER lumen by a specific carrier protein (Bishop & Bell, 1985), serve as a source of chylomicrons phospholipid. Chylomicron phospholipids consist mainly of lecithin (70-100%).The fatty acids composition of phospholipids differs from those present in diet and remain relatively constant despite large variations in the composition of dietary fatty acids (Goodman, 1965). The phospholipid is found exclusively in surface coat around the chylomicron particle (covering almost the entire chylomicron surface, >80%), which also contains apolipoproteins (covering remaining 20% of the surface) (Zilversmith, 1965), 70% of free cholesterol and a small amount of TAG. The phospholipids along with the apolipoproteins, stabilise the chylomicron particle.

Cholesterol

Cholesterol, both free and as cholesterol ester, is the other principal component of chylomicrons. After transfer from intestinal lumen to enterocyte cytoplasm, free cholesterol is esterified in the SER by acyl-CoA cholesterol acyltransferase (ACAT) (Clark & Tercyak, 1984) prior to incorporation into chylomicrons. Cholesteryl ester fatty acid composition has little relationship to dietary fatty acids (Goodman, 1965). Chylomicron cholesteryl esters are rich in oleic and linoleic acids (Whyte & Dumount, 1963; Redgrave & Dunne, 1975).

2.2.4.2 Chylomicron apolipoprotein composition

Although apolipoproteins comprise only 1-2% of the mass of

chylomicrons, they are a complex mixture and have important roles in the chylomicron clearance and metabolism (*ie.* apo B48, apo CII, apo CIII and apo E). One well-established apolipoproteins function involves their capacity to stabilise the lipoprotein particles in the circulation by providing a hydrophobic side for interaction with lipids and hydrophilic surface to interact with physiological fluids. Besides their lipid binding properties, apolipoproteins are also required as co-factors and ligands of various enzymes and receptors involved in lipid transport. Most of the known plasma apoproteins are present and include apo B (10%), apo A IV (10%), apo E (5%) apo AI (15-35%) and apo C (45-50%) (Bisgaier & Glickman, 1983). Apolipoprotein B-48, AI and AIV are synthesised in the enterocytes, while apolipoprotein CII, CIII and apo E, appear to be acquired by the chylomicron in the circulation. Half of the plasma apoprotein is immunologically related to high density lipoproteins (HDL) (Glickman & Kirsch, 1973).

Apo B48

One of the major apolipoprotein of chylomicron is apo B48. Apo B48 is synthesised in the rough endoplasmic reticulum of enterocyte and incorporated into nascent chylomicrons. Unlike other apoproteins apo B-48 remains within the core of the chylomicron from the time of assembly of the particle in RER (of enterocyte) until uptake and removal by the liver.

Apo CII

Apo CII is synthesised primarily in the liver and is a requisite co-factor for lipoprotein lipase (LPL). Enrichment of chylomicrons with apo CII results in faster removal from plasma by the liver (Hussain *et al.,* 1997), probably due to increased rates of triglycerides hydrolysis. However, overexpression of apo CII in transgenic mice results in hypertriglyceridaemia (Shachter *et al.,* 1994) suggesting that chronic increase in apo CII affects the clearance of remnant lipoprotein. Individuals with inherited apo CII deficiency show massive fasting chylomicronaemia, episodic abdominal pain and recurrent pancreatitis (a clinical expression indistinguishable from familial LPL deficiency).

Apo CIII

Apo CIII is a surface component of chylomicrons with a poorly defined metabolic role. It has been suggested that the presence of apo CIII may inhibit the interaction of chylomicron and VLDL remnants with hepatic receptors, thus modulating their uptake by the liver (Mahley *et al.,* 1984). Apo CIII may also inhabit LPL activation

by apo CII and activate LCAT *in vitro*, but the physiological implications of these *in vitro* observations are still unclear.

Apo E

Apo E is a constituent of chylomicrons and its major physiological role is to mediate the interaction of lipoproteins with cell receptors, both the LDL receptor and the LDL receptor related protein (LRP), thus modulating the *in vivo* catabolism of various lipoprotein species (Mahley *et al.,* 1984). Type III hyperlipoproteinaemia, characterised by an impaired chylomicron and VLDL remnant catabolism, is most frequently associated with homozygosity for apo E2, an apo E isoform that is defective in its *in vitro* binding to LDL receptor (Mahley *et al.,* 1984).

The extent to which enterocyte apoprotein synthesis is regulated by chronic or acute dietary lipid intake and/or dietary lipid type is not clear. Animal studies suggest that sustained changes in acute transmucosal triacylglycerol flux or in background dietary lipid have no effect on the synthesis of apo AI and apo B-48 (Davidson *et al.,* 1986, Davidson *et al.,* 1987).

2.2.5 Chylomicron Assembly

Chylomicron assembly has not been well characterised as that of VLDL in liver. One reason for this neglect could be the absence of suitable culture model for intestinal studies. The following discussion on the assembly of chylomicrons is mostly based on the information obtained from liver derived cells which synthesise either apo B100 ($HepG_2$, human hepatoma cell line) or both apo B100 and apo B48 (rat hepatoma, McA-RH 7777cells and primary hepatocytes) containing lipoproteins.

2.2.5.1 Transcription of apo B gene

Apo B48 is a structural protein required for the assembly of chylomicrons. Transcription and then translation of apo B gene results in the formation of apo B48. The human apo B gene is 43 kb long and contains 29 exons and 28 introns. In the intestine the transcription of apo B gene results in the formation of two stable species of 14 and 7Kb mRNAs (Giannoni *et al,* 1994), with the larger form predominating generally (Giannoni *et al,* 1994). After transcription, apo B mRNA undergoes splicing and polyadenylation similar to all eukaryotic transcripts.

2.2.5.2 Post-transcriptional editing of apo B mRNA

Although the human intestine synthesises and secretes small amounts of apo B100, the majority of adult intestinal apo B mRNA is edited post-transcriptionally (81-97%) resulting in the synthesis and secretion of predominantly apo B48 (Chen *et al*,1987, Powell *et al*, 1987). In this intranuclear process, a cytidine at nucleotide 6666 on apo B mRNA is deaminated to uracil (Powell *et al*, 1987). Thus instead of glutamine codon (CAA) at amino acid position 2153, a stop codon (UAA) is produced which leads to the production of apo B48 (the amino-terminal half of apo B100). The enzyme responsible for the deamination of cytosine in apo B mRNA has been identified, cloned, and functionally expressed (Teng *et al*, 1993), and is called as apobec-1 (apo B mRNA-editing enzyme catalytic polypeptide).

Apo B mRNA editing activity is developmentally regulated in humans and rats (Hodges & Scott, 1992; Chan, 1993) as the fetal intestine is unable to edit the apo B message as compared to adult intestine (Teng *et al*, 1990). This is in accordance with the synthesis of apo B100 in the fetal intestine and apo B48 by the adult intestine (Teng *et al*, 1990). The developmental regulation of editing activity correlates with the expression of apo bec-1 (Funahshi *et al*, 1995; Giannoni *et al* 1995). The editing activity is not altered by hormonal or dietary manipulations in the intestine.

2.2.5.3 Translational and post-translational processing of apo B and chylomicron assembly

In the human intestine both 14 and 7kb forms of edited mRNAs are translated into a single polypeptide of 2152 amino acids called apo B48, which then combines with lipids and forms intestinally derived triglyceride-rich lipoproteins called chylomicrons. On the basis of current knowledge, Hussain *et al* (1996) suggested a working model for chylomicron assembly. According to this model the nascent apo B polypeptide is either incorporated into the rough ER membrane or is cotranslationally lipidated to form a lipid poor primordial lipoprotein particle of HDL size. Lipidation results in the release of primordial lipoprotein particle into the lumen of rough endoplasmic reticulum, where it is either degraded (if insufficiently lipidated) or is subsequently enriched with neutral lipids (core expansion), mainly triglycerides, resulting in the formation of nascent lipoproteins of VLDL and chylomicron size. The nascent lipoproteins are then transported to golgi apparatus for further modifications involving carbohydrates (glycosylations) (Kessler *et*

al, 1975) and possibly lipids. The lipoprotein particles are then concentrated in secretory vesicles and released into the intracellular space and subsequently enter intestinal lacteals and are transported into lymph (Sabesin & Frase, 1977). Electron microscopic studies (Tytgat *et al.* 1971; Sabesin & Frase, 1977; Christensen *et al.* 1983; Swift *et al.* 1984) have shown that, after a high lipid meal, smooth ER in enterocytes become lipid-filled, and lipoprotein particles can be visualised in rough ER, the golgi complexes, and in the secretory vesicles opposed to basolateral membrane.

The mechanisms involved in the cotranslational transfer of neutral lipids to nascent apo B are not clear. It has been suggested that as nascent apo B is cotranslationally translocated across the rough endoplasmic reticulum (RER), it interacts with the regions in RER where neutral lipid and phosphatidylcholine synthesis occurs, allowing the protein to interact cotranslationally with newly synthesised lipids (Boren *et al*, 1992). It has been suggested that microsomal triglyceride transfer protein (MTP) takes part in the transfer of triglycerides, cholesterylesters and phospholipids into nascent particles (Jamil *et al*, 1995). Mutations in the MTP gene cause the genetic disorder abetalipoproteinaemia (Wetterau *et al*, 1992), which is characterised by an absence of apo B-containing lipoproteins in plasma but normal biosynthesis of apo B (Lackner *et al*, 1986; Bouma *et al*, 1990). Hence it appears that MTP is involved in the initial lipidation of nascent apo B, which results in the formation of nascent lipoprotein particles of HDL size.

Similarly the mechanisms and factors involved in the "core expansion" of nascent lipoprotein particles in the lumen of RER are poorly understood. The "core expansion" of nascent lipoprotein particles does not seem to involve MTP (Glickman *et al*, 1986). Immunoelectron microscopic studies (Alexander *et al*, 1976) of rat hepatocytes suggest the presence of pre-formed neutral lipid droplets in smooth ER that do not contain immonoreactive apo B. Whereas immunoreactive apo B is seen in RER and nascent VLDL particles (with immunoreactive apo B) in smooth termini of the RER. This suggests that the nascent lipoprotein particles are expanded to triglyceride-rich lipoprotein particles after fusion with lipid droplets at the junction between smooth and rough ER (Alexander *et al*,1976; Hamilton *et al.,* 1998). However, as the majority of TAG synthesis occur in the cytoplasmic side of smooth ER, the mechanisms involved in the transport of TAG molecules or droplets across the smooth ER membrane are not known.

2.2.6 Microsomal Triglyceride transfer protein (MTP) and apo B lipoprotein assembly.

MTP is a heterodimeric protein consisting of a large 97 kDa sub-unit and 58 kDa multifunctional enzyme, protein disulphide isomerase (PDI). MTP is localised in the lumen of endoplasmic reticulum in liver and intestine (Wetterau & Zilversmith, 1984; Gregg & Wetterau, 1994). MTP is the functional protein in the co-translational transfer of TAG, cholesteryl esters, and phospholipids in to apo B particles (Jamil *et al.* 1995). It has been hypothesised that MTP plays a key role in the prevention of intracellular degradation of apo B. The larger sub-unit rescues apo B from intracellular degradation by translocating lipid into the nascent protein on RER and PDI sub-unit by mediating proper folding of large hydrophobic apo B molecule during translation (Wetterau *et al.* 1992; Gordon *et al.* 1994).

The intestinal expression of MTP is responsive to dietary lipid (Lin *et al.* 1994). Type of lipid also appears to be important. It has been shown that in hamsters, diets enriched in triolein alone or triolein together with linoleic acid-rich safflower oil produce lower levels of hepatic MTP mRNA than those enriched with mixtures of triolein and saturated fatty acids-rich lipids such as trimyristin (Bennett *et al.,* 1995). Based on the studies showing that the MTP activity varies in parallel with the rate of apo B secretion by both liver and intestine (Gregg & Wetterau, 1994), it is has been proposed that in normal humans, varied expression of MTP may be responsible for the post-transcriptional regulation of apo B secretion (Du *et al.* 1996). Thus the use of MTP inhibitors (BMS-200150, Jamil *et al.,* 1996; 4-bromo-methaqualone, Haghpassand *et al.,* 1996) may be a unique tool for interference with the initial step(s) of lipoprotein assembly and may prove to be an efficient way of decreasing the synthesis and secretion of apo B containing lipoproteins from intestine and liver.

Patients with abetalipoproteinaemia synthesise apo B normally but are unable to secrete TAG-rich lipoproteins from liver or intestine (Lackner *et al.* 1986), thus resulting in absence of apo B containing lipoproteins in circulation and undetectable plasma triacylglycerols. These patients also lack detectable MTP activity and large subunit due to defect in the gene coding for the larger sub-unit of MTP (Sharp *et al.* 1993). This discovery of a defect in MTP in these patients further supports the view that substrate delivery to nascent apo B is critical for TAG-rich lipoprotein assembly (Sharp *et al.* 1993)

and MTP plays a key role in the rescue of apo B from intracellular degradation by lipidating the nascent apo B which is required for its translocation across the ER membrane. Recently, a study has shown that specific inhibition of MTP activity in CaCo-2 cells with BMS-200150 resulted in reduced secretion of triglyceride rich lipoproteins, predominantly apo B100 (d < 1.006 g/ml) with reduced triglyceride content (Van Greevenbroek *et al.,* 1998). This further suggests that MTP is necessary for the initial assembly of TAG-rich lipoproteins, however, the exact function of MTP in later "core expansion" of nascent lipoprotein particles is still poorly understood.

2.2.7 Regulation of the secretion of apo B containing lipoproteins

As discussed in section 2.2.5, the following discussion on the regulation of secretion of chylomicrons is mostly based on the information obtained from liver derived cells.

Transcription of apo B does not seems to play a major role in the production of apo B as the steady state concentrations of hepatic apo B mRNA do not change significantly, under some conditions which alter apo B secretion by several fold (Pullinger *et al.* 1989). Evidence to date suggests that modulation of the secretion of apo B100 is regulated post-transcriptionally (Bostrom *et al.* 1988; Dashti *et al.* 1989; Pullinger *et al.* 1989; Moberly *et al.* 1990). In HepG2 cells, up to 70-80% newly synthesised apo B is degraded by ER proteases with in the first 20 minutes after its synthesis (Dixon *et al.* 1991). Translocation of apo B across the endoplasmic reticular membrane appears to be one of the control points in the regulation of apo B secretion (Davis *et al.* 1990; Du *et al.* 1994; Rusinol *et al.* 1993; Rusinol & Vance, 1995). Choline analogue phosphotidylmonomethylethanolamine inhibits VLDL and apo B secretion by inhibiting the translocation of apo B across the ER membrane in to the lumen, stimulating intracellular degradation and increasing the susceptibility of apo B to ER proteases (Rusinol *et al.* 1993). This shows that the translocation of nascent apo B across the ER membrane determines its fate of either secretion or degradation.

Availability of lipids appears to determine sorting of apo B to degradation because inhibition of intracellular degradation by itself does not result in increased secretion of apo B (Dixon *et al.* 1991), however, availability of lipids coupled with inhibition of intracellular degradation results in increased secretion of apo B-containing lipoproteins. A close positive correlation between hepatic TAG concentration and apo B secretion has

been reported in many studies (Arbeeny *et al.* 1992; Gibbons *et al.* 1992). Benoist & Grand-Perret, (1996) showed that in primary hepatocytes the triglyceride availability, rather than triglyceride synthesis, was rate limiting in lipoprotein synthesis. Similarly, sorting of lipoproteins in ER lumen to degradation also appears to be related to the degree of lipidation of the particles (Boren *et al.* 1993b; Rustaeus *et al.* 1995) because the less dense particles assembled in HepG2 cells are secreted and the more dense particles in the lumen of secretary pathway appear to be retained and degraded in the cell (Boren *et al.* 1992; Boren *et al.* 1993a). In hepatic G2 cells supplementation of medium with oleate (Bostrom *et al.* 1988) and chylomicron remnants (Craig *et al.* 1988) effectively stimulates TAG synthesis, elevates intracellular TAG concentration and enhances secretion of apo B containing lipoproteins. Triascin D, a potent inhibitor of fatty acyl-CoA acyltransferase and triglyceride synthesis, blocks the stimulation of apo B secretion from HepG2 cells by oleic acid and results in corresponding increase in apo B degradation.

The exact mechanisms by which apo B secretion is modulated by lipid availability remains to be further elucidated, however, it has been suggested that increased availability of lipids presumably permits folding of apo B in to a conformation that protects it from degradation by ER proteases or allows for its continuing of translocation across the bilayer in to the ER lumen (Sakata *et al.* 1993).

2.2.8 Chylomicron metabolism

After assembly in the endoplasmic reticulum (ER), chylomicrons move to the golgi apparatus (Sabesin & Frase, 1977) for glycosylation (Kessler *et al.*, 1975) and final processing. From the golgi apparatus secretory vesicles, each containing several chylomicrons, the chylomicrons bud off and move to the basolateral enterocyte plasma membrane. The chylomicrons are then released into the intracellular space and subsequently enter intestinal lacteals and are transported into lymph (Sabesin & Frase, 1977). From lymph the chylomicrons pass through the thoracic duct to the general circulation through the subclavian vein and result in postprandial lipaemia. The first organ they encounter are the lungs but they rapidly enter the capillaries of muscle, heart, mammary gland, adipose tissue, and other important tissues.

The entry of chylomicrons in the blood ensures the availability of dietary lipids to the peripheral tissues before the particles are cleared by the liver. Within the circulation the chylomicrons are subjected to apolipoprotein exchange and lipolysis, and they are

converted to chylomicron remnants (Mahley & Hussain, 1991; Cooper, 1992). The chylomicron remnants are then rapidly removed by liver (Redgrave, 1970).

Exchange of apolipoproteins in circulation

After entering the systemic circulation chylomicrons acquire more apo CII, CIII and apo E. These apoproteins are transferred from HDL with free and esterified cholesterol and phospholipid. Some apo AI and AIV are transferred from chylomicron on to HDL at this stage. Exchange of apolipoproteins between lipoprotein particles occur concomitant with lipolysis and is not well characterised because of its rapidity and complexity.

Chylomicron-TAG hydrolysis

After acquiring apoproteins in the systemic circulation, the chylomicrons attach to the capillary endothelial cells (Blanchette-Mackie & Scow, 1971). Here, following activation by apoprotein CII (Bier & Havel, 1970) they are exposed to the enzyme LPL, the enzyme which hydrolyses of most of the TAG in chylomicron and VLDL (Mahley & Hussain, 1991; Cooper, 1992; Brunzell, 1995). LPL also hydrolyses monoglycerides and phospholipid but does so very slowly.

LPL is synthesised in the parenchymal cells of extrahepatic tissues including adipose tissue, skeletal and cardiac muscle, and is translocated through the interstitium and the endothelial cell layer to its physiological site of action in the luminal surface of capillary endothelium, where it is bound to heparan sulphate proteoglycan chains. Not all LPL found in tissues participates in triacylglycerol hydrolysis. Only that fraction of enzyme protein, the so called functional LPL which is localised on the endothelial surfaces of blood vessels has contact with circulating triacylglycerol- rich lipoproteins (Eckel, 1989). Binding of LPL to the lipoprotein surface is an obvious prerequisite, however, the molecular mechanisms for binding of LPL to the lipoprotein surface are mostly unknown. Choi *et al.,* (1995) reported that LPL interacts with amino terminal fragment of apo B, which might modulate binding of LPL to apo B containing lipoproteins. The results of another study demonstrated that lipid emulsion droplets without any apo lipoprotein bind LPL as efficiently as VLDL (Carrero *et al.,* 1996). These results suggest that both the lipid moiety, probably phospholipids, and apo B account for the LPL-lipoprotein interaction. The importance of lipolysis by LPL is well documented in type I hyperlipoproteinaemia which is characterised by a massive accumulation of

chylomicrons in plasma due to deficiency in LPL activity (Brunzell, 1995). Overexpression of the LPL in mice results in rapid clearance of chylomicrons and a decrease in VLDL levels (Liu, 1994). Hence LPL activity is an important determinant of the extent and duration of postprandial lipaemia. Studies in animals have shown a correlation between the activity of LPL in the tissue and its uptake of fatty acids from chylomicron (Taskinen, 1987).

The regulation of LPL activity is complex and is different in different tissues. LPL activity in adipose tissue is high during the postprandial state but is down regulated during fasting, presumably to provide a large amount of fatty acids for lipid deposition during the postprandial state. In contrast no effects of fasting and feeding have been seen on LPL expression in skeletal muscle or cardiac muscle. Extensive evidence exists to indicate that insulin is predominantly responsible for the effects of fasting and feeding on adipose tissue LPL (Semenkovich *et al.,* 1989; Raynolds *et al.,* 1990). A positive relationship exists between plasma insulin levels and the fasting LPL activities in adipose tissue of rodents and humans. Insulin resistance in adipose tissue has been suggested to be the cause of hypertriglyceridaemia in obese patients with type II diabetes (Jeppesen *et al.,* 1995a; Eckel *et al.,* 1995; Potts *et al.,* 1995). In smooth muscle LPL is down regulated in response to the insulin in normal individuals (Laville *et al.,* 1996), however, it is unregulated in obese subjects with type II diabetes (Yost *et al.,* 1995). Thus tissue specific LPL regulation by identical effectors is different under various metabolic conditions. Insulin increases LPL activity in adipose tissue, whereas in short term infusion studies, insulin seems to decrease LPL activity in skeletal muscle (Farese *et al.,* 1991; Richelsen *et al.,* 1993). Hence, an inverse regulation of LPL by insulin seems to exists between adipose tissue and muscle thereby promoting lipid uptake in the adipose tissue after a meal and simultaneously reducing lipid uptake in muscle tissue (Richelsen *et al.,* 1993). Further more, LPL is also regulated by the local concentrations of fatty acids (Peterson *et al.,* 1990). In vitro studies have demonstrated that increased fatty acids will inhibit the action of LPL (Bengtsson & Olivecrona, 1980), will displace it from endothelial surface (Saxena *et al.,* 1989), and will separate the CII-LPL complex which is essential for triacylglycerol hydrolysis (Saxena & Goldberg, 1990).

The size and triacylglycerol content of lipoprotein also affects the susceptibility of hydrolysis by LPL. Increasing the size and triacylglycerol content of lipoprotein particle increases its susceptibility to hydrolysis by LPL (Fisher *et al.,* 1995).

This effect is most likely related to greater surface concentration of apo CII in large TAG-rich particles. Apo CII is an enzyme which acts as a co-lipase by increasing the interaction of enzyme with the substrate at the interface of lipoprotein. Furthermore chylomicrons are the preferred substrate for LPL than VLDL (Van Beek *et al.,* 1998), probably because of their larger size.

Other lipases can also hydrolyse chylomicrons and have similar effects on the clearance of remnants. For example lipolysis of chylomicrons with hepatic lipase (Shafi *et al.,* 1994; Ji *et al.*, 1994a), phospholipase A (Borensztajn & Kotlan, 1981) or bacterial lipase (Skottova *et al.,* 1995) results in the generation of chylomicron remnants that can acquire apo E and are cleared rapidly from plasma. This suggests that lipolysis by any enzyme may be sufficient for their clearance from plasma. Lipolysis probably serves to reduce the size of the particles since chylomicrons are excluded from entering the Space of Disse (Fraser *et al.,* 1995) because of their large size. Peripheral lipolysis, not only reduces the size of chylomicrons, but it may also results in a change in conformation of resident apo E molecules and help acquisition of other apo E molecules from plasma. These modifications facilitates the entry of these particles into the Space of Disse.

There are different fates of fatty acids which are released after the hydrolysis of chylomicron-TAG by LPL. If the released fatty acids are not immediately transferred to the tissues such as adipocytes, they will bind to albumin (for their solubilization and efficient transport), leave the capillary and enter the general circulation. The fatty acids which are taken up by the tissues binds to cytosolic fatty acid binding proteins which are thought to function in solubulizing or buffering intracellular fatty acids or possibly in shuttling them to different cellular compartments (Coe & Bernlohr, 1998). Regarding fatty acid uptake across the plasma membrane, it is thought that fatty acids could transfer passively across the phospholipid bilayer and the uptake is governed by the molar ratio of fatty acid to albumin in the circulation and cellular fatty acid metabolism (Zakim, 1996). Recently it has been suggested that there are two components of fatty acid transport: a) a simple diffusion component that increases in significance as the concentration of unbound fatty acid is increased and b) a protein-mediated component that would be a major contributor at low physiological unbound fatty acid concentration (Abumrad *et al.,* 1998).

In adipose tissue acylation stimulation protein (ASP) plays an important role in the uptake of fatty acids after they are released by LPL hydrolysis of triglyceride-rich lipoproteins. Increase in intracellular adipocyte TAG synthesis may be an integral step in

the efficient energy storage (Frayn *et al.,* 1995) and increased uptake of NEFA. In vitro studies have shown that ASP is a major determinant of rate of adipocyte TAG synthesis (Baldo *et al.,* 1993; Cianflone *et al.,* 1994). Both *in vitro* (Cianflone *et al.,* 1994; Cianflone *et al.,* 1995) and postprandial *in vivo* (Saleh *et al.,* 1998) studies have shown that ASP is produced by adipocytes. Moreover generation of ASP appears to be accelerated both *in vivo* and vitro by chylomicrons and there is a significant relationship between ASP generation and net fatty acid uptake by adipocytes in the postprandial period (Saleh *et al.,* 1998). The above discussion suggests that ASP plays an important role in the clearance and storage of NEFA by adipose tissue. The clearance of NEFA from circulation is important. Reduced rate of fatty acid clearance by adipose tissue will result in increased capillary fatty acid concentrations that would inhibit LPL activity and diminish TAG clearance from plasma (Peterson *et al.,* 1990). Moreover, increased fatty acid flux might lead to insulin resistance by variety of effects on liver and skeletal muscle (Bjorntorp, 1994), as well as stimulating the increased secretion of apo B100 lipoprotein particles.

Chylomicron-remnant formation

As the core of triacylglycerol becomes progressively smaller, surface materials (phospholipid, cholesterol, and apoproteins CII and CIII) are transferred to HDL to maintain stability of chylomicron particle. Apolipoprotein AI and AII are also rapidly distributed to HDL fraction of plasma (Redgrave & Small, 1979). Chylomicron remnants retain apolipoproteins B48 (Elovson *et al.,* 1988), E and CIII (Chen & Reaven, 1991). The transfer of apo CII, together with increasing inaccessibility of core triacylglycerols for the LPL active site, results in cessation of further triacylglycerol removal. Thus after most (90%) of chylomicron have been hydrolysed, the affinity of LPL for chylomicron is weakened and a partly degraded chylomicron is released back into circulation as a chylomicron remnant. Chylomicron remnants are smaller (comprising ~4% of the original chylomicron mass), and are relatively protein enriched particles with a surface coating of free cholesterol and phospholipid.

In the circulation remnant particles acquire more apo E and this is an important signal for their removal from the plasma (Mahley, 1991). Mutations and deficiencies in apo E results in type III hyperlipoproteinaemia characterised by the presence of cholesterol and triglyceride-rich remnant lipoproteins (Mahaley & Stanley, 1995). Under normal conditions the availability of apo E is probably not rate limiting, but acute administration (Mahley *et al.,* 1989) and chronic overexpression (Linton *et al.,* 1995) of

apo E results in increased clearance of plasma lipoproteins and decreased levels of plasma cholesterol. The importance of apo E is also evident from studies in transgenic mice that do not express apo E (Ishibashi *et al.,* 1994); as they accumulate remnants in their plasma and are susceptible to atherosclerosis. Accumulation of remnants in apo E deficient mice or hypercholesterolaemic animal models can be corrected by acute or sustained increases in plasma apo E levels (Mahley *et al.,* 1989; Linton *et al.,* 1995).

Chylomicrons and their remnants are also remodelled in circulation due to the effects of CETP. CETP facilitates the exchange of TAG in the chylomicron and chylomicron remnant particles for cholesteryl esters in HDL (Chen & Reaven, 1991).

2.2.9 Chylomicron-remnant uptake/clearance by liver

Following remodelling of chylomicrons by LPL and CETP, the resulting remnant particles are cleared by hepatic low density lipoprotein receptor (LDL-R) and LDL receptor-related protein (LDL-RP) pathways.

Low-density Lipoprotein Receptor pathway

The LDL-R plays a central role in the clearance of plasma lipoproteins. Since this receptor binds lipoproteins containing apo B100 and/or apo E, it is responsible for the bulk clearance of plasma lipoproteins such as apo B48 and apo E containing chylomicron remnants, apo B100 and apo E containing intermediate density lipoproteins (VLDL remnants) and apo B100 containing LDL. The importance of LDL-R in the metabolism of triglyceride-rich lipoproteins is understood by the pathological changes observed in the patients with familial hypercholesterolaemia (FH), a genetic defect of LDL-R (Goldstein & Brown, 1989). In FH, there is poor removal of apo B100 containing lipoproteins with the resulting increase in LDL in circulation. On the other hand, chylomicron remnant clearance was normal in FH (Rubinsztein *et al.,* 1990). Similarly in genetically modified mice lacking LDL receptor, there is a distinct accumulation of apo B100 containing particles, however, the hepatic clearance of cholesteryl esters contained in intravenously injected chylomicrons was not impaired as compared with that in wild-type mice (Herz *et al.,* 1995). Taken together, these observations indicates the presence of an alternate apo E specific receptor pathway for clearance of chylomicron remnants (Rubinsztein *et al.,* 1990; Ishibashi *et al.,* 1993).

The LDL receptor-related protein pathway

LDL receptor related protein (LDL-RP) is suggested to be an alternate or a

remnant receptor because it binds apo E containing lipoproteins *in vitro* and mediates the cellular uptake of apo E enriched particles in familial hypercholesterolaemic fibroblasts (Beisiegel *et al.,* 1989). Several other *in vitro* studies have also established that LDL-RP is involved in the binding and uptake of apo E-enriched lipoproteins (Mahley *et al.,* 1994; Mahley, 1996). The ability of LDL-RP to clear chylomicron remnants is dependent on an interaction with proteoglycans in the Space of Disse (Ji *et al.,* 1993). LDL-RP activity is enhanced in the presence of hepatic lipase (Ji *et al.,* 1994a) or enrichment of particles with apo E (Ji *et al.,* 1993). The apo E content of chylomicron remnants may also allow them to be cleared by the LDL-R (Hussain *et al.,* 1997).

Relative contribution of both receptor pathways to chylomicron remnant clearance

Studies in the knockout mouse have shown that either the LDL-R or the activity of LDL-RP alone was sufficient to clear chylomicron remnants normally. De Faria *et al.,* (1996) used infusion of purified receptor associated protein (specific inhibitor of ligand binding to LDL-RP) or anti-LDL-R antibodies to block either uptake pathway specifically and to measure the plasma clearance of intravenous injected remnant particles. The results of these experiments suggested that ~ 55% of the remnants were taken up by the LDL-R and ~ 20% by LDL-RP. The residual uptake was attributed to non-receptor mediated pathways.

Initial binding sites for remnants are non-receptor binding sites

Although endocytosis of chylomicron remnant is mediated through hepatic lipoprotein receptors, a number of studies suggest that a non-receptor binding site is responsible for initial binding of remnant particles to the hepatocellular surface. In LDL receptor knockout mice, when the LDL-RP activity was blocked by intravenous infusion of LDL-RP antagonists, the binding of remnants to hepatic parenchyma was not affected but endocytosis into hepatocytes was delayed (Herz *et al.,* 1993). It is suggested that this initial binding site was heparan sulphate proteoglycans (HSPG) on the cell surface. This is supported by the finding that treatment of cells by heparinase dramatically reduces their ability to bind remnant particles *in vitro* (Ji *et al.,* 1993), and intravenous infusion of heparinase significantly delays liver uptake of remnant particles *in vivo* (Ji *et al.,* 1994b). It is suggested that hepatocytes actively promote this sequestration of remnant particles by secreting non-lipoprotein associated apo E, which is bound uniformly on their basolateral surface and interact with lipoprotein particles (Hamilton *et al.,* 1990). Treatment of cells

with heparinase releases this apo E and significantly impairs their ability to internalise remnant particles (Ji *et al.,* 1994b; Ji *et al.,* 1995). Furthermore addition of apo E to apo E-deficient chylomicrons from intestinal lymph of rats dramatically increase their rate of removal by perfused rat livers (Windler *et al.,* 1980). Hence both apo E associated with chylomicron particles and with heparan sulphate proteoglycans helps in the initial binding of chylomicrons.

Secretion of hepatic lipase, which binds both HSPG and lipoprotein particles, could have a similar effect (Ji *et al.,* 1995). Removal of hepatic lipase (together with apo E) by pre perfusion of livers with heparin or direct inhibition of hepatic lipase with specific antiserum inhibited the initial removal of chylomicrons up to 60% (Shafi *et al.,* 1994). Furthermore substantially impaired clearance of preformed rat chylomicron remnants by the liver of hepatic lipase knock-out mice have also been reported (Qiu *et al.,* 1998). Hepatic lipase could alter the conformation of apo E on chylomicron remnant (Brasaemle *et al.,* 1993), thereby increasing its affinity for endocytic receptors. Moreover, it could also serve as ligand for LDL-RP. Taken together, these results suggest that HSPG, apo E and hepatic lipase appear to fulfil a critical role in the sequestration or capture of remnants.

Willnow (1997) suggested a two step model for remnant particles clearance. According to this model, chylomicron remnants enter Space of Disse from the circulation through the fenestrated endothelium of the liver. There majority of remnant lipoproteins initially bind to HSPG molecules anchored on the hepatic surface. This binding is mediated by apo E and hepatic lipase bound remnants are further metabolised by hepatic lipase and by enrichment with apo E, secreted by the liver. Subsequently the particles are internalised by the LDL-R and LDL-RP recognising apo E. In addition some of the remnant particles are probably bound and internalised by LDL-R directly without prior binding to hepran sulphate proteoglycan (HSPG).

Factors influencing the receptor mediated remnant removal

Factors which appear to be important in regulating receptor mediated remnant removal include lipoprotein lipase, hepatic lipase, apo E phenotype and presence of apo CIII.

LPL has been found to efficiently mediate binding of lipoproteins to cell surfaces and to LDL-RP under cell culture conditions (Beisiegel *et al.,* 1991). This supports the

previously proposed idea that lipase could have a role in receptor mediated uptake of chylomicron remnants in liver. Skottova *et al* (1995) investigating the effects of LPL on the clearance of chylomicrons during the perfusion of rat livers, concluded that any lipase causing lipolysis of chylomicron can stimulate their clearance by liver, but that LPL has an additional effect on the removal, which is not dependent on its catalytic activity.

Hepatic lipase is a glycoprotein and is synthesised in hepatocytes, secreted and bound to hepatocyte and hepatic endothelial surfaces (Sanan *et al.,* 1997). Several studies (Daggy & Bensadoun, 1986; Demacker *et al.,* 1988; Sultan *et al.,* 1989, Sultan *et al.,* 1990) have suggested that hepatic lipase may play a role in chylomicron remnant removal. It has shown that inhibition of HL by specific antibodies inhibited the plasma clearance and hepatic uptake of chylomicron remnants in rats (Sultan *et al.,* 1990; Shafi *et al.,* 1994). Similarly, *in vivo* studies had reported that inhibition of HL by specific antibodies resulted in plasma accumulation of partially degraded phospholipid-rich lipoproteins, presumably of intestinal origin (Daggy & Bensadoun, 1986). Recently it has been shown that in apo E deficient mice the phospholipolysis of chylomicrons by hepatic lipase generates remnant particles that are rapidly cleared from circulation by the liver (Crawford *et al.,* 1999). Accumulation of remnants also occur in human subjects with hepatic lipase deficiency. Taken together, these studies shows that the hydrolysis of chylomicron lipids by LPL and then by hepatic lipase may facilitate exposure of the receptor binding domain of apo E for interaction with LDL receptor and LDL-RP (Brasaemle *et al.,* 1993). Furthermore, the extent of apo E self-association, which is dependent on the degree of hydrolysis of triglyceride rich particles, can also control remnant interaction with LRP (Dergunov & Rosseneu, 1994). Other studies have suggested that hepatic lipase can facilitate chylomicron remnant uptake independently of lipolysis by acting as a ligand that mediates the cellular binding and uptake of the remnants. Diard *et al.,* (1994) have shown that heat-inactivated hepatic lipase also promotes the uptake of chylomicron remnant particles by rat hepatocytes. Furthermore, this ligand function is apparent only when HL is attached in excessive amounts either to the remnant particle (Ji *et al.,* 1997) or to the surface of hepatocytes (Dichek *et al.,* 1998).

Genetic variation at the apo E locus results in three isoforms of apo E in human population and are referred to as E2, E3 and E4. The frequencies of these isoforms in Caucasian populations is approximately 0.08, 0.77 and 0.15 respectively (Ordovas & Schaefer, 1999). The avidity of binding of apo E isomers to receptors is in the order of

E4>E3>E2. Population studies have shown that total cholesterol, LDL cholesterol and apo B are highest in individuals carrying apo E4 isoform, intermediate in those with apo E3 and lowest in those with apo E2 (Ordovas & Schaefer, 1999). Subjects homozygous for E2 such as type III hyperlipoproteinaemia have elevated levels of chylomicron remnants because of decreased affinity for the receptors.

Plasma apo CIII is a component of chylomicrons, VLDL and HDL and is synthesised primarily in liver and to lesser extent in intestine. In vitro apo CIII inhibits LPL (Krauss *et al.,* 1973) and also inhibits the binding apo E containing lipoproteins to the LDL receptor, but not to the LRP receptor (Ordovas & Schaefer, 1999). A study has shown that postprandial hypertriglyceridaemia was abolished in homogenous mice lacking apo CIII (Maeda *et al.,* 1994). It is therefore suggested that apo CIII appears to displace apo E or to interact with and obscure the apo E ligand. It is further suggested that binding of chylomicron remnant to hepatic receptors require prior dissociation of apo CIII.

2.3 Dietary and physiological factors influencing postprandial lipaemia

Before discussing dietary and physiological factors influencing postprandial lipaemia it is important to highlight a) the methods of quantification of postprandial lipaemia b) limitations of current methods in differentiation between exogenous and endogenous lipids and lipoproteins in circulation as lipoproteins are quasi-discrete structures that do not have a single characteristic density or a distant stoichiometry of lipids and proteins and c) difficulty in comparing the results of different trials on postprandial lipid metabolism due to variability in meal size, composition and/or experimental design.

Postprandial lipaemia is quantified by determining the time to reach peak concentration, concentration at the peak, and the total and incremental areas under the concentration : time curves for TAG in total plasma, retinyl esters and apo B48 in TGRL fraction. A simple method to examine the postprandial lipid metabolism is to measure the changes in total plasma TAG concentrations or in different fractions of TGRL separated after ultracentrifugation. However, measuring simply postprandial triglyceride concentrations does not give information about the source of triglycerides in circulation *ie.* chylomicrons or VLDL. Similarly, due to similarities in size and density of exogenous and endogenous lipoproteins (chylomicrons and VLDL) and their remnants (chylomicron remnant and IDL), it is difficult to have complete separation of these particles by

ultracentrifugation. Majority of studies have measured TAG, and/or retinyl palmitate and/or apo B levels in chylomicrons (Sf >1000) and chylomicron remnants (Sf <1000) fractions. The measurement of TAG, retinyl palmitate or apo B in these fractions allows to separate the metabolism of large chylomicrons (Sf >1000) from small chylomicrons, chylomicrons with partially hydrolysed TAG and chylomicron remnants, however, this method does not allow the interpretation regarding the metabolism of chylomicron remnants exclusively. Studies have also employed measurements of retinyl esters in TAG-rich lipoprotein fractions Sf > 400, Sf 60-400 and Sf 20-60 in order to trace the metabolism of chylomicrons, VLDL and their remnants respectively, however, there are few studies which have used these extensive lipoprotein separations.

A number of techniques have been employed in order to distinguish between intestinally-derived and liver-derived triglyceride rich lipoproteins in circulation. These techniques include retinyl ester labelling of chylomicrons after the ingestion of lipid-rich meal supplemented with retinyl palmitate or retinyl esters and measurement of apo B48 (chylomicrons) and apo B100 (VLDL) concentrations in the triglyceride-rich lipoprotein fractions. Both of these techniques have certain shortcomings.

Retinyl palmitate labelling (RP) have been used frequently to examine the metabolism of intestinally-derived lipoprotein (chylomicrons) as it is incorporated into the core of newly synthesised chylomicrons and serve as a marker of chylomicron and chylomicron remnants. It is assumed that RP is a) totally absorbed from intestine b) exchange minimally between the lipoprotein particles and c) do not re-secrete after the uptake of remnant particles by liver. Retinyl palmitate technique has been criticised for using as a marker of intestinally derived lipoproteins due to delay in absorption and association with apo B100 lipoproteins. A number of studies have shown that peak RP concentrations are delayed as compared to peaks of apo B48 and triglyceride levels (Krasinski *et al.,* 1990b; Boerwinkle *et al.,* 1994; Karpe *et al.,* 1995a; Lemieux *et al.,* 1998, Tanaka *et al.,* 1998). This delay in RE peak might represent delay in RE absorption/secretion at the level of gut or delay in RE hydrolysis by LPL as compared to TAG in the circulation. Moreover, the way vitamin A is given in test meal also affects its absorption and appearance in the circulation (Krasinski *et al.,* 1990). For example, vitamin A (retinyl esters) given in the oily preparations must be hydrolysed to retinol and solublized by bile salts before taken up by enterocytes (Lewis *et al.*, 1947), where as retinol given in water soluble preparations can be taken up directly. There is also evidence

that during later postprandial period significant amounts of RP are transferred on to other lipoproteins such as VLDL and LDL (Krasinski *et al.,* 1990b; Cohn *et al.,* 1993).

Since apo B48 is exclusively attached to chylomicron particle and it is not transferred to other particles, it is therefore an ideal marker for intestinally derived particles such as chylomicrons. However, the primary disadvantage for apo B48 is that it is the marker of particle carrying TAG but not the amount of TAG carried by these particles. Higher or lower concentrations of measured apo B48 may be misleading. As there is only one apo B per particle, it is possible that few TAG-rich particles (*ie.* decreased apo B48 concentration) may contain more TAG than many TAG-poor particles (*ie.* increased apo B100 concentration). Number of studies have shown approximately 80% increase in apo B100 after the lipid intake (Cohn *et al.,* 1988a; Schneeman *et al.,* 1993; Karpe *et al.,* 1993a), however, 80% of triglyceride was accounted for by proteins containing apo B48 (Cohn *et al.,* 1993).

Another problem in the postprandial studies is the difficulty in comparing the results of trials on postprandial lipid metabolism. Most of the trials are performed with test meals with highly variable size, composition and/or experimental design (Karpe, 1997). In some studies the test meal is composed of only lipids is highly artificial as normal meals always contains some carbohydrate. Carbohydrates in the meals are important as they stimulate insulin release which is a potent lipolytic regulator. Similarly, in a number of studies cream has been used as the only component of the meal. This is a disadvantage in postprandial lipid metabolism studies as dairy lipids contains a substantial amount of short and medium chain fatty acids, which cannot form chylomicrons and the saturated fatty acid content is high as well. Moreover, many studies have used large amount of lipid in the test meal e.g. 60 g lipid per m^2. High amount of lipid in test meal is highly artificial as compared to normal physiological intake and might result in delayed gastric emptying and interference with absorption. Finally little attention has been given to control the preceding diet, physical activity and alcohol intake.

It is emphasised that interpretation and significance of some of the findings as described in the following sections should be made in the context of methodological constraints and variability in meal composition and study design as discussed above. The purpose of this section is to emphasise that the effect of these dietary and physiological factors on postprandial lipaemic response (primary outcome variable) can be minimised if controlled in experimental design.

2.3.1 Dietary factors influencing postprandial lipaemia

Dietary lipid load

A number of studies have shown that increasing the lipid content of standard test meals results in increased postprandial lipaemia in a dose related manner. The greater the lipid content of the meal the greater the postprandial triacylglycerolaemia. Murphy *et al* (1995a) studied the postprandial lipid responses to meals of various lipid contents containing 20, 40 or 80% of lipid but identical carbohydrate and protein content in young men. Their results showed that there was a significantly higher peak triacylglycerol response to the high lipid meal compared to low lipid meal. Similarly, Jeppesen *et al.,* (1995b) showed that postprandial plasma TAG increased in approximately linear fashion as the oral lipid load was increased (5, 40 and 80 g lipid loads). Recently Dubious *et al* (1998) studied the effects of five mixed meals containing 0, 15, 30, 40, and 50 g lipid on the postprandial lipaemia and lipoproteins in normalipidaemic adults. Their results showed that increasing the amount of lipid in the usual range of ingestion per meal (0-50 g) led to step-wise increases in the postprandial rise of chylomicron and serum triacylglycerol.

Effect of meal protein and carbohydrate composition

Few studies have dealt with the effects of meal protein and carbohydrates on postprandial lipaemia in healthy subjects. A study on the effect of protein ingestion on postprandial lipaemia or triglyceride clearance in normalipidaemic young men and women, showed that dietary protein did not significantly affect postprandial lipaemia or chylomicron triglyceride clearance (Cohen, 1989).

Studies have shown that various carbohydrates affect the postprandial lipaemic response in different ways. Addition of sucrose to a lipid-rich meal amplifies and prolongs the postprandial triacylglycerolaemic response compared to same lipid dose in the absence of sucrose (Grant *et al.,* 1994). Similarly the inclusion of sucrose instead of isocaloric amount of glucose resulted in amplified postprandial triacylglycerolaemic response (Mann *et al.,* 1971). These authors suggested that higher insulin response after glucose containing meal might have resulted in increased LPL stimulation and thus increasing clearance of postprandial TRL. Similarly, the study by Cohen & Schall, (1988) showed that the addition of 50g glucose to a 40g lipid meal did not change the lipaemic response as compared to 40g lipid meal alone, However when 100g sucrose was added to

the lipid meal, the lipaemic response was amplified. The authors proposed that it was the fructose component of sucrose which had the hyper-triacylglycerolaemic effect as fructose might have increased the influx of TAG in to the circulation from liver.

Effect of dietary fiber (non-starch polysaccharides)

A number of researchers have studied the effects of dietary fibers on postprandial lipaemia and lipoproteins in healthy subjects (Abraham & Mehta, 1988; Redard *et al.,* 1990; Cara *et al.,* 1992; Dubious *et al.,* 1993; Dubious *et al.,*1995; Sandstrom *et al.,* 1994; Anderson *et al.,* 1995). When dietary fibers from different sources were added to the test meals, lowering of chylomicron and/or plasma triglycerides were observed with oat bran, wheat fiber and germ and pea fiber (Cara *et al.,* 1992; Dubious *et al.,* 1993; Sandstrom *et al.,* 1994). On the other hand, following a two week supplementation of the diet with soluble fibers such as oat bran and psyllium, the supplementation of test meals had no effects (Abraham & Mehta, 1988) or resulted in an increased postprandial triacylglycerolaemia (Anderson *et al.,* 1995; Dubois *et al.,* 1995). This discrepancy could be due to the fact that carbohydrates and fibers can slow gastric emptying. Some fibers can decrease and/or delay lipid emulsification and lipolysis of dietary lipid as well as slow the rate of intestinal absorption. Moreover, carbohydrates can stimulate triglyceride-rich lipoprotein lipolysis as a result of increased LPL activity due to increased postprandial insulin concentrations whereas the reported reduced elevation in postprandial insulinaemia might explain the opposite effect observed after chronic fiber supplementation. More studies of better experimental designs are needed to examine the acute/chronic effects of dietary fiber on plasma lipid and lipoprotein concentrations.

Effect of dietary fatty acid composition

Studies, testing the effects of modifying the fatty acid composition of both standard test meal and background diets, have shown that acute and habitual intake of meals and diets containing unsaturated fatty acids results in lowering of fasting and postprandial triglyceride responses (Demacker *et al.,* 1991; Levy *et al.,* 1991; Zampelas *et al.,* 1994b; Lai & Ney, 1995; Yahiah *et al.,* 1996; Williams, 1998). The magnitude of postprandial lipaemic response is inversely related to the degree of saturation of lipid in the test meal (Nestel *et al.,* 1970). Phan *et al* (1999) determined the TAG and cholesteryl ester clearance from butter fat, fractions of butter and other dietary lipids by reinjecting radiolabelled lymph chylomicrons in to recipient rats. Their results showed that clearance

of cholesteryl ester in chylomicrons derived from coca butter, palm oil and butter fat was slower than clearance of cholesteryl ester from chylomicron derived from safflower oil. Similarly, animal experiments in general agree with human studies in that higher postprandial plasma TAG levels and slower chylomicron removal were noted with the ingestion of saturated relative to unsaturated lipids (Green *et al.,* 1984; Groot *et al.,* 1988; Van Heek & Zilversmit, 1990).

Little is known about the effect of monounsaturated fatty acids, especially, oleic acid, on the extent of postprandial lipaemia. Recently, Jackson *et al.,* (1999) studied the effects of three test meals (40 g lipid, 150 g CHO) of varying monounsaturated (MUFA) and saturated fatty acids (SFA) on the postprandial responses of triglyceride-rich lipoprotein fraction. There results suggested that substitution of meal SFA with MUFA (12-24% as MUFA) offers no advantage in terms of attenuation of total lipaemic response and no reduction in the circulating levels of intestinally-derived lipoproteins. Similarly two other previous studies have also shown no difference in postprandial triglyceride response for meals containing SFA versus MUFA (Roche & Gibney, 1997; Roche *et al.,* 1998). On the other hand, Thomsen *et al* (1999) studied the effects of test meals (80 g lipid, 50 g CHO) rich in saturated and monounsaturated fatty acids on postprandial responses of chylomicron rich fraction. Their results showed that chylomicron-rich fraction TAG concentrations (2-6 h postprandial period) were significantly higher after the butter fat meal than the olive oil meal, however, the differences were not significant for the area under the curves for two meals.

A greatest reduction in the concentration of plasma triacylglycerol has been shown the most consistent effect noted in people using diets rich in long chain n-3 polyunsaturated fatty acids from fish oils (Goodnight *et al.,* 1982; Zampelas *et al.,* 1993; Zampelas *et al.,* 1994b). Demarker and co-workers (1991) investigated the effects increasing PUFA content of background diet for a period of two weeks on chylomicron triacylglycerol and apo B-48 response and showed a marked reduction in the area under the apo B48 and chylomicron triacylglycerol response curves on the PUFA diet compared with the usual background diet. There results indicated that more remnants of TAG-rich lipoproteins were accumulated postprandially with ingestion of saturated versus the unsaturated lipid diets. Similarly, Weintraub *et al.,* (1988) examined the effects of fish oils rich in n-3 PUFA on postprandial lipid metabolism. Their results showed that postprandial chylomicron and chylomicron remnant responses were most markedly attenuated in

subjects given an acute test meal enriched in n-3 PUFA when their background diets also contained n-3 PUFA

A number of mechanisms may be responsible for the blunted responses to n-3 PUFA-containing oils including direct effects on chylomicron metabolism such as reduced rates of absorption and/or resynthesis of triacylglycerol rich in n-3 fatty acids in the enterocyte and increased rate of clearance of n-3 fatty acids containing lipoproteins by LPL mediated lipolysis (Harris *et al.,* 1997). There is also the possibility of indirect effects on chylomicron concentration through decreased VLDL synthesis and/or secretion from the liver, and thereby reduced competition for clearance between endogenous and exogenous dietary lipids. Recently, it has been shown that dietary fish oils interfere with the assembly of initial VLDL precursor particles in the RER lumen and thus target apo B for degradation, with a net result of inhibition of VLDL secretion (Kendrick & Higgins, 1999). Despite all of this knowledge about n-3 PUFA, the question remains is why does chronic n-3 fatty acid supplementation reduce postprandial chylomicronaemia and what are the exact biochemical mechanisms responsible for their triacylglycerol lowering effects? Moreover, it is not known that which of the major n-3 fatty acids is responsible for fish oil effect and how does n-3 fatty acids affect atherosclerosis itself rather than affecting atherosclerosis risk factors? Finally, further research is also needed to compare individual saturated fatty acid of interest for their effect on postprandial plasma lipid and lipoprotein metabolism.

Effect of position of individual fatty acids in triacylglycerol structure.

Although dietary fatty acid configuration had no substantial effect on fasting lipoprotein concentrations, it can influence postprandial lipoprotein concentrations. The postprandial effects of different isomers of stearate containing triacylglycerols have been extensively studied (Redgrave *et al.,* 1988; Mortimer *et al.,* 1988; Mortimer *et al.,* 1992; Mortimer *et al.,* 1994). Chylomicron remnants from rats fed 1, 3-dioleoy, 2-stearoyl glycerol (OSO, saturated fatty acid at *sn-2*) were cleared significantly more slowly from the plasma than from the rats fed 1, 2-dioleoyl, 3-stearoyl glycerol (OOS, monounsaturated fatty acid at *sn-2* position) (Redgrave *et al.,* 1988). As both chylomicron TAG and cholesteryl ester clearance were slower the authors suggested that the hydrolysis of TAG and the uptake of chylomicron remnants by liver were retarded. In another study, Mortimer *et al.* (1994) suggested that triacylglycerols in which saturated chains are present in the *sn-1* or *sn-3* position are hydrolysed more poorly *in vivo*, than those in

which they are situated in the *sn-2* position.

The effects of positional distribution of dietary palmitic acid on postprandial lipoprotein metabolism have also been examined (Zampelas *et al.,* 1994a; Salter *et al.,* 1994; Pufal *et al.,* 1995). In rats, the enrichment of chylomicron TAG with *sn-2* position palmitic acid appeared not to affect the extent of lipaemia following a meal (Pufal *et al.,* 1995). Summers *et al* (1998) studied the effect of different structured triacylglycerols rich in palmitic acid either at position *sn-1* or the *sn-1*,3 positions on the clearance of chylomicron TAG from circulation. The results of that study demonstrated that in human subjects, the stereospecific position of the fatty acyl chains on the structured TAG had no measurable effect on the postprandial clearance chylomicron TAG from circulation.

2.3.2 Effects of Physiological factors on postprandial lipaemia

Age and gender effects

There are few studies which have specifically addressed the issue of the effect of physiological factors such as age and gender on the postprandial lipaemia. Cohn *et al.,* (1988) found that the magnitude of postprandial triglyceridaemia was dependent on age and gender with males and elderly subjects tend to have higher postprandial triglyceridaemic response than females and younger subjects after the same lipid load. Fasting plasma TAG concentration was twice as greater in older subjects than in younger subjects, but this difference was not statistically significant. However, markedly consistent associations were found between the basal plasma triacylglycerol level and the extent of postprandial lipaemia (Nestel, 1964; Grundy & Mok, 1976). Differences between subjects due to age, and between the sexes, largely disappeared when the effect of fasting plasma triacylglycerols on postprandial lipaemia was studied. The low fasting TAG levels and postprandial lipaemic response of females in the study of Cohn *et al.,* (1988) could be mediated through greater LPL activity. Measurement of LPL activity in biopsied human muscle and fat showed that females had more LPL in tissue than males (Kashyap *et al.,* 1983). Recently, it has been suggested that the increased triglyceride-rich lipoprotein response in the elderly individuals might be due to reduced clearance of triglyceride-rich lipoproteins (Cassader *et al.,* 1996; Borel *et al.,* 1998).

Exercise effects

Several studies have indicated that exercise reduces postprandial lipaemia

(Sady *et al.,* 1988; Cohen *et al.,* 1989; Weintrab *et al.,* 1989; Podl *et al.,* 1994; Hardman, 1998). However, studies measuring postprandial TAG concentrations during initial 8 hours after consuming a high-lipid breakfast showed that postprandial TAG concentrations were higher when men exercised for 30 minute starting 1 hour after the meal than a rested control trial (Klein *et al.,* 1992). On the other hand, studies measuring postprandial TAG concentrations in response to dietary lipid consumed the day after exercise resulted in postprandial lipaemia one third lower than on a no-exercise control trial (Aldered *et al.,* 1994). This suggest that the benefits of exercise in terms of reduction in postprandial lipaemia occurs some hours after the session of exercise (*ie.* during the period of recovery after exercise). The reasons for increased postprandial lipaemia before recovery period after exercise could be due to a positive influence of exercise on intestinal activity and a negative effect on splanchnic and hepatic blood flow. Furthermore exercise induced changes in LPL activity also appear to be delayed.

The studies on chronic effects of exercise suggested that physical exercise conditioning reduces the magnitude of postprandial lipaemia in endurance trained men than in control (Cohen *et al.,* 1989; Merrill *et al.,* 1989; Hartung *et al.,* 1993; Isherwood, 1996). The lower postprandial lipaemia in endurance trained men could be due to larger well-vascularised muscle mass with more capillaries around each muscle fiber and more capillaries per unit cross-sectional area of muscle (Ingjer, 1979) and higher LPL activity in trained muscle (Kiens & Lithell, 1989). Furthermore endurance trained men possess a larger proportion of type I highly oxidative muscle fibres (Costill *et al.,* 1976), which could also enhance their total body response to lipid tolerance test as muscle LPL activity has been reported to be strongly related to the proportion of type I fibers in needle biopsy samples of human skeletal muscles (Jacobs *et al.,* 1982). Moreover, differences in muscle blood flow between athletes and others could also contribute to differences in TAG uptake through increasing the exposure of LPL in this tissue to substrate. In this respect, one study reported higher blood flow in the skeletal muscle of athletes as compared to sedentary individuals (Ebeling *et al.,* 1993).

The major determinant of exercise-induced decrease in postprandial lipaemia appears to be enhanced clearance of TAG-rich lipoproteins from plasma. In male distance runners, clearance of intravenous TAG was 76% higher than the morning after a marathon run than when measured 24 hour before the race (Sady *et al.,* 1986). Similarly other studies have reported increases of 22 and 66% in removal rates of intravenous lipid

administered the morning after a 3-hour exercise session (Dufaus *et al.,* 1981; Annuzzi *et al.,* 1987).

LPL activity association with age, gender, and exercise

The lipoprotein lipase activity may be an important determinant of variation in postprandial lipid response with age, gender and fasting triacylglycerol levels and increased lipoprotein lipase activity may also explain the lower postprandial lipaemia associated with acute and chronic exercise. Weintraub *et al* (1987a & 1987b) demonstrated that LPL activities were significantly higher in younger subjects (< 30 years) than in older subjects (> 40 years). These authors also showed a tendency towards higher activity of LPL in women than in men, although the differences were not statistically significant in men ($p>0.05$). Several authors have also shown increased LPL activity in post heparin plasma (Peltonen *et al.,* 1981; Isherwood, 1996), in adipose tissue (Nikkila *et al.,* 1978) and in skeletal muscle (Svedenhag *et al* 1983) in endurance trained men. It is apparent that changes in LPL activity may be responsible in part for the variation in postprandial lipaemia which is observed between individuals under different conditions. Further studies are needed to observe the dietary and hormonal influences on the regulation of LPL activity in human subjects.

Effect of body composition

Studies have shown that postprandial TAG-rich lipoprotein metabolism is disturbed in obesity. A study by Lewis *et al.,* (1990) showed that obese (BMI, 43.7 $\pm$ 2.81 kg/m^2), normolipidaemic subjects had a 3.35 fold higher postprandial response as compared to normal weight subjects (BMI, 23.6 $\pm$ 0.72 kg/m^2). Furthermore the obese subjects also demonstrated a 1.63 fold greater cumulative increment in plasma retinyl palmitate compared to normal weight controls, suggesting accumulation of TAG-rich lipoproteins. Finally the obese group was normolipidaemic but their fasting TAG concentrations were significantly higher (~ 2 fold) than controls ($p<0.001$) The increased hypertriglyceridaemia in obesity was suggested to be due to increased production and decreased clearance of triglycerides (Kissebah *et al.,* 1989).

In order to investigate the mechanism of disturbed TAG-rich lipoprotein clearance in obesity, Potts *et al.,* (1995) studied the extraction of TAG from plasma and triglyceride rich lipoproteins in subcutaneous adipose tissue in controls and obese subjects before and after a mixed meal. These authors concluded that decreased triglyceride-rich lipoprotein

TAG clearance in adipose tissue was related to the elevated plasma TAG concentrations in obese group. Recently, Binnert *et al* (1998) studied the metabolism of exogenous lipid by tracing 1-^{13}C oleate metabolism in control and obese normaltriglyceridaemic subjects. The results of their study showed that the appearance of label in chylomicron-TAG was similar in both the groups, however, the appearance of label in non-esterified fatty acid fraction was dramatically reduced in obese group compared with the control. The decreased appearance of label in non-esterified fatty acid fraction in obese could be due to greater uptake at the level of adipocytes which is shown to be more metabolic active than subcutaneous tissue. The results of Binnert *et al.* (1998) study are different than Potts *et al.,* (1995) who showed decreased removal of chylomicron-TAG in obese subjects. Difference between the two studies could be related to differences in subjects in terms of metabolic profiles between these two studies. Obese subjects in Binnert *et al.* (1998) study were chosen to have normal fasting TAG concentrations whereas in Potts *et al* (1995) study the fasting TAG concentrations were almost twofold higher in obese than controls. Taken together, the results of these two studies suggest that obesity in the absence of fasting hypertriglyceridaemia has no abnormal effect on the postprandial lipid metabolism.

Recent evidence suggests that abdominal fat deposition is strongly related to the incidence of CVD. Wideman *et al* (1996) studied postprandial lipid metabolism in obese men with abdominal fat patterning and men of desirable of weight. These authors observed significantly increased postprandial lipid metabolism in obese men with abdominal fat patterning to desirable weight men matched for weight and habitual physical activity. Although intra-abdominal fat accounts less than 20% of body fat it is a major determinant of fasting and postprandial lipid availability because of its physiologic (such as high lipolytic rate and insulin resistance) and anatomic properties (such as portal drainage) (Carey, 1998). Increased visceral fat correlates with an adverse metabolic profile such as hyperinsulinaemia, decreased glucose tolerance, dyslipidaemia, and increased blood pressure (Pieris *et al.,* 1989). Moreover, hyperinsulinaemia and insulin resistance are established features of obesity (Ferrannini *et al.,* 1997). Finally abdominal fat patterning influences insulin sensitivity even in non-obese people (Carey, 1998). Further studies are needed to explore the mechanisms responsible for increased postprandial triglyceridaemia and reduced HDL cholesterol in obesity

2.4 Insulin resistance, diabetic dyslipidaemia and risk of CHD

This section describes insulin resistance, role of lipids in developing insulin resistance, diabetic dyslipidaemia and the associated abnormalities in lipoprotein composition and metabolism. It also focuses on the relationship of insulin resistance with the hypertriglyceridaemia and cardiovascular disease risk in Type II diabetes.

2.4.1 Insulin Resistance

The definition of hyperinsulinaemia is difficult and varies between populations because of ethnic variability in insulin concentrations. A simple definition of insulin resistance is the decreased ability of insulin, endogenous or exogenous, to suppress the hepatic glucose production and to enhance glucose clearance, particularly in the skeletal muscle (Walker, 1995). Another definition is that, hyperinsulinaemia represents a situation in which plasma insulin is higher than the expected for a given plasma glucose concentration, and may occur in the presence of normalglycaemia or hyperglycaemia (Zimmet, 1993). Hyperinsulinaemia may result both from increase insulin secretion by the pancreas and from reduced hepatic insulin extraction. Insulin resistance has also been defined in more generic terms by Kahn, (1986), it exists whenever normal concentration of insulin produce a less than normal biological response. In individuals who retain insulin secretory function, particularly non-diabetics, there is relatively linear relationship between measures of insulin resistance and plasma insulin concentrations (Reaven, 1988; Reaven *et al.,* 1989), *ie.* the more resistant, the greater the magnitude of hyperinsulinaemia.

These definitions are useful , but, restrictive as these does not include other aspects of insulin resistance such as effects of insulin on lipid metabolism.

2.4.2 Insulin resistance and dyslipidaemia

Hyperinsulinaemia and insulin resistance have been related to dyslipidaemia (Grag *et al.,* 1988; Haffner *et al.,* 1992; Laws *et al.,* 1991). Insulin resistance is associated with increased serum triglyceride and reduced HDL cholesterol concentrations (Laakso *et al.,* 1987; Reaven, 1988; Laakso *et al.,* 1990; Manolio *et al.,*1990), small dense LDL particles (Reaven *et al.,* 1992; Reaven *et al.,* 1993; Selby *et al.,* 1993), increased apo B and decreased A1; and postprandial lipaemia (Patsch *et al.,* 1992; Schrezenmeir *et al.,* 1993). The relationship between insulin concentrations and TAG and HDL cholesterol concentrations has also been established in population studies (Fontbonne *et al.,* 1989; Mitchell *et al.,* 1992; Godsland *et al.,* 1992). The consistent positive associations between insulin and plasma

triglycerides or VLDL and negative associations between insulin and HDL cholesterol have remained significant even when adjusted for covariates, such as obesity and age, and appear to be consistent in both genders and among populations of various nationalities and races.

2.4.3 Role of lipids in the development of insulin resistance

It is not clear that whether insulin resistance causes dyslipidaemia or is a consequence of dyslipidaemia, however, a number of studies suggests that lipids play an important role in the development of insulin resistance. Randle *et al.* (1963) was the first who demonstrated that the increased availability of fatty acids decreased carbohydrate oxidation in isolated perfused rat hearts and proposed a glucose fatty acid cycle. He proposed that the glucose/fatty acid cycle might cause the alterations in the insulin action in type II diabetes. According to that cycle, increased availability of free fatty acids in the circulation increases lipid oxidation and produces an increase in intramuscular aceteyl-CoA and citrate content which decreases the activities of enzymes pyruvate dehydrogenase and phosphofructokinase. This results in the accumulation of glucose 6-phosphate, which in turn, inhibits hexokinase, glucose uptake and glucose oxidation. In later studies, infusions of triglyceride emulsions (Intralipid) have confirmed this inhibitory effect of increased lipid oxidation on glucose oxidation (Gomez *et al.,* 1972; Balasse *et al.,* 1974) and also on glucose storage (Thiebaud *et al.,* 1982; Ferrannini *et al.,* 1983; Bonadonna *et al.,* 1989; Felly *et al.,* 1989). It has been suggested that the inhibitory effect of NEFA on non-oxidative glucose metabolism may result from the inhibition of glycogen synthase (Felber *et al.,* 1993). Furthermore, it has been shown that elevated NEFA concentrations first inhibited glucose oxidation (with in 1 to 2 hours) and later inhibited insulin-mediated glucose uptake (generally after 4 hours; Boden & Chen, 1995; Boden, 1997). NEFA also has a stimulatory effect on liver gluconeogenesis (Gonzales-Manchin *et al.,* 1989; Morand *et al.,* 1993; Boden, 1997). Finally, acute decreases in NEFA concentrations by antilipolytic agents was also accompanied by an increase in insulin-mediated glucose uptake (Balasse &Neef, 1973). Hence NEFA plays an important role in the development of insulin resistance and glucose intolerance, however, the cellular and molecular mechanisms responsible for NEFA inhibition of glucose transport or phosphorylation or inhibition of muscle glycogen synthase activity are unknown.

Studies have shown that loss of significant amount of weight either by caloric restriction or by gastric bypass surgery improves insulin sensitivity and glucose tolerance. Normalisation of plasma glucose has been demonstrated in obese diabetic patients after weight loss by hypocaloric diet (Beck-Nelson *et al.,* 1979; Hughes *et al.,* 1984) or in obese

patients by gastric bypass surgery (Long *et al.,* 1994; Pories *et al.,* 1995; Cowan & Buffington, 1998). These authors suggested that the weight loss itself induced the improvement of insulin sensitivity. With weight loss enhanced insulin action is reflected in both an increase in insulin ability to suppress hepatic glucose output and an improvement in insulin mediated glucose uptake in to peripheral tissues (Henry *et al.,* 1986). Virtually all aspects of peripheral glucose uptake have shown improvements with weight loss (Albu *et al.,* 1995; Pi-Sunyer, 1996). However, the precise mechanisms underlying this improvement have not yet been identified (Albu *et al.,* 1995)

Studies have also shown that loss of weight by biliopancreatic diversion surgery (BPD) also results in normalization in insulin resistance and glucose metabolism due to lipid malabsorption and improvement in serum lipids. Disappearance of hypertriglyceridaemia and type II diabetes have been reported after BPD (Mingrione *et al.,* 1997; Scopinaro *et al.,* 1998; Mingrione *et al.,* 1999). Mingrione *et al* (1997) suggested that normalization of insulin resistance and glucose metabolism may not entirely be a result of weight loss *per se*, but the lowered serum fatty acids and triglycerides after BDP may significantly contribute to markedly significant glucose uptake observed 3 months after operation. Furthermore, it has also been shown that reducing plasma levels of triglycerides with gemfibrozil, without changing body mass reduced the insulin resistance of hypertriglyceridaemia (Steiner, 1991). These studies suggests the role of hypertriglyceridaemia in the development of insulin resistance, however, the underlying mechanisms are remain to be determined.

2.4.4 Diabetic dyslipidaemia

The prevalence of dyslipidaemia is very common in type II diabetics as compared to insulin dependent diabetes (IDDM). The major lipid abnormality in type II diabetics is elevated plasma triglycerides (hypertriglyceridaemia) which is usually associated with decreased HDL concentrations (Betteridge, 1994a). In San Antonio Heart Study, 23% had hyperlipidaemia or low HDL cholesterol (Haffner *et al.,* 1992). It is important to note that these abnormalities in lipid metabolism are already present at the stage of impaired glucose tolerance (IGT) which precedes type II diabetes (Laakso, 1995) and persists in type II diabetic patients established on hypoglycaemic therapy (Stern *et al.,* 1992). Type II diabetes is often associated with obesity but this does not fully account for the triglyceride elevation.

It is re-emphasised that interpretation and significance of some of the findings as described in the following sub-sections should be made in the context of

methodological constraints and variability in meal composition and study design as discussed in introduction to section 2.

Alteration in postprandial chylomicron metabolism

Postprandial chylomicron metabolism is altered in type II diabetics. Decreased chylomicron remnants clearance have been reported in diabetic animals by some investigators, but not others (Redgrave & Snibson, 1977; Levy *et al.,* 1985; Feingold *et al.,* 1987; Redgrave and Callow, 1990). Hypertriglyceridaemic (HTG) type II diabetics have a greater postprandial chylomicron response (Lewis *et al.,* 1991; Tan *et al.,* 1995; Curtin *et al.,* 1996) and greater increase in postprandial chylomicron derived remnant particles (Lewis *et al.* 1991; Tan *et al.* 1992; Curtin *et al.,* 1996) than non diabetic subjects. Normal triglyceridaemic (NTG) type II diabetics have also demonstrated greater total postprandial TAG response in chylomicron and non-chylomicron fractions (Reznik *et al.* 1996) and excess chylomicron remnants (Chen *et al.,* 1993. Tan *et al.,* 1995, Attia *et al.,* 1995) after a lipid load. Type II diabetic patients under adequate nutritional and hypoglycaemic therapy have also displayed wider and delayed blood TAG response after the lipid load than non diabetic subjects despite a fasting TAG frequently below 2g/l (Cavallero *et al.,* 1992; Cavallero *et al.,* 1994). Several case control studies have indicated that the magnitude of postprandial lipaemia is a significant risk factor for CHD (Havel, 1994; Cohn, 1994). Patsch and colleagues (1992) showed that postprandial TAG levels 6 and 8 hours after a standardised lipid load were highly discriminatory between CHD cases and control male subjects. The area under the postprandial TAG curve and the maximal TAG increase were significantly higher in CHD cases than in control cases following correction for differences in fasting TAG ($P \leq .008$). Interestingly, postprandial lipoprotein clearance is improved markedly with fibrate administration; this phenomenon has been reported in CHD patients (Simpson *et al.,* 1990), in subjects with mild hypertriglyceridaemia and hypoalphalipoprotinemia (Simo *et al.,* 1993) and in patients with type II diabetes (Syvanne *et al.,* 1993). However, as yet there are no trials in diabetic and non diabetic patients designed specifically to determine the effect of triglyceride lowering on the primary and secondary prevention of CHD (Betteridge, 1996). Furthermore, the causes of enhanced postprandial chylomicron response and delayed clearances of chylomicron remnants are not known and remains to be determined. At present there are no direct and practical means for measuring the presence of chylomicron and VLDL remnants, techniques need to be developed and validated for getting clear picture of remnant lipoprotein metabolism.

Alteration in VLDL metabolism and composition

Since high concentrations of VLDL is a consistent finding in type II diabetics (Albrink *et al.,* 1963), one of the determinants of diabetic hyperlipidaemia may be the over-production of VLDL triglyceride (Ginsberg & Grundy, 1982; Dunn *et al.,* 1984; Howard *et al.,* 1987a). This increase in the production of VLDL triglycerides is more pronounced in diabetic patients whose TAG values are very high. Over-production of VLDL triglycerides is primarily attributed to the increased flow of substrates, particularly free fatty acids to liver because of increased lipolysis in adipose tissue due to resistance of this tissue to insulin. In addition, individuals with type II diabetes appear to have a defect in clearance of VLDL triglyceride (Howard *et al.,* 1987a; Howard *et al.,* 1987b) that parallels the degree of hyperglycemia. Lipoprotein lipase (LPL) activity is also decreased in individuals with type II diabetes, especially those with moderate to severe hyperglycemia who exhibit both an insulin deficiency and insulin resistance (Taskinen, 1987a).

The metabolism of VLDL apo B may also be altered in type II diabetes. Subjects with type II diabetes have a decreased fractional catabolic rate for VLDL apo B100 similar to that for VLDL triglyceride (Kissebah *et al.,* 1982; Howard *et al.,* 1987b). Over-production of VLDL apo B occurs in type II diabetes; this over-production may be further increased by obesity (Kissebah *et al.,* 1982; Howard *et al.,* 1987b). Although obese diabetic subjects have a higher VLDL apo B production than do lean individuals, in obese non diabetic subjects, VLDL apo B production may already be maximally stimulated (Howard *et al.,* 1987b). Thus, the extent of over-production of VLDL triglyceride may be greater than that of apo B in type II diabetics, situation that results in the production of larger triglyceride rich VLDL particles.

The large triglyceride-rich particles appear in conjunction with remnant VLDL particles that are enriched in cholesterol and contribute to atherogenesis (Schonfeld *et al.,* 1974; Patti *et al.,* 1991). Other changes in VLDL composition that occur in type II diabetes include elevated level and glycation of apo E, which may inhibit binding to the B/E receptor (Fielding *et al.,* 1986; Eto *et al.,* 1986). Furthermore, apo C:apo E ratio in VLDL is decreased in type II diabetes (Klein *et al.,* 1990). This has implications for VLDL metabolism, since apo C controls the activity of LPL (Cryer, 1981), and apo E influences the affinity for binding to receptors (Mahley, *et al.,* 1981). Diabetic VLDL enhance cellular lipid accumulation in peritoneal macrophages (Klein *et al.,* 1990).

Alteration in LDL metabolism and composition

Despite the increased risk of CHD in type II diabetics, LDL cholesterol concentrations are often similar to those of control subjects (Gordon *et al.,* 1977; Barrett-Connor *et al.,* 1982). However, some studies have also reported a slight increase in LDL cholesterol concentrations in diabetes (Mancini *et al.,* 1980; Howard *et al.,* 1984). Reduced concentrations of LDL in diabetics might be due to decreased conversion of increased VLDL to IDL and then to LDL due to due to decreased LPL activity (Kissebah *et al.,* 1982). On the other hand, relatively high LDL concentrations in type II diabetics with hyperglycaemia have been reported to be due to reduced fractional catabolic rate of LDL apo B. Impaired clearance of LDL has been attributed to insulin resistance because LDL binding is stimulated by insulin (Chait *et al.,* 1979; Kissebah *et al.,* 1983).

Since LDL cholesterol concentrations are generally similar to that of controls, metabolic and compositional abnormalities in LDL in type II diabetes might be related to atherogenesis in this group. Smaller, higher density LDL particles (Known as subclass B) are more prevalent in type II diabetic subjects with hypertriglyceridaemia and insulin resistant (Feingold *et al.,* 1992; Haffner *et al.,* 1994). Approximately 70% of variability in LDL density distribution is explained by plasma triglyceride (McNamara *et al.,* 1987). Lower concentration of larger more buoyant LDL-I and increased concentration of small dense LDL-III particles are found in hypertriglyceridaemic (TAG>2.3 mmol/l) non obese type II diabetes patients when compared with NTG diabetics and non diabetic controls. These findings are consistent with the reports of altered LDL density distribution in morbidly obese type II diabetic women (BMI > 40 kg/m^2) and obese hypertriglyceridaemic type II diabetic patients with poor glycaemic control (Barakat *et al.,* 1990; James & Pometta 1991). Diabetic with documented coronary artery disease were found to have a shift in LDL density compared with patients without coronary disease (Tilly-Kiesi *et al.,* 1992).

A possible explanation for the alteration in LDL density distribution is lipid exchange promoted by hypertriglyceridaemia with the transfer of TAG to LDL via cholesterol ester transfer protein (CETP) in exchange for cholesteryl ester. Triglyceride enriched LDL is a substrate for lipase activity resulting in lipid poor, protein rich particles (Griffin 1995). Kinetic studies of VLDL cascade have suggested that large TAG rich VLDL give rise to small dense LDL (Caslake *et al.,* 1992). Insulin resistance possibly acting through increased hepatic VLDL production is associated with small dense LDL. Non diabetic subjects with LDL subclass pattern B have been found to be more insulin resistant than those with pattern

A (Raeven *et al.,* 1993; Selby *et al.,* 1993). In type II diabetic patients, LDL size and peak density relate to insulin resistance (Stewart *et al.,* 1993) and insulin resistance correlated inversely with LDL-1 and positively with LDL-III (Tan *et al.,* 1995).

Other abnormalities in composition of LDL include increased TAG content and elevated level of free cholesterol (Howard & Howard, 1994). Another important change is the extent glycation of apo B, which is thought to have a significant effect on the metabolic process of LDL. Glycation in the range of 2-5% may reduce LDL metabolism by 5 to 25% (Steinbrecher & Witztum, 1984). Some studies have also suggested that glycated LDL is taken up by macrophage scavenger receptors because of its oxidative modification and thus contribute to foam cell formation (Lopes-Virella *et al.,* 1988; Hunt *et al.,* 1990).

Alteration in HDL metabolism and composition

Decrease in HDL concentrations are generally found in type II diabetics (Hollenbeck *et al.,* 1986). The reduction is mostly in the HDL2 subfraction (Laakso *et al.,* 1985). Although the causative mechanisms have not been isolated with certainty, it is believed that as the HDL levels increase during lipolysis therefore impaired triglyceride rich lipoproteins clearance and decreased lipoprotein lipase activity might act to decrease HDL accretion. Furthermore, increased hepatic lipase (HL) activity, associated with type II diabetes, may be a factor in lowering HDL levels by accelerating HDL catabolism (Harno *et al.,* 1980).

Changes in HDL composition also occur in type II diabetes. These changes include, increased concentration of TAG (Biesbrook *et al.,* 1982) and nonenzymatic glycation. The low cholesterol and high TAG content in HDL could be explained by increased exchange of triglyceride-rich lipoprotein TAG for cholesteryl esters in HDL. Non enzymatic glycation of HDL might impede receptor binding and intracellular egress of cholesterol.

2.4.5 Triglyceride-rich lipoproteins, insulin resistance and CHD risk in type II diabetics

Since type II diabetes is characterised by increased insulin resistance, the existence of multiple cardiovascular risk factors in Type II diabetics suggest the importance of type II diabetes in relation to CVD. It has been shown that insulin sensitivity is related to coronary heart disease risk factors (especially high triglycerides) in type II subjects (D'Agostino & Haffner, 1996). CHD is not only confined to patients with frank diabetes, and there is evidence that the risk of CHD is increased amongst individuals with normal glucose tolerance who have the highest plasma glucose concentrations after a glucose load (Fuller *et*

al., 1980; Vaccaro *et al.,* 1992). Although hyperinsulinaemia may maintain glucose tolerance in individuals who have a defect in insulin stimulated glucose uptake, endogenous hyperinsulinaemia has been shown to be associated with increased risk of CHD (Ducimetiere *et al.,* 1980). The results of The Paris Prospective Study (Fontbonne *et al.,* 1991; Casassus *et al.,* 1992) have consistently supported the hypothesis that insulin resistance is associated with a higher risk of CHD mortality. The Paris Prospective Study found that during mean follow-up period of 11 years, baseline TAG was significantly higher in men with glucose intolerance or type II diabetes who died of CHD ($P \leq .006$) This association which persisted after adjustment for other risk factors such as smoking, systolic blood pressure, body mass index (BMI), and cholesterol (Fontbonne, *et al.,* 1989). Studies have shown that chylomicrons and VLDL are atherogenic in experimental animals and in humans with type III hyperlipoproteinaemia (Mahley, 1985). Recent studies have also suggested that individuals with angiographically verified CHD have a greater increase in chylomicron remnants in postprandial plasma than control subjects without coronary lesions (Simpson *et al.,* 1990). In NIDDM, high concentrations of very low density lipoproteins (VLDL) are consistently found (Bierman *et al.,* 1966), and their composition is also altered resulting in particularly large and triglyceride-rich particles with an increased triglyceride : apo B ratio (Haffner *et al.,* 1984). In type II diabetic patients, hypertriglyceridaemia also reflects greater increase in chylomicron derived remnants than non diabetic control subjects (Curtin *et al.,* 1996). The role of remnant particles in atherogenesis is supported by *in vitro* studies demonstrating foam cell formation when incubated with macrophages (Van Lenten *et al.,* 1985). Furthermore, in angiographic studies remnant particle concentration correlates with atheroma progression (Krauss *et al.,* 1987). These studies provide strong evidence that triglyceride rich lipoproteins play an important role in the development of CHD in type II diabetes.

Emphasis is usually placed on high concentrations of LDL cholesterol when metabolic risk factors for CHD are considered. However, CHD can occur in the absence of hypercholesterolaemia (Reaven & Laws, 1990). Furthermore, over-emphasis on cholesterol lowering in CHD prevention to the exclusion of triglycerides is misleading (Durrington, 1998). Such a view is further supported by two recent coronary-angiographic trials involving fibrate drugs (Ericsson *et al.,* 1996; Frick *et al.,* 1997). In the first of these trials, the Bezafibrate Coronary Atherosclerosis Intervention Trial (BECAIT, Ericsson *et al.,* 1996), the decrease in the rate of progression achieved with bezafibrate was comparable with that reported in similar trials involving statins despite only a 9% decrease in serum cholesterol

with bezafibrate. In the second and larger of these trials The Lopid Coronary Angiography Trial (LOCAT; Frick *et al.,* 1997) in patients with low HDL cholesterol and relatively modest dyslipidaemia after coronary artery bypass surgery, gemfibrozil reduced coronary disease progression in native vessels and in the grafts themselves. If the mechanisms by which lipid-lowering drugs decreased CHD events were simply mediated through the total LDL cholesterol concentrations, then statins would have a markedly greater effects than fibrates in decreasing CHD risk. The probable explanation is that not all LDL is atherogenic (Durrington & Illingworth, 1998). IDL (d 1.006-1.019 g/ml) and probably chylomicron remnants are likely to be highly atherogenic, because they can be taken up directly without modification by arterial-wall monocyte-macrophages (Gianturco *et al.,* 1994). LDL itself is taken up only slowly by macrophages unless it has undergone oxidative modification and can enter via scavanger receptors or oxidised LDL receptors (Witztum & Steinberg, 1991). Hypertriglyceridaemic subjects tend to have small and dense LDL particles (Austin *et al.,* 1988; Austin *et al.,* 1990). A preponderance of small dense LDL has been associated with coronary artery disease (Austin *et al.,* 1988). Several studies have suggested that small dense small dense LDL level might also form part of insulin resistance syndrome (Reaven *et al.,* 1993; Haffner *et al.,* 1995).The small dense LDL subclass (d 1.040-1.053 g/ml) appears to be the most susceptible to oxidation (De Graaf *et al.,* 1991; Chait *et al.,* 1993) and it is less readily cleared by physiological LDL receptors (Galeano *et al.,* 1994). The LDL in the range density 1.020-1.040 g/ml may thus be less critical to atherogenesis and lowering its concentration, although impressive in terms of the effect of serum cholesterol, may have little effect on CHD risk (Durrington & Illingworth, 1998). The small dense LDL contributes little to total cholesterol and decreases in its concentration will not be detectable from routine serum lipid estimations. Decrease in intermediate density lipoprotein (IDL) will produce a decrease in serum cholesterol, but again this lipoprotein does not contribute as much as 1.020-1.040 g/ml density LDL to serum cholesterol. Fibrate drugs are known to decrease both IDL and chylomicron remnants (Bhatnagar *et al.,* 1992) and small dense LDL (Gaw *et al.,* 1994), which may explain why they decrease CHD incidence to a similar extent to statins , even though their effect on 1.020-1.040 g/ml density LDL are so much less (Durrington & Illingworth, 1998).

2.5 Stable isotopes in metabolic research

As stable isotope tracer methodology was used in the studies presented in this thesis, the main purpose of this section is to give brief introduction about stable isotopic

studies and to familiarise the reader with the basic concepts and the instruments involved in the stable isotope tracer methodology.

2.5.1 Introduction

Stable isotopes have been used to study the *in vivo* metabolism of substrates as early as 1930s (Schonheimer & Rittenberg, 1935a,b). However, the widespread utilisation of stable isotopes for clinical research and diagnostics is a relatively new development. This expansion in the use of stable isotopes has been due to advances in the synthesis of suitable substrates, reduced cost of tracers and analytical methods available for their determination in biological fluids (Cummings & Watts, 1996; Seal, 1997). This later factor includes techniques for isolation, and if necessary, derivatization of metabolites present at very low concentration and the accurate determination of enrichment approaching natural abundance levels (Seal, 1997). The major advantage to the use of stable isotopes over radioactive alternatives is that they are not radioactive and presents no risk to human subjects and experimenters. Moreover, with the recent advances in the sensitivity and precision of the instrumentation, only small amount of tracer can be used to elevate enrichments significantly above natural abundance levels. The principal difficulty in the use of stable isotopes is the cost of stable isotopes, expensive instrumentation and time consuming analytical methods.

Currently stable isotopes are used for diagnostic purpose such as detection of helicobacter pylori infection or measurement of gastric emptying, as well as for research questions including determination of turnover rates of substrates such as amino acids, glucose and fatty acids, as well as measurement of energy consumption, distribution volumes of particular metabolites, the elucidation of metabolic pathways and pharmokinetic studies (Koletzo *et al*, 1998).

2.5.2 Characteristics of Stable Isotopes:

A chemical element's atomic number is the number of protons in the nucleus of each of its atom. Atoms of the same atomic number but different atomic weights are called isotopes. The difference in the atomic weight is due to the differences in the number of neutrons in the atom.

Elements can exist in both stable and unstable (radioactive) forms. Elements of biological interests, such as carbon (C) and nitrogen (N), have two or more stable isotopes, with the lightest of these present in much greater abundance than the others. Stable isotopes

of C and N are used as biological tracers. These two elements are found in earth, the atmosphere, and all living things. Each has a heavy isotope (^{13}C, and ^{15}N) with the natural abundance of ~ 1% or less, and a light isotope (^{12}C and ^{14}N) that makes up all the rest in the case of nitrogen or nearly all in the case of carbon (carbon has also a radioactive isotope ^{14}C).

Element	Isotope	Abundance
Carbon	^{12}C	98.89
	^{13}C	1.11
Nitrogen	^{14}N	99.63
	^{15}N	0.37

2.5.3 Isotope Ratio Mass Spectrometer (IRMS)

A sample's isotopic composition is measured by determining the ratios of the stable isotope masses being examined, e.g., $^{13}C/^{12}C$. The ratios are measured on a device called isotope ratio mass spectrometer (IRMS). IRMS produces charged particles (ions) of the stable isotopes being examined and then separates these ions on the basis of their differing mass/charge ratio (m/z). A basic mass spectrometer consists of a combustion tube, reduction tube, water scrubber, GC column, mass separator containing flight tube and ion source, analyser and a detector.

Combustion tube

The main function of combustion tube is to convert the sample into gas (N_2 or CO_2). The combustion tube consists of oxidation catalyst (chromium oxide granules) and is held at 1000 ^{0}C. A sample is admitted into the combustion tube along a pulse of pure oxygen. All combustible material in the sample are flash-burned and the resulting gaseous combustion products [CO_2, N, and nitrogen oxides (Nox) and water] are swept out by a constant stream of non reactive helium carrier gas. These gases then enter into the reduction tube.

Reduction tube

The reduction tube contains copper oxide wires and is maintained at a temperature of 600 ^{0}C. In the reduction tube all the molecules of Nox give up their oxygen to hot copper and emerge as pure nitrogen (N_2). Reduction of Nox to N_2 is very important because N_2O can produce mass 44 and mass 45 ions in the ion source which interfere with carbon isotope ratio measurement on CO_2. The sample is then passed through water scrubber.

Water scrubber and Gaschromatograph Column (GC)

The water scrubber is a nafion tube containing magnesium perchlorate, which removes water vapours from the sample. The sample then passes through the GC column to separate the N_2 and CO_2 and permits these two gases to reach the mass spectrometer at different times. N_2 eludes first from the GC column followed by CO_2.

Mass separator containing flight tube and ion source

A needle-type splitter valve admits small portion of pure gasses (~ 1%) into one end of a highly evacuated flight tube containing an ion source. A hot filament in the ion source generates a beam of electrons for bombarding gas molecules. As the N_2 and CO_2 gas molecules undergo bombardment from the electron beam, some of them lose an outer electron and becomes positively charged molecular ions. These ions are then collimated into a focused beam and accelerated into the flight tube. The ion beam then enters into the analyser section of the mass spectrometer.

The analyser

The analyser contains electromagnet which creates a strong magnetic field. The magnetic field then deflects and separates ions according to their momentum [the product of their mass times their velocity (mv)]. Light ions are deflected more because of its smaller mass (small mv) and heavier ions are deflected less because of their large mass (large mv). The separated masses then reach the detector of mass spectrometer

The detector

The detector of mass spectrometer contains different collectors placed so as to receive masses 28, 29, and 30 for N_2 and 44, 45, and 46 for CO_2. Here the impact of these ions is translated into a recordable signal that is collected for data processing and analysis.

2.5.4 Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS)

GC-C-IRMS is a device in which GC is directly connected to the combustion furnace of IRMS. GC separates the sample in to individual components and IRMS measures the stable isotopic enrichments.

Prior to GC-C-IRMS analysis, the sample must be converted to derivatives that are thermally stable, chemically inert and volatile at temperatures below approximately 300 ^{0}C (Cummings & Watts, 1996). Since GC is used for the separation of fatty acids, methyl esters

are the most widely used derivative. Sulphuric acid in methanol is used to convert TAG into fatty acid methyl esters. Free fatty acids (FFA) produced by sulphuric acid treatment are methylated by methanol. Methylated fatty acids are non-polar and can be separated by GC.

The basic principal of GC involves the introduction of a sample into an "injector chamber", where high resolution separation of the volatile compounds occur (Cummings & Watts, 1996). By heating the "injector chamber" the derivatized sample is vapourized and is then swept through GC column by a inert carrier gas (helium) flowing simultaneously through the GC. GC separates the components of the sample by their differential migration through a two-phase system consisting of mobile phase (inert gas, helium, carrying the vapourized sample) and a stationery phase (a fixed porous sorbent). The total amount of time that an analyte spends in the system is called the retention time, which depends on analyte partition coefficient (the ratio of solute in the stationery phase to solute in the mobile phase). The separated analytes than enters directly into combustion tube of IRMS as described above.

2.5.5 Naturally labelled tracers

Carbon isotopes are fractioned to a small degree during biochemical processes (Smith & Epstein, 1971). For example, plants that fix CO_2 via photosynthesis by the Calvin C_3 pathway (Quayle *et al*, 1954) contain relatively less ^{13}C than plants utilising the C_4 Hatch-Slack pathway (Hatch & Slack, 1970). Glucose derived from cane and corn falls into C_4 plant food chain. Since the diet of Europeans is derived from C_3 plant food chain, their breath $^{13}CO_2$ enrichment rises when a corn sugar meal is given. Hence, the relatively ^{13}C enriched corn sugar is naturally labelled (Lacroix *et al*, 1973). Use of this effect has allowed an extensive series of end product studies of glucose metabolism using "shelf glucose" as tracer (Lefebvre, 1979). Because the diet of Americans is derived largely from a C_4 plant food chain, this naturally-labelled approach is not possible there.

2.5.6 Measurement Notation

Following are the different units in which the results of stable isotope enrichments studies are reported:

2.5.6.1 Atom Percent

Results from environmental and agricultural studies using isotopically enriched tracers are usually reported in units of atom percent (At%). This value gives the absolute number of a given isotope in 100 atoms of the total element.

$$\text{At\% } ^{13}C = \frac{^{13}C}{^{12}C + ^{13}C} * 100$$

A sample containing 1 At% has 1% of ^{13}C and 99% of ^{12}C

2.5.6.2 Atom Percent Excess and Mole Percent Excess

Medical tracer studies of human physiology are most often reported in units of atom percent excess (APE). This specify the increase in the isotopic abundance above the background level or natural abundance level.

APE = At% at time t - At% at baseline

For example, (1-^{13}C)palmitic acid at 90 APE means that approximately 90% of C atoms at carbon 1 are ^{13}C and remaining 10% of C atoms at position 1 are at natural abundance *ie.* 1.11 At%.

Mole% excess = 17 APE

Where, 17 is the number of C atoms in derivitized molecules *Ie.* methylated (1-^{13}C)palmitic acid.

2.5.6.3 Delta (δ)

Studies examining stable isotopes at or near natural abundance levels are usually reported as δ, a value given in parts per thousand or per mil ($^0/_{00}$). δ values are not absolute abundances but differences between sample readings and one or another of the widely used natural abundance standards (which are considered δ=0). For example air for nitrogen, At% ^{15}N = 0.3663033; Pee Dee Belemnite (PDB) for C, At% ^{13}C = 1.1112328. A $\delta^{13}C$ value of zero is equal to 1.112328 At% ^{13}C.

Absolute isotopic ratios are measured for the sample and standard and the relative δ values is calculated according to the following equation:.

$$\delta^{13}C\ (^0/_{00}) = \frac{(^{13}C/^{12}C)\text{ sample} - (^{13}C/^{12}C)\text{ standard.}}{(^{13}C/^{12}C)\text{ standard}} * 1000$$

The common reference for $\delta\ ^{13}C$, the Chicago PDB Marine Carabonate Standard, was obtained from a Cretaceous marine fossil Belemnitella americna, from the Pee Dee formation in the South Carolina. This material has a higher $^{13}C/^{12}C$ ratio than nearly all other natural

carbon based substances. For convenience it is assigned a $\delta^{13}C$ value of zero, giving all the other naturally-occurring samples negative δ values, which range from ~ 0 $^{0}/_{00}$ to ~ -110 $^{0}/_{00}$ relative to PDB standard.

For example,

Natural abundance	^{13}C stool	= -27 $^{0}/_{00}$
	^{13}C breath	= -25 $^{0}/_{00}$
	^{13}C cane sugar	= -11.65 $^{0}/_{00}$
	^{13}C beet sugar	= -25.96 $^{0}/_{00}$
	^{13}C Corn flour	= -11.20 $^{0}/_{00}$

2.5.7 Applications stable isotopes in lipid and lipoprotein research

Stable isotopes have been extensively used to study the metabolism of apo B100 (particles carrying lipids) using stable isotopically labelled amino acids (Cryer *et al.,* 1986; Cortner *et al.,*1992; Walsh *et al.,* 1991, Packard, 1995; Cummings & Watts, 1996;), however, few studies have addressed the issue of lipid metabolism (Hachey, *et al.,* 1987; Hellerstein *et al.,*1994; Binnert *et al.,*1996, Brossard, *et al.,*1996; Rhee *et al.,*1997; Binnert *et al.,*1998). In some of these lipid studies (Binnert *et al.,*1996; Binnert *et al.,*1998) test meal is composed of lipids only and thus are highly artificial as they lack CHO which stimulates insulin (a potent lipolytic regulator in the postprandial state). Further stable isotope studies are needed to trace the metabolism of lipids as present as core of apo B100 of apo B48 particles in various hyperlipidaemic conditions such as type II diabetes, obesity, familial hyperlipidaemias etc.

2.6 Summary and conclusions from literature of review

After digestion and absorption of ingested lipid in the lumen of intestine (2.2.2.2), synthesis of chylomicrons takes place in enterocyte (2.2.5) to transport dietary lipid and lipid soluble vitamins in to the circulation. Synthesis of apo B48 is required for the assembly of chylomicrons (2.2.5), which is produced by the translation of a post-transcriptionally edited apo B100 mRNA (2.2.5.3). The apo B48 is cotranslationally integrated in to endoplasmic reticulum and is lipidated and result in the formation of nascent HDL-size chylomicrons (2.2.5.3). MTP is essential for the Lipidation of nascent apo B48 (2.2.6). The HDL-size chylomicrons then increase their size by the addition of neutral lipids in to the core. The availability of lipids coupled with inhibition of intracellular degradation of apo B results in increased secretion of apo B-containing

lipoproteins (2.2.7). Chylomicrons enter the blood, acquire apo CII and apo E and the triglycerides present in the particles are hydrolysed by endothelial cell bound LPL, resulting in the generation of chylomicron remnants (2.2.8). Remnants acquire more apo E from plasma and are further metabolised by hepatic lipase and then cleared from the circulation by the liver by LDL receptor and LDL receptor related protein (2.2.9). Since, most of this information regarding chylomicron assembly is obtained from liver derived cells, hence the exact mechanisms involved in the assembly of larger chylomicron particle from the shorter apo B48 remains to be elucidated. Furthermore the role of proteins involved during the chylomicron assembly (e.g. apobec-1, MTP and apo AIV) needs to be further studied in detail, for further development of efficient inhibitory tools against these proteins for reducing the postprandial lipaemia.

Dietary factors can modify postprandial triacylglycerolaemia (2.3.1). Both saturated lipid and lipid load result in higher postprandial lipaemia. Addition of sucrose to lipid-rich meal amplifies and prolong the postprandial. The n-3 PUFA from fish oils have a potent hypotriacylglycerolaemic effect as compared to n-6 PUFA and saturated lipids but the biochemical mechanisms responsible for the triacylglycerol lowering effect of n-3 fatty acids are not known. It is also not known that which of the n-3 fatty acids is responsible for the fish oil effect. Since most of the studies compared the effect of meals high in fatty acid class of interest (saturated fatty acid rich meal and n-3 PUFA rich meal), thus more postprandial studies are needed to determine the potency with which each fatty acid (rather than diet rich in specific classes of fatty acids) affect the postprandial plasma lipids as well as the mechanisms that account for their marked different effects. Furthermore better methodological tools are needed to distinguish the exogenous and endogenous sources of individual fatty acids for better understanding their effects. This information obtained from these studies will be useful in making dietary recommendations for individual fatty acids that may further reduce the risk of chronic diseases. The acute/chronic effects of dietary fiber on plasma lipid and lipoprotein concentrations are inconclusive. More studies are needed in this respect. The effects of dietary triacylglycerol structure on the hydrolysis of chylomicron TAG and clearance of the potentially atherogenic particles, chylomicron remnants , is another important area for further studies. Dietary influences on the regulation of LPL activity in human subjects needs further investigation.

Physiological factors such as age and body composition also affect the

postprandial lipoprotein metabolism (2.3.2). However, there are few studies describing the effect of age and obesity on the postprandial lipid metabolism. Further studies, with better methodological tools to distinguish between exogenous and endogenous sources of lipid in circulation, are needed for better understanding of the causes responsible for high postprandial TAG responses in these groups.

There is strong evidence that triglyceride rich lipoproteins play an important role in the development of CHD. Several modifications in lipid and lipoprotein concentrations and compositions (that appear to be atherogenic) have been described in type II diabetics and therefore may contribute to the increased risk of CHD (2.4.4). It is not clear that whether insulin resistance causes dyslipidaemia or is a consequence of dyslipidaemia (2.4.3). The precise mechanisms of elevated triglyceride- rich lipoproteins, lowered HDL, increased apo B and altered postprandial lipaemia in type II diabetics are not clear. At present there are no direct and practical means for measuring the presence of chylomicron and VLDL remnants, techniques need to be developed and validated for getting clear picture of remnant lipoprotein metabolism. Further postprandial studies are needed with techniques to distinguish between exogenous and endogenous lipids and lipoprotein metabolism in order to understand the mechanisms responsible for diabetic dyslipidaemia. This will help in determining ways of correcting these abnormalities with diets or drugs.

Chapter 3

Methodology

3.1 Introduction

The methodology described in this chapter is divided in to three sections. The section 3.2 describes the recruitment of subjects and general protocol for the studies. The section 3.3 describes analytical methods involved in the study including blood sampling, chylomicron-rich fraction isolation, and lipid extraction from chylomicron-rich fraction. It also gives the details of ^{13}C enrichment measurements in stool and breath samples (CF-IRMS) and blood samples (GC-C-IRMS, Orchid system). The section 3.4 describes indirect calorimetry, anthropometric measurements, and data analysis and presentation.

3.2 Subjects and experimental protocol

3.2.1 Subjects

Six to eight subjects were recruited for each of the three studies [a) healthy young men for repeatability study, b) healthy middle-aged men for age-effects study, c) normaltriglyceridaemic and hypertriglyceridaemic type II diabetics for diabetic study] from the university student and staff population and the Diabetic Department of Southampton General Hospital. Except for diabetic group, all the subjects were healthy and normal lipidaemic (fasting TAG less than 1.7 mmol/l; fasting HDL cholesterol greater than 1.04 mmol/l and fasting total cholesterol less than 6.4 mmol/l). Normaltriglyceridaemic type II diabetics had fasting TAG less than 1.7 mmol/l and hypertriglyceridaemic type II diabetics had fasting TAG greater than 2.0 mmol/l plasma. Apart from diabetics all the subjects had fasting blood glucose values less than 6.5 mmol/l). Further details of the subjects are described in relevant chapters

All the subjects refrained from alcohol consumption and abstained from volitional exercise three days prior to the study. All the subjects were non-smokers.

Healthy subjects had no known history of diseases like gastrointestinal, liver, renal, diabetes and hypertension. Healthy subjects were also not using any medications affecting lipid metabolism. Informed consent was obtained from all subjects and the study protocol was approved by the Ethical Committee of Southampton and South West Hampshire Health Commission.

3.2.2 Experimental Protocol

The duration of study was two days in each of the above mentioned study groups, except for repeatability study which lasted for nine days (including five days of stool collection). In repeatability study each subject took part in two identical trials separated approximately by six weeks interval.

For each study, an information sheet explaining the significance and details of the study was distributed among the tentative subjects. Subjects agreeing taking part in the study were given an appointment at their convenience for screening purpose except for diabetics who were recruited by clinician. On the screening day, fasting blood samples were withdrawn (for the analysis of plasma TAG, total cholesterol, and HDL cholesterol) and medical history (relating to GIT and liver problems and medications taken) was recorded. Initial measurements of basal metabolic rate (by indirect calorimetry) and body composition (by bioelectrical impedance) were also made. Subjects fulfilling the study criteria (as mentioned in subject recruitment section) signed the consent form and were given an appointment date for the study trial. A letter explaining the procedures involved was sent to their general practitioners for their information.

In order to minimise the effect of preceding diet on the postprandial lipid metabolism, a controlled diet was provided to all subjects (except for diabetics) for three days prior to the study (Appendix 8.1A). Because of the practical constraints, the diabetics were just given a standard evening meal prior to the study day. The controlled diet was fixed to provide 10 MJ energy with additional energy requirements provided by incremental component (Appendix 8.1B). Both the diet and incremental component had fixed composition which was typical of present UK diet for adult men (Gregory *et al.* 1990). The controlled diet was composed of 40% energy from fat, 45% energy from carbohydrate (58% starch, 42% sugar) and 15% energy from protein. All the subjects were advised to avoided volitional exercise and alcohol intake for 3 days before the study day. Only in the repeatability study, a baseline stool sample was collected (in a stool kit

provided) before the study day. The reason for not collecting stool samples in other studies is described in chapter 4.

On the evening before the study day, the subjects participating in the repeatability study were admitted to Clinical Nutrition and metabolism unit where they consumed a standardised meal by 1900 hours and then fasted thereafter. The subjects participating in the age-effect and diabetic studies were delivered standardised evening meal to their homes and were admitted to CNMU during the morning of the study day.

During the morning of the study day (at 0800 hours), the subjects consumed a [1,1,1-^{13}C]tripalmitin within a lipid-casein-glucose-sucrose emulsion and test meal (10mg/kg body weight tripalmitin, 99 atom percent excess; tracer technologies Inc. Somerville, MA). The test meal was of fixed quantity, containing 3.2 MJ energy, 90.3 g carbohydrate, 35.7 g lipid and 27.2 g protein. Both the emulsion and the test meal composition was typical of present UK diet for adults (Gregory *et al.* 1990). At 1400 hours, a second identical unlabelled meal was served in order to observe the effect of second meal on the release of labelled fatty acid (in CRF-TAG) as ingested in previous meal. No additional foods or liquids were allowed during the study except for bottled mineral water. The study day meal menu and its composition are given in Appendix 8.2A and 8.2B respectively. The ^{13}C enrichment of the test meal and the effect of test meal on ^{13}C enrichment of breath CO_2 have been validated and found to be close to natural ^{13}C abundance of -25 δ PDB (Appendix 8.3A and 8.3B respectively)

Venous blood samples were collected in heparinized tubes from an indwelling cannula before and at hourly intervals for ten hours after the label administration. Similarly, Breath samples were collected in breath collection bags (Quintron, Milwaukee, WI) before and at hourly intervals for 10 hours after the label administration. Three breath samples at each time points were transferred into evacuated glass tubes (Exetainers; Labco, High Wycombe). Similarly whole body CO_2 excretion was determined by indirect calorimetry (GEM, Europa Scientific) before the test meal and then at hourly intervals for ten hours after the test meal.

In the repeatability study a stool sample was collected before the study day in order to measure baseline ^{13}C excretion. After the label administration, all stools passed were collected for a period of 5 days. Stools were collected separately into polyethylene bags and were kept frozen until analysis.

3.3 Analytical Methods

3.3.1 Blood Sampling

I0 ml blood samples were collected into heparinized sterile tubes (Vacutainer, Becton Dickinson, Meylan Cedex, France) for the analysis of insulin, TAG, NEFA, ^{13}C PA in NEFA and ^{13}C PA in CRF-TAG. For plasma glucose analysis, 2ml blood samples were collected in sterile tubes containing sodium fluoride and potassium oxalate (Vacutainer, Becton Dickinson, Meylan Cedex, France). Plasma was recovered instantly by centrifugation (2500 g, 15 min) at 4 ^0C. For the separation of chylomicron-rich fraction, 3 ml plasma was stored in fridge with preservatives such as PMSF (10 mmol/l, dissolved in 2-propanol), EDTA (0.5 m, at pH 7.4) and trasylol (10,000 KIE/ml, Trasylol, Bayer, Leverkusen, Germany). These preservatives were added immediately to plasma at the final concentrations of 1 µl/ml, 2 µl/ml, and 5 µl/ml plasma respectively. Chylomicron rich fraction was separated next morning. The remaining plasma (~ 1.5 ml) was stored in freezer at -35 ^0C for analysis for plasma TAG, NEFA, ^{13}C enrichment in NEFA and insulin.

3.3.2 Lipoprotein fractionating

Chylomicron-rich fraction was separated by differential density gradient ultracentrifugation. Three ml plasma was adjusted to density 1.10 kg/l by adding sodium bromide solution (d 1.42 kg/l, containing 0.1 m EDTA) to a final concentration of 0.5 ml of sodium bromide per ml of plasma. For density gradient formation, 4 ml of density adjusted plasma was gently poured in coated Beckman Ultraclear tubes (Beckman, Palo Alto, CA). That 4 ml density adjusted plasma was then sequentially layered with 3 ml each of d 1.065, d 1.020, and d 1.006 kg/l NaCL. The layering process was done very gently by pouring solutions along the walls of Beckman tube through a fine gauge needle. Ultracentrifugation was performed in a Beckman SW40 Ti swinging bucket rotor at 40,000 rpm for 32 min at 15 °C. After centrifugation, the top ~ 2.0 ml cloudy gradient containing CRF-TAG was aspired with the help of glass pipette and was stored in freezer for lipid extraction and fatty acid methyl ester preparation (FAME) for Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) analysis.

Plasma TAG, glucose and NEFA concentrations were measured using colorimetric assays (For TAG and glucose, Sigma Chemical Co., St. Louis, MO; and for NEFA, Wako Chemical GmbH, Germany). Plasma insulin concentrations were measured by radio-immuno assay.

3.3.3 Lipid extraction from plasma and chylomicron-rich fraction

Lipids were extracted from the plasma and chylomicron-rich fraction according to Folch *et al.,* (1957) method using chloroform/methanol (2:1, vol./vol.). This method involved mixing the sample with chloroform-methanol and washing the organic phase with saline solution. The lower phase (chloroform) contained lipids and the upper phase (aqueous and methanol) contained water and non-lipid material. The lower phase was removed into clean glass tubes with glass pipettes.

NEFA and TAG in the extracted lipid phase were then separated by thin layer chromatography (TLC) on 60 G silica gel plates using hexane/diethyl ether/acetic acid (70:30:1.8 by vol.) as a mobile phase (Stein and Smith, 1982) in a glass solvent tank. TLC provides separation of a wide variety of compounds with different polarities on a single plate. In TLC silica gel is a polar adsorbant. Polar lipids (fatty acids, phospholipids) adsorb more tightly due to polar interactions than non-polar lipids (TAG, cholesterol eaters). The most non-polar lipids therefore migrate at the fastest rates and the polar lipids at the slowest rates. When the solvent front was close to the top of the plate, the plate was removed from the solvent tank and evaporated under vacuum hood. TAG and NEFA spots on the TLC plates were visualised with dyes rhodamine B and flourescein respectively (0.05% in ethanol). TAG and NEFA spots were then scraped off the plate in to the screw capped tubes and treated with 2% sulphuric acid in methanol and toluene overnight at 50 ^{0}C for fatty acid methyl ester (FAME) preparation. Next morning, the samples were treated with neutralising reagent (25g $KHCO_3$ and 34.55g K_2CO_3 in 500 ml distilled water) and hexane and centrifuged at 2000 rpm for 10 minutes. The solvent containing FAME was then removed in to mini vials and was evaporated (~ 0.1 ml) under dry nitrogen, sealed and stored in freezer (-20 ^{0}C) for later determination of ^{13}C enrichment by GC-C-IRMS.

3.3.4 Stool and breath sample analysis

Stools were weighted and homogenised with distilled water, and aliquots were then freeze-dried overnight (Genevac, Ipswich, UK) to a constant weight. Samples of freeze-dried stool (~2mgs) were weighted into tin capsules (Elemental Microanalysis, Okehampton, UK) for analysis in duplicate. These samples were then loaded into carousel (65 space) with 3 reference samples (2.0 $\pm$ 0.1 mg beet sugar) initiating the run followed by the repeated series of 6 samples inter-spaced with 3 reference samples and finishing

with two reference samples. These reference samples had similar ^{13}C abundance as compared to the baseline stool samples. ^{13}C enrichment of whole dried stool was then determined by Continuous Flow-Isotope Ratio Mass Spectrometry (CF-IRMS; 20/20 IRMS with G/S/L interface; Europa Scientific Ltd., Crewe, UK). For that purpose, the carousel containing the samples was dropped in to the quartz combustion chamber maintained at temperature of 1000 ^0C. The combustion chamber consisted of oxidation catalyst (chromium oxide granules) followed by copper oxide wire (for oxidation of hydrocarbons) and silver wool (to trap sulphur and halogens). A pulse of pure oxygen combusted the sample which resulted into a mixture of gases containing CO_2, N_2, N_{ox} and H_2O. The produced gases were than carried in a flow of pure helium (60ml/sec) through a reduction tube (maintained at 600 ^0C) containing copper oxide wires for reducing nitrogen oxides (N_{ox}) to nitrogen. The sample was then passed through water scrubber (containing magnesium perchlorate) for the removal of water. The sample then passed through GC column (kept at 125 ^0C) to separate gases. Only small proportion (1%) of the GC effluent entered into the ion source of Isotope Ratio-Mass Spectrometer, while the remainder was passed to the atmosphere. In the ion source the sample was bombarded with electrons to generate positively charged CO_2 ions. These charged ions were then passed through magnetic field and were deflected according to their charge to mass ratio. The Isotope Ratio Mass Spectrometer was equipped with triple collector for simultaneous recording of ions of three different masses e.g. m/z = 44, 45, and 46, which represented $^{12}C^{16}O^{16}O$, $^{13}C^{16}O^{16}O$ or $^{12}C^{16}O^{16}O$ and $^{12}C^{18}O^{16}O$ respectively. Data processing and enrichment measurement were determined using the software provided by the manufacturer.

The ^{13}C enrichment of stool samples was expressed as the isotopic ratio delta ($^0/_{00}$), as defined in the following equation:

$$^{13}C \text{ enrichment } (^0/_{00}) = \frac{^{13}C/^{12}C_{SAMPLE} - ^{13}C/^{12}C_{PDB}}{^{13}C/^{12}C_{PDB}} \times 1000$$

Since delta units had no physiological value, excretion of ^{13}C in stools was expressed as percentage of administered dose, determined by using the equations of Schoeller et al (1981). Total excretion of ^{13}C in stool over the study period was calculated as the sum of the individual days. Appendix 8.4C shows equations for the calculations of % ^{13}C excretion in stool.

Breath samples were loaded into an auto-sampler rack (capacity 220

samples). Three reference breath samples (10ml of 5% CO_2, 95% N_2 gas mix, BOC Gases, Manchester, UK) initiated the run followed by repeated series of 6 samples inter-spaced with three reference samples and finishing with two reference samples.^{13}C enrichment of breath samples was determined by CF-IRMS (20/20 IRMS with G/S/L interface; Europa Scientific Ltd., Crewe, UK). Breath samples were injected into the combustion chamber and carried in the flow of helium through the reduction tube, water scrubber, GC column, mass spectrometer and results recorded as described above.

The proportion of the ^{13}C label excreted as breath $^{13}CO_2$ (expressed as percent administered ^{13}C dose) was determined using the equations of Watkins *et al.* (1982). Total excretion of $^{13}CO_2$ (over the 10 hour study period) was calculated from the area under curve of a graph plotted for time versus % administered dose per hour (Matthews *et al.*, 1990) using Simpson's rule formula (Borowski & Borwein, 1989). Total exogenous lipid oxidation for the period of 10 hour was calculated in relation to the percentage of ^{13}C palmitic acid oxidised over that period. It was assumed that the oxidation of the tracer (^{13}C palmitic acid) was similar to that of the tracee (total exogenous palmitic acid or total exogenous fat). Appendix 8.4D shows the equations for the calculation of ^{13}C excretion in breath.

3.3.5 Blood sample analysis

The ^{13}C enrichment of FAME was determined by IRMS (Europa Scientific Ltd, Crewe, UK) interfaced with a gas chromatograph (5890, Hewlett Packard, Palo, Alto, CA). FAME were evaporated to dryness with dry nitrogen and reconstituted with 15 µl hexane. One µl FAME in hexane was injected on to the capillary gas chromatography column (BPX-70 column, 30 x 0.33mm 0.d., 0.25 film thickness, 5% phenyl methyl silicone) for the separation of fatty acids. The injector port temperature was set at 290 ^0C and the detector set at 250 ^0C. Individual compounds separated by the GC column were introduced sequentially in a constant stream of helium (at flow rate of 2 ml/ min) into combustion furnace containing platinized copper oxide powder (Elemental Microanalysis) maintained at 800 ^0C. In the combustion furnace each compound was converted to CO_2, H_2O, and/or nitrogen oxide gases (N_{ox}). The water was removed during passage of the combustion products through the water trap (hygroscopic ion exchange membrane, Perma Pure Inc., Toms River, N.Y.). The gases produced in combustion chamber were passed through reduction furnace, where N_{ox} was converted to N_2. The reduction of N_2O was important because N_2O can produce mass 44 and mass 45 ions in the ion source which

interfere with carbon isotope ratio measurement on CO_2. Only a small fraction of gas stream (carrying CO_2 and N_2) from reduction furnace was introduced into the ion source of IRMS, while rest of the gas stream was diverted to atmosphere. In the ion source the sample was bombarded with electrons to generate positively charged CO_2 ions. These charged ions were then passed through magnetic field and were deflected according to their charge to mass ratio. The Isotope Ratio-Mass Spectrometer was equipped with triple collector for simultaneous recording of ions of three different masses e.g. m/z = 44, 45, and 46, which represented $^{12}C^{16}O^{16}O$, $^{13}C^{16}O^{16}O$ or $^{12}C^{16}O^{16}O$ and $^{12}C^{18}O^{16}O$ respectively. Data processing and enrichment measurement were determined using the software provided by the manufacturer. Ions at m/z 44, 45 and 46 were continuously recorded until the return of the 44 signal to baseline value. Enrichments of FAME obtained from plasma samples was calculated with reference to the known enrichment of external standard tricosanoic acid methyl ester [δ ^{13}C -32.45 $^{0}/_{00}$ versus Pee Dee Beleminte (PDB)] (Figure 3.2a and 3.2b).

The ^{13}C enrichment of blood samples was expressed as the isotopic ratio delta ($^{0}/_{00}$), as defined in the following equation:

$$^{13}C \text{ enrichment } (^{0}/_{00}) = \frac{^{13}C/^{12}C_{SAMPLE} - ^{13}C/^{12}C_{PDB}}{^{13}C/^{12}C_{PDB}} \times 1000$$

Where PDB (Pee Dee Belemnite) refers to the ^{13}C abundance of an international standard, which is being set at zero. Most natural foods like corn based products etc and biological material contain less ^{13}C than standard PDB therefore there enrichment values are negative.

Since δ units had no physiological value therefore the results were expressed as the concentration (μg) of ^{13}C palmitic acid per ml plasma. The concentrations (μg) of ^{13}C palmitic acid was obtained by reference to peak produced by GC-C-IRMS for internal standards such as C 17:0 TAG and C 21:0 fatty acid, for CRF-TAG palmitic acid and NEFA palmitic acid respectively. The internal standards were dissolved in chloroform-methanol (2:1), and the aliquots containing 60 μg were dispensed in to tubes containing CRF-TAG and NEFA fractions before lipid extraction procedure (Appendix 8.4A). Percentage of enriched palmitic acid was calculated through a established relationship between δ enrichments and % ^{13}C in palmitic acid. The relationship was linear; r = 1.000 with y = 53.80X - 31.57) (Appendix 8.4B). Concentration of enriched

palmitic acid was then calculated from the values of % enriched palmitic acid by using concentrations of total palmitic acid. (Appendix 8.4B shows the equations involved in the calculations of CRF-TAG ^{13}C PA and NEFA ^{13}C PA).

3.4 The natural ^{13}C abundance measurement of the test meal

A test meal was homogenised with 100g of distilled water and six aliquots were freeze-dried to a constant weight. Samples from each aliquot of freeze-dried meal were weighed in to tin capsules for analysis in duplicate for enrichment of ^{13}C by IRMS.

3.5 Indirect calorimetry

Indirect calorimetry (GEM-Indirect calorimeter, Europa Scientific) was used to measure oxygen consumption (VO_2) and CO_2 production (VCO_2), as these can be used to quantify the rate of fat oxidation and carbohydrate oxidation (Frayn, 1983) and estimate metabolic rate (Ferrannini, 1988). The GEM used a ventilated hood placed over the subject's head to provide subject with room air for inspiration at a constant flow rate (40 litres /min) and expired breath in to calorimeter for gaseous analysis. Measurements were made for 20 minute period before the ingestion of test meal and then at hourly intervals for 10 hours after the ingestion of test meal, while subjects were lying in supine position in a room maintained at room temperature. The system was calibrated automatically by software controlled programme every day by using 5% CO_2, 95% O_2 gas mix (BOC) and validated every month for flow rate and respiratory exchange ratio by the burning of pure ethanol.

The rate of net lipid oxidation was estimated by using the equation of Frayn (1983) as follows :

$$\text{Lipid (g/min)} = 1.67\ VO_2 - 1.67\ VCO_2 - 1.92N$$

$$\text{CHO (g/min)} = 4.55\ VCO_2 - 3.21\ VO_2 - 2.87N$$

Where VO_2 and VCO_2 are in litres/min, and N represents grams of urinary nitrogen/min.

The value of N was estimated for the present study as the urinary nitrogen was not obtained. It is possible to use an assumed value of N excretion without inducing a large error for substrate oxidation rates, particularly if the subjects are on controlled diets. Since the rate of N excretion is usually a reflection of protein intake (Wolfe,1992), on most normal diets, normal volunteers are in N balance over the course of day, meaning that N intake equals N excretion. Since our subjects were on controlled diets, and were healthy,

the average daily nitrogen intake over the four day study period was used for repeatability study and average N intake for study day was used for age effects and diabetic studies as a measure of nitrogen excretion.

3.6 Antropometeric measurements

Weight was measured in kilograms to the nearest 100 g on electronic digital portable scales (Soehnle Digital S, CMS weighing equipment Ltd., London, UK) in light clothing without shoes. Height was measured in meters to the nearest 1cm using an electronic digital portable stadiometer (Digi-Rod, CMS weighing equipment Ltd; London UK) without shoes and with the subject standing as straight, arms held loosely by their sides, and heels touching the backboard of the stadiometer. The head was positioned in the Frankfurt plane. The stadiometer was self calibrating. Body mass index [(BMI = weight/height^2 (kg/m^2)] was calculated and body fat mass and lean mass were determined by measuring bioelectrical impedance (Bodystat 1500, Bodystat Ltd., Isle of Man, UK). The subjects were measured fasted while they were lying in supine position with their legs and arms splayed and not touching other parts of the body. Two electrodes were positioned on wrist and two on ankle on the right hand side of the body. A small electric current (500 μA) was passed between the electrodes and the impedance to the flow was measured.

3.7 Data analysis and presentation

Data analysis was carried out using a statistical data analysis package (SPSS for Windows, SPSS Inc., Chicago, IL, USA). Area under the curve was calculated by using graphical package, Fig-P. Results were expressed as Mean ± SEM. Comparisons of mean was done by paired t-test in repeatability study and independent sample t-test for age effect study. Analysis of variance (ANOVA) was used to compare means in diabetic study. Tukey HSD test was used for post-hoc analysis of ANOVA results. Correlation between different variables was established by determining Pearson correlation coefficient. Statistical significance will be assumed at 5% level of significance. The graphical representation of results was illustrated for Means (SEM).

Figure 3.1 Schematic diagram of Continuous Flow Isotope Ratio-Mass Spectrometer (20/20 IRMS with G/S/L interface, Europa Scientific) for breath $^{13}CO_2$ enrichment measurement.

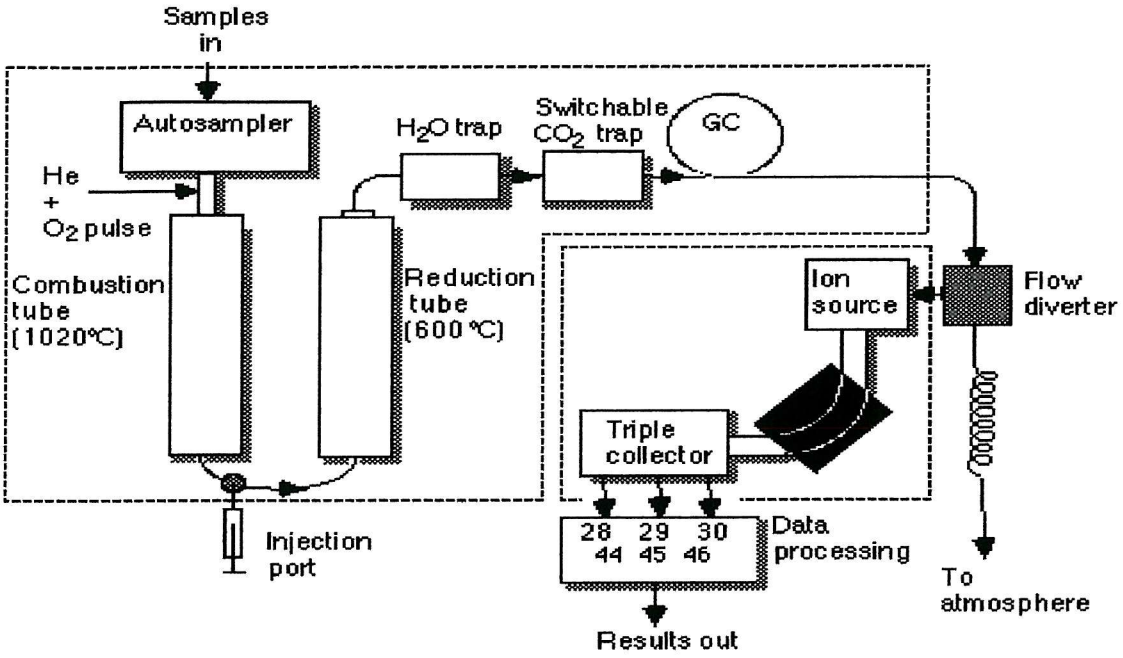


Figure 3.2a Overview of Gaschromatograph Isotope Ratio-Mass Spectrometer (Orchid System, Europa Scientific) for ^{13}C enrichment measurement in fatty acid methyl esters.

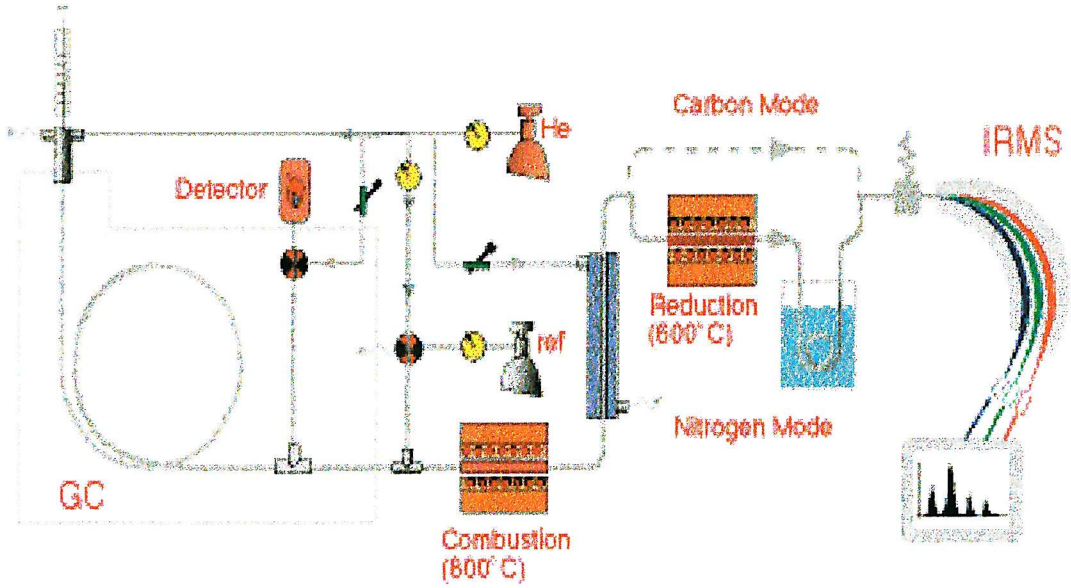
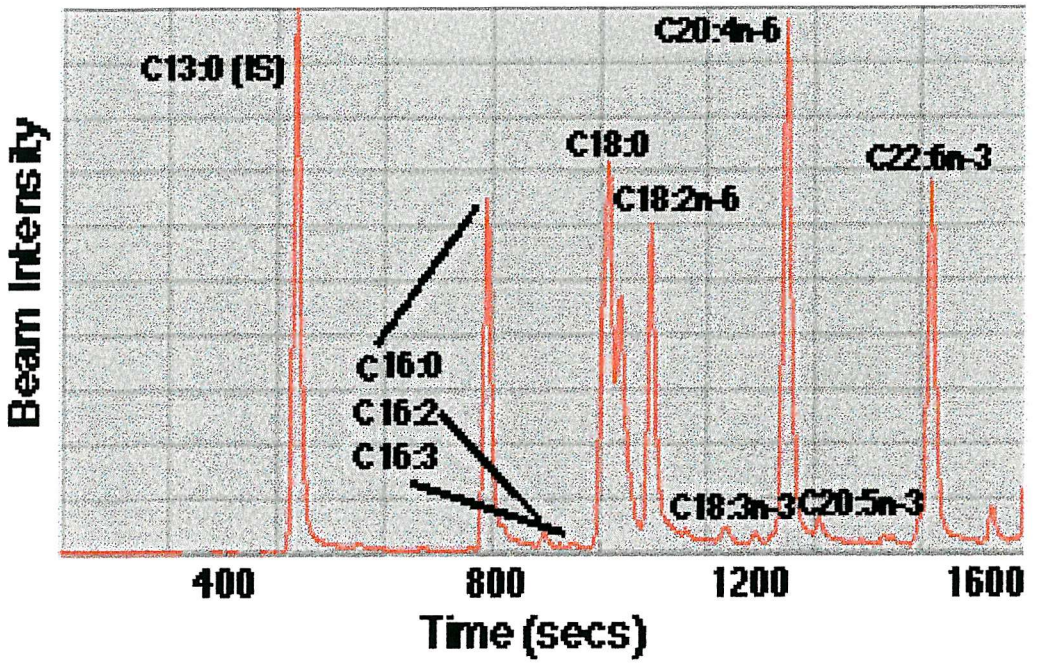


Figure 3.2b Sample of Mass Spectrometer chromatogram by Europa Scientific showing peaks for different fatty acids including palmitic acid (C 16:0).



CHAPTER 4

Validation of Stable Isotope Tracer Methodology Protocol and Repeatability of Postprandial Lipid Metabolism.

4.1 Introduction

High fasting concentrations of plasma triacylglycerol (TAG) is considered to be a risk factor for CVD (Hokanson & Austin, 1996). Since most humans eat 4 to 6 times per day, they are in postprandial state most of their life. The dynamic processes occurring in the postprandial state are particularly important for atherogenesis because the lipid transport system is subjected to physiological challenge at each intake of fatty meal. In this regard, it has been demonstrated that increased plasma levels of postprandial lipids are associated with an increased risk of CVD (Simpson *et al.,* 1990; Groot *et al.,* 1991; Patsch *et al.,* 1992; Karpe *et al.,* 1994b).

The mechanisms responsible for increased plasma levels of postprandial lipids in CVD are not clear partly because of the methodological problems of differentiation between exogenous (test meal) and endogenous fat (liver) in circulation. Retinyl esters and apo B48 are commonly used as a marker of the particles of TGRL of intestinal origin in humans. As discussed in chapter 1 (section 1.1), these markers have been criticised for the delay in its absorption and only serving as a marker of particle of triglyceride-rich lipoproteins (TGRL) of intestinal origin but not of its lipid. The alternate methodology to trace the metabolism of exogenous fat through different pools of the body is stable isotope tracer methodology. Stable isotope methods are now increasingly used to study lipid metabolism because of its safety as compared to radioactive alternates, marked improvements in analytical technologies and better availability of suitable tracers. Since the relative mass difference between ^{12}C and ^{13}C is small and physiochemical characteristics of the two isotopes are almost identical therefore the probability of clinical side effects caused by the administration of ^{13}C is very low. The toxicity of ^{13}C has been

investigated in detail for yeast, algae, plants and mice; however, growth and reproduction remained normal in these organisms even when 50% enrichment of the body pool was maintained for several months.

Gas Chromatography- combustion-Isotope Ration Mass Spectrometry (GC-C-IRMS; Europa Scientific Ltd., Crew, UK) is a relatively new commercial system, which can measure stable isotope enrichments in blood and stool samples. Previously the validity (in terms of precision) of continuous flow-IRMS (CF-IRMS) has been addressed for the measurements of stable isotopic enrichments in breath, stool and urine (20/20 IRMS with G/S/L interface; Jones, 1996). However the validity of GC-C-IRMS system has not yet been addressed for the measurement of stable isotopic enrichment and concentration in blood samples. Since GC-C-IRMS was used through this research, therefore validation of this system was required for providing a level of confidence in the results obtained. Similarly, postprandial lipid metabolism has been studied previously by many researchers but it is not known that whether the results of postprandial lipid metabolism are repeatable if observed for the same subject on two separate occasions under identical conditions. Hence two studies were conducted with a primary objective of addressing the issue of validity of current tracer methodology protocol and repeatability of postprandial lipid metabolism. The following questions were addressed by these studies:

Part A

1. What is the between-sample variability in ^{13}C palmitic acid enrichment and concentration in chylomicron-rich fraction TAG (CRF-TAG)? (To validate the lipid extraction procedures in terms of precision).
2. What is the within-sample variability in ^{13}C palmitic acid enrichment and concentration in CRF-TAG? (To validate the GC-C-IRMS measurements in terms of precision).

Part B

3. To what extent are the primary outcome measures of lipid absorption and postprandial lipid metabolism repeatable?

4.2 Chapter outline

The following chapter describes both the validation and repeatability studies. It includes a) characteristics of subjects including fasting profiles of various variables, b) results of a study describing the precision of GC-C-IRMS measurements of isotopic

enrichments and tracer fatty acid concentrations and precision in lipid extraction procedures c) results of a study describing the repeatability of the responses of postprandial glucose, lipid (including ^{13}C PA responses in CRF-TAG), energy expenditure (EE), net lipid oxidation (net Lox) and breath $^{13}CO2$ excretion, e) discussion and f) summary.

Part A Validation study

4.3 Methodology

4.3.1 Subjects

The study was conducted on a single lean, healthy male subject in the Clinical Nutrition and Metabolic Unit, Southampton General Hospital. The subject was normal lipidaemic with his fasting TAG and cholesterol levels in normal range.

4.3.2 Study Protocol

After an overnight fast, the subject consumed [,1,1,I$^{13}C_3$]tripalmitin (10mg/kg body weight) as an emulsion and part of test meal (3MJ energy, with 36.9%, 51.8%, and 11.3% energy from fat, carbohydrate and protein respectively). Venous blood samples were collected before the ingestion of the labelled fat and at 2 hours following the label administration.

The between-sample variability of CRF-TAG ^{13}C PA concentration was determined by the processing of 9 independent blood samples obtained 2 hours postprandially. The within-sample variability of CRF-TAG ^{13}C PA concentration was determined by injecting nine separate aliquots of the same CRF-TAG sample (as extracted @ 2 hours postprandially) into GC-C-IRMS.

4.4 RESULTS

The between-sample variability in δ enrichment ($^0/_{00}$) and CRF-TAG ^{13}C PA concentration (μg/ml plasma) in nine independent aliquots of CRF are shown in Table 4.1. The mean (SD) and coefficient of variance for between-sample variability in ^{13}C PA δ enrichment were 574 (7.1) ($^0/_{00}$) and 1.24 % respectively. The mean (SD) and coefficient of variance for between-sample variability in CRF-TAG ^{13}C PA concentration were 3.71 (0.30) (μg/ml plasma) and 8.09 % respectively. The variability in δ enrichment expressed as $^0/_{00}$ was smaller than the variability in the mass of labelled fatty acid (1.2 *v* 8 %). These results shows that δ enrichments were high, and variability in δ enrichments and palmitic acid concentrations were low in case of the between-sample variability.

The within-sample variability in δ enrichment ($^0/_{00}$) and CRF-TAG ^{13}C PA concentration (ug/ml plasma) in nine separate GC-C-IRMS injections from the same aliquot of CRF are shown in Table 4.2. The mean (SD) and coefficient of variance for within-sample variability in ^{13}C PA δ enrichment were 580 (5.5) ($^0/_{00}$) and 0.96 % respectively. The mean (SD) and coefficient of variance for within-sample variability in CRF-TAG ^{13}C PA concentration were 4.08 (0.15) (μg/ml plasma) and 3.68 % respectively. The variability in δ enrichment expressed as $^0/_{00}$ was smaller than the variability in the mass of labelled fatty acid (0.96 *v* 3.68 %). Again these results demonstrates that there was less variability in δ enrichments and palmitic acid concentrations in CRF-TAG.

Table 4.1 The between-sample variability in δ enrichment and CRF-TAG ^{13}C PA concentration in nine independent aliquots of CRF obtained from a single subject two hours after consuming the test meal

Sample #	CRF-TAG ^{13}C PA Enrichment ($^{0}/_{00}$)	CRF-TAG ^{13}C PA Concentration (μg/ml plasma)
1	568.84	3.66
2	566.85	3.89
3	572.53	3.99
4	570.98	3.69
5	573.82	3.22
6	582.87	3.66
7	584.25	3.97
8	570.16	3.22
9	584.28	3.74
Mean (SD)	574.04 (7.1)	3.71 (0.30)
Coefficient of variance (%)	1.24	8.09

Table 4.2. Within-sample variability in δ enrichment and CRF-TAG ^{13}C PA concentration in nine separate GC-C-IRMS injections from the same aliquot of CRF obtained from a single subject two hours after consuming the test meal

Sample #	CRF-TAG ^{13}C PA Enrichment ($^{0}/_{00}$)	CRF-TAG ^{13}C PA Concentration (μg/ml plasma)
1	577.87	4.12
2	590.70	4.01
3	583.00	4.07
4	586.37	4.00
5	577.83	4.09
6	574.64	4.09
7	574.20	4.30
8	578.81	4.19
9	576.92	3.74
Mean (SD)	579.98 (5.58)	4.08 (0.15)
Coefficient of variance (%)	0.96	3.68

4.5 Discussion

The present study describes for the first time the validity of GC-C-IRMS system for the precision of measurements of stable isotopic enrichments and concentrations in CRF-TAG fraction.

4.5.1 Between and within-sample variability in ^{13}C enrichments in CRF-TAG palmitic acid

High levels of ^{13}C enrichments in palmitic acid suggests that the dose of tracer fatty acid given in the test meal (10mg/kg body weight) was sufficient to achieve high levels of enrichments. Low between and within-sample variability (with coefficient of variances of 0.96% and 1.24% respectively) confirms the precision of lipid extraction procedures and the ability of GC-C-IRMS to repeatedly measure ^{13}C enrichments with considerable confidence. In the current study, the between and within-sample variability could be attributed to high ^{13}C enrichment levels in palmitic acid. Previously study by Jones (1996) had also observed that variability in ^{13}C enrichment values of enriched stool samples was larger than the ^{13}C abundance measurements of baseline stool samples. The between-sample variability in enrichments was higher than within-sample variability enrichments. The higher between-sample ^{13}C enrichment variability was simply because of natural variability in 9 independent CRF-TAG samples injected once in to GC-C-IRMS as opposed to a single CRF-TAG sample injected 9 times into GC-C-IRMS. Moreover higher between-sample enrichment variability can also be partly attributed to the variation in GC-C-IRMS tuning between two different days as the samples in that case were not analysed on the same day. Subsequent to this study, a new version of GC-C-IRMS analysis protocol was developed which analysed the samples in single batches.

4.5.2 Between and within-sample variability in CRF-TAG ^{13}C PA concentration

Similarly, low between and within-sample variability (with coefficient of variances of 3.68% and 8.09% respectively) confirms the precision of lipid extraction procedures and the ability of GC-C-IRMS to repeatedly measure ^{13}C concentration with confidence. Some of these observed variability in ^{13}C PA concentrations could be attributed to post processing of the sample e.g. the peak areas were shaded manually and the postprocessor was not performing appropriate baseline corrections.

Both of these factors can contribute to the variability in the area calculated for the palmitic acid. The variability in the area calculated for the mass of palmitic acid is an important factor affecting the variability in ^{13}C palmitic acid concentrations. Subsequent to this study a new improved version of postprocessor was introduced to minimise the variability in area calculated for the mass of palmitic acid. Higher between-sample variability in ^{13}C PA concentrations were expected as compared to within-sample variability due the greater number of samples involved and different days of processing as discussed in previous paragraph.

4.6 Summary

The results of the validation study illustrates that with precision in sample extraction procedures and sample derivatization, the GC-C-IRMS system can measure ^{13}C PA enrichments and concentrations (in chylomicron-rich fraction TAG) with low between-sample and within-sample variability. Based on the results of this study a new standardised protocol for tracer studies was developed and used in the subsequent studies of this thesis.

Part B Repeatability study

4.7 Methodology

4.7.1 Subjects

For this study 6 healthy male subjects were recruited from Southampton university population. Informed consent was obtained from all the subjects and the study protocol was approved by the Ethical Committee of Southampton and South West Hampshire Health Commission.

The characteristics of the subjects and their fasting profiles in repeatability study are described in Table 4.3. The fasting profiles of the subjects in both the trials were similar.

Trial 2 showed a trend of higher fasting TAG, NEFA and CHO oxidation than trial 1, however, the differences were highly non significant. On the other hand trial 1 showed a trend of higher basal energy expenditure and net lipid oxidation than trail 2, however, the differences were highly non significant.

4.7.2 Study protocol

The subjects participated in two identical trials (separated approx. 4-6 weeks apart). The duration of each trial was nine days. Subjects followed the general protocol as described in chapter 3 (section 3.2). Briefly, three days prior to study, subjects consumed a standard control diet and abstained from alcohol consumption and refrained from volitional exercise. On the evening before the study day, subjects were admitted in Clinical Nutrition and Metabolism unit and finished their standard evening meal by 1900 hours and then fasted there after except for drinking water.

Next morning around 0700 hours, fasting blood and breath samples were collected and baseline basal metabolic rate was measured for 20 minutes. At 0800 hours the subjects consumed a ^{13}C labelled TAG (tripalmitin) in an emulsion and a standard test meal. At 1400 hours another unlabelled emulsion and test meal were given. Blood and breath samples were collected before the ingestion of ^{13}C label and at hourly intervals until ten hours after the ingestion of test meal. Whole body CO_2 excretion was determined by indirect calorimetry before the ingestion of label and then at hourly intervals for ten hours after the ingestion of test meal. During the study, no additional food or liquids were allowed except for bottled mineral water.

A baseline stool sample was collected on the day before the label administration (i-e on day three of the trial) in order to measure baseline ^{13}C excretion. Thereafter, all stools passed were collected for a period of 5 days. Stools were collected separately into polyethylene bags and were frozen immediately.

4.8 Results

4.8.1 Stool ^{13}C excretion

The mean AUC for stool ^{13}C excretion (% administered dose) in trials 1 and 2 are shown in Table 4.4. Total ^{13}C excretion in stool was very low in both trials. There was a trend towards higher AUC for ^{13}C excretion in stools in trial 2 than trial 1 but the differences did not attain statistical significance ($p>0.81$). The difference between trials (T1-T2) was -0.10 (-0.22) % administered dose.

4.8.2 Blood variables

4.8.2.1 Postprandial plasma glucose responses

The time courses of changes in plasma glucose concentrations after the first and second meal were similar in trials 1 and 2 (Figure 4.1). In trial 2, postprandial glucose concentrations were significantly higher after the first meal ($p<0.05$). In both the trials, there was a modest glycaemic response after the first meal. After the second meal, a peak was observed at 1 hour in trial 2 and at 2 hour in trial 1. After the peaks the decline in glucose concentrations was similar in both the trials. At 4 hours after second meal, glucose concentrations were still higher than fasting levels in both the trials.

The mean AUC for glucose responses over the 10-hour postprandial period in trials 1 and 2 are shown in Table 4.4. Postprandial AUC for plasma glucose concentration was significantly higher in trial 2 than trial 1 ($p<0.04$). The incremental increase in glucose AUC was also higher in trial 2 than trial 1, however, the differences did not reach the significance level ($p=0.07$). The difference between trials (T1-T2) was -5.0 (0.30) mmol/l for glucose AUC and -3.6 (-0.20) mmol/l for incremental increase in glucose AUC.

4.8.2.2 Postprandial plasma NEFA responses

The time courses of changes in plasma NEFA concentrations following the first and second meals were similar in trials 1 and 2 (Figure 4.2). After the first meal, both the trials showed a sharp decline in NEFA concentrations with the nadir reached at 1 hour. After the nadir, both the trials showed a gradual increase in NEFA concentrations to return near to fasting levels at 6 hours in both the trials. After the second meal, suppression of NEFA was slower in both the trials with the nadir

reached 3 hour later in both the trials. Trial 2 showed decreased suppression of NEFA than trial 1 after the second meal.

The mean AUC for NEFA responses over the 10-hour postprandial period were similar in trials 1 and 2 (Table 4.4). The incremental increase in NEFA AUC was also similar for both the trials (Table 4.4). The difference between trials (T1-T2) was -0.10 (0.0) mmol/l for NEFA AUC and -3.0 (-0.40) mmol/l for the incremental increase in NEFA AUC.

4.8.2.3 Postprandial plasma TAG responses

The time courses of changes in plasma TAG concentrations following the first and second meal in trials 1 and 2 are shown in Figure 4.3. After the first meal, the peak concentration was reached earlier in trial 1 (2h) than trial 2 (3h). Before attaining the peak concentration, trial 1 showed a trend towards higher TAG concentrations than trial 2. After the peaks, the decline in plasma TAG concentrations was slower but similar in both the trials. At 6 hours after the first meal, the plasma TAG concentrations returned to near fasting concentrations in both trials. After the second meal, the peak was reached earlier in trial 1 (1h) than trial 2 (2h). After the peak, trial 1 showed a trend of lower TAG concentrations than trial 2. In both trials, after the second meal, the TAG response was smaller than that observed after the first meal. In both the trials plasma TAG concentrations remained higher than fasting levels at 4 hours after the second meal.

The mean AUC for TAG responses in trials 1 and 2 over the 10 hour postprandial period are shown in Table 4.4. Postprandial AUC for plasma TAG were similar in both trials. The incremental increase in TAG AUC was higher in trial 1 than trial 2 but the differences did not attain statistical significance. The difference between trials (T1-T2) was -0.10 (0.60) mmol/l for TAG AUC and 0.40 (0.0) mmol/l for incremental increase in TAG AUC.

4.8.2.4 Postprandial CRF-TAG ^{13}C PA responses

The time courses of postprandial CRF-TAG ^{13}C PA responses following the first and second meal in trials 1 and 2 are shown in Figure 4.4. In both trials, ^{13}C PA appeared in CRF-TAG within the first hour but the peak was reached earlier in trial 2 (2h) than trial 1(3h). At peak, the concentration of ^{13}C PA was similar in both the trials. Thereafter the pattern of ^{13}C PA concentration was similar in both trials. At 6

hours after the first meal, ^{13}C PA concentrations were still higher than fasting levels in both the trials. After the second unlabelled meal, the peak ^{13}C PA concentration occurred at 1 hour in both trials. The concentration of ^{13}C PA at peak was higher in trial 2 than trial 1. After the second peak, ^{13}C PA started to decline in both trials but the concentrations remained higher than fasting levels at 4 hours after the second meal.

The mean AUC for CRF-TAG ^{13}C PA responses in trials 1 and 2 over the 10 hour postprandial period are shown in Table 4.4. Postprandial AUC for CRF-TAG ^{13}C PA concentration was similar in both the trials. The difference between trials (T1-T2) was -0.20 (0.10) μg/ml plasma.

4.8.2.5 Effect of second meal on ^{13}C palmitic acid appearance as ingested in the previous meal

After the second meal (served 6 hours after the first meal), the peak mean ^{13}C palmitic acid concentration in CRF-TAG increased by 39% from 0.77 μg/ml plasma to 1.14 μg/ml plasma. The peak occurred at 1 hour after the administration of second meal. After the peak the concentration of ^{13}C palmitic acid remained lower than the concentrations observed immediately before the administration of second test meal.

4.8.3 Energy expenditure and substrate oxidation

4.8.3.1 Energy expenditure

The time courses of changes in energy expenditure/kg lean mass following the first and second meal in trials 1 and 2 are shown in Figure 4.5. The time courses for EE were similar for both trials over the postprandial period. In trial 1, peak EE has reached 2 hours and 1 hour following the first and second meals respectively. In trial 2, peak EE were reached at 1 and 2 hour following the first and second meals respectively. In both the trials, energy expenditure values declined gradually after the peak and returned near to baseline levels at 6 hours after first meal but remained higher than baseline values at 4 hours after second meal.

The mean AUC for energy expenditure in trials 1 and 2 over the 10 hour postprandial period are shown in Table 4.5. Both the trials showed similar AUC for energy expenditure. The incremental increase in EE AUC (the thermic effect of food, TEF) was higher in trial 2 than trial 1, however, the differences did not attain the

statistical significance. The difference between trials (T1-T2) was -0.20 (0.10) kcal/kg lean mass for energy expenditure AUC and -0.51 (0.0) kcal/kg lean mass incremental increase in EE.

4.8.3.2 CHO oxidation

The time courses of changes in CHO oxidation/kg lean mass following the first and second meal in trials 1 and 2 are shown in Figure 4.6. In both the trials, there was a sharp increase in postprandial CHO oxidation with peak in CHO oxidation reached at 1 hour in trial 2 and at 2 hours in trial 1. The peak CHO oxidation was similar in both trials. After the peaks, CHO oxidation started to decline gradually until 6 hours where the values were significantly lower than baseline levels in both trials ($p<0.05$). After the second meal, postprandial CHO oxidation increased sharply in both the trials with the peak at 1 hour in trial 1 and at 2 hours in trial 2. At 4 hours after the second meal, CHO oxidation was higher than baseline values in both trials.

The mean AUC for CHO oxidation in trials 1 and 2 over the 10 hour postprandial period are shown in Table 4.5. Postprandial AUC for CHO oxidation AUC was higher in trial 1 than trial 2, however, the differences were not significant ($p=0.25$). The postprandial AUC for incremental increase in CHO oxidation AUC was higher in trial 2 than trial 1, however, the differences were not significant ($p=0.22$). The difference between trials (T1-T2) was 0.06 (0.0) kcal/kg lean mass for CHO oxidation AUC and -0.15 (0.08) kcal/kg lean mass for incremental increase in CHO oxidation AUC.

4.8.3.3 Net lipid oxidation

The time courses of changes in net lipid oxidation/kg lean mass following the first and second meal in trials 1 and 2 are shown in Figure 4.7. There was a trend towards higher postprandial net lipid oxidation (net Lox) in trial 2 than trial 1, however, the differences over the postprandial time course were not statistically significant ($p>0.15$). In trial 1, there was an initial decline in postprandial net Lox and the values went down below baseline levels at 2-hour time point. Thereafter, a sharp increase in net Lox was observed and the peak was reached at 4 hours after first meal. Whereas in trial 2, there was a step-wise increase in postprandial net Lox and the peak was reached at 5 hours after the first meal. After the peaks, both the trials followed a decline in net Lox but the trial 2 values remained higher than baseline levels at 4

hours after second meal. Second meal did not result in increase in net Lox in both the trials.

The mean AUC for net Lox in trials 1 and 2 over the 10 hour postprandial period are shown in Table 4.5. Postprandial AUC for net Lox tended to be higher in trial 2 than trial 1, however, the differences were not statistically significant (p=0.25). The incremental increase net Lox over the baseline levels was significantly higher in trial 2 than trial 1 (p<0.04). The difference between trials (T1-T2) was -0.05 (0.00) kcal/kg lean mass for net Lox oxidation and -0.11 (0.00) kcal/kg lean mass for incremental increase in net lipid oxidation AUC.

4.8.3.4 Breath $^{13}CO2$ recovery

The time courses of breath $^{13}CO_2$ recovery/kg lean mass following the first and second meal in trials 1 and 2 are shown in Figure 4.8. In both the trials ^{13}C label appeared in the breath within the first hour of the label administration. ^{13}C label in the breath then continued to increase and the peak was reached at 4 hours in trial 2 and at 5 hours in trial 1. After the peaks, ^{13}C label in breath started to decline gradually in both the trials but remained higher than baseline levels at 4 hours after second meal. Trial 1 showed a trend towards higher breath $^{13}CO_2$ recovery in the later half of postprandial period.

The mean AUC for breath $^{13}CO_2$ appearance in trials 1 and 2 over the 10 hour postprandial period are shown in Table 4.5. Postprandial AUC for breath $^{13}CO_2$ appearance was similar in both the trials. The difference between trials (T1-T2) was -0.01 (-0.01) % administered dose/kg lean mass.

4.8.3.5 Endogenous and exogenous lipid oxidation

Total exogenous lipid oxidation was calculated in relation to percentage of ^{13}C oxidised over the 10 hour period. Total endogenous lipid oxidation was calculated by subtracting exogenous lipid oxidation from net Lox. The mean AUC for endogenous lipid oxidation/kg lean mass trials 1 and 2 over the 10 hour postprandial period are shown in Table 4.5. Postprandial AUC for endogenous lipid oxidation tended to be higher in trial 2 than trial 1 but the differences were not significant (p=0.07). The difference between trials (T1-T2) was -0.05 (0.01) kcal/kg lean mass.

The mean AUC for exogenous Lox/kg lean mass in trials 1 and 2 over the 10 hour postprandial period are shown in Table 4.5. Postprandial AUC for exogenous

Lox was similar in both the trials. The difference between trials (T1-T2) was 0.01 (0.0) kcal/kg lean mass.

Table 4.3 Physical characteristics and fasting parameters of subjects.

Characteristics	Young men Trial 1 (n=6) Mean (SE)	Young men Trial 2 (n=6) Mean (SE)
Age (yrs)	25.6 (1.8)	25.6 (1.8)
Weight (kg)	74.2 (3.9)	73.4 (3.9)
Height (m)	1.8 (0.0)	1.8 (0.0)
BMI (kg/m^2)	23.2 (1.3)	23.5 (1.3)
Fat mass (kg)	10.1 (3.0)	NM
Lean mass (kg)	63.9 (2.5)	NM
Fasting glucose (mmol/l)	5.5 (0.1)	5.6 (0.1)
Fasting TG (mmol/l)	0.56 (0.1)	0.61 (0.09)
Fasting NEFA (mmol/l)	0.36 (0.02)	0.40 (0.06)
Basal EE (kcal/kg lean mass)	1.21 (0.03)	1.18 (0.02)
Basal net Lox (kcal/kg lean mass)	0.039 (0.007)	0.032 (0.007)
Basal CHO ox (kcal/kg lean mass)	0.151 (0.015)	0.160 (0.016)

NM, not measured; EE, energy expenditure; CHO, carbohydrate; Ox, oxidation.

Table 4.4 Mean AUC for stool and various blood parameters over the 10-hour postprandial period.

Group	AUC stool ^{13}C PA (% AD)	AUC Glucose (mmol/l.10h) Mean (SE)	AUC Δ Glucose (mmol/l.10h) Mean (SE)	AUC NEFA (mmol/l.10h) Mean(SE)	AUC Δ NEFA (mmol/l.10h) Mean(SE)	AUC TAG (mmol/l.10h) Mean (SE)	AUC ΔTAG (mmol/l.10h) Mean (SE)	AUC ^{13}CPA In CRF-TAG (ug/ml.10h) Mean (SE)
Young men T1	1.0 (0.15)	58 (2.0)	3.8 (1.2)	2.2 (0.1)	-1.4 (0.3)	8.7 (1.9)	3.1 (0.7)	18.3 (3.8)
Young men T2	1.1 (0.37)	63 (1.7)	7.4 (1.4)	2.3(0.1)	-1.7 (0.7)	8.8 (1.3)	2.7 (0.7)	18.5 (3.7)
Difference (T1-T2)	-0.10 (-0.22)	-5.0 (0.30) *	-3.6 (-0.20)	-0.10 (0.0)	-0.30 (-0.40)	-0.10 (0.60)	0.40 (0.0)	-0.20 (0.10)

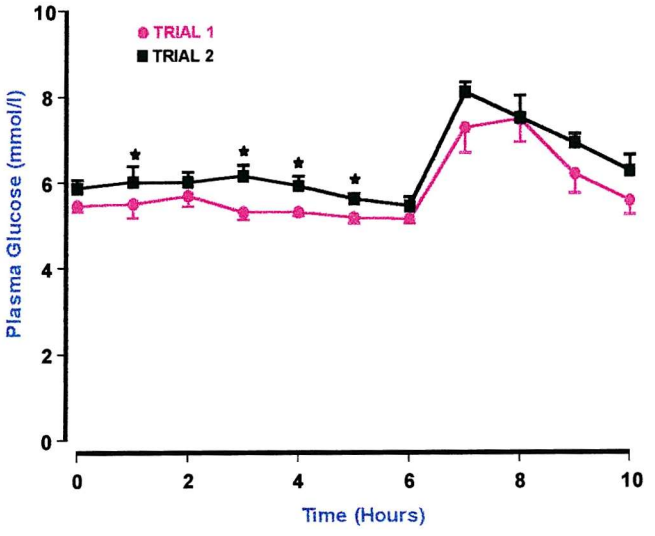
AUC, area under the curve; NEFA, non-esterified fatty acids; TAG, triacylglycerol; PA, palmitic acid; CRF, chylomicron-rich fraction; Δ, increment increase above the base line; *, means are significantly different between trials ($p<0.05$)

Table 4.5 Mean AUC for EE and substrate oxidation over the 10-hour postprandial period.

Group	AUC EE Kcal/kg LM.10h Mean (SE)	AUC Δ EE Kcal/kg LM.10h Mean (SE)	AUC CHO ox gm/kg LM.10h Mean (SE)	AUC Δ CHO ox gm/kg LM.10h Mean (SE)	AUC net Lox gm/kg LM.10h Mean (SE)	AUC Δ net Lox gm/kg LM.10h Mean (SE)	AUC Breath $^{13}CO_2$ % AD/kg LM.10h Mean (SE)	AUC Endo Lox gm/kg LM.10h Mean (SE)	AUC Exo Lox gm/kg LM.10h Mean (SE)
Young men T1	13.43 (0.3)	1.32 (0.3)	2.06 (0.1)	0.40 (0.18)	0.43 (0.05)	0.05 (0.06)	0.33 (0.02)	0.20 (0.05)	0.24 (0.02)
Young men T2	13.63 (0.2)	1.83 (0.3)	2.00 (0.1)	0.55 (0.10)	0.48 (0.05)	0.16 (0.06)	0.32 (0.03)	0.25 (0.04)	0.23 (0.02)
Difference (T1-T2)	-0.02 (0.1)	-0.51 (0.0)	0.06 (0.0)	-0.15 (0.08)	-0.05 (0.00)	-0.11 (0.00) *	0.01 (-0.01)	-0.05 (0.01)	0.01 (0.00)

AUC, area under the curve; EE, energy expenditure; LM, lean mass; Δ, increment above the base line; CHO, carbohydrate; ox, oxidation; Lox, lipid oxidation; AD, administered dose; Endo, endogenous; Exo, exogenous; *, means are significantly different between trials ($p<0.05$)

Figure 4.1 Time courses of changes in plasma glucose concentrations (mmol/l) after the first and second meal in trials 1 and 2.



*, means are significantly different between trials at these time points ($p<0.05$).

Figure 4.2 Time courses of changes in plasma NEFA concentrations (mmol/l) after the first and second meal in trials 1 and 2.

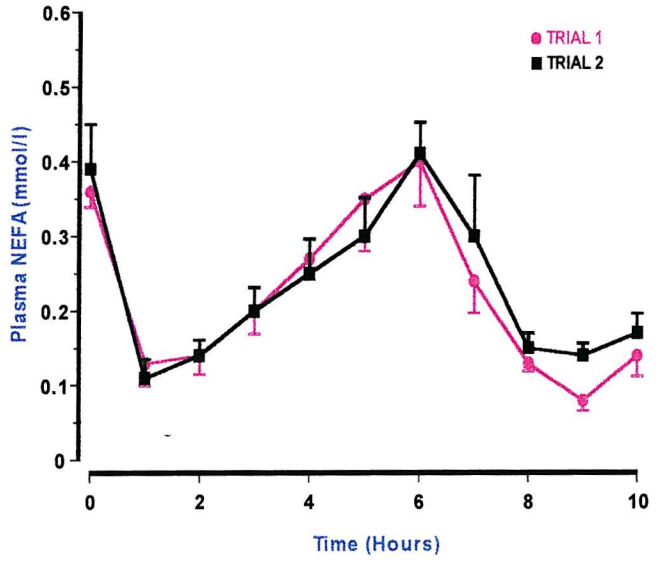


Figure 4.3 Time courses of changes in plasma TAG concentrations (mmol/l) after the first and second meal in trials 1 and 2.

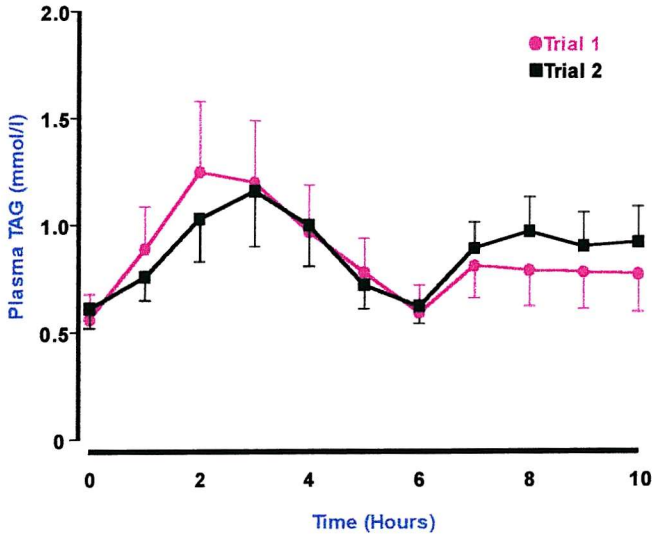


Figure 4.4 Time courses of changes in CRF-TAG ^{13}C PA concentrations (μg/ml) after the first and second meal in trials 1 and 2.

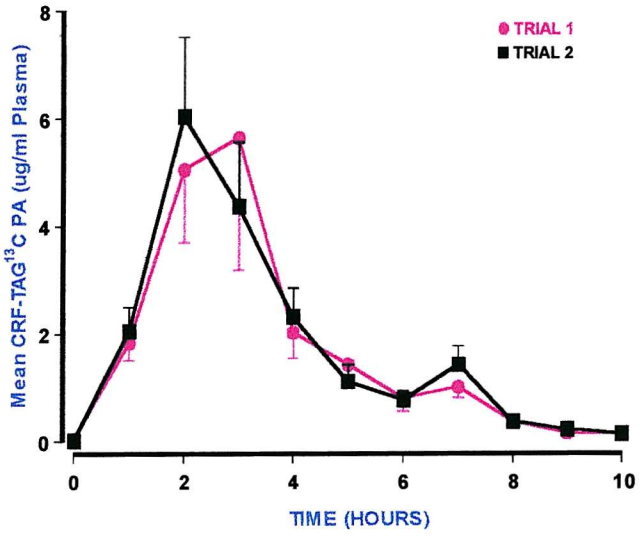


Figure 4.5 Time courses of changes in energy expenditure (kcal/kg lean mass) after the first and second meal trials 1 and 2.

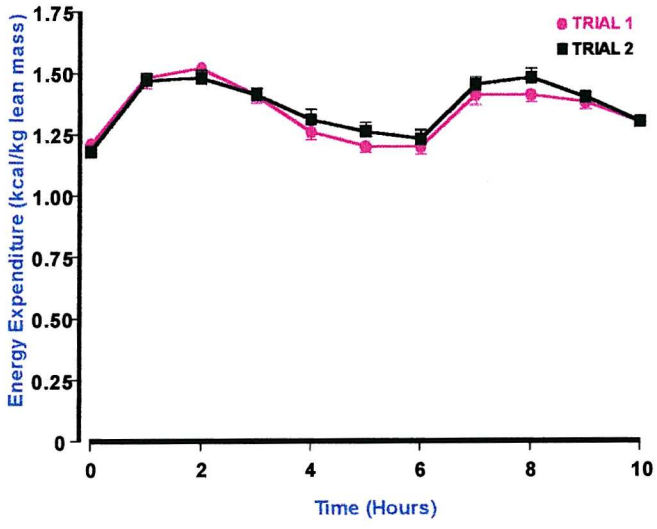


Figure 4.6 Time courses of changes in carbohydrate oxidation (gm/kg lean mass) after the first and second meal trials 1 and 2.

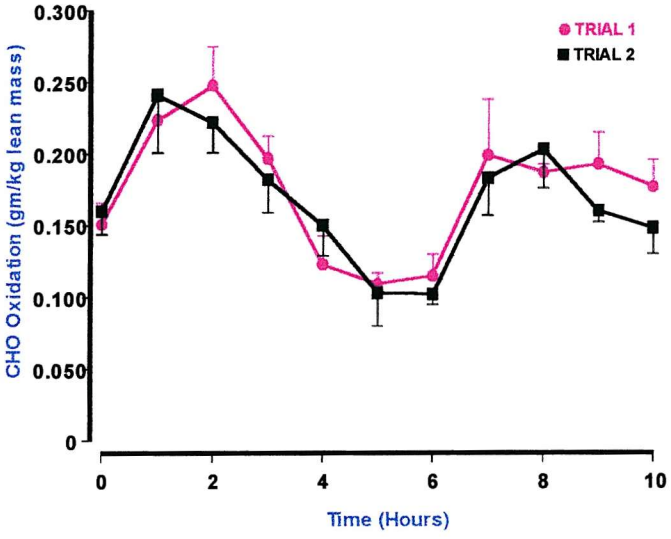


Figure 4.7 Time courses of changes in net lipid oxidation (gm/kg lean mass) after the first and second meal inn trials 1 and 2.

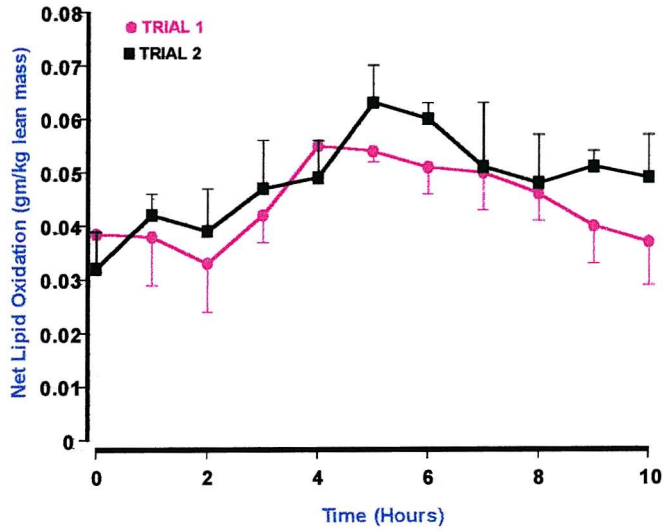


Figure 4.8 Time courses of changes in breath $^{13}CO2$ excretion (% administered dose) after the first and second meal in trials 1 and 2.

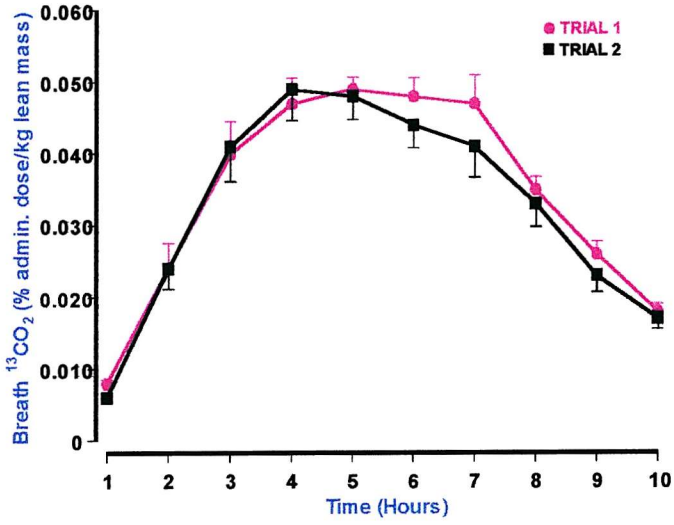
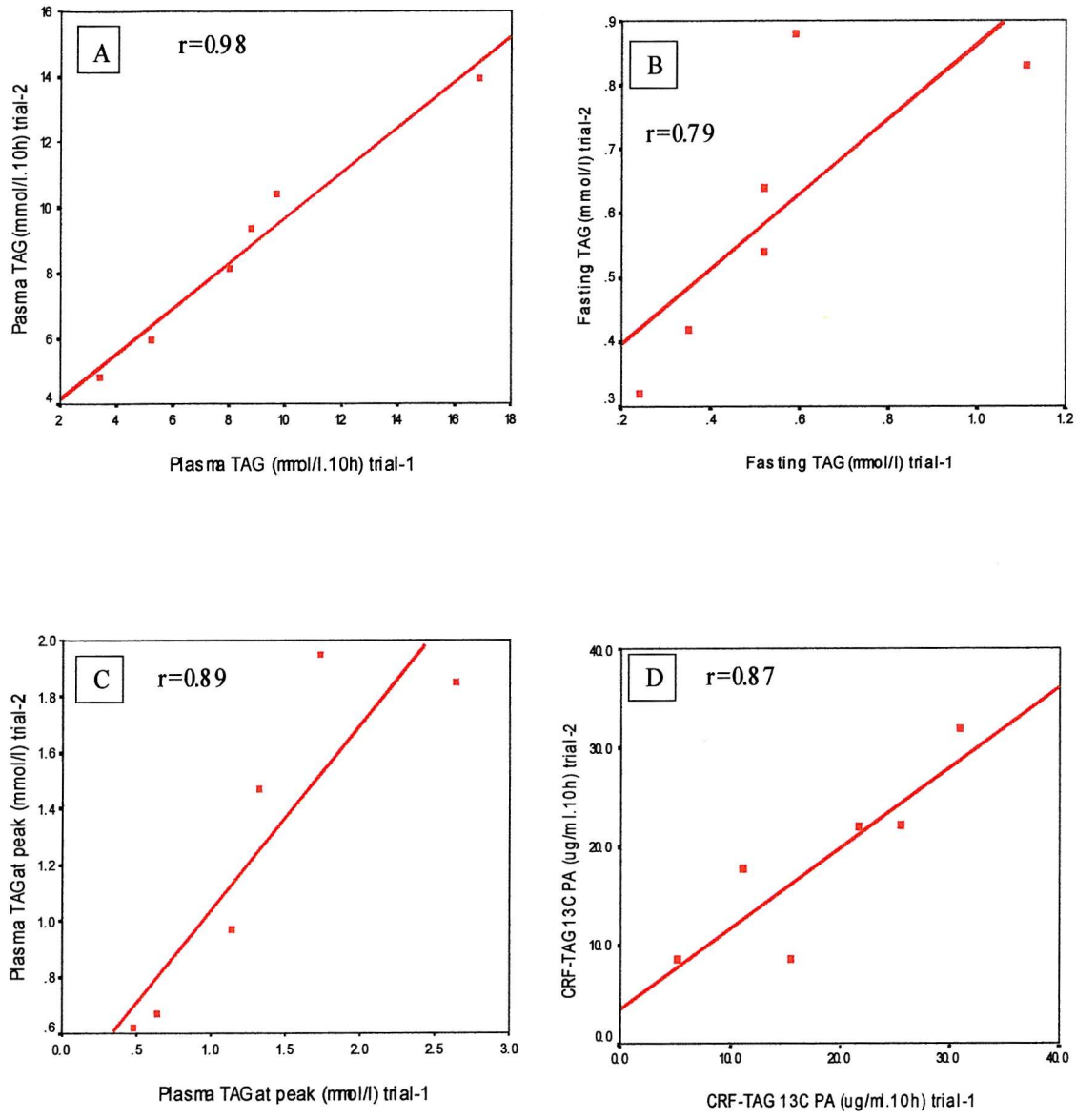


Figure. 4.9 Scatter plots for plasma TAG and CRF-TAG ^{13}C PA in trial I and trial 2 for healthy young men



4.9 Discussion

The present study describes for the first time the repeatability of postprandial lipid metabolism by tracing the metabolism of exogenous lipid [(1,1,1 $^{13}C_3$) tripalmitin] through different pools of the body following the intake of two standard test meals (in sequence) while controlling for evening meal and physical activity. The fat content of the test meals represented typical amounts of fat as used in normal daily life. In this study, particular attention was given to the (a) postprandial lipid metabolism in circulation and (b) postprandial energy expenditure and substrate oxidation.

The major finding of the present study was that the stool ^{13}C excretion and postprandial lipid metabolism were highly repeatable. However, postprandial glucose and incremental increase in net Lox responses were not very repeatable.

4.9.1 Stool ^{13}C excretion.

The results of the present study also demonstrated that the absorption of ^{13}C palmitic acid was almost complete (99%), as only 1% of the administered label was recovered in the stools.

The results of the present study suggests that ^{13}C excretion in stools was repeatable as the differences observed between two identical trials were small and did not attain statistical significance ($p>0.80$). These results are not comparable to a previous study (Jones, 1996), which observed large differences (although statistically insignificant) in total ^{13}C excretion in stools between two identical trials (10.7 *v* 25.25,% administered dose). Furthermore the total ^{13}C excretion (% admin. dose) in stool was very large in the study reported by Jones (1996) as compared to the present study. The differences could be attributed to the differences in methodologies between the two studies, such as gender (men *v* women), type of substrate (1,1,1-^{13}C tripalmitin *v* 1-^{13}C palmitic acid) and the way of its presentation (incorporation in to emulsion *v* incorporation in to butter). Presentation of tracer as a fatty acid rather than TAG and mixing it with butter rather than incorporating it into emulsion might have resulted in decreased absorption in Jones (1996) study.

4.9.2 Postprandial responses of plasma TAG, NEFA, ^{13}C palmitic acid in CRF-TAG and glucose.

The postprandial responses of plasma TAG and NEFA and ^{13}C palmitic acid in CRF-TAG were repeatable as the differences in the AUC for these blood lipid parameters were highly non significant. Furthermore, the strong correlation observed for TAG AUC

(r=0.98, Figure 4.9A), fasting TAG (r=0.79, Figure 4.9B), peak plasma TAG concentrations (r=0.89, Figure 4.9C) and CRF-TAG ^{13}C PA (Figure 4.9D) between two trials showed that the postprandial responses for these variables were highly repeatable. There appears to have been only one another published study which has determined the repeatability of postprandial plasma TAG responses (Brown *et al.,* 1992). The results of that study also showed that the postprandial plasma TAG responses are repeatable. However the correlation of the plasma triglyceride between repeat visits at fasting and 3.5 hours were less strong compared to that observed in the present study ($r = 0.67$ *v* $r = 0.79$ for fasting TAG level and $r = 0.69$ *v* $r = 0.89$ for 3.5 hour/peak TAG response). The less correlation as observed for fasting and 3.5 hours plasma TAG in the previous study might be due to lack of control of preceding diet and level of physical activity in that study. Previous studies have shown that both preceding diet and level of physical activity affects the postprandial lipid metabolism.

In contrast, higher fasting and postprandial glucose concentrations in trial 2 than trial 1 suggest that fasting and postprandial glucose concentrations are not very repeatable. The issue of repeatability of postprandial glucose metabolism does not appear to have been addressed previously. However, in Dutch adults (Mooy *et al.,* 1996) intra-individual variation of glucose concentrations was determined. An oral glucose tolerance test was repeated during a 2-6 week interval and the intra-individual variation for two hour plasma glucose was found to be 16.7%. This suggest that postprandial glucose metabolism is subject to considerable intra-individual variability.

The repeatability of postprandial lipid responses after a test meal (while controlling for preceding diet and level of physical activity) as observed in the present study suggests that the metabolic and physiological handling of dietary lipids is determined by individual's own defined capability (metabolic competence). Furthermore it appears that individual's defined capability of handling dietary lipids is partly modified by preceding diet and level of physical activity as the postprandial lipid responses observed in another study were not very repeatable when the preceding diet and level of physical activity were not controlled. The practical implication of the repeatability of lipid responses as observed in the present study is that reliable postprandial lipaemia tests can be conducted in clinical settings after the intake of standard test meal (while controlling for the preceding diet and physical activity) in order to identify individuals with increased risk for CVD.

Serum triglyceride is considered as a potential risk factor for CVD. However, there is striking evidence for intra-individual variability of fasting triglyceride levels, even if the standardisation of blood sampling procedures (overnight fasting) and laboratory techniques are used (Jacobs & Barret-Connor, 1982; Namboodiri *et al.,* 1984; Brenner & Heiss, 1990). Therefore it has been suggested that fasting TAG levels should be regarded as extremely "rough" diagnostic tool and interpreted with caution (Brenner and Heiss, 1990). On the other hand, the results of the present study indicated that the fasting plasma TAG levels were very repeatable when the preceding diet and level of physical activity were controlled.

4.9.3 Effect of second unlabelled meal on the appearance of label in CRF-TAG as ingested in the previous meal.

Previous studies have observed the presence of an early postprandial peak in plasma TAG concentration, when successive meals have been consumed (Fielding *et al.,* 1996; Peel *et al.,* 1993). Fielding *et al.,* (1996) used test meals of different fatty acid composition to distinguish between lipid in first meal and second meal. The results of that study showed that the early postprandial peak (1 hour after the second meal) was found to contain large proportion of fatty acids from the first meal, whereas at later time points the fatty acids in chylomicron-TAG resembled more closely to that in the second meal. These authors suggested that the second meal resulted in the release of preformed chylomicrons from the first meal which might be stored at their site of synthesis in the enterocyte or in lymphatics. The results of the present study also suggests that some preformed chylomicrons containing ^{13}C palmitic acid were released from the enterocytes and lymphatics after the intake of second meal. However in present study, the increase in the CRF-TAG ^{13}C palmitic acid concentration was only 39% of the concentration immediately before the intake of second meal. Whereas, in Fielding *et al.,* (1996) study the increase in linoleic acid was 144% (approx.). The differences between the two studies could be attributed to differences in experimental designs such as age and test meal composition. The subjects in Fielding *et al.,* (1996) study were significantly older than this study. It might be possible that increased age results in greater retention of exogenous fat at the level of gut or lymphatics than young men. This issue is further discussed in chapter 5 (section 5.5.1.5).

4.9.4 Energy expenditure and substrate oxidation

The results of energy expenditure and substrate oxidation (CHO oxidation, net lipid oxidation, breath $^{13}CO_2$ appearance, and exogenous lipid oxidation) were repeatable as the differences in AUC for these parameters did not attain statistical significance. The exception is endogenous lipid oxidation (p=0.07). The results of breath $^{13}CO_2$ excretion are comparable to a previous study (Jones, 1996). However the results of that study showed larger intra-individual variability (although not statistically significant) between two identical trials despite the fact that the breath $^{13}CO_2$ excretion was corrected for losses of tracer in stool (24.90 *v* 19.40, % absorbed dose). Higher intra-individual variability between two identical trials in the study of Jones (1996) could be related to the effect of menstrual cycle (hormonal changes) which was not controlled.

In contrast, significantly higher incremental increase in net Lox in trial 2 than trial 1 ($p<0.04$) suggests that it is not very repeatable. Net Lox was lower in trial 1 than trial 2 because of higher trend of basal net Lox in trial 1. Further contribution in lower incremental increase in net Lox in trial 1, comes from postprandial net Lox values which went down below the baseline levels until 2 hours post first meal and did not increase to great extent. Higher basal net Lox values in trial 1 could be due to problems in indirect calorimetry measurements and/or lack of proper rest before basal metabolic rate measurements. However both of these possibilities are less likely as the indirect calorimeter is calibrated every month and in both the trials subjects stayed overnight and were rested properly before the basal metabolic rate measurements.

4.10 Summary

The results of the repeatability study suggests that GI handling and postprandial lipid metabolism is fairly repeatable especially for the group as a whole. Minimal amount of ^{13}C label appearance in stool suggests that determination of ^{13}C excretion in stools might not be required for the correction for ^{13}C label excreted in breath CO_2 (as % absorbed dose) in the future studies using this protocol. Finally, the results of the present study also demonstrates that GC-C-IRMS tracer methodology can be successfully used to study the postprandial metabolism of exogenous fat in order to get better insight into the underlying mechanisms responsible for disturbed lipid metabolism in conditions such as insulin resistance and diabetes.

CHAPTER 5

Age-related changes in Postprandial Lipid Metabolism

5.1 Introduction

Ageing is characterised by deleterious changes in body composition (Baumgartner *et al.,* 1991) and alterations in CHO/lipid metabolism commensurate with a increase risk of CVD. The changes in body composition include a decrease in fat free mass and an increase in fat mass (Poehlman *et al.,* 1993; Heymsfield *et al.,* 1994). An increase of fat mass typically occurs between age 30 and 70, and the ratio of fat mass over lean body mass increases throughout human life (Cohn *et al.,* 1980; Andres *et al.,* 1985). Moreover ageing is associated with redistribution of fat towards intra-abdominal compartment (Busby *et al.,* 1992; Poehlman *et al.,* 1993). This increase in fat mass, even in adults whose weight remains relatively remains constant, is associated with increased risks of several diseases including CVD, and type II diabetes (Colditz *et al.,* 1995; Willett *et al.,* 1994).

The mechanisms responsible for the age-associated changes in the body composition are not well defined, however, it is suggested that the loss of fat free mass is partly due to physical inactivity (Poehlman *et al.,* 1993; Heymsfield *et al.,* 1994). The increase in fat mass and, more important, its redistribution to intra-abdominal compartment are also not well understood.

Ageing is also associated with alterations in glucose and lipoprotein metabolism (Munro, 1988). Cohan *et al.* (1988b) have found that older subjects have greater postprandial triglyceridaemia after a lipid-rich meal than do young subjects. The exact mechanisms of this increase in postprandial triglyceridaemia are not known partly because of the methodological problems in differentiating between exogenous and endogenous TAG in circulation. However, indirect methods, such as retinyl ester infusion studies, suggests that increased postprandial triglyceridaemia in older subjects is due to delayed plasma clearance of intestinal TGRL (Krasinski *et al.,* 1990a). Since hepatic receptors recognise chylomicron

remnants rather than chylomicrons (Carrella & Cooper, 1979), hydrolysis of chylomicron TAG by LPL is a potentially rate limiting step in the removal of intestinal TGRL. Hence, delayed clearance of plasma retinal esters in older subjects might be due to diminished catabolism of chylomicrons due to decreased activity of LPL and/or increased competition with endogenous lipoprotein at the level of LPL. Several studies have shown that heparin induced LPL activity decreases significantly with increasing age in humans (Nikkila & Niemi, 1957; Bradows & Campbell, 1972; Krauss *et al.,* 1974; Huttenen *et al.,* 1976).

Human and animal ageing are also characterised by progressive increase in fasting and postprandial insulin levels (Reaven & Reaven, 1985; Barzilai & Rossetti, 1996) with concomitant increase in rates of obesity. The presence of insulin resistance, altered lipoprotein metabolism, obesity and diabetes in advanced age suggests that insulin resistance plays a central role in the disturbance of lipid metabolism. If the insulin action is disturbed after the ingestion of meals, series of co-ordinated changes in TAG metabolism will not occur thus resulting in postprandial triglyceridaemia due to the accumulation of TGRL and their remnants. For example, in the insulin resistant state it may be hypothesised that, there will be a) decreased suppression of the entry of endogenous TAG in to circulation (due to decreased suppression of free fatty acids from adipose tissue and decreased suppression of VLDL production) and b) decreased TAG clearance from the plasma (due to decreased activation of LPL and remnant receptors and greater competition between chylomicrons and VLDL at the site of LPL and remnant receptors). This will result in postprandial triglyceridaemia due to the accumulation of TGRL and their remnants.

Since increasing age is associated with insulin resistance and disturbed postprandial lipid metabolism, hypertriglyceridaemia and the association circulating lipids may have with insulin resistance formed the basis of the central hypothesis of this study. The hypothesis states that as the age increases, levels of lipids in circulation also increase depending upon the individual's metabolic competence for clearing lipids from circulation, levels of physical activity and types and amounts of lipids and CHO in diet. The levels of lipids in circulation will then determine the level of insulin resistance, the risk of developing obesity, type II diabetes and atherosclerosis (Figure 1.1, Chapter 1).

Since the mechanisms responsible for disturbed postprandial lipid metabolism in advanced age are still unclear, the current cross-sectional study was initiated to examine the age-related changes on the postprandial processing of exogenous TAG in young versus middle-aged men. The main objective of the study was to examine the handling of exogenous

TAG both at the levels of circulation and oxidation. ^{13}C labelled tripalmitin was used in the test meal and was traced through different pools of the body (for example, appearance of tracer in chylomicron rich fraction TAG, non-esterified fatty acids fraction and in breath (Figure 1.2, Chapter 1).

Within the context of the hypothesis, the following questions were addressed by this study:

a) To what extent do age related changes influence the handling of exogenous lipid handling at the levels of the circulation and oxidation?

b) To what extent do age related changes influence the secretion of labelled TAG as ingested in the previous meal?

5.2 Chapter outline

The following chapter describes a) characteristics of subjects including fasting profiles of various variables, b) changes in postprandial glucose, insulin and lipid responses including ^{13}C PA responses in CRF-TAG and NEFA fraction, c) changes in postprandial energy expenditure (EE), net lipid oxidation (net Lox) and breath $^{13}CO2$ excretion, d) discussion and e) summary.

5.3 Methodology

5.3.1 Subjects

Eight healthy, middle-aged men aged between 47 and 58 years, were recruited from Southampton area. All the subjects had fasting TAG and cholesterol levels in normal range. None of the subjects were taking lipid lowering therapy or any other drugs affecting lipid metabolism such as thiazides, beta-blockers, insulin etc. None of the subjects suffered from hypertension or had evidence of hepatic, renal or thyroid disease. These subjects were compared against the results obtained for young men in trial 1 as described in chapter 4. Informed consent was obtained from all subjects and the study protocol was approved by the Ethical Committee of Southampton and South West Hampshire Health Commission.

The characteristics of the subjects and their fasting profiles are described in Table 5.1. Middle-aged men were not only older, but also had a greater fat mass than young controls ($p<0.05$). It should be noted that although middle-aged men had relatively similar weights and BMI compared to young controls, they had significant changes in body composition characterised by greater fat mass. Since this study examined the effect of age-related changes (including changes in body composition) on postprandial lipid metabolism, the fat mass was not controlled between the groups.

Similarly, middle-aged men had a trend towards higher BMI, fasting TAG, NEFA, glucose, and insulin concentrations than young controls ($P>0.05$). Young men showed a trend towards higher basal energy expenditure and basal net lipid oxidation/kg lean mass than middle-aged men ($p>0.05$). On the other hand, middle-aged men showed a trend of higher basal CHO oxidation/kg lean mass than young controls ($p>0.05$).

5.3.2 Study Protocol

Subjects followed the general protocol as described in chapter 3 (section 3.2). Briefly, three days prior to study subjects consumed a standard control diet and abstained from alcohol consumption and refrained from volitional exercise. On the evening before the study day, subjects were advised to finish their standard evening meal by 1900 hours and then fast their after except for drinking water.

Next morning around 0700 hours the subjects were admitted to CNMU. Baseline blood and breath samples were collected and baseline basal metabolic rate was measured for 20 minutes. At 0800 hours the subjects consumed a ^{13}C labelled TAG (tripalmitin) in

an emulsion and a standard test meal. At 1400 hours another unlabelled emulsion and test meal were given. Blood and breath samples were collected before the ingestion of ^{13}C label and at hourly intervals until ten hours after the ingestion of test meal. Whole body CO_2 excretion was determined by indirect calorimetry before the ingestion of label and then at hourly intervals for ten hours after the ingestion of test meal. During the study, no additional food or liquids were allowed except for bottled mineral water.

5.4 RESULTS

5.4.1 Blood variables

5.4.1.1 Postprandial plasma glucose responses

The time courses of changes in plasma glucose concentrations after the first and second meal are shown in Figure 5.1. Middle-aged men showed a trend towards higher postprandial glucose concentrations than young controls. The differences were only statistically significant at the later time points after the first meal ($p<0.05$). In both the groups, there was a marginal glycaemic response after the first meal. After the second meal, a peak was observed at 1 hour in middle-aged men and at 2 hour in young controls. After the peak the decline in glucose concentrations was slower in middle-aged men than controls. At 4-hour time point post second meal, glucose concentrations were still higher (7%) than fasting levels in middle aged men.

The Mean AUC for glucose responses over the 10-hour postprandial period are shown in Table 5.2. Postprandial AUC for plasma glucose concentration was significantly higher (12%) in middle-aged men than young controls ($p<0.04$). Incremental increase in glucose AUC was 79% higher in middle-aged men than controls, however, the differences between the groups were not statistically significant ($p=0.16$).

The association between plasma glucose and various anthropometric and metabolic parameters are shown in Appendix 8.6. Fasting plasma glucose was significantly positively correlated to postprandial glucose AUC ($r=0.74$); postprandial insulin AUC ($r=0.59$); incremental increase in insulin AUC ($r=0.59$); postprandial NEFA AUC ($r=0.55$) and fasting TAG ($r=0.64$) and postprandial TAG AUC ($r=0.64$) ($p<0.05$). Fasting glucose was also positively correlated to fat mass ($r=0.44$), fasting NEFA ($r=0.40$) and fasting insulin ($r=0.46$) concentrations, however, the association did not reach the statistical significance ($p=0.12$).

The Postprandial glucose AUC was significantly positively correlated to fat mass ($r=0.68$); fasting glucose ($r=0.73$) and incremental increase in glucose AUC ($r=0.55$); postprandial insulin AUC ($r=0.70$) and incremental increase in insulin AUC ($r=0.73$); postprandial NEFA AUC ($r=0.57$); fasting TAG ($r=0.52$) and postprandial TAG AUC ($r=0.57$), incremental increase in TAG AUC ($r=0.51$) and CRF-TAG ^{13}C PA AUC ($r=0.52$) ($p<0.05$). Postprandial glucose AUC was also positively correlated to fasting insulin ($r=0.44$), however, the relationship did not reach the significance ($p=0.10$).

Incremental increase in glucose AUC was significantly positively correlated to postprandial glucose AUC (r=0.55) and incremental increase in NEFA AUC (r=0.50) ($p<0.05$). Incremental increase in glucose AUC was also positively correlated to fat mass (r=0.46) and postprandial CRF-TAG ^{13}C PA AUC (r=0.45), however, the association did not reach the statistical significance (p=0.11).

5.4.1.2 Postprandial plasma insulin responses

The time courses of changes in plasma insulin concentrations following the first and second meal are shown in Figure 5.2. Middle-aged men showed a trend of higher postprandial insulin concentrations than young controls. The differences between groups were only statistically significant at the 6 hour time point post first meal ($p<0.05$). In middle-aged men and young controls, the peaks were reached at 1 hour following the first meal and 1 and 2 hour respectively following the second meal. In both the groups, insulin responses at peaks were higher after second meal than first meal. Both after the first and second peaks, the insulin concentrations followed a declining trend and the values reached near to baseline levels at 6 hours post first meal but remained higher than baseline levels at 4 hours post second meal (232% and 137% in middle-aged men and young men respectively).

The mean AUC for insulin responses over the 10-hour postprandial period are shown in Table 5.2. Postprandial AUC for plasma insulin concentration was 49% higher in middle-aged men than controls, however, the differences between groups did not reach the statistical significance (p=0.18). Incremental increase in insulin AUC was 54% higher in middle-aged men than controls, however, the differences between groups did not reach the statistical significance (p=0.17).

The association between plasma insulin and various anthropometric and metabolic parameters are shown in Appendix 8.6. Fasting plasma insulin was significantly positively correlated to fat mass (r=0.51); postprandial insulin AUC (r=0.84) and incremental increase in insulin AUC (r=0.75); postprandial NEFA AUC (r=0.65) and fasting TAG (r=0.57) ($p<0.05$). Fasting insulin was also positively correlated to fasting glucose (r=0.46) and postprandial glucose AUC (r=0.44); fasting NEFA (r=0.42) and postprandial TAG AUC (r=0.40), however, the association did not reach the significance (p=0.14).

Postprandial insulin AUC was significantly positively correlated to fat mass (r=0.58); fasting glucose (r=0.59) and postprandial glucose AUC (r=0.70); fasting insulin

(r=0.84) and incremental increase in insulin AUC (r=0.98); postprandial NEFA AUC (r=0.77); fasting TAG (r=0.69) and postprandial TAG AUC (r=0.58); and postprandial CRF-TAG ^{13}C PA AUC (r=0.60) ($p<0.05$). Postprandial insulin AUC was negatively correlated to breath $^{13}CO2$ excretion (r= -0.37)and exogenous lipid oxidation (r= -0.37), however, the association did not reach the statistical significance (p=0.18).

Incremental increase in insulin AUC was significantly positively correlated to fat mass (r=0.57); fasting glucose (r=0.59) and postprandial glucose AUC (r=0.73); fasting insulin (r=0.75) and postprandial insulin AUC (r=0.98); postprandial NEFA AUC (r=0.76); fasting TAG (r=0.69) and postprandial TAG AUC (r=0.60) and CRF-TAG ^{13}C PA AUC (r=0.66) ($p<0.05$). Incremental increase in insulin AUC was negatively correlated to breath $^{13}CO2$ excretion (r= -0.37) and exogenous lipid oxidation (r= -0.37), however, the association did not reach the statistical significance (p=0.18).

5.4.1.3 Postprandial plasma NEFA responses

The time courses of changes in plasma NEFA concentrations following the first and second meals are shown in Figure 5.3. After the first meal, both the groups showed a sharp decline in NEFA concentrations with nadir reached at 1 hour. Since middle-aged men had higher fasting NEFA concentrations, drop in NEFA concentrations at nadir was less in this group than controls. After the nadir, both the groups showed gradual increase in NEFA concentrations with the peak reached at 6 hour. At peak, NEFA concentrations were significantly higher (29%) in middle-aged men than controls ($p<0.05$). The increase in NEFA concentration was higher than fasting levels in both the groups (29% and 20% for middle-aged men and young men respectively). After the second meal, suppression of NEFA was slower in both the groups with the nadir reached three hour later. Middle-aged men showed decreased suppression of NEFA than young controls after the second meal.

The mean AUC for NEFA responses over the 10-hour postprandial period are shown in Table 7.2. Postprandial AUC for plasma NEFA concentration was significantly higher (32%) in middle-aged men than young controls ($p<0.02$). Incremental increase in NEFA AUC was similar for both the groups.

The association of plasma NEFA and various anthropometric and metabolic parameters are shown in Appendix 8.6. Fasting plasma NEFA was significantly positively correlated to fasting TAG (r=0.53) ($p<0.05$). Fasting NEFA was also positively correlated to fasting glucose (r=0.40); fasting insulin (r=0.42); postprandial NEFA AUC (r=0.40) and

postprandial TAG AUC (r=0.44), however, the relationship did not reach the significance (p=0.14). Fasting NEFA was significantly negatively correlated to incremental increase in NEFA AUC (r= -0.84) and postprandial CHO oxidation AUC (r= -0.53) ($p<0.05$). Fasting NEFA was also negatively correlated to basal CHO oxidation (r= -0.39), however, the relationship did not reach the statistical significance level (p=0.0.14)

Postprandial NEFA AUC was significantly positively correlated to fat mass (r=0.72); fasting glucose (r=0.55) and postprandial glucose AUC (r=0.57); fasting insulin (r=0.65) and postprandial insulin AUC (r=0.77); incremental increase in insulin AUC (r=0.76); fasting TAG (r=0.78) and postprandial TAG AUC (r=0.73) and CRF-TAG ^{13}C PA AUC (r=0.54) ($p<0.05$). Postprandial NEFA AUC was also positively correlated to fasting NEFA (r=0.40), however, the relationship did not reach the statistical significance (p=0.14). Postprandial NEFA AUC was significantly negatively correlated to breath $^{13}CO_2$ excretion (r= -0.53) and exogenous lipid oxidation (r= -0.53) ($p<0.05$). Postprandial NEFA AUC was also negatively correlated to CHO oxidation AUC (r= -0.47) and incremental increase in CHO oxidation AUC (r= -0.49), however, the relationship did not reach the statistical significance (p=0.08)

Incremental increase in NEFA AUC was significantly positively correlated to incremental increase in glucose AUC (r=0.50) ($p<0.05$). Incremental increase in NEFA AUC was also positively correlated to basal CHO oxidation (r=0.49), however, the relationship did not reach the statistical significance (p=0.07). Incremental increase in NEFA AUC was significantly negatively correlated to fasting plasma NEFA (r= -0.84), breath $^{13}CO2$ excretion (r= -0.52) and exogenous lipid oxidation (r= -0.52) ($p<0.05$).

5.4.1.4 Postprandial plasma TAG responses

The time courses of changes in plasma TAG concentrations following the first and second meal are shown in Figure 5.4. The time courses for plasma TAG were similar for both the groups over the postprandial period. However, there was a trend towards higher magnitude and duration of postprandial TAG responses in middle-aged men than young controls. The differences between the groups were only statistically significantly at later time points and after the second meal ($p<0.05$). In both the groups, the peaks were reached at 2 hour after the first meal and at 1 hour after the second meal. After the peaks, the decline in plasma TAG concentrations was significantly slower in middle-aged men than controls. In young men, at 6 hour post first meal, the plasma TAG concentrations came

down near to fasting levels. Where as in middle-aged men, the plasma TAG concentrations were 37% higher than fasting levels at 6-hour time point. In young men, after the second meal, the TAG response was smaller than that observed after the first meal. Where as in middle-aged men, the TAG response was of the same magnitude as observed after the first meal. In both the groups plasma TAG concentrations remained higher than baseline levels at 4 hours post second meal (59% and 36% in middle-aged men and young men respectively).

The mean AUC for TAG responses over the 10 hour postprandial period are shown in Table 5.2. Postprandial AUC for plasma TAG concentration was 46% higher in middle-aged men than young controls, however, the differences between the groups did not reach the statistical significance ($p=0.11$). Similarly incremental increase in TAG AUC was 35% higher in middle-aged men than young controls but the differences between the groups were not statistically significant ($p=0.20$).

The association of plasma TAG and various anthropometric and metabolic parameters are shown in Appendix 8.6. Fasting plasma TAG was significantly positively correlated to fasting glucose ($r=0.64$) and postprandial glucose AUC ($r=0.52$); fasting insulin ($r=0.57$) and postprandial insulin AUC ($r=0.69$); incremental increase in insulin AUC ($r=0.69$); fasting NEFA ($r=0.53$) and postprandial NEFA AUC ($r=0.78$); postprandial TAG AUC ($r=0.96$); incremental increase in TAG AUC ($r=0.57$) and postprandial CRF-TAG ^{13}C PA AUC ($r=0.65$) ($p<0.05$). Fasting TAG was also positively correlated to fat mass ($r=0.45$), however, the relationship did not reach the statistical significance ($p=0.09$).

Postprandial TAG AUC was significantly positively correlated to fasting glucose ($r=0.63$) and postprandial glucose AUC ($r=0.57$); postprandial insulin AUC ($r=0.58$) and incremental increase in insulin AUC ($r=0.60$); postprandial NEFA AUC ($r=0.73$); fasting TAG ($r=0.96$) and incremental increase in TAG AUC ($r=0.77$) and postprandial CRF-TAG ^{13}C PA AUC ($r=0.71$) ($p<0.05$). Postprandial TAG AUC was also positively correlated to fat mass ($r=0.44$); fasting insulin ($r=0.40$) and fasting NEFA ($r=0.44$), however, the relationship did not reach the statistical significance ($p=0.14$).

Incremental increase in TAG AUC was significantly positively correlated to postprandial glucose AUC ($r=0.51$), fasting TAG ($r=0.57$) and postprandial TAG AUC ($r=0.77$) and CRF-TAG ^{13}C PA AUC ($r=0.65$) ($p<0.05$). Incremental increase in TAG

AUC was also positively correlated to fasting plasma glucose (r=0.42), however, the relationship did not reach the statistical significance (p=0.12).

5.4.1.5 Postprandial CRF-TAG ^{13}C PA responses

The time courses of postprandial CRF-TAG ^{13}C PA responses following the first and second meal are shown in Figure 5.5. In both the groups, ^{13}C PA appeared in CRF-TAG within first hour and the peaks were reached at 2 hours following the label administration. At peak, the concentration of ^{13}C PA was similar in both the groups. After the peak, decline of ^{13}C PA concentration was significantly slower in middle-aged men than young controls ($p<0.05$). In both the groups, ^{13}C PA concentrations were still higher than fasting levels at 6-hour time point post first meal (60 fold and 18 fold in middle-aged men and young men respectively). After the second unlabelled meal, the peak ^{13}C PA concentration occurred at 1 hour in both the groups. The concentration of ^{13}C PA at peak was significantly higher (245%) in middle-aged men than controls ($p<0.05$). After the second peak the decline of ^{13}C PA was significantly slower in middle-age men than young controls ($p<0.05$). In both the groups, ^{13}C PA concentrations remained higher than baseline levels at 4 hours post second meal (11 fold and 2 fold in middle-aged men and young men respectively).

The mean AUC for CRF-TAG ^{13}C PA responses over the 10 hour postprandial period are shown in Table 5.2. Postprandial AUC for CRF-TAG ^{13}C PA concentration was 29% higher in middle-aged men than young controls, however, the differences were not statistically significant (p=0.27).

The association of postprandial CRF-TAG ^{13}C PA and various anthropometric and metabolic parameters are shown in Appendix 8.6. Postprandial CRF-TAG ^{13}C PA AUC was significantly positively correlated to postprandial glucose AUC (r=0.52); postprandial insulin AUC (r=0.60) and incremental increase in insulin AUC (r=0.66); postprandial NEFA AUC (r=0.54); fasting TAG (r=0.65) and postprandial TAG AUC (r=0.71) and incremental increase in TAG AUC (r=0.65) ($p<0.05$). Postprandial CRF-TAG ^{13}C PA AUC was also positively correlated to incremental increase in glucose AUC (r=0.45) and NEFA ^{13}C PA AUC (r=0.44), however, the relationship did not reach the statistical significance (p=0.12).

5.4.1.6 Postprandial plasma NEFA ^{13}C PA responses

The time courses of postprandial plasma NEFA ^{13}C PA responses following the first and second meal are shown in Figure 5.6. Following the administration of labelled meal, the peak NEFA ^{13}C PA concentration occurred at 2 hour for young men and at 3 hour for middle-aged men. Peak ^{13}C PA concentrations were similar in both groups. Young men showed higher NEFA ^{13}C PA concentrations before the peak. Where as middle-aged men showed higher NEFA ^{13}C PA concentrations after the peak. After the peak, in young men, NEFA ^{13}C PA concentrations declined gradually until 6 hour but the values were still higher than baseline levels. Where as in middle-aged men, NEFA ^{13} C PA declined to a small extent until 6 hours after the first meal. After the second unlabelled meal, in young men, a small peak was observed for NEFA ^{13}C PA concentrations at 1 hour followed by a sharp decline. Where as in middle-aged men, no peak was observed rather a sharp decline occurred in NEFA ^{13}C PA concentrations. Data of ^{13}C NEFA concentrations should be interpreted with caution because the PA peaks detected by GC-IRMS were very small due to little concentration of PA in NEFA fraction, especially after the postprandial suppression of NEFA concentrations. Very small peaks can give erroneous results in terms of isotopic enrichments and substrate concentrations.

The mean AUC for plasma NEFA ^{13}C PA responses over the 10-hour postprandial period are shown in Table 5.2. Postprandial AUC for plasma NEFA ^{13}C PA concentration was non-significantly higher (14%) in middle-aged men than young controls ($p>0.05$).

The association of postprandial NEFA ^{13}C PA and various anthropometric and metabolic parameters are shown in Appendix 8.6. NEFA ^{13}C PA was significantly positively correlated to incremental increase in EE AUC ($r=0.50$) ($p<0.05$). NEFA ^{13}C PA was also positively related to CRF-TAG ^{13}C PA ($r=0.44$), however, the relationship did not reach the statistical significance ($p>0.12$).

5.4.2 Energy expenditure (EE) and substrate oxidation

5.4.2.1 Energy expenditure

The time courses of changes in EE/kg lean mass following the first and second meal are shown in Figure 5.7. Young men showed trend towards higher EE during the early hours of postprandial period than middle-aged men. In young men, the peaks were reached at 2 and 1 hour following the first and second meals respectively. In middle-aged men, the peak was reached at 1 hour following both first and second meals. In both the

groups, EE values declined gradually after the peak and returned near to baseline levels at 6 hours post first meal but remained higher than baseline values at 4 hours post second meal (10% and 8% in middle-aged men and young men respectively).

The mean AUC for EE over the 10 hour postprandial period are shown in Table 5.3. Young men showed a trend towards higher (4%) postprandial AUC for EE than middle aged men but the differences were statistically non-significant (p=0.29). The incremental increase in EE AUC or the thermic effect of food (TEF) was similar between the two groups.

The association of EE and various anthropometric and metabolic parameters are shown in Appendix 8.6. Basal EE was significantly positively correlated to postprandial EE (r=0.85) (p<0.01).

Postprandial EE was significantly positively correlated to basal EE (r=0.85) (p<0.05). Postprandial EE was also positively correlated to breath $^{13}CO2$ oxidation (r=0.48) and exogenous lipid oxidation (r=0.48), however, the relationship did not reach the statistical significance (p=0.07).

Incremental increase in EE or TEF was significantly positively correlated to NEFA ^{13}C PA AUC (r=0.50) (p<0.05).

5.4.2.2 CHO oxidation

The time courses of changes in CHO oxidation/kg lean mass following the first and second meal are shown in Figure 5.8. Young men showed a trend towards higher postprandial CHO oxidation than middle aged-men. In both the groups, there was a sharp increase in postprandial CHO oxidation with the peak reaching at 1 hour for middle-aged men and at 2 hour for young controls. The peak CHO oxidation was 16% higher in young men than middle-aged men. After the peaks, CHO oxidation started to decline until 6 hours where the values were significantly lower than baseline levels in both the groups (p<0.05). The decline in CHO oxidation was sharper in young men than middle-aged men. After the second meal, postprandial CHO oxidation increased sharply and the peaks were reached at 1 hour in both the groups.

The mean AUC for CHO oxidation over the 10 hour postprandial period are shown in Table 5.3. Postprandial AUC for CHO oxidation was significantly higher (44%) in young men than middle-aged men (p<0.05). Similarly incremental increase in CHO oxidation AUC were significantly higher in young men than middle-aged men (p<0.05).

The association of CHO oxidation and various anthropometric and metabolic parameters are shown in Appendix 8.6. Basal CHO oxidation was significantly positively correlated incremental increase in net Lox AUC (r=0.69) ($p<0.05$). Basal CHO oxidation was also positively correlated incremental increase in NEFA AUC (r=0.49) and postprandial CHO oxidation AUC (r=0.38), however, the relationship did not reach the statistical significance (p=0.16). Basal CHO oxidation was significantly negatively correlated to incremental increase in CHO oxidation AUC (r= -0.61) and basal net Lox (r= -0.87) ($p<0.05$). Basal CHO oxidation was also negatively correlated Basal NEFA (r= -0.39); postprandial net Lox (r= -0.47); breath $^{13}CO_2$ excretion (r= -0.38); exogenous lipid oxidation (r= -0.38) and endogenous lipid oxidation (r= -0.37), however, the relationship was not statistically significant (p=0.17).

Postprandial CHO oxidation AUC was positively correlated to basal CHO oxidation (r=0.38) and incremental increase in CHO oxidation AUC (r=0.50), however, the relationship did not reach the statistical significance (p=16). Postprandial CHO oxidation was significantly negatively correlated to fat mass (r= -0.52); postprandial NEFA AUC (r= -0.53); incremental increase in NEFA AUC (r= -0.47); postprandial net Lox (r= -0.71) and endogenous Lox (r= -0.76) ($p<0.05$).

Incremental increase in CHO oxidation AUC was significantly positively correlated basal net Lox (r=0.60); breath $^{13}CO2$ excretion (r=0.59) and exogenous lipid oxidation (r=0.59) ($p<0.05$). Incremental increase in CHO oxidation was also positively correlated to postprandial CHO oxidation (r=0.50), however, the relationship did not reach the statistical significance (p=0.06). Incremental increase in CHO oxidation was significantly negatively correlated to fat mass (r= -0.71); basal CHO oxidation (r= -0.61) and incremental increase in net Lox (r= -0.90) ($p<0.05$). Incremental increase in CHO oxidation AUC was also negatively correlated to postprandial glucose AUC (r= -0.49) and postprandial NEFA AUC (r= -0.49), however, the relationship did not reach the statistical significance (p=0.07).

5.4.2.3 Net lipid oxidation

The time courses of changes in net lipid oxidation/kg lean mass following the first and second meal are shown in Figure 5.9. There was a trend towards higher postprandial net lipid oxidation in middle-aged men than controls; however, the differences between the groups over the postprandial time course were not statistically significant ($p>0.05$). In

young men, there was an initial decline in postprandial net Lox until 2-hour time point. Thereafter, a sharp increase in net Lox was observed and the peak was reached at 4 hour post first meal. Where as in middle-aged men, postprandial net Lox remained same near the baseline levels until 1 hour, and thereafter a step-wise sharp increase was observed and the peak was reached at 6 hour post first meal. At peak, net Lox was 14% higher in middle-aged men than controls. After the peaks, both the groups followed a decline in net Lox but the values remained higher than baseline levels at 4 hours post second meal (45% and 8% in middle-aged men and young men respectively). Second meal did not result in increase in net Lox in both the groups.

The mean AUC for net lipid oxidation over the 10 hour postprandial period are shown in Table 5.3. Postprandial AUC for net lipid oxidation was 16% higher in middle-aged men than young controls, however, the differences between the groups were not statistically significant ($p=0.34$). The incremental increase in net lipid oxidation over the baseline levels was 3.5 fold higher in middle-aged men than young controls, however, the differences between the groups could not reach the statistical significance ($p=0.10$).

The association of net lipid oxidation (net Lox) and various anthropometric and metabolic parameters are shown in Appendix 8.6. Basal net Lox was significantly positively correlated to incremental increase in CHO oxidation AUC ($r=0.60$), postprandial net Lox AUC ($r=0.61$) and endogenous lipid oxidation AUC ($r=0.50$) ($p<0.05$). Basal net Lox was also positively correlated to breath $^{13}CO_2$ excretion ($r=0.42$) and exogenous lipid oxidation ($r=0.42$), however, the relationship was not statistically significant ($p=0.12$). Basal lipid oxidation was significantly negatively correlated to basal CHO oxidation ($r= -0.87$) and incremental increase in net Lox AUC ($r= -0.71$) ($p<0.05$).

Postprandial net Lox AUC was significantly positively correlated to basal net Lox AUC ($r=0.61$) and endogenous Lox AUC ($r=0.97$) ($p<0.05$). Postprandial net Lox AUC was significantly negatively correlated to postprandial CHO oxidation AUC ($r= -0.71$) ($p<0.05$). Postprandial net Lox AUC was also negatively correlated to basal CHO oxidation ($r= -0.47$), however, the relationship did not reach the statistical significance ($p=0.08$).

Incremental increase in net Lox AUC was significantly positively correlated to basal CHO oxidation ($r=0.68$) ($p<0.05$). Incremental increase in net Lox AUC was also positively correlated to fat mass ($r=0.45$), however, the relationship did not reach the

statistical significance (p=0.09). Incremental increase in net Lox AUC was significantly negatively correlated to incremental increase in CHO oxidation AUC (r= -0.90) and basal net Lox (r= -0.71) (p<0.05). Incremental increase in net Lox AUC was also negatively correlated to breath ^{13}CO2 excretion AUC (r= -0.48) and exogenous Lox AUC (r= -0.48), however, the relationship did not reach the statistical significance (p=0.07).

5.4.2.4 Breath ^{13}CO2 recovery

The time courses of breath $^{13}CO_2$ recovery/kg lean mass following the first and second meal are shown in Figure 5.10. Breath $^{13}CO_2$ recovery was towards higher trend in young men than middle-aged men after the first meal. In both the groups ^{13}C label appeared in the breath within the first hour of the label administration. ^{13}C label in the breath then continued to increase and the peak was reached at 5 hours post first meal in young men and at I hour post second meal in the middle-aged men. After the peak ^{13}C label in breath started to decline but remained higher than 1 hour levels at 4 hour post second meal (2 fold and 1 fold in middle-aged men and young men respectively). After the second meal a trend of higher breath $^{113}CO_2$ excretion was observed in middle-aged men than controls.

The mean AUC for breath $^{13}CO_2$ appearance over the 10 hour postprandial period are shown in Table 5.3. Postprandial AUC for breath $^{13}CO_2$ appearance was 10% higher in young men than middle-aged men, however, the differences were not statistically significant (p=0.21).

The relationship of breath ^{13}CO2 recovery and various anthropometric and metabolic parameters are shown in Appendix 8.6. Breath ^{13}CO2 AUC was significantly positively correlated to incremental increase in CHO oxidation AUC (r=0.59) (p<0.05). Breath $^{13}CO_2$ was also positively correlated to EE AUC (r=0.48) and basal net Lox AUC (r=0.42), however, the relationship did not reach the statistical significance (p=0.12). Breath ^{13}CO2 AUC was significantly negatively correlated to fat mass (r= -0.75); lean mass (r= -0.68); postprandial NEFA AUC (r= -0.53) and incremental increase in NEFA AUC (r= -0.52) (p<0.05). Breath ^{13}CO2 AUC was also negatively correlated to incremental increase in net Lox AUC (r= -0.48), however, the relationship did not reach the statistical significance (p=0.07).

5.4.2.5 Endogenous and exogenous lipid oxidation

The mean AUC for endogenous lipid oxidation/kg lean mass over the 10 hour postprandial period are shown in Table 5.3. Postprandial AUC for endogenous lipid oxidation was 43% higher in middle-aged men than young controls, however, the differences were not statistically significant (p=0.22).

The mean AUC for exogenous Lox/kg lean mass over the 10 hour postprandial period are shown in Table 5.3. Postprandial AUC for exogenous Lox was 9% higher in young men than middle-aged men, however, the differences were not statistically significant (p=0.21).

The association of endogenous and exogenous Lox/kg lean mass and various anthropometric and metabolic parameters are shown in Appendix 8.6. Endogenous Lox AUC was significantly positively correlated to lean mass (r=0.52); basal net Lox (r=0.50) and postprandial net Lox AUC (r=0.97) ($p<0.05$). Endogenous Lox AUC was also positively correlated to fat mass (r=0.40) but the relationship was not statistically significant (p=0.14). Endogenous Lox was significantly negatively correlated to postprandial CHO oxidation AUC (r= -0.76) ($p<0.05$).

Exogenous Lox was significantly positively correlated to incremental increase in CHO oxidation AUC (r=0.59) ($p<0.05$). Exogenous Lox was also positively correlated to EE AUC (r=0.48) and basal net Lox(r=0.42), however, the relationship did not reach the statistical significance (p=0.12). Exogenous Lox AUC was significantly negatively correlated fat mass (r= -0.75); lean mass (r= -0.68); postprandial NEFA AUC (r= -0.53) and incremental increase in NEFA AUC (r= -0.52) ($p<0.05$). Exogenous Lox AUC was also negatively correlated to incremental increase in net Lox AUC (r= -0.48), however, the relationship did not reach the statistical significance (p=0.07).

Table 5.1 Physical characteristics and fasting parameters of subjects.

Characteristics	Young men (n = 7) Mean (SE)	Middle-aged men (n = 8) Mean (SE)
Age (yrs)	25.6 (1.8)	52.1 (1.3) *
Weight (kg)	74.1 (3.5)	80.8 (3.9)
Height (m)	1.8 (0.0)	1.8 (0.0)
BMI (kg/m^2)	23.2 (1.1)	25.8 (1.3)
Fat mass (kg)	10.1 (3.0)	18.0 (1.8) *
Lean mass (kg)	63.9 (2.5)	62.8 (2.4)
Fasting TG (mmol/l)	0.59 (0.1)	0.90 (0.2)
Fasting NEFA (mmol/l)	0.35 (0.02)	0.42 (0.04)
Fasting insulin (mU/l)	6.2 (0.6)	8.3 (1.7)
Basal EE (kcal/kg lean mass)	1.20 (0.04)	1.15 (0.04)
Basal net Lox (kcal/kg lean mass)	0.039 (0.006)	0.033 (0.008)
Basal CHO ox (kcal/kg lean mass)	0.146 (0.014)	0.155 (0.020)

EE, energy expenditure; CHO, carbohydrate; Ox, oxidation; *, significant difference between groups ($p<0.05$)

Table 5.2 Mean AUC for various blood parameters over the 10-hour postprandial period.

Group	AUC Glucose (mmol/l.10h) Mean (SE)	AUC Δ Glucose (mmol/l.10h) Mean (SE)	AUC Insulin (mU/l.10h) Mean (SE)	AUC Δ Insulin (mU/l.10h) Mean (SE)	AUC NEFA (mmol/l.10h) Mean(SE)	AUC Δ NEFA (mmol/l.10h) Mean(SE)	AUC TAG (mmol/l.10h) Mean (SE)	AUC ΔTAG (mmol/l.10h) Mean (SE)	AUC ^{13}CPA In CRF-TAG (ug/ml.10h) Mean (SE)	AUC ^{13}CPA In NEFA (ug/ml.10h) Mean (SE)
Young men	59 (1.9)	4.3 (1.1)	247 (25)	185 (23)	2.2 (0.1)	-1.3 (0.3)	9.3 (1.7)	3.4 (0.7)	19.3 (3.4)	8.7 (1.1)
Middle-aged Men	66 (2.5) *	7.7 (1.9)	367 (78)	285 (64)	2.9 (0.2) *	-1.3 (0.4)	13.6 (1.9)	4.6 (0.6)	24.8 (3.3)	9.9 (1.1)

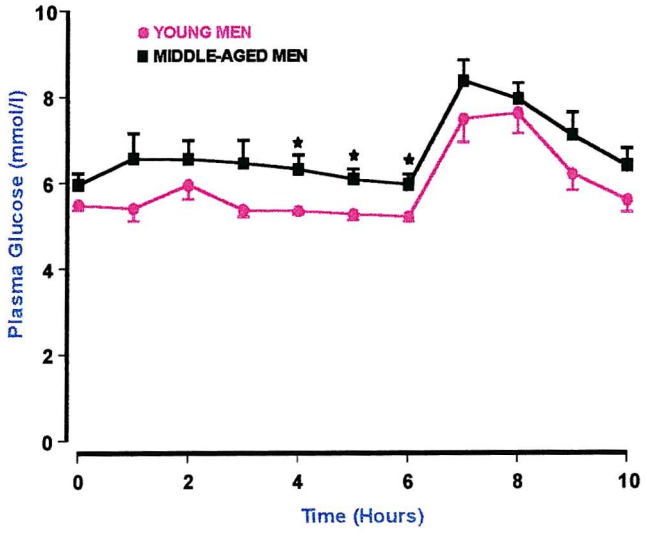
AUC, area under the curve; NEFA, non-esterified fatty acids; TAG, triacylglycerol; PA, palmitic acid; CRF, chylomicron-rich fraction; Δ, increment increase above the base line; *, significant difference between groups ($p<0.05$)

Table 5.3 Mean AUC for EE and substrate oxidation over the 10-hour postprandial period.

Group	AUC EE kcal/kg LM.10h Mean (SE)	AUC Δ EE Kcal/kg LM.10h Mean (SE)	AUC CHO ox gm/kg LM.10h Mean (SE)	AUC Δ CHO ox gm/kg LM.10h Mean (SE)	AUC net Lox gm/kg LM.10h Mean (SE)	AUC Δ net Lox gm/kg LM.10h Mean (SE)	AUC Breath $13CO_2$ % AD/kg LM.10h Mean (SE)	AUC Endo Lox gm/kg LM.10h Mean (SE)	AUC Exo Lox gm/kg LM.10h Mean (SE)
Young men	13.42 (0.2)	1.41 (0.2)	2.10 (0.1)	0.60 (0.09)	0.44 (0.04)	0.04 (0.05)	0.33 (0.01)	0.21 (0.04)	0.24 (0.02)
Middle-aged Men	12.92 (0.4)	1.41 (0.2)	1.46 (0.1) *	-0.09 (0.15) *	0.51 (0.06)	0.18 (0.06)	0.30 (0.02)	0.30 (0.06)	0.22 (0.01)

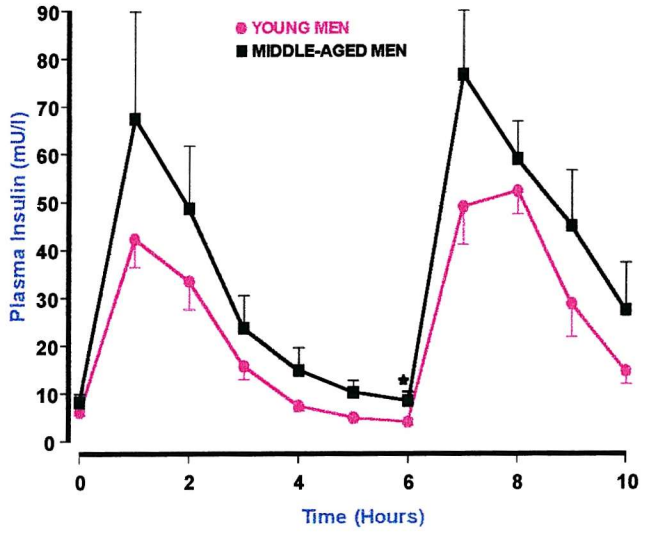
AUC, area under the curve; EE, energy expenditure; LM, lean mass; Δ, increment above the base line; CHO, carbohydrate; ox, oxidation; Lox, lipid oxidation; AD, administered dose; Endo, endogenous; Exo, exogenous; *, significant difference between groups ($p<0.05$)

Figure 5.1 Time courses of changes in plasma glucose concentrations (mmol/l) after the first and second meal for healthy middle-aged men and young controls.



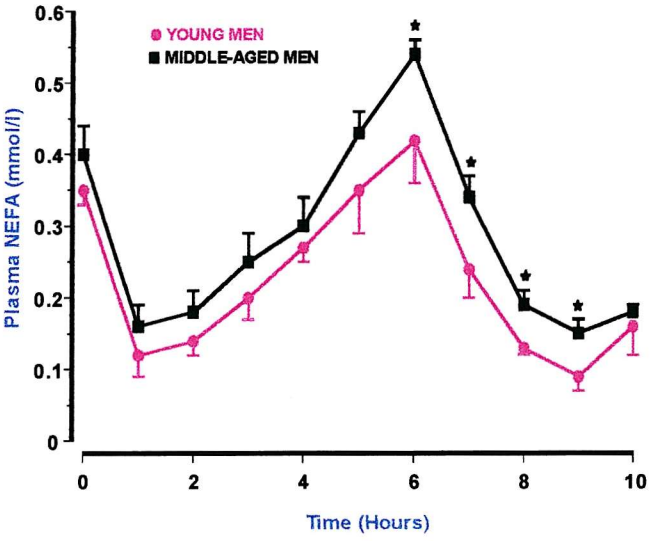
*, significant differences between groups ($p<0.05$).

Figure 5.2 The Time courses of changes in plasma insulin concentrations (mU/l) after the first and second meal for healthy middle-aged men and young controls.



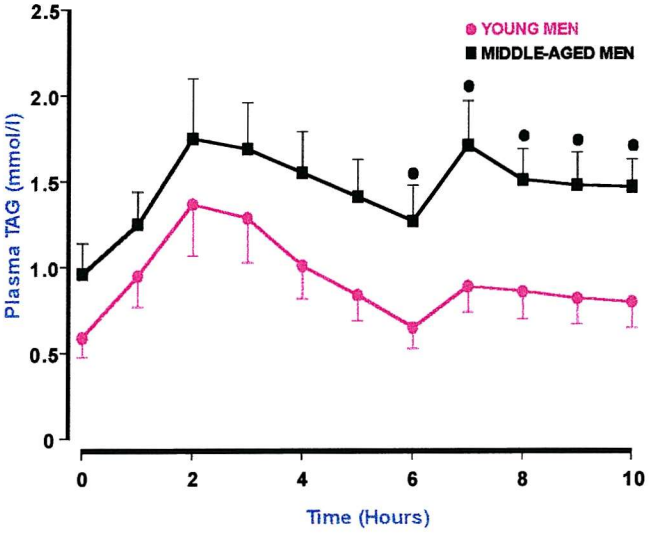
*, significant difference between groups ($p<0.05$).

Figure 5.3 Time courses of changes in plasma NEFA concentrations (mmol/l) after the first and second meal for healthy middle-aged men and young controls.



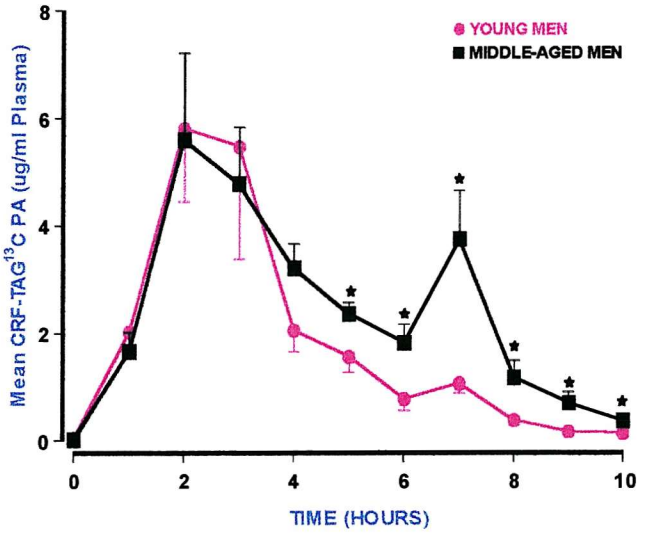
*, significant differences between groups ($p<0.05$).

Figure 5.4 Time courses of changes in plasma TAG concentrations (mmol/l) after the first and second meal for healthy middle-aged men and young controls.



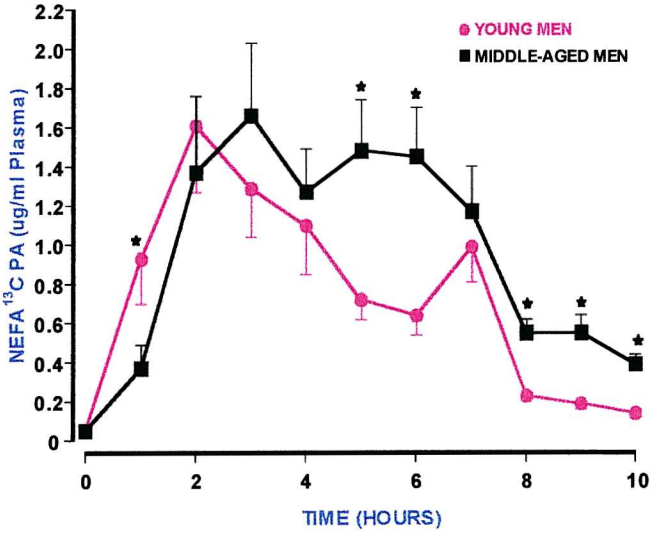
*, significant differences between groups ($p<0.05$).

Figure 5.5 Time courses of changes in CRF-TAG ^{13}C PA concentrations (μg/ml) after the first and second meal for healthy middle-aged men and young controls.



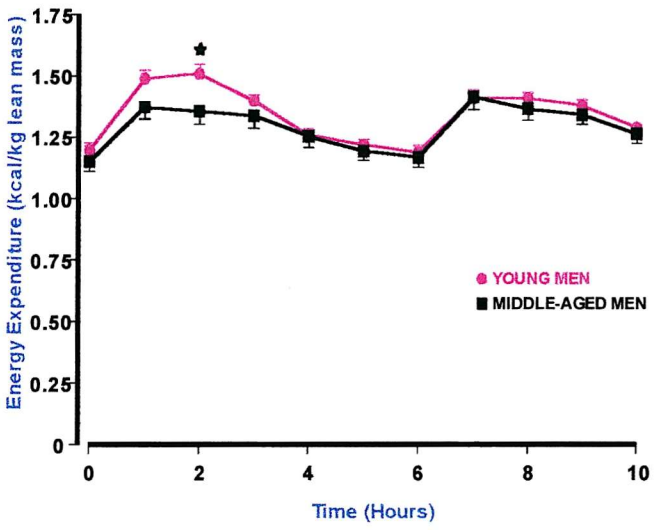
*, significant differences between groups ($p<0.05$).

Figure 5.6 Time courses of changes in NEFA ^{13}C PA concentrations (μg/ml) after the first and second meal for healthy middle-aged men and young controls.



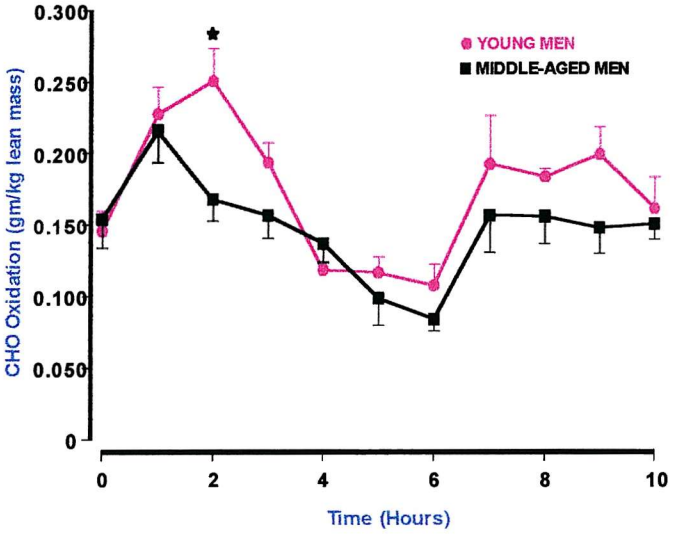
*, significant differences between groups ($p<0.05$).

Figure 5.7 Time courses of changes in energy expenditure (kcal/kg lean mass) after the first and second meal for healthy middle-aged men and young controls.



*, significant differences between groups ($p<0.05$).

Figure 5.8 Time courses of changes in carbohydrate oxidation (gm/kg lean mass) after the first and second meal for healthy middle-aged men and young controls.



*, significant differences between groups ($p<0.05$).

Figure 5.9 Time courses of changes in net lipid oxidation (gm/kg lean mass) after the first and second meal for healthy middle-aged men and young controls.

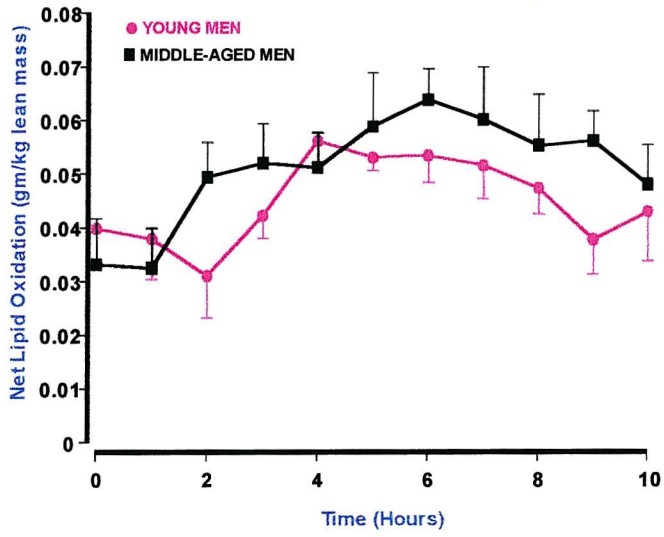
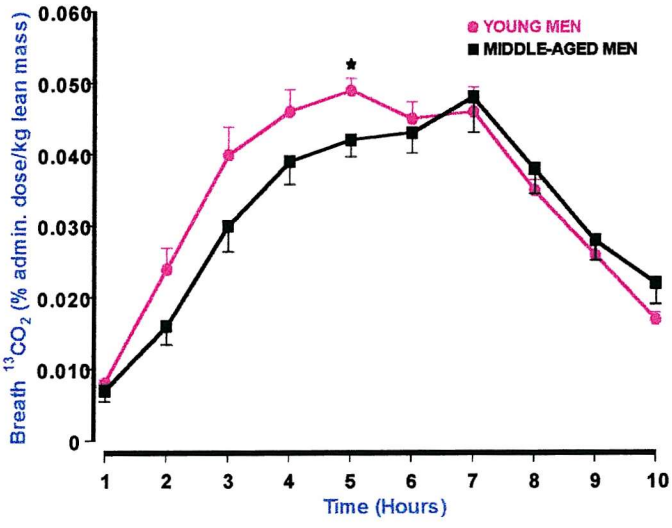


Figure 5.10 Time courses of changes in breath $^{13}CO_2$ excretion (% administered dose) after the first and second meal for healthy middle-aged men and young controls.



*, significant differences between groups ($p<0.05$).

Figure 5.11 Scatter plots for various variables in the study. Red squares, young men; Green squares, middle-aged men.

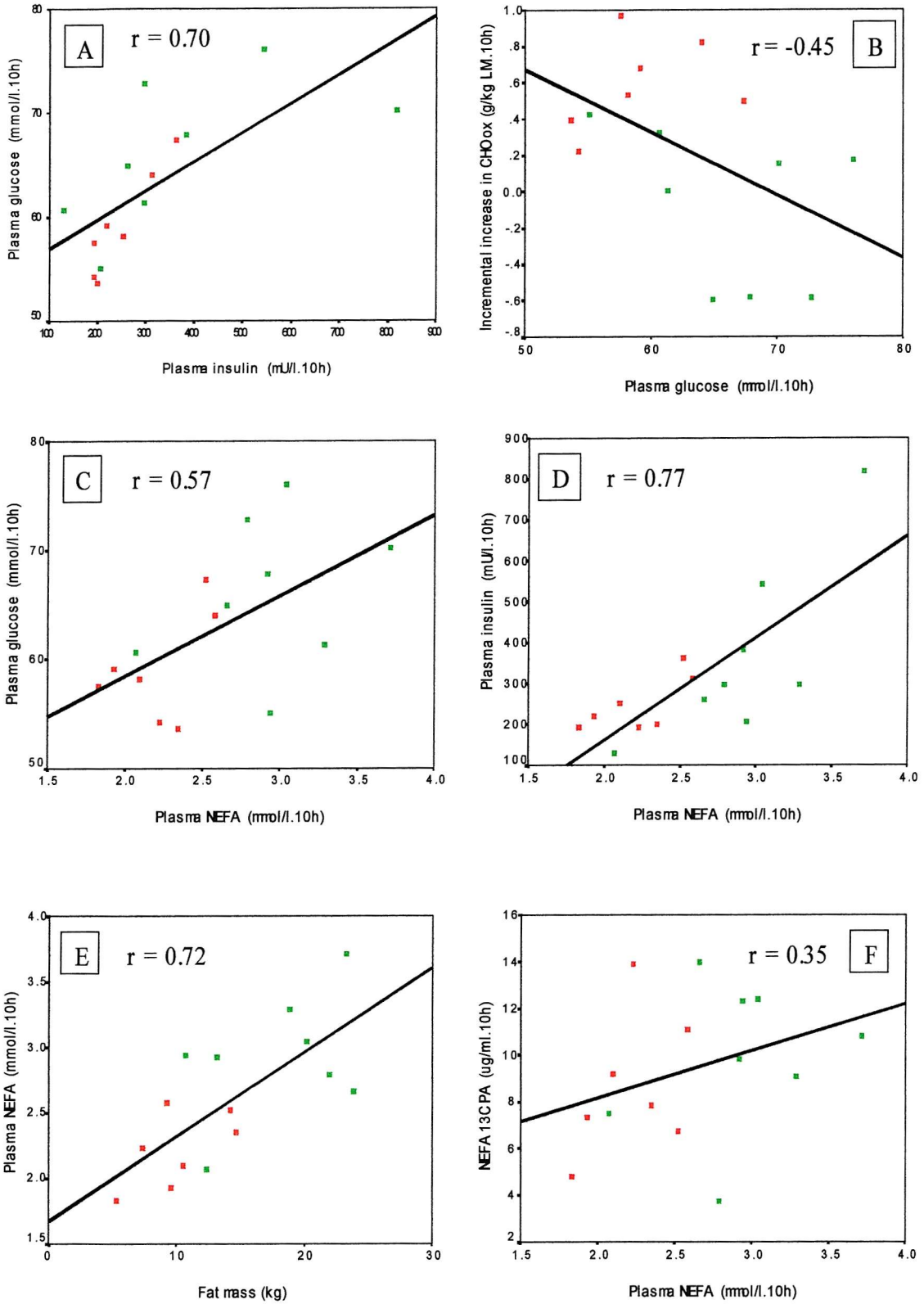


Figure 5.11 Continued....

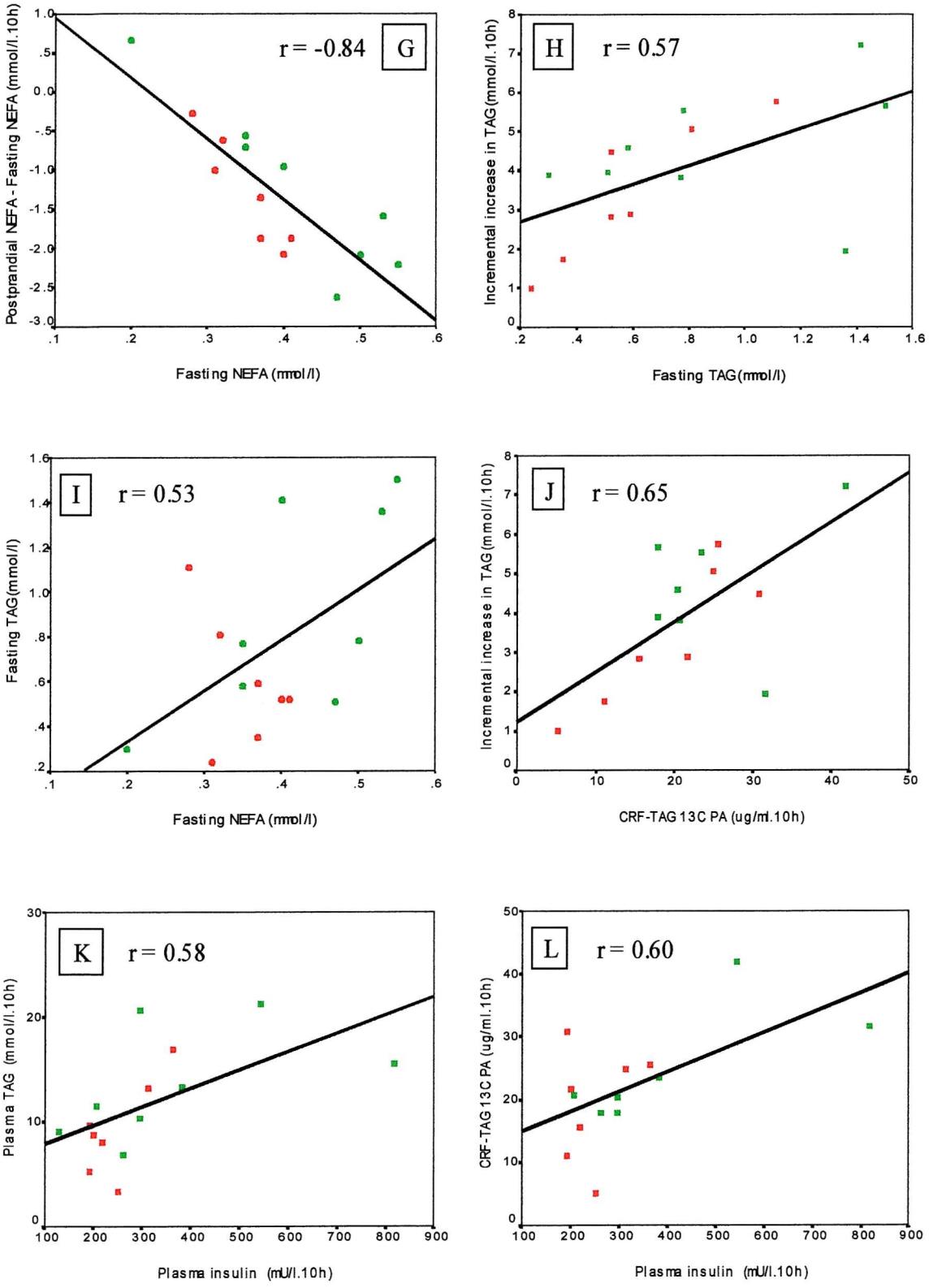


Figure 5.11 Continued....

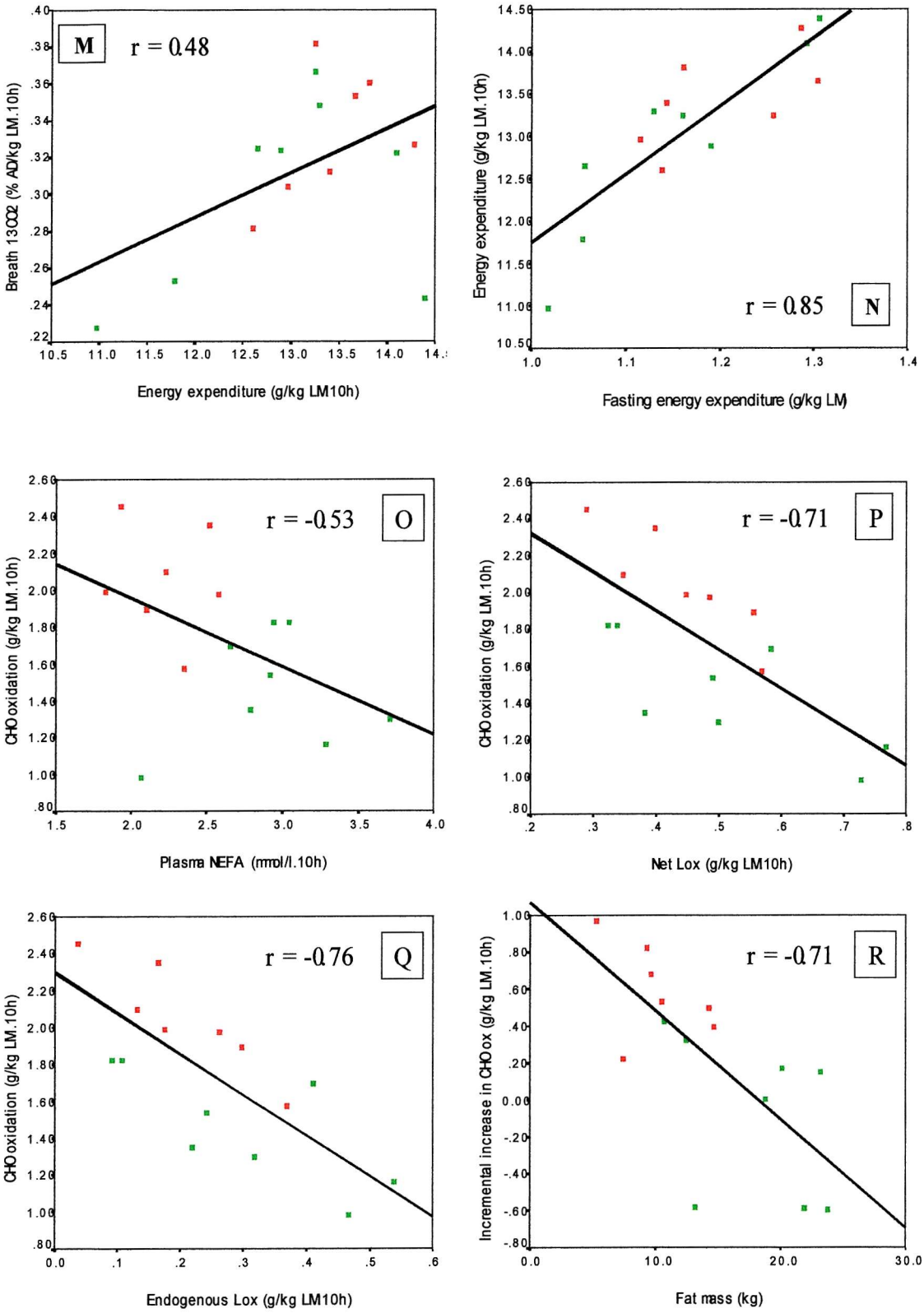
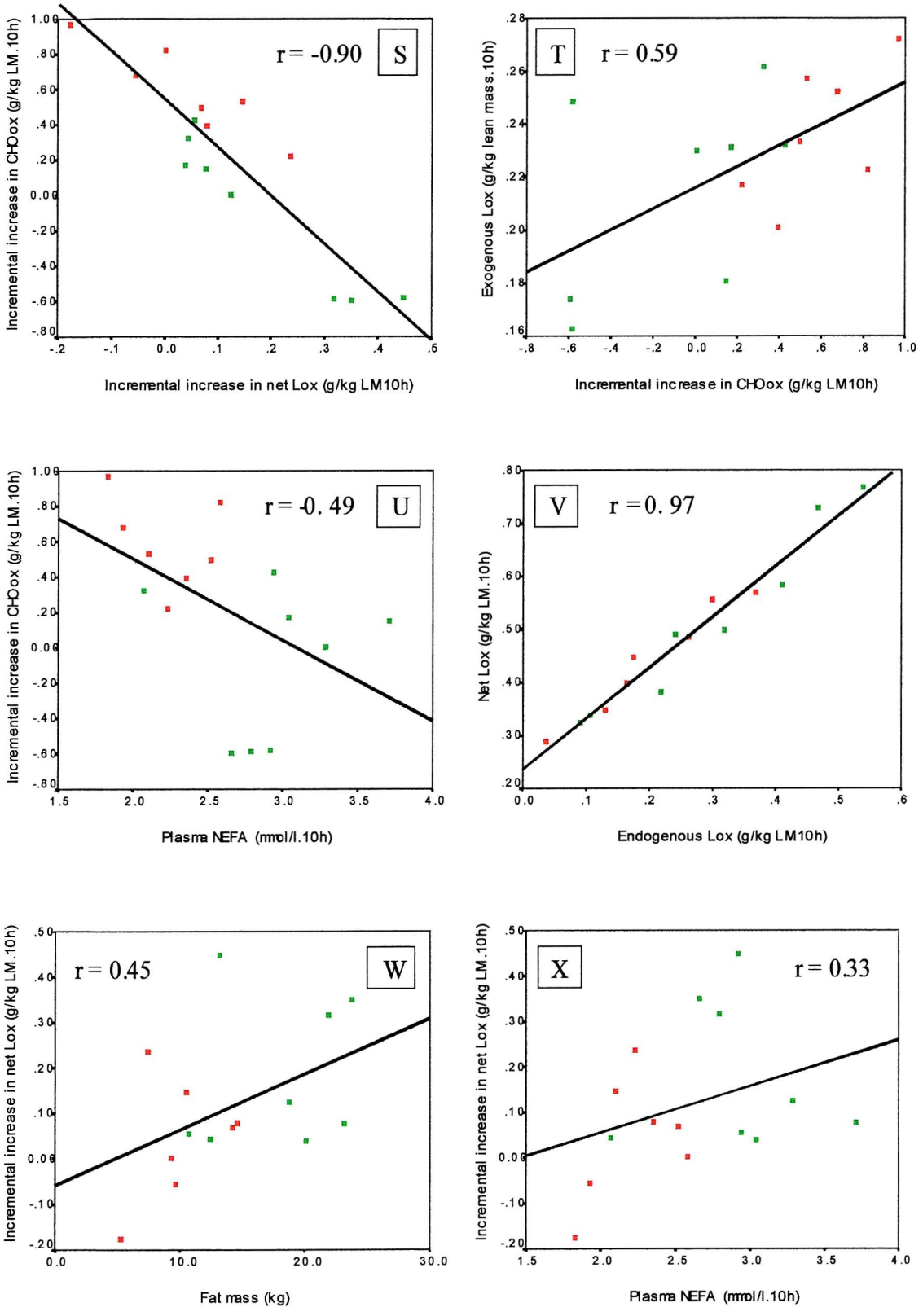


Figure 5.11 Continued....



5.5 Discussion

The present study describes for the first time the age-related changes on the postprandial processing of exogenous TAG in young versus middle-aged men by tracing the metabolism of exogenous lipid [(1,1,1 $^{13}C_3$) tripalmitin] through different pools of the body following the intake of two standard test meals (in sequence) while controlling for evening meal and physical activity. The lipid content of the test meals represented more typical amounts of lipid as used in normal daily life. In this study, particular attention was given to the (a) postprandial lipid metabolism in circulation and (b) postprandial energy expenditure and substrate oxidation.

The major finding of the present study was that increasing age was associated with abnormalities in postprandial lipid metabolism such as decreased postprandial clearance of exogenous TAG from circulation and decreased exogenous lipid oxidation. The other abnormalities observed with increasing age were higher postprandial glucose and insulin concentrations; higher fasting and postprandial TAG and NEFA concentrations and decreased postprandial CHO oxidation.

5.5.1 Fasting and postprandial responses of plasma glucose, insulin, NEFA, TAG and ^{13}C palmitic acid in chylomicron TAG and free fatty acid fraction.

5.5.1.1 Plasma glucose

Elevated fasting glucose and postprandial glucose concentrations in middle-aged men than control suggests impaired insulin mediated glucose disposal with increasing age. Many previous studies also report that glucose utilisation declines with age (Rosenthal *et al.,* 1982; Rowe *et al.,* 1983; Pagano *et al.,* 1984). Since the middle-aged men had higher postprandial insulin concentrations, a significant positive correlation between postprandial insulin AUC and postprandial glucose AUC (r=0.70, p=0.001; Figure 5.11A) suggests that part of impaired glucose disposal in middle aged men might be due to insulin resistance.

The impaired insulin mediated glucose disposal in middle-aged men could be at the level of decreased uptake and oxidation of glucose by muscle. The observation of lower CHO oxidation in middle-aged men than young control and a negative correlation between postprandial glucose AUC and incremental increase in CHO oxidation AUC (r= -0.49, p=0.07; Figure 5.11B) suggests that decreased glucose uptake and oxidation might be partly responsible for increased postprandial glucose concentrations in middle-aged men.

Lower non-oxidative glucose disposal inside the cell (*ie.* glycogen synthesis and lactate formation) can also play a role in postprandial hyperglycemia. The modest glycaemic response in both middle-aged men and young controls after the first meal and not after the second meal suggests that there was increased non-oxidative glucose disposal (*ie.* increased glycogen synthesis) when the glycogen stores were depleted after longer fasting period. However, after the first meal, since the glycaemic response was higher in middle-aged men than controls, decreased glycogen synthesis in middle-aged men might also be partly responsible for their higher postprandial glucose concentrations. There is also evidence that insulin resistance is also a consequence of a reduced glucose deposition as glycogen in muscle, which makes up 40% of body weight (Beck-Nielsen *et al.,* 1992).

Finally, increased hepatic glucose output due to increased gluconeogenesis in insulin resistant state might also result in increased postprandial glucose concentrations. The observation of significantly higher fat mass and postprandial NEFA concentrations in middle-aged men than controls and positive correlation of NEFA AUC to a) glucose AUC (r=0.57, p=0.03), b) insulin AUC (r=0.77, p=0.001) and c) fat mass (r=0.72, p=0.001) (Figure 5.11 C, D, and E respectively) suggests that higher NEFA concentrations along with insulin resistance in middle-aged men might have contributed to higher glucose concentrations due to increased hepatic gluconeogenesis and glucose output. Results of one recent study also supports this idea that impaired suppression of plasma NEFA levels after glucose ingestion lead to higher rate of hepatic glucose output and systemic glucose delivery (Kruszynska *et al.,* 1997). It is suggested that NEFA provide energy for gluconeogenesis, thus resulting in increased hepatic glucose output (Foley, 1992).

5.5.1.2 Plasma insulin

In this study, insulin resistance was not determined directly, rather relative concentrations of insulin were used as the proxy for the degree of insulin resistance. It has been previously shown that in subjects with normal and abnormal glucose tolerance, fasting insulin concentration is the best marker of insulin resistance as determined by whole body glucose uptake using euglycaemic hyperinsulinaemic clamp technique (Laakso, 1993). Since middle aged men had higher fasting and postprandial insulin concentrations than young controls, they were considered more insulin resistant relative to young controls. Higher insulin concentrations are also confirmed by previous studies showing that human and animal ageing are characterised by progressive increase in fasting and postprandial insulin levels (Reaven & Reaven, 1985; Barzilai & Rossetti, 1996). In this

study the exact mechanism of hyperinsulinaemia was not known but it could be due to higher insulin production by pancreatic beta cells or decreased clearance by liver or both of these.

Insulin resistance in middle-aged men relative to young men was further evident from their higher fasting NEFA concentration and lesser suppression of NEFA in the postprandial state. A strong positive relationship between postprandial insulin AUC and postprandial NEFA AUC (r=0.77, p=0.001; Figure 5.11C) also suggest that part of lack of suppression of NEFA could be due to insulin resistance.

It was not clear from this study whether higher NEFA concentrations were cause or consequence of relatively higher insulin resistance in middle-aged men. Some studies have shown that plasma NEFA concentrations are largely determined by the action of insulin to suppress adipocyte lipolysis, by suppressing hormone sensitive lipase (Weiland et al, 1980; Yki-Jarvinen & Taskinen, 1988) and promoting re-esterification of NEFA (Coppack *et al.,* 1992). While other studies have shown that increased plasma NEFA produce peripheral insulin resistance in diabetic subjects (Boden & Chen, 1995) and can also stimulate acute insulin secretion (Felber & Vanotti, 1994). Recently it has been found in dogs that NEFA can impair hepatic insulin extraction *in vivo* at high and low insulin levels, an effect that may contribute to peripheral hyperinsulinaemia of obesity (Wisenthal *et al.,* 1999). It is also suggested that free fatty acids or products of free fatty acids may decrease the number of surface receptors by increasing the rate of insulin receptor internalisation, decreasing the rate of receptor recycling, or both.

5.5.1.3 Plasma NEFA and ^{13}C PA in NEFA fraction

Middle aged-men had significantly higher circulating NEFA (as AUC) than young controls ($p<0.05$). Since insulin plays an important role in the suppression of NEFA in postprandial state, higher NEFA concentrations in middle-aged men might be due to their increased insulin resistance relative to young controls. There are number of mechanisms by which insulin lowers plasma NEFA levels e.g. inhibition of TAG hydrolysis in the adipose cell to FFA and glycerol (Havel, 1965) and together with glucose, promotion of re-esterification of FFA in to TAG in adipose cells (Wolfe & Peters, 1987). Hence, the mechanisms responsible for higher postprandial NEFA concentrations in middle-aged men might be a) decreased adipocyte suppression of NEFA and/or b) decrease entrapment of NEFA by tissues (such as adipose and muscle) after hydrolysis by LPL. In middle-aged

men, the observation of lesser decrease in NEFA concentration along with higher increase in ^{13}C PA in NEFA fraction suggest that higher postprandial increase in NEFA was due to both lesser adipocyte suppression of NEFA and poor entrapment of ^{13}C PA by tissues after hydrolysis of CRF-TAG by LPL. A weak non significant correlation between NEFA AUC and ^{13}C PA in NEFA (r=0.35, p=0.20; Figure 5.11F) suggests that higher NEFA AUC in middle-aged men might be predominantly due to decreased suppression of NEFA rather than decreased entrapment of ^{13}C PA. Furthermore, a strong negative correlation between incremental increase in NEFA AUC and fasting NEFA concentrations (r=-0.84; p=0.001; Figure 5.11 G) suggest that higher NEFA AUC in middle-aged men might be predominantly due to lesser suppression of higher basal NEFA concentrations.

5.5.1.4 Plasma TAG and CRF-TAG ^{13}C PA

Middle-aged men had higher fasting TAG than young controls, however, the differences between the groups were not statistically significant (p=0.14). Fasting TAG, which reflect the size of VLDL pool, is known to be a strong determinant of postprandial TAG increment in normal subjects (Cohn *et al.,* 1988b). In this study a significant correlation between fasting TAG and postprandial TAG increment (r=0.57, p=0.03; Figure 5.11H) also suggest the contribution of VLDL in the postprandial lipaemia. Furthermore, a significant positive correlation between fasting NEFA and fasting TAG (r=0.53, p=0.01; Figure 5.11 I) suggest that part of the source of TAG might be VLDL as fasting NEFA serves as a source of FA for VLDL TAG synthesis. However, this relationship only explain part of variation in fasting plasma TAG to be due to VLDL. This raises the question that whether the fasting TAG in the insulin resistant state represents the size of the pool of VLDL and/ or VLDL remnants or both VLDL and chylomicron and their remnant? Similarly, to what extent is the TAG in fasting VLDL is derived from lipid within the meal?

Since the postprandial TAG concentrations continually increase after every meal in the insulin resistant state due to decreased clearance and greater entry in to circulation of TAG eaten in the previous meal along with the TAG eaten in the recent meal (as observed in middle-aged men), it is suggested that fasting TAG might reflect both VLDL and chylomicron and their remnants. It is further suggested that, in the insulin resistant state, after a evening dinner the residence time of exogenous lipid concentration in the circulation might further prolong in to the late fasting state as the physical activity (which helps to clear lipids from circulation) is minimal during the night. Finally, increased

incorporation of exogenous fatty acids (from chylomicron remnants and exogenous NEFA) in the VLDL TAG in liver might further result in higher TAG concentrations containing exogenous TAG (as insulin resistance fails to shut the release of VLDL in the postprandial state). Previous studies have shown that NTG type II diabetic patients and hypertriglyceridaemic patients had increased amounts of fasting apoB-48 (Karpe *et al.,* 1993b; Curtin *et al.,* 1996). Further stable isotopically labelled TAG studies are needed to confirm the presence of chylomicron and their remnants in the fasting state by giving labelled TAG with the regular meals for 24 hours and then measuring CRF-TAG ^{13}C PA in the morning after 12-h fasting.

Postprandial TAG AUC was also tended to be higher in middle-aged men than young controls, however, the differences were not statistically significant ($p=0.12$). The results of the present study support the results of a previous study (Cohn *et al.,* 1988) which also found higher plasma TAG responses in older subjects than young controls (66 years *v* 29 years). Since plasma TAG does not gives the relative contribution of endogenous and exogenous TAG in circulation, the present study used ^{13}C labelled TAG to trace the exogenous lipid in circulation. Tracer fatty acid data of this study showed that CRF-TAG ^{13}C PA was also tended to be higher in middle-aged men than young controls, however, the differences were not statistically significant ($p=0.27$). Furthermore, a significant positive correlation of incremental increase in TAG with CRF-TAG ^{13}C PA ($r=0.65$, $p<.01$; Figure 5.11 J) suggest that part of the increase in postprandial TAG concentration was due to CRF-TAG. Previous studies, using retinyl ester as the marker of chylomicron, have also shown that chylomicron retinyl esters response was higher in older men than young controls (Krasinski *et al.,* 1990a; Borrel *et al.,* 1998).

Higher trend of postprandial CRF-TAG ^{13}C PA responses in middle-aged men can be due to a) delayed absorption of TAG b) increased production and release of chylomicron in to circulation c) decreased clearance of chylomicron TAG at the level of LPL and chylomicron remnant at the level of hepatocyte. The sharp increase in CRF-TAG tracer FA during the initial postprandial period in middle-aged men suggests that delayed absorption or delayed gastric emptying in the middle-aged men was not a factor in higher postprandial lipaemia in this group. A previous study, using retinyl ester as the marker of chylomicron, observed lower chylomicron TAG and retinyl ester concentrations over the initial hours after the meal intake in the elderly and suggested that appearance of lipids and vitamin A was slightly delayed in these subjects due to lower gastric emptying rate in

elderly (Borel *et al.,* 1998). This difference between the two studies could be a) due to higher age of the subjects in Borel *et al.* (1998) study than the present study (64-72 years *v* 47-58 years) and/or b) due to differences in methodologies for tracing exogenous lipids. Previously, the use of vitamin A had been criticised for its delay in absorption. As the peak tracer fatty acid responses were not different between the two groups, it is suggested that the higher postprandial tracer fatty acid response in middle-aged men might not be due to enhanced formation and secretion of chylomicrons.

The decline of tracer fatty acid in CRF-TAG after the peak both after the first and second meal was significantly slower in middle-aged men than young controls. This suggests that the trend of higher postprandial lipaemia in middle-aged men was due to slower clearance of exogenous lipoproteins especially after the second meal due to greater entry in to circulation of chylomicrons containing TAG not only from the most recent meal but also from the previous meal. The delayed clearance of chylomicrons from the plasma is supported by the previous studies showing delayed clearance of retinyl esters in triglyceride-rich lipoprotein fraction in older subjects (Krasinski *et al.,* 1990a; Borrel *et al.,* 1998).

The slower clearance of exogenous TAG might be due to a) insulin resistance related decreased LPL activity b) greater competition between chylomicron and chylomicron remnants with VLDL for their TAG lipolysis by LPL and c) greater competition between chylomicron and VLDL remnants for removal by the liver, which is a saturable process (Berr,1992). LPL is an insulin dependent enzyme, the activity of which is decreased with increasing age (Huttunen *et al.,* 1976; Mahley *et al.,* 1981; Wilson & Chan, 1983), in obesity (Eckel, 1987), and in insulin resistant subjects (Chen *et al.,* 1994; Knudsen *et al.,* 1995). Since middle-aged men had relatively higher BMI (25.8 *v* 23.2 kg/m^2) and fat mass (18 *v* 10 kg) and were relatively insulin resistant (because of relatively higher fasting and postprandial insulin concentrations) than young controls, the decreased activity of LPL might explain the slower clearance of CRF-TAG ^{13}C PA in this group. Furthermore, a correlation of postprandial insulin AUC with postprandial TAG AUC (r=0.58, p=0.02) and CRF-TAG ^{13}C PA (r=0.60, p=0.02; Figure 5.11 K & L) suggest that slower clearance may be a consequence of insulin resistance related decreased activation of LPL activity. It has recently been shown that the proportion of retinyl ester was higher in the chylomicron fraction (sf > 1000) than sf < 1,000 fraction. This would

support the suggestion of slower lipolysis of chylomicrons by LPL in elderly subjects (Borel *et al.,* 1998).

In the insulin resistant state a greater competition between chylomicrons and VLDL for their TAG removal might also be responsible for slower CRF-TAG ^{13}C PA removal in middle-aged men. The antilipolytic effect of a mixed meal in adipose tissue is reduced in obesity (Roust & Jensen, 1993) and insulin resistance (Frayn, 1993). It has been shown that intra-abdominal adipocyte have a higher sensitivity to the stimulation of lipid mobilisation processes, and that the antilipolytic effect of insulin on these cells is less than on the subcutaneous adipocytes. Failure to suppress both the supply of NEFA from adipose tissue to liver after a meal and VLDL synthesis and release in the insulin resistant state result in sustained VLDL production in the postprandial state (Malmstrom *et al.,* 1997a). Furthermore, NEFA which have not been trapped after the hydrolysis by LPL might also result in greater VLDL production after their uptake by liver in insulin resistant state. Since middle-aged men had significantly greater fat mass and higher postprandial insulin levels than young controls, the overproduction of VLDL along with decreased activation of LPL in the postprandial period might result in greater competition between endogenous and exogenous TRL for the same lipolytic pathway. Furthermore a positive correlation between postprandial insulin AUC and NEFA AUC ($r=0.77$, $p=0.001$) (Figure 5.11 D) suggest that lack of suppression of NEFA might have resulted in greater VLDL production thus leading to greater competition with chylomicrons for their TAG to be removed by LPL. Previous studies have also suggested that rate of supply of NEFA to the liver is the major factor controlling hepatic TAG secretion (Byrne *et al.,* 1991, Kissebah *et al.,* 1974). Hence, in the insulin resistant state (such as increasing age) decreased suppression of NEFA after every meal during a day might result in higher VLDL production and secretion and greater competition with chylomicrons for their TAG to be removed by LPL.

Although chylomicron remnants were not measured in this study, impaired hepatic uptake of chylomicron remnant, might indirectly cause accumulation of intestinally derived particles, as chylomicron remnant removal appears to be the rate-limiting step of postprandial TAG clearance in humans (Berr, 1992). Studies in rats also suggest that chylomicron and VLDL remnants compete for the same removal process (Cooper *et al.,* 1982). Hence, decreased clearance of chylomicrons could also be due to decreased removal of chylomicron remnants by liver as a result of greater competition between

VLDL and chylomicron remnants. Finally, delayed clearance of CRF-TAG in middle-aged men might also be due to diminished hepatic remnant receptor activity. However, one study failed to establish a clear relationship between ageing and chylomicron remnant receptor activity (Mahley *et al.,* 1981).

In summary, it is suggested delayed clearance of CRF-TAG ^{13}C PA in middle aged men might be related that impaired lipolytic processing of chylomicrons due to lower LPL activation by insulin and greater competition between chylomicron and VLDL for their TAG removal or ineffective removal of remnant particles by hepatic receptors.

5.5.1.5 Effect of second unlabelled meal on the appearance of labelled FA in CRF-TAG as ingested in the previous meal.

The administration of second unlabelled meal resulted in significantly higher appearance of previously ingested labelled FA in the CRF-TAG fraction in middle-aged men than young controls. Previous studies reported similar findings, when successive meals were consumed (Peel *et al.,* 1993; Fielding *et al.,* 1996). In the study of Fielding *et al.* (1996) the early postprandial peak (at 1 hour after the second meal) was found to contain large proportion of fatty acids (linoleic acid) from the first meal, whereas at later time points the fatty acids in chylomicron-TAG resembled more closely to that in the second meal. These authors suggested that the second meal resulted in the release of preformed chylomicrons from the first meal which might be stored at their site of synthesis in the enterocyte or in lymphatics. The results of the present study also suggested that preformed chylomicrons containing ^{13}C palmitic acid were released due to the administration of second meal. Significantly higher concentration of CRF-TAG ^{13}C PA and significantly slower clearance of CRF-TAG ^{13}C PA later postprandial period in middle-aged men suggests that decreased clearance of chylomicrons might have resulted in negative feed back to gut thus slowing down chylomicron entrance in to the circulation and holding them back at the level of lymphatics and/or enterocyte (because of its high surface area). Ingesting further meals might result in releasing of held back chylomicrons along with those containing TAG from the most recent meal. The holding back of chylomicrons might represent a protective mechanism to prevent further exceeding the metabolic competence of clearing TAG from circulation when there is a problem of TAG clearance as in insulin resistant state.

5.5.2 Energy expenditure and substrate oxidation

5.5.2.1 Energy Expenditure and Thermic Effect of Food

The postprandial EE/kg lean mass was only lower during the initial hours of postprandial period after the first meal in middle-age men than young controls. The AUC for postprandial EE/kg lean mass was lower in middle-aged men than young controls, however, the differences were between the groups were not statistically significant (p=0.27). Previously, it has also been shown that rates of fasting and postprandial EE are smaller in aged than young controls (Vaughan *et al.,* 1991). The exact mechanisms by which age effect the energy expenditure are not known (Weyer *et al.,* 1999). Since the young men had higher trend towards breath $^{13}CO_2$ excretion, a positive correlation, although non significant (r=0.48, p=0.07), between postprandial EE energy expenditure and breath $^{13}CO_2$ suggest that the trend of higher postprandial EE in young men might be associated with increased exogenous lipid oxidation (Figure 5.11 M). Furthermore, since the young men had higher trend towards basal EE, a strong positive correlation between basal EE and postprandial EE AUC (r=0.85, p=0.001) suggests that basal EE is the major determinant of postprandial EE in this group of subjects (Figure 5.11 N).

Incremental increase in energy expenditure/kg lean mass or thermic effect of food (TEF) was not different between the two groups. There is a debate regarding the effect of ageing on TEF. Previous studies suggest either decreased TEF in older persons (Schutz *et al.,* 1984; Schwartz *et al.,* 1990; Throne & Wahren, 1990) or no age-related change (Bloesch *et al.,* 1988; Fukagawa *et al.,* 1991; Visser *et al.,* 1995). One possible explanation for this difference is that TEF does decrease with ageing but is not detected in some studies due to methodological error. For example previous studies have used only one small test meal, whereas impaired thermogenesis might be observed more readily after the consumption of large meals that challenge the capacity of tissues contributing to TEF to respond maximally. Alternatively, some factor other than age may have accounted for positive results in some studies. Recently the effect of large test meals was investigated to study TEF in young and older women (Melanson *et al.,* 1998). The results of that study strongly suggested that there was no decrease in TEF with increased ageing in women for meal sizes ranging from 0-4184 kJ.

5.5.2.2 CHO oxidation

Postprandial CHO oxidation and incremental increase in CHO oxidation were significantly higher in young controls than middle-aged men ($p<0.05$). One previous study has also shown a trend of lower glucose oxidation in older men compared to young men (Toth *et al.,* 1996). Although the factors and mechanisms underlying reduced glucose oxidation are not known, but a number of plausible mechanisms exists. Randle *et al.* (1963) postulated that increased availability of NEFA will direct metabolism towards lipid oxidation and away from glucose oxidation. Kim *et al.,* (1995) had demonstrated that glucose uptake was decreased after 2 hours of *in vivo* infusion of fatty acids. As the postprandial CHO oxidation was significantly lower in middle-aged men, the observation of a significant negative correlation of CHO oxidation AUC with a) postprandial NEFA AUC ($r= -0.53$, $p=0.05$), b) postprandial net Lox AUC ($r= -0.71$, $p=0.001$) and c) endogenous Lox AUC ($r= -0.76$, $p=0.001$), suggest that Randle hypothesis appears to explain at least part of the variances in decreased CHO oxidation observed in middle-aged men (Figure 5.11 O, P & Q). Furthermore, it has also been speculated that reduced glucose oxidation in NIDDM is merely a consequence of reduced glucose transport in to the cells with less glucose available to traverse the oxidative pathway (Thorburn *et al.,* 1990). Significantly higher glucose AUC in middle-aged men than controls suggest that part of the decreased CHO oxidation in middle-aged men might be due to decreased glucose transport to the cells.

In this study, the incremental increase in CHO oxidation AUC was strongly and inversely correlated to fat mass ($r= -0.71$, $p=0.001$), incremental increase in net Lox AUC ($r= -0.90$, $p=0.001$) and positively correlated to exogenous lipid oxidation ($r=0.59$, $p=0.02$) (Figure 5.11 R, S & T). Moreover, postprandial NEFA was negatively correlated to incremental increase in CHO oxidation AUC ($r= -0.49$, $p=0.06$; Figure 5.11U). Since, the middle-aged men had higher fat mass, lower incremental increase in CHO oxidation as well as lower exogenous Lox and trend of higher net Lox, the above relationships suggest that rate of net lipid oxidation is an important determinant of CHO oxidation and that increase in net Lox and decline in exogenous Lox, is in part, responsible for the decrease in CHO oxidation in middle-aged men. In summary, these results suggests that decreased CHO oxidation in middle-aged men appears to be a consequence of insulin resistance and high fat mass in this group.

5.5.2.3 Lipid oxidation

Young men showed a trend towards higher basal net lipid oxidation/kg lean mass compared to that seen in middle-aged men. Previous investigators, examining age related differences in basal lipid oxidation between older and younger volunteers, have reported conflicting results in this respect. Felber & co-workers (1987) found no differences in basal lipid oxidation between older and younger volunteers, whereas Bonadonna *et al.* (1994) reported higher rates of basal lipid oxidation in older individuals and Calles-Escandon *et al.,* (1994) reported lower fasting lipid oxidation in older individuals. Results of one of these studies suggested that ageing *per se* does not result in the decrease in lipid oxidation in elderly women but the decreased lipid oxidation is more related to the alterations in body composition, especially the decrease in fat free mass (Calles-Escandon *et al.,* 1994). While the results of other study suggested that lipid oxidation is more related to fat mass than to lean body mass in obese women (Schutz *et al.,* 1992). Further studies, with large sample population, are needed to resolve this controversy.

In contrast to basal net Lox, middle-aged men showed a trend towards higher postprandial net Lox and incremental increase in net Lox than young controls. There are not many studies examining the effect of age on postprandial lipid oxidation. The results of one recent study showed decreased postprandial Lox in older women (Melanson *et al.,* 1997). The differences between this study and the previous study could due to differences in gender (men *v* women), age [52.1(1.3) *v* 72.3(2.1 years)] and size of the meal (3247 *v* 4184 kJ). In the present study, a strong positive correlation between postprandial net Lox and endogenous Lox suggest that increase in net Lox in middle-aged men was mainly due to increase in endogenous Lox ($r=0.97$, $p=0.001$; Figure 5.11 V). Despite the trend towards higher postprandial and incremental net Lox, middle aged men showed a trend of lower exogenous Lox than young controls. This again suggests that increases in net Lox in middle-aged men are mainly due to endogenous Lox. The source of higher endogenous Lox could be higher fat mass and higher circulating NEFA. Although middle-aged men had significantly higher fat mass and postprandial NEFA concentrations, a weak and non significant positive correlation of incremental increase in net Lox with fat mass ($r= 0.45$, $p=0.09$) and NEFA AUC ($r=0.33$, $p=0.22$) suggest that only a small part of increased net Lox in middle-aged men might be due to increased availability of NEFA due to higher fat mass (Figure 5.11 W & X).

Decreased exogenous Lox in middle-aged men could be due to a) decreased hydrolysis of CRF-TAG ^{13}C PA, b) decreased entrapment of released ^{13}C PA by skeletal muscle after hydrolysis and c) increased storage of ^{13}C PA in muscle or adipose tissue. Since middle-aged men had a trend of higher CRF-TAG ^{13}C PA and NEFA ^{13}C PA in circulation than young controls, both decreased hydrolysis of CRF-TAG ^{13}C PA and decreased entrapment of NEFA ^{13}C PA might be responsible for decreased exogenous Lox in middle-aged men.

In the present study, the incremental increase in net Lox was positively correlated to fat mass ($r= 0.45$, $p=0.09$) and negatively correlated to incremental increase in CHO ox ($r= -0.90$, $p=0.001$; Figure 5.11 W & S). Since, middle-aged men had higher fat mass and lower incremental increase in CHO oxidation AUC, the above relationships suggest that rate of lipid oxidation is an important determinant of CHO oxidation and that increase in net Lox, is in part, responsible for the decrease in CHO oxidation in middle-aged men.

5.6 Summary

The results of the present study suggests that presence of higher fasting TAG (a marker for decreased metabolic competence for clearing lipids) and insulin resistance with increasing age results in a) decreased postprandial clearance of exogenous TAG from circulation b) decreased exogenous lipid oxidation c) increased appearance of exogenous fatty acids in NEFA fraction and d) greater entry in to circulation of chylomicrons containing lipids from the previous meal along with those containing lipids from the most recent meal. Since the men in the increased age group had significantly higher fat mass along with higher fasting TAG, it was not clear that whether the age *per se* or higher fat mass or their mutual interaction resulted in impaired postprandial lipid metabolism in this group.

CHAPTER 6

Postprandial Lipid Metabolism in Hyper-Triglyceridaemic and Normal-Triglyceridaemic Type II Diabetics

6.1 Introduction

Patients with type II diabetes have high rates of vascular disease (Betteridge, 1994; Fuller *et al.* 1996; Yudkin *et al.,* 1996). Several studies have shown that mortality due to coronary artery disease (CAD) is two to four times greater in individuals with diabetes than in those without diabetes (Kleinman *et al.,* 1991; Wilson & Kannel, 1992). Although cardiovascular risk is markedly increase in type II diabetes the excess morbidity cannot be explained by conventional risk factors (Taskinen, 1990). However, hypertriglyceridaemia in type II diabetes has been found to qualify as an independent predictor of CAD among type II diabetes in both cross-sectional (West *et al.,* 1983) and prospective studies (Fontbone *et al.,* 1989). The mechanisms underlying fasting hypertriglyceridaemia in type II diabetes are unclear.

Patients with diabetes mellitus also exhibit abnormal triglyceride response in the postprandial state. There is some disagreement regarding the postprandial triglyceride response in normal-triglyceridaemic (NTG) type II diabetic. For example, Reznik *et al.* (1996) and Cavallero *et al.,* (1992, 1994) demonstrated that NTG type II diabetics exhibited greater postprandial TAG response as compared to controls. On the other hand other researchers (Lewis *et al.,* 1991; Tan *et al.,* 1995; Cooper *et al.,* 1996) failed to show differences in postprandial TAG response in NTG type II diabetics versus controls. While there is some inconsistency regarding postprandial lipid metabolism in NTG type II diabetes, it is established that the magnitude of postprandial hyperlipidaemia is greater in hypertriglyceridaemic (HTG) type II diabetes. As our understanding of physiology of postprandial lipaemia is poor, the mechanisms responsible for the postprandial hyperlipidaemia in type II diabetes are also not very clear. It is not clear that whether there is a defect in exogenous or endogenous or both exogenous and endogenous lipoprotein metabolism in HTG type II diabetes at the levels of a) synthesis and

secretion of TAG-rich particles into the circulation, b) clearance of TAG from the particle by lipoprotein lipase and c) uptake of remnant particles by liver. There are not many studies seeking to examine the relative role of both endogenous and exogenous lipids in postprandial hypertriglyceridaemia. Studies seeking the role of exogenous lipid metabolism have found that hypertriglyceridaemic (HTG) type II diabetic patients show a greater postprandial chylomicron response (Lewis *et al.,* 1991; Tan *et al.,* 1995; Curtin *et al.,* 1996) and greater increase in postprandial chylomicron derived remnant particles (Lewis *et al.*. 1991; Tan *et al.*. 1992; Curtin *et al.,* 1996) than non diabetic subjects. While studies seeking to examine the relative role of both endogenous and exogenous lipids in postprandial hypertriglyceridaemia suggest that there are abnormalities of both forms (Curtin *et al.,* 1994).

Since type II diabetes is associated with insulin resistance and disturbed postprandial lipid metabolism, hypertriglyceridaemia and the association circulating lipids may have with insulin resistance formed the basis of hypothesis for this study which is a part of central hypothesis for this thesis. The hypothesis states that as age increases levels of lipids in circulation also increase depending upon the individual's metabolic competence for clearing lipids from circulation, levels of physical activity and types and amounts of lipids and CHO in diet. The levels of lipids in circulation will then determine the level of insulin resistance, the risk of developing obesity, type II diabetes and atherosclerosis. Figure 1.1, Chapter 1).

The primary difficulty in determining how secretion and clearance of TGRL from gut and liver are integrated in the postprandial state (especially in insulin resistant conditions) is the difficulty in differentiation between exogenous and endogenous lipids in circulation by traditional methodologies. This is discussed in detail in chapter 1 (section 1.1). Use of stable isotope tracer methodology can provide a best available option to study the metabolism of exogenous fat.

Since the mechanisms responsible for disturbed postprandial lipid metabolism are not clear, the current study was initiated to examine the effects of type II diabetes on the postprandial metabolism of exogenous fat. The main objective of the study was to examine the handling of exogenous fat both at the levels circulation and oxidation. ^{13}C labelled tripalmitin was used in the test meal and was traced through different pools of the body (for example, appearance of tracer in chylomicron rich fraction TAG, non-esterified fatty acids fraction and in breath (Figure 1.2, chapter 1).

With in the context of hypothesis, following major question was answered by this study: To what extent does type II diabetes influence the exogenous lipid handling at the level of circulation and oxidation?

6.2 Chapter outline

The following chapter describes a) characteristics of subjects including fasting profiles of various variables, b) changes in postprandial glucose, insulin and lipid responses including ^{13}C PA responses in CRF-TAG and NEFA fraction, c) changes in postprandial energy expenditure (EE), net lipid oxidation (net Lox) and breath $^{13}CO_2$ excretion, d) discussion and e) summary.

6.3 Methodology

6.3.1 Subjects

For the present current study 12 newly diagnosed Type II diabetic patients were recruited from diabetic outpatient clinics in Southampton. None of the patients were taking lipid lowering therapy or any other drugs affecting lipid metabolism such as thiazides, beta-blockers, insulin etc. None of the patients suffered from hypertension, or had evidence of hepatic, renal or thyroid disease. These patients were further classified in to normaltriglyceridaemic or hypertriglyceridaemic depending on their fasting TG levels. These patients were compared against the middle-aged men as described in chapter 5. Informed consent was obtained from all subjects and the study protocol was approved by the Ethical Committee of Southampton and South West Hampshire Health Commission.

The characteristics of the subjects described in Table 6.1. The mean age was significantly higher in NTG diabetics than HTG diabetics and controls ($p<0.05$). HTG diabetics had significantly higher weight (25%), BMI (36%), fat mass (63%), lean mass (19%) and fasting plasma TAG (~ 3.5 fold) than NTG diabetics ($p<0.05$). Similarly, HTG diabetics had significantly higher weight (48%), BMI (53%), fat mass (~1.5 fold), lean mass (21%) and fasting plasma TAG (~ 5 fold) than controls ($p<0.05$). Moreover, HTG diabetics had significantly higher fasting insulin (> 2 fold) and glucose concentrations (76%) than controls ($p<0.05$). Three subjects in the control group had plasma glucose levels on the borderline according to the recent diagnostic criteria for diabetes. Although HTG and NTG diabetics had higher fasting NEFA concentrations than controls but the differences could not reach the statistical significance between HTG and NTG diabetics versus controls ($p=0.08$ and 0.20 respectively). There were no differences in basal energy expenditure/kg lean mass, basal net lipid oxidation/kg lean mass and basal CHO oxidation/kg lean mass between the three groups.

6.3.2 Study Protocol

The subjects followed general protocol as described in chapter 3 (section 3.2). Briefly, on the evening before the study day, subjects were delivered a standard evening meal around 1800 hours and were advised to finish it by 1900 hours and then fast thereafter except for drinking water.

Next morning around 0700 hours the subjects were admitted to CNMU. Baseline blood and breath samples were collected and baseline basal metabolic rate was measured for

20 minutes. At 0800 hours the subjects consumed a ^{13}C labelled TAG (tripalmitin) in an emulsion and a standard test meal. At 1400 hours another unlabelled emulsion and test meal was given. Blood and breath samples were collected before the ingestion of ^{13}C label and at hourly intervals until ten hours after the ingestion of test meal. Similarly whole body CO_2 excretion was determined by indirect calorimetry before the ingestion of label and then at hourly intervals for ten hours after ingesting the test meal. During the study, no additional food or liquids were allowed except for bottled mineral water.

6.4 RESULTS

6.4.1 Blood variables

6.4.1.1 Postprandial plasma glucose responses

The time courses of changes in plasma glucose concentrations after the first and second mixed meal are shown in Figure 6.1. As expected postprandial glucose concentrations were higher in HTG and NTG Type II diabetics than controls. The differences were only significant between HTG diabetics and controls ($p<0.05$). In both HTG and NTG Type II diabetics the peaks were reached at 1 and 2 hours following the first and second meal. On the other hand, in controls peak was only observed after second meal at 1 hour and there was a modest glycaemic response after the first meal.

The mean AUC for glucose responses over the 10 hour postprandial period are shown in Table 6.2. Postprandial AUC for plasma glucose concentration was significantly higher (73%) in HTG type II diabetics than controls ($p<0.003$; Appendix 8.5, ANOVA Tables). AUC for plasma glucose concentration was also higher in HTG type II diabetics than NTG Type II diabetics but the differences did not reach the significance level ($p=0.10$). Postprandial AUC for plasma glucose concentration was also 37% higher in NTG Type II diabetics than controls, however, the differences were not statistically significant ($p=0.42$). Incremental AUC for glucose was highest in HTG diabetics (12.2 mmol/l.10h) and lowest in NTG diabetics (5.8 mmol/l.10h), however, the differences were not significant significant.

The association between plasma glucose and various anthropometric and metabolic parameters are shown in Appendix 8.7. Fasting plasma glucose was significantly positively correlated to fat mass ($r=0.60$); postprandial glucose AUC ($r=0.97$) and incremental increase in glucose AUC ($r=0.49$); fasting insulin ($r=0.70$); fasting NEFA ($r=0.46$) and postprandial NEFA AUC ($r=0.79$); fasting TAG ($r=0.78$) and postprandial TAG AUC ($r=0.76$); incremental increase in TAG AUC ($r=0.47$), and postprandial CRF-TAG ^{13}C PA AUC ($r=0.55$) ($p<0.05$). Fasting glucose was also positively correlated to insulin AUC ($r=0.37$), however, the association did not reach the statistical significance ($p=0.10$). Fasting glucose was negatively correlated to breath $^{13}CO_2$ AUC ($r= -0.35$), however, the association did not reach the statistical significance ($p=0.12$).

The postprandial glucose AUC was significantly positively correlated to fat mass ($r=0.56$); fasting glucose ($r=0.97$) and incremental increase in glucose AUC ($r=0.66$); fasting plasma insulin ($r=0.68$); fasting NEFA ($r=0.45$) and postprandial NEFA AUC ($r=0.79$);

fasting TAG (r=0.78) and postprandial TAG AUC (r=0.77); incremental increase in TAG AUC (r=0.50), and postprandial CRF-TAG ^{13}C PA AUC (r=0.59) ($p<0.05$). Postprandial glucose AUC was also positively correlated to insulin AUC (r=0.38), however, the association did not reach the statistical significance (p=0.10). Postprandial glucose AUC was negatively associated to breath $^{13}CO_2$ (r= -0.38) and exogenous fat oxidation (r= -0.38), however, the association did not reach the statistical significance (p=0.10).

The incremental increase in glucose AUC was significantly positively correlated to fasting glucose (r=0.49) and postprandial glucose AUC (r=0.66); incremental increase in NEFA AUC (r=0.46); fasting TAG (r=0.45) and postprandial TAG AUC (r=0.47) and postprandial CRF-TAG ^{13}C PA AUC (r=0.49) ($p<0.05$). Incremental increase in glucose AUC was also positively correlated to incremental increase in TAG AUC (r=0.40), however, the association did not reach the statistical significance level (p=0.08).

6.4.1.2 Postprandial plasma insulin responses

The time courses of changes in plasma insulin concentrations following the first and second meal are shown in Figure 6.2. Depending upon the fasting insulin concentration, there was a graded postprandial insulin response, with highest responses in HTG and lowest in controls. In HTG, NTG Type II diabetics and controls the peaks were reached at 1, 2 and 1 hour respectively following the first meal and 2, 3 and 1 hour respectively following the second meal. After the first meal, at the later time points, the insulin concentrations were significantly higher in HTG Type II diabetics than controls ($p<0.05$), while the differences were not statistically significant between two diabetic groups and between NTG Type II diabetics and controls ($p>0.05$).

The mean AUC for insulin responses over the 10 hour postprandial period are shown in Table 6.2. Postprandial AUC for plasma insulin concentration was significantly higher (72%) in HTG type II diabetics than NTG diabetics (p=0.04). Similarly, postprandial AUC for plasma insulin concentration was significantly higher (1.5 fold) in HTG type II diabetics than the controls (p=0.002; Appendix 8.5, ANOVA Tables). The differences in insulin AUC were not statistically significant between NTG Type II diabetics and controls ($p>0.40$). Incremental increase in insulin AUC above the base line was significantly higher (1.5 fold) in HTG type II diabetics than controls (p=0.001), however, the differences between two diabetic groups did not reach the significance level (p=0.06). Differences between NTG diabetics and controls were also statistically not significant (p=0.65; Appendix 8.5, ANOVA Tables).

The association between plasma insulin and various anthropometric and metabolic parameters are shown in Appendix 8.7. Fasting plasma insulin was significantly positively correlated to fat mass (r=0.79); lean mass (r=0.55); fasting glucose (r=0.70) and postprandial glucose AUC (r=0.67); postprandial insulin (r=0.80) and incremental increase in insulin AUC (r=0.68); fasting NEFA (r=0.57) and postprandial NEFA AUC (r=0.75); fasting TAG (r=0.69) and postprandial TAG AUC (r=0.73); incremental increase in TAG AUC (r=0.69) and CRF-TAG ^{13}C PA AUC (r=0.54) ($p<0.05$). Fasting insulin was significantly negatively associated to breath $^{13}CO2$ (r= -0.44) and exogenous fat oxidation (r= -0.44) ($p<0.05$).

The postprandial insulin AUC was significantly positively correlated to fat mass (r=0.63); fasting insulin (r=0.80) and incremental increase in plasma insulin AUC (r=0.98); fasting NEFA (r=0.52) and postprandial NEFA AUC (r=0.58); fasting TAG (r=0.51) and postprandial TAG AUC (r=0.55); incremental increase in TAG AUC (r=0.57) and postprandial CRF-TAG ^{13}C PA AUC (r=0.55) ($p<0.05$). Postprandial insulin AUC was also positively correlated to lean mass (r=0.43), fasting glucose (r=0.37) and postprandial glucose AUC (r=0.38), however, the relationship did not reach the statistical significance (p=0.11).

The incremental increase in insulin AUC was significantly positively correlated to fat mass (r=0.54); fasting insulin (r=0.67) and postprandial plasma insulin AUC (r=0.98); fasting NEFA (r=0.48) and postprandial NEFA AUC (r=0.49); fasting TAG (r=0.47) and postprandial TAG AUC (r=0.46); incremental increase in TAG AUC (r=0.49) and CRF-TAG ^{13}C PA (r=0.50) ($p<0.05$).

6.4.1.3 Postprandial plasma NEFA responses

The time courses of changes in plasma NEFA concentrations following the first and second meals are shown in Figure 6.3. After the first meal, both HTG and NTG Type II diabetics showed slower suppression of NEFA concentrations with nadir reached at 2 hours later than the controls in HTG diabetics and 3 hours later than controls in NTG diabetics. After the second meal, suppression of NEFA was slower in all the three groups with the nadir reached at 3 hour in HTG diabetics and controls and at 4 hours in NTG diabetics. Both after the first and second meals, the suppression of NEFA was slower in two diabetic groups than controls. Both after the first and second meals, NEFA concentrations were significantly lower (except fasting and 1 hour time point) at time points before and at nadir in controls than two diabetic groups ($p<0.02$). It is interesting to note the lack of suppression of NEFA in NTG diabetics immediately after the second meal.

The mean AUC for NEFA responses over the 10 hour postprandial period are shown in Table 6.2. Postprandial AUC for plasma NEFA concentration was significantly higher (49%) in HTG type II diabetics than controls ($p<0.01$) (Appendix 8.5, ANOVA Tables). The differences in NEFA AUC were not statistically significant between two diabetic groups and between NTG Type II diabetics and controls ($p=0.35$ and $p=0.14$, respectively). Incremental increase in plasma NEFA AUC was higher in controls than HTG and NTG type II diabetics, however, the differences between the three groups were not statistically significant ($p>0.51$) (Appendix 8.7, ANOVA Tables).

The association between plasma NEFA and various anthropometric and metabolic parameters are shown in Appendix 8.7. Fasting plasma NEFA was significantly positively correlated to fat mass ($r=0.46$); fasting glucose ($r=0.46$) and postprandial glucose AUC ($r=0.45$); fasting insulin ($r=0.57$) and postprandial insulin AUC ($r=0.52$); incremental increase in insulin AUC ($r=0.48$); postprandial NEFA AUC ($r=0.63$); fasting TAG ($r=0.52$) and postprandial TAG AUC ($r=0.50$); and fasting net lipid oxidation (net Lox) ($r=0.57$) ($p<0.05$). Fasting NEFA was also positively correlated to incremental increase in CHO oxidation AUC ($r=0.42$), however, the relationship did not reach the statistical significance ($p=0.07$). Fasting NEFA was significantly negatively correlated to incremental increase in NEFA AUC ($r= -0.90$) and incremental increase in net Lox AUC ($r= -0.50$) ($p<0.05$). Fasting NEFA was also negatively correlated to incremental increase in EE ($r= -0.38$) and basal CHO oxidation ($r= -0.41$), however, the relationship did not reach the statistical significance ($p>0.07$)

Postprandial NEFA AUC was significantly positively correlated to fat mass ($r=0.52$); fasting glucose ($r=0.79$) and postprandial glucose AUC ($r=0.79$); incremental increase in glucose AUC ($r=0.46$); fasting insulin ($r=0.75$) and postprandial insulin AUC ($r=0.58$); incremental increase in insulin AUC ($r=0.49$); fasting plasma NEFA ($r=0.63$); fasting TAG ($r=0.65$) and postprandial TAG AUC ($r=0.64$) and postprandial CRF-TAG ^{13}C PA AUC ($r=0.53$) ($p<0.05$). Fasting NEFA was also positively correlated to incremental increase in TAG AUC ($r=0.43$) and CHO oxidation AUC ($r=0.40$), however, the relationship did not reach the statistical significance ($p>0.06$).

The incremental increase in NEFA AUC was significantly positively correlated to basal CHO oxidation ($r=0.53$) and incremental increase in net Lox ($r=0.49$) ($p<0.05$). Incremental increase in NEFA AUC was also positively correlated to incremental increase in EE ($r=0.42$), however, the relationship did not reach the statistical significance ($p=0.07$).

Incremental increase in NEFA AUC was significantly negatively correlated to fasting plasma NEFA (r=0.79), and basal net Lox (r=0.79) and postprandial net Lox (r=0.79) ($p<0.05$). Incremental increase in NEFA AUC was also negatively correlated to incremental increase in CHO oxidation AUC (r= -0.90) and endogenous net Lox AUC (r= -0.41), however, the relationship did not reach the statistical significance ($p>0.07$).

6.4.1.4 Postprandial plasma TAG responses

The time courses of changes in plasma TAG concentrations following the first and second meal are shown in Figure 6.4. Both the magnitude and duration of postprandial TAG response was higher in HTG diabetics than NTG diabetics and controls. In HTG and NTG Type II diabetics the peaks were reached at 3 and 2 hour after the first and second meals respectively. Where as in controls the peak plasma TAG concentrations were reached at 2 and 1 hour following the first and second meal respectively. Both after the first and second meals, the plasma TAG responses were significantly higher at all time points in HTG diabetics than NTG diabetics and controls ($p<0.004$). The differences were statistically not significant between NTG Type II diabetics and controls. In all the three groups, postprandial plasma TAG concentrations were not increased to a great extent as compared to baseline levels.

The mean AUC for TAG responses over the 10 hour postprandial period are shown in Table 6.2. Postprandial AUC for plasma TAG concentration was significantly higher (3 fold) in HTG type II diabetics than NTG diabetics ($p<0.0001$; Appendix 8.5, ANOVA Tables). Similarly, postprandial AUC for plasma TAG concentration was significantly higher (3.5 fold) in HTG type II diabetics than controls ($p<0.0001$; Appendix 8.5, ANOVA Tables). The incremental increase in TAG AUC was also significantly higher (70%) in HTG type II diabetics than NTG diabetics ($p<0.004$; Appendix 8.7, ANOVA Tables). The incremental increase in TAG AUC was also significantly higher (62%) in HTG type II diabetics than controls ($p<0.004$; Appendix 8.7, ANOVA Tables).

The association between plasma TAG and various anthropometric and metabolic parameters are shown in Appendix 8.7. Fasting plasma TAG was significantly positively correlated to fat mass (r=0.70); lean mass (r=0.50); fasting glucose (r=0.78) and postprandial glucose AUC (r=0.78); incremental increase in glucose AUC (r=0.45); fasting insulin (r=0.69) and postprandial insulin AUC (r=0.51); incremental increase in insulin AUC (r=0.47); fasting NEFA (r=0.52) and postprandial NEFA AUC (r=0.65); postprandial TAG

AUC (r=0.98); incremental increase in TAG AUC (r=0.69); postprandial CRF-TAG ^{13}C PA AUC (r=0.55) ($p<0.05$). Fasting TAG was also positively correlated to endogenous Lox AUC (r=0.36), however, the relationship did not reach the statistical significance (p=0.11). Fasting TAG was significantly negatively correlated to breath ^{13}CO2 excretion (r= -0.51) and exogenous lipid oxidation (exo Lox) (r= -0.51) ($p<0.05$).

The postprandial TAG AUC was significantly positively correlated to fat mass (r=0.73); lean mass (r=0.53); fasting glucose (r=0.76) and postprandial glucose AUC (r=0.77); and incremental increase in glucose AUC (r=0.47); fasting insulin (r=0.73) and postprandial insulin AUC (r=0.55); incremental increase in insulin AUC (r=0.49); fasting NEFA (r=0.49) and postprandial NEFA AUC (r=0.64); fasting plasma TAG (r=0.98); incremental increase in TAG AUC (r=0.79) and postprandial CRF-TAG ^{13}C PA AUC (r=0.57) ($p<0.05$). Postprandial TAG AUC was significantly negatively correlated to breath ^{13}CO2 excretion (r= -0.54) and exo Lox (r= -0.54) ($p<0.05$).

The incremental increase in TAG AUC was significantly positively correlated to fat mass (r=0.67); lean mass (r=0.55); fasting glucose (r=0.47) and postprandial glucose AUC (r=0.50); fasting insulin (r=0.69) and postprandial insulin AUC (r=0.57); incremental increase in insulin AUC (r=0.47); fasting TAG (r=0.69) and postprandial TAG AUC (r=0.79) and postprandial CRF-TAG ^{13}C PA AUC (r=0.51) ($p<0.05$). Incremental increase in TAG AUC was also positively correlated incremental increase in glucose AUC (r=0.40) and NEFA AUC (r=0.43), however, the relationship did not reach the statistical significance ($p>0.06$). Incremental increase in TAG AUC was significantly negatively correlated to breath ^{13}CO2 excretion (r= -0.50) and exo Lox (r= -0.50) ($p<0.05$).

6.4.1.5 Postprandial CRF-TAG ^{13}C PA responses

The time courses of postprandial CRF-TAG ^{13}C PA responses following the first and second meal are shown in Figure 6.5. In all the three groups, ^{13}C PA appeared in CRF-TAG with in the first hour of the label administration. Both the magnitude and duration of Postprandial CRF-TAG ^{13}C PA response was higher in HTG diabetics than NTG diabetics and controls. As ^{13}C PA concentration was initially rising continuously in HTG diabetics, the peak ^{13}C PA concentration in this group occurred one hour later than NTG diabetics and controls (3 hour *v* 2 hour). After the peak, decline of ^{13}C PA concentration in HTG diabetics was slower than NTG diabetics and controls. In all the three groups, ^{13}C PA concentrations were still higher than fasting levels at 6-hour time point following the label administration.

After the second unlabelled meal, both peak ^{13}C PA concentrations and the timing of the peaks were similar in all the three groups. After the second peak the decline of ^{13}C PA was slower in HTG diabetics than NTG diabetics and controls. In all the three groups, CRF-TAG ^{13}C PA concentrations remained higher than fasting levels at 4-hour time point post second meal.

The mean AUC for CRF-TAG ^{13}C PA responses over the 10-hour postprandial period are shown in Table 6.2. Postprandial AUC for CRF-TAG ^{13}C PA concentration was significantly higher (70%) in HTG type II diabetics than NTG diabetics ($p<0.03$; Appendix 8.5, ANOVA Tables). Similarly, postprandial AUC for CRF-TAG ^{13}C PA concentration was significantly higher (62%) in HTG type II diabetics than controls ($p<0.03$; Appendix 8.5, ANOVA Tables). There were no statistically significant differences between NTG diabetics and controls.

The association between postprandial CRF-TAG ^{13}C PA and various anthropometric and metabolic parameters are shown in Appendix 8.7. Postprandial CRF-TAG ^{13}C PA AUC was significantly positively correlated to fasting glucose ($r=0.55$) and postprandial glucose AUC ($r=0.59$); incremental increase in glucose AUC ($r=0.49$); fasting insulin ($r=0.54$) and postprandial insulin AUC ($r=0.55$); incremental increase in insulin AUC ($r=0.50$); postprandial NEFA AUC ($r=0.53$); fasting TAG ($r=0.55$) and postprandial TAG AUC ($r=0.57$) and incremental increase in TAG AUC ($r=0.51$) ($p<0.05$).

6.4.1.6 Postprandial plasma NEFA ^{13}C PA responses

The time courses of postprandial plasma NEFA ^{13}C PA responses following the first and second meal are shown in Figure 6.6. Unexpectedly, both the magnitude and duration of Postprandial plasma NEFA ^{13}C PA response was higher in middle-aged men than HTG and NTG diabetics. The peak plasma NEFA ^{13}C PA concentration occurred at 3 hour in all the three groups. Decline of ^{13}C PA concentration in middle-aged men was slower than HTG and NTG diabetics. After the second unlabelled meal, there was slight increase in NEFA ^{13}C PA concentrations in middle-aged men and NTG diabetics but not in HTG diabetics. After the second meal the decline of NEFA ^{13}C PA was again slower in middle-aged men than HTG and NTG diabetics. Data of ^{13}C NEFA concentrations should be interpreted with caution because the PA peaks detected by GC-IRMS were very small due to little concentration of PA in NEFA fraction, especially after the postprandial suppression of NEFA concentrations.

Very small peaks can give erroneous results in terms of isotopic enrichments and substrate concentrations.

The mean AUC for plasma NEFA ^{13}C PA responses over the 10 hour postprandial period are shown in Table 6.2. Postprandial AUC for plasma NEFA ^{13}C PA concentration was significantly higher (2 fold) in middle-aged men than NTG type II diabetics. Postprandial AUC for plasma NEFA ^{13}C PA concentration was also higher (44%) in middle-aged men than HTG type II diabetics, however, the differences were not statistically significant (p=0.29) (Appendix 8.5, ANOVA Tables).

The association between postprandial NEFA ^{13}C PA and various anthropometric and metabolic parameters are shown in Appendix 8.7. NEFA ^{13}C PA was significantly negatively correlated to fat mass (r= -0.43) ($p<0.05$).

6.4.2 Energy expenditure and substrate oxidation

6.4.2.1 Energy expenditure

The time courses of changes in energy expenditure/kg lean mass following the first and second meal are shown in Figure 6.7. There were no differences in the EE over the postprandial period between the three groups. In HTG diabetics, NTG diabetics and controls the peaks were reached at 1, 2 and 1 hour respectively following both the first and second meals. Six hours post first meal, the energy expenditure values returned near to baseline levels in all the three groups. Four hour post second meal, EE values remained higher than baseline levels in all the three groups.

The mean AUC for energy expenditure/kg lean mass over the 10 hour postprandial period are shown in Table 6.3. HTG diabetics showed a trend towards higher values for postprandial energy expenditure (13.27 kcal/kg leanmass.10h), while controls showed a trend towards lower values (12.92 kcal/kg leanmass.10h). The differences between the groups were statistically not significant (p>0.80) (Appendix 8.5, ANOVA Tables).. The incremental increase in energy expenditure above the baseline levels or the thermic effect of food (TEF) was highest in controls (1.41 kcal/kg leanmass.10h) and lowest in NTG diabetics (1.14 kcal/kg leanmass.10h), however, the differences between the three groups were not statistically significant (p>0.62) (Appendix 8.5, ANOVA Tables)..

The association between energy expenditure (EE) and various anthropometric and metabolic parameters are shown in Appendix 8.7. Basal EE was significantly positively

correlated to postprandial EE (r=0.91); basal net Lox (r=0.58) and postprandial net Lox (r=0.61) and endogenous lipid oxidation (endo Lox) (r=0.57) ($p<0.05$).

The postprandial EE was significantly positively correlated to basal EE (r=0.91); basal net Lox (r=0.46) and postprandial net Lox (r=0.60) and endo Lox (r=0.53) ($p<0.05$).

The incremental increase in EE or TEF was positively correlated to incremental increase in net Lox (r=0.38), however, the association was not statistically significant ($p=0.10$). Incremental increase in EE AUC was negatively correlated to fasting NEFA (r= -0.38) and incremental increase in NEFA AUC (r= -0.42); basal EE (r= -0.42) and basal net Lox (r= -0.39), however, the association was not statistically significant ($p>0.07$).

6.4.2.2 CHO oxidation

The time courses of changes in CHO oxidation/kg lean mass following the first and second meal are shown in Figure 6.8. Although peak CHO oxidation (at 1 hour) was similar in all the three groups, but there was a trend of higher CHO oxidation after the peak until 6 hours post first meal in NTG and HTG diabetics and lower in controls. At 6 hours the CHO oxidation was lower than baseline levels for all the three groups. After the second meal, there was a trend of higher CHO oxidation at peak (at 1 hour) and at later time points in NTG diabetics than HTG diabetics and controls. The differences in CHO oxidation over the postprandial time course were statistically not significant.

The mean AUC for CHO oxidation/kg lean mass over the 10 hour postprandial period are shown in Table 6.3. Postprandial AUC for CHO oxidation was highest (1.70 g/kg leanmass.10h) in NTG diabetics lowest (1.46 g/kg leanmass.10h) in controls, however, the differences between the groups were not statistically significant ($p>0.18$; Appendix 8.5, ANOVA Tables). The incremental increase in CHO oxidation over the baseline levels was also highest (0.29 g/kg leanmass.10h) in NTG diabetics and lowest (-0.09 g/kg leanmass.10h) in controls, however, the differences between the groups were statistically not significant ($p>0.13$; Appendix 8.5, ANOVA Tables).

The association between CHO oxidation and various anthropometric and metabolic parameters are shown in Appendix 8.7. Basal CHO oxidation was significantly positively correlated incremental increase in NEFA AUC (r=0.53); postprandial CHO oxidation AUC (r=0.52) and incremental increase in net Lox AUC (r=0.77) ($p<0.05$). Basal CHO oxidation was significantly negatively correlated to incremental increase in CHO oxidation AUC (r= -0.79) and basal net Lox (r= -0.77) ($p<0.05$). Basal CHO ox was also negatively correlated

fasting NEFA (r= -0.40), however, the relationship did not reach the statistical significance (p=0.07).

The postprandial CHO oxidation was significantly positively correlated to basal CHO oxidation (r=0.52) (p<0.05). Postprandial CHO ox AUC was also positively correlated NEFA AUC (r=0.40), however, the relationship did not reach the statistical significance (p=0.08). Postprandial CHO oxidation was significantly negatively correlated to postprandial net Lox AUC (r= -0.59) and endogenous Lox AUC (r= -0.54) (p<0.05).

The incremental increase in CHO oxidation AUC was significantly positively correlated to basal net Lox (r=0.64) (p<0.05). Incremental increase in CHO oxidation was also positively correlated fasting NEFA (r=0.42), however, the relationship did not reach the statistical significance (p=0.07). Incremental increase in CHO oxidation AUC was significantly negatively correlated to basal CHO oxidation (r= -0.79) and incremental increase in net Lox AUC (r= -0.94) (p<0.05). Incremental increase in CHO oxidation was also negatively correlated incremental increase in NEFA AUC (r= -0.38), however, the relationship did not reach the statistical significance (p=0.09).

6.4.2.3 Net lipid oxidation

The time courses of changes in net lipid oxidation/kg lean mass following the first and second meal are shown in Figure 6.9. There was a trend towards higher postprandial net Lox in HTG diabetics and controls versus NTG diabetics. At I hour after the first meal, the decline in net Lox was highest in NTG diabetics and lowest in controls, whereas, there was no decline in net Lox in HTG diabetics. After the decline the net Lox continued to rise until 6 hours in all the three groups and the values were higher than baseline levels. After the second meal net Lox declined in all the three groups. After the decline a peak was observed only in HTG and NTG type II diabetics at 2 hour and then a further decline until 4 hour in all the three groups. At 4 hour post second meal net Lox returned near to baseline levels in NTG diabetics but was still higher than baseline levels in HTG diabetics and controls. The differences in net lipid oxidation over the postprandial time course were highly non-significant.

The mean AUC for net lipid oxidation over the 10 hour postprandial period are shown in Table 6.3. HTG diabetics showed a trend towards higher (0.55 gm/kg leanmass.10h) postprandial net lipid oxidation whereas NTG diabetics showed a trend towards lower postprandial net Lox (0.45 gm/kg leanmass.10h), however, the differences between the three

groups were not statistically significant (Appendix 8.5, ANOVA Tables). Controls showed a trend towards higher (0.18 gm/kg leanmass.10h) incremental increase in net lipid oxidation whereas NTG diabetics showed a trend towards lower (0.03 gm/kg leanmass.10h) incremental increase in net Lox, however, the differences between the three groups were not statistically significant.

The association between net lipid oxidation and various anthropometric and metabolic parameters are shown in Appendix 8.7. Basal net Lox was significantly positively correlated to fasting NEFA (r=0.57); basal EE (r=0.58) and postprandial EE AUC (r=0.46); incremental increase in CHO oxidation AUC (r=0.64); postprandial net Lox AUC (r=0.70) and endogenous lipid oxidation (r=0.64) ($p<0.05$). Basal lipid oxidation was significantly negatively correlated to incremental increase in NEFA AUC (r= -0.65); basal CHO oxidation (r= -0.77) and incremental increase in net Lox AUC (r= -0.75) ($p<0.05$).

The postprandial net Lox was significantly positively correlated to lean mass (r=0.43); basal EE (r=0.61) and postprandial EE AUC (r=0.60); basal net Lox (r=0.70) and endogenous Lox (r=0.95) ($p<0.05$). Postprandial net Lox was significantly negatively correlated to incremental increase in NEFA AUC (r= -0.45) and postprandial CHO oxidation AUC (r= -0.59) ($p<0.05$). Postprandial net Lox AUC was also negatively correlated to fasting CHO oxidation (r= -0.34), however, the relationship was not statistically significant (p=0.15).

The incremental increase in net Lox AUC was significantly positively correlated to incremental increase in NEFA AUC (r=0.49) and basal CHO oxidation (r=0.77) ($p<0.05$). Incremental increase in net Lox AUC was significantly negatively correlated to fasting NEFA (r= -0.50), incremental increase in CHO oxidation (r= -0.94) and basal net Lox (r= -0.75) ($p<0.05$).

6.4.2.4 Breath $^{13}CO_2$ recovery

The time course of recovery of label on breath (oxidation of labelled fat) following the first and second meal are shown in Figure 6.10. Breath $^{13}CO_2$ recovery was highest in NTG diabetics and lowest in HTG diabetics. In all the three groups ^{13}C label appeared in the breath with in the first hour of the label administration. ^{13}C label in the breath then continued to increase until 6 hours when the second unlabelled meal was given. Peak ^{13}C label in breath was observed at 1 hour post second meal in all the three groups. At the peak and an hour before and after the it, breath $^{13}CO_2$ recovery was significantly lower in HTG diabetics than NTG diabetics ($p<0.05$). After the peak ^{13}C label in breath started to decline but remained

higher than baseline levels at 4 hour post second meal. During the later time points post second meal a trend towards higher enrichment of label in breath was observed in NTG diabetics than HTG diabetics and controls.

The mean AUC for breath $^{13}CO_2$ appearance over the 10 hour postprandial period are shown in Table 6.3. Postprandial AUC for breath $^{13}CO_2$ appearance was highest (0.34 % administered dose/kg leanmass.10h)in NTG diabetics and lowest (0.25 % administered dose/kg leanmass.10h) in HTG diabetics, however, the differences were only significant between NTG and HTG diabetics ($p<0.05$; Appendix 8.5, ANOVA Tables).

The association between breath $^{13}CO_2$ recovery and various anthropometric and metabolic parameters are shown in Appendix 8.7. Breath $^{13}CO_2$ was significantly negatively correlated fat mass (r= -0.61); lean mass (r= -0.72); fasting insulin (r= 0.44); fasting TAG (r= -0.51) and postprandial TAG AUC (r= 0.54); incremental in TAG AUC (r= -0.51) and endogenous lipid oxidation (r=0.54) ($p<0.05$). Breath $^{13}CO_2$ was also negatively correlated to fasting glucose (r= -0.35) and postprandial glucose AUC (r= -0.38), however, the relationship did not reach the statistical significance ($p>0.08$).

6.4.2.5 Endogenous and Exogenous lipid oxidation

The mean AUC for endogenous lipid oxidation over the 10 hour postprandial period are shown in Table 6.3. Postprandial AUC for endogenous lipid oxidation was highest (0.38 gm/kg leanmass.10h) in HTG diabetics and lowest (0.38 gm/kg leanmass.10h) in NTG diabetics, however, the differences between the three groups were not statistically significant ($p>0.13$).

The mean AUC for exogenous lipid oxidation over the 10 hour postprandial period are shown in Table 6.3. Postprandial AUC for exogenous lipid oxidation was highest in NTG diabetics (0.24 gm/kg leanmass.10h) and lowest (0.18 gm/kg leanmass.10h) in HTG diabetics, however, the differences were only significant between HTG and NTG diabetics ($p<0.05$) (Appendix 8.5, ANOVA Tables).

The association between endogenous and exogenous Lox and various anthropometric and metabolic parameters are shown in Appendix 8.7. Endogenous Lox was significantly positively correlated to lean mass (r=0.61); basal EE (r=0.57) and postprandial EE AUC (r=0.53); basal net Lox (r=0.63) and postprandial net Lox AUC (r=0.95) ($p<0.05$). Endogenous Lox was also positively correlated to fat mass (r=0.39); fasting TAG (r=0.36) and incremental increase in TAG (r=0.35), however, the association did not reach the

statistical significance ($p>0.09$). Endogenous Lox was significantly negatively correlated to postprandial CHO oxidation AUC ($r= -0.55$); breath $^{13}CO_2$ AUC ($r= -0.54$) and exogenous lipid oxidation ($r= -0.54$) ($p<0.05$). Endogenous Lox was also negatively correlated to incremental increase in NEFA AUC ($r= -0.41$), however, the relationship did not reach the significance level ($p=0.07$).

Exo Lox was significantly negatively correlated fat mass ($r= -0.61$); lean mass ($r= -0.72$); fasting insulin ($r= 0.44$); fasting TAG ($r= -0.51$) and postprandial TAG AUC ($r= 0.54$); incremental in TAG AUC ($r= -0.51$) and endogenous lipid oxidation ($r=0.54$) ($p<0.05$). Exo Lox was also negatively correlated to fasting glucose ($r= -0.35$) and postprandial glucose AUC ($r= -0.38$), however, the relationship did not reach the statistical significance ($p>0.08$).

Table 6.1 Physical characteristics and fasting parameters of subjects

Characteristics	HTG Type II Diabetics (n = 6) Mean (SE)	NTG Type II Diabetics (n = 6) Mean (SE)	Middle-aged men (n = 8) Mean (SE)
Age (yrs)	50.7 (0.9)	61.3 (2.6) [b,c]	52.1 (1.3)
Weight (kg)	119.5 (7.2) [a, b]	95.3 (4.9)	80.8 (3.9)
Height (m)	1.7 (0.03)	1.8 (0.03)	1.8 (0.02)
BMI (kg/m^2)	39.5 (1.6) [a, b]	29.1 (2.5)	25.8 (1.3)
Fat mass (kg)	43.5 (3.8) [a, b]	26.7 (3.5)	18.0 (1.8)
Lean mass (kg)	76.1 (3.7) [a, b]	63.9 (3.1)	62.8 (2.4)
Fasting TG (mmol/l)	5.3 (1.1) [a, b]	1.2 (0.1)	0.9 (0.1)
Fasting NEFA (mmol/l)	0.62 (0.05)	0.58 (0.09)	0.42 (0.04)
Fasting insulin (mU/l)	27.0 (2.8) [a]	20.9 (5.6) [c]	8.3 (1.7)
Fasting glucose (mmol/l)	10.2 (1.4) [a]	7.7 (0.4)	5.8 (0.3)
Basal EE (kcal/kg lean mass)	1.19 (0.05)	1.18 (0.05)	1.15 (0.04)
Basal net Lox (kcal/kg lean mass)	0.041 (0.007)	0.042 (0.007)	0.033 (0.008)
Basal CHO ox (kcal/kg lean mass)	0.156 (0.015)	0.142 (0.010)	0.155 (0.020)

EE, energy expenditure; CHO, carbohydrate; Ox, oxidation;
a, HTG *v* controls; b, HTG *v* NTG; c, NTG *v* controls.
Lower case letters shows that the means are significantly different between the groups ($p<0.05$)

Table 6.2 Mean AUC for various blood parameters over the 10-hour postprandial period.

Group	AUC Glucose (mmol/l.10h) Mean (SE)	AUC Δ Glucose (mmol/l.10h) Mean (SE)	AUC Insulin (mU/l.10h) Mean (SE)	AUC Δ Insulin (mU/l.10h) Mean (SE)	AUC NEFA (mmol/l.10h) Mean(SE)	AUC Δ NEFA (mmol/l.10h) Mean(SE)	AUC TAG (mmol/l.10h) Mean (SE)	AUC ΔTAG (mmol/l.10h) Mean (SE)	AUC ^{13}CPA In CRF-TAG (ug/ml.10h) Mean (SE)	AUC ^{13}CPA In NEFA (ug/ml.10h) Mean (SE)
Middle-aged Men	66 (2.5)	7.7 (1.9)	367 (78)	285 (64)	2.9 (0.2)	-1.3 (0.4)	13.6 (1.9)	4.6 (0.6)	24.8 (3.3)	9.9 (1.1)
NTG Type II Diabetics	83 (6.0)	5.8 (2.6)	559 (75)	395 (41)	3.7 (0.3)	-2.0 (0.8)	16.7 (1.7)	4.6 (0.9)	23.6 (3.2)	4.8 (1.0)[c]
HTG Type II Diabetics	114 (16.9)[a]	12.2 (3.7)	963 (153)[a, b]	716 (142)[a]	4.2 (0.4)[a]	-2.0 (0.6)	67.3 (12.5)[a, b]	13.9 (3.2)[a, b]	40.2 (5.2)[a, b]	6.9 (2.0)

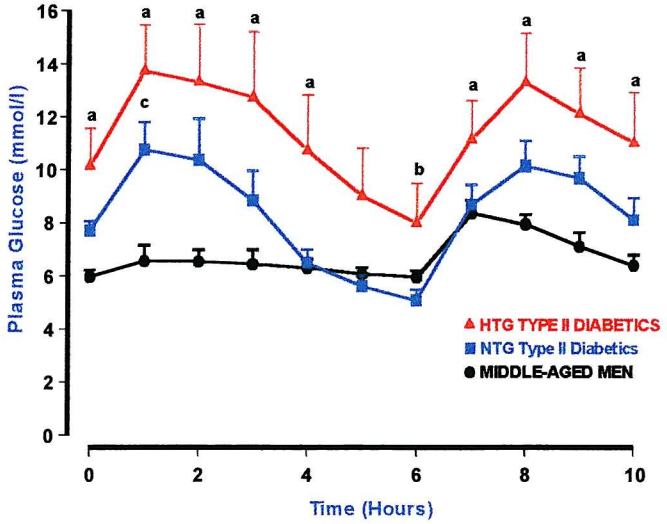
AUC, area under the curve; PA, palmitic acid; CRF, chylomicron-rich fraction
HTG, hypertriglyceridaemic; NTG, normaltriglyceridaemic, Δ, increment above the base line;
a, HTG *v* controls; b, HTG *v* NTG diabetics; c, NTG *v* controls.
Lower case letters shows that the differences between the groups were statistically significant ($p<0.05$).

Table 6.3 Mean AUC for EE and substrate oxidation over the 10 hour postprandial period

Group	AUC EE kcal/kg LM.10h Mean (SE)	AUC Δ EE kcal/kg LM.10h Mean (SE)	AUC CHO ox gm/kg LM.10h Mean (SE)	AUC Δ CHO ox gm/kg LM.10h Mean (SE)	AUC net Lox gm/kg LM.10h Mean (SE)	AUC Δ net Lox gm/kg LM.10h Mean (SE)	AUC Breath $13CO_2$ % AD/kg LM.10h Mean (SE)	AUC Endo Lox gm/kg LM.10h Mean (SE)	AUC Exo Lox gm/kg LM.10h Mean (SE)
Middle-aged Men	12.92 (0.4)	1.41 (0.2)	1.46 (0.1)	-0.09 (0.15)	0.51 (0.06)	0.18 (0.06)	0.30 (0.02)	0.30 (0.06)	0.22 (0.01)
NTG Type II Diabetics	12.96 (0.4)	1.14 (0.2)	1.70 (0.08)	0.29 (0.05)	0.45 (0.04)	0.03 (0.03)	0.34 (0.03)	0.20 (0.06)	0.24 (0.02)
HTG Type II Diabetics	13.27 (0.4)	1.39 (0.2)	1.61 (0.06)	0.05 (0.13)	0.55 (0.04)	0.14 (0.06)	0.25 (0.02)[b]	0.38 (0.05)	0.18 (0.01)[b]

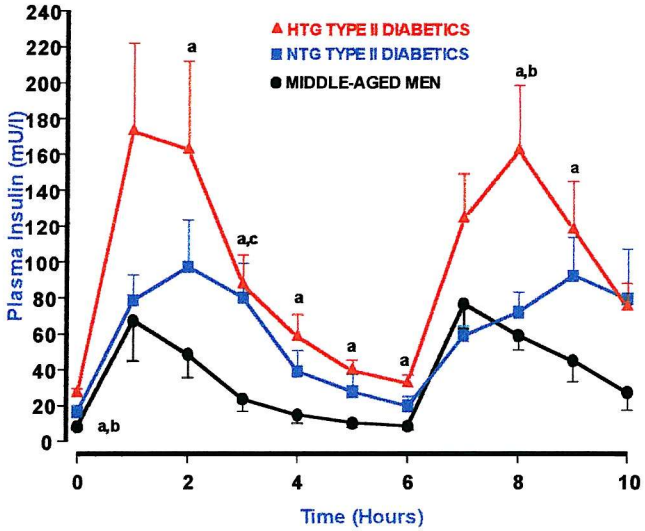
AUC, area under the curve; EE, energy expenditure; LM, lean mass; Δ, increment above the base line; CHO, carbohydrate; ox, oxidation; Lox, lipid oxidation; AD, administered dose; endo, endogenous; exo, exogenous; b, HTG *v* NTG diabetics. Lower case letters shows that the differences between the groups were statistically significant ($p<0.05$).

Figure 6.1 Time courses of changes in plasma glucose concentrations (mmol/l) after the first and second meal in HTG and NTG type II diabetics and controls.



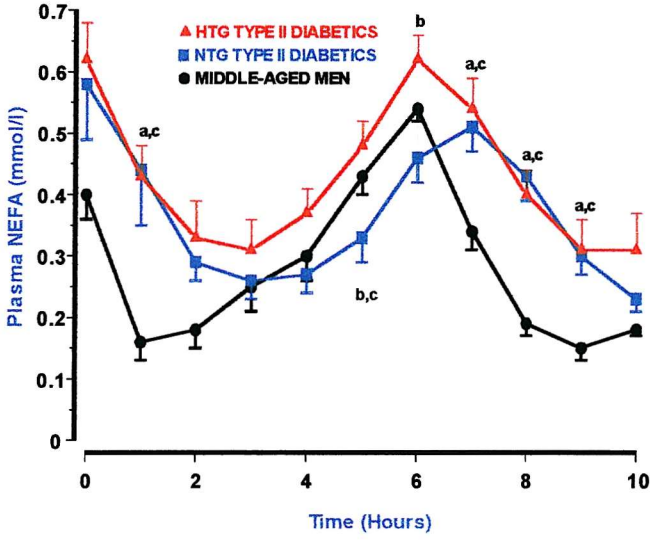
a, HTG *v* controls; b, HTG *v* NTG; c, NTG *v* Controls. Lower case letters shows that the differences between the groups were statistically significant ($p<0.05$).

Figure 6.2 Time courses of changes in plasma insulin concentrations (mU/l) after the first and second meal in HTG and NTG type II diabetics and controls.



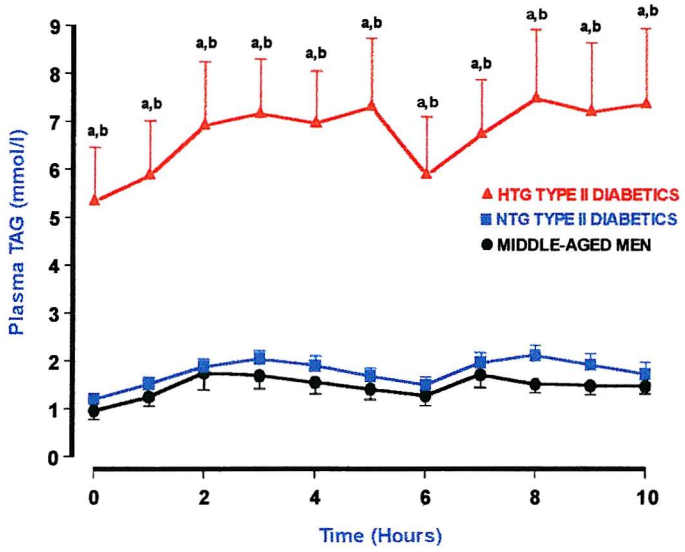
a, HTG *v* controls; b, HTG *v* NTG; c, NTG *v* Controls. Lower case letters shows that the differences between the groups were statistically significant ($p<0.05$).

Figure 6.3 Time courses of changes in plasma NEFA concentrations (mmol/l) after the first and second meal in HTG and NTG type II diabetics and controls.



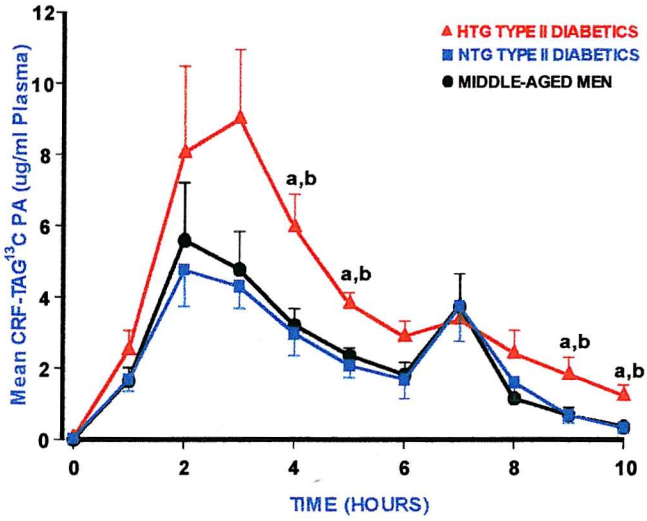
a, HTG Vs controls; b, HTG Vs NTG, c, NTG VS controls. Lower case letters shows that the differences between the groups were statistically significant ($p<0.05$)

Figure 6.4 Time courses of changes in plasma TAG concentrations (mmol/l) after the first and second meal in HTG and NTG type II diabetics and controls.



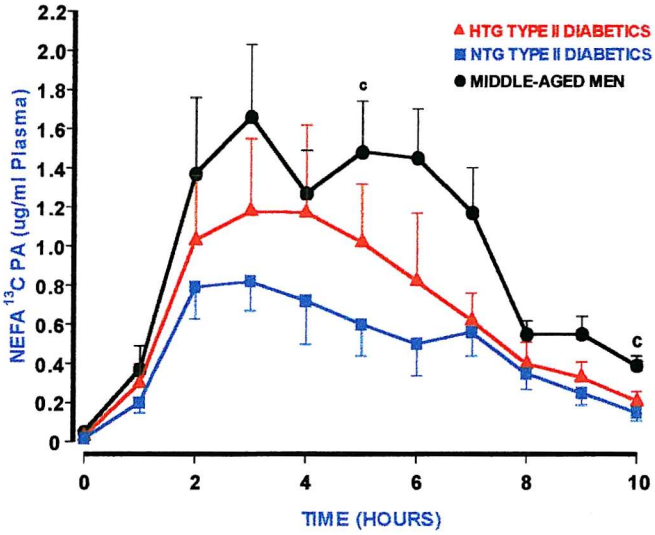
a, HTG *v* controls; b, HTG *v* NTG; c, NTG *v* Controls. Lower case letters shows that the differences between the groups were statistically significant ($p<0.05$).

Figure 6.5 Time courses of changes in CRF-TAG ^{13}C PA concentrations (μg/ml) after the first and second meal in HTG and NTG type II diabetics and controls.



a, HTG *v* controls; b, HTG *v* NTG; c, NTG *v* Controls. Lower case letters shows that the differences between the groups were statistically significant ($p<0.05$).

Figure 6.6 Time courses of changes in NEFA ^{13}C PA concentrations (μg/ml) after the first and second meal in HTG and NTG type II diabetics and controls.



a, HTG *v* controls; b, HTG *v* NTG; c, NTG *v* Controls. Lower case letters shows that the differences between the groups were statistically significant ($p<0.05$).

Figure 6.7 Time courses of changes in energy expenditure (kcal/kg lean mass) after the first and second meal in HTG and NTG type II diabetics and controls.

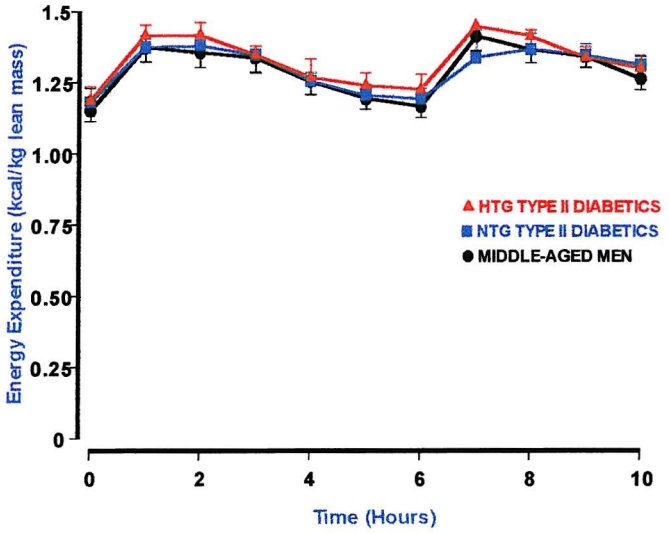


Figure 6.8 Time courses of changes in carbohydrate oxidation (gm/kg lean mass) after the first and second meal in HTG and NTG type II diabetics and controls.

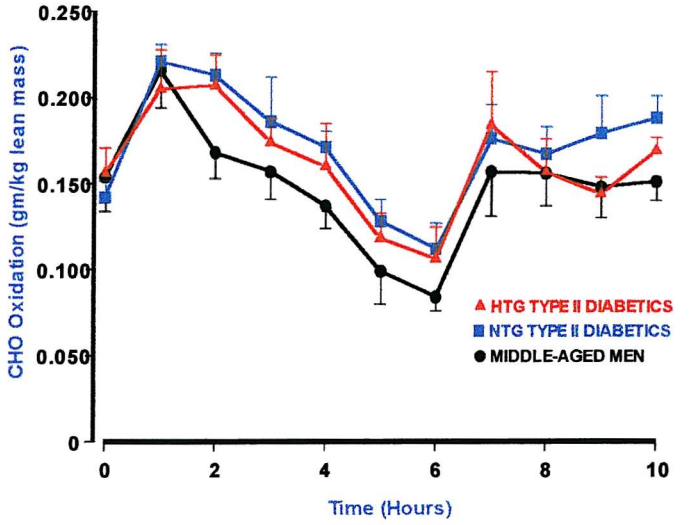


Figure 6.9 Time courses of changes in net lipid oxidation (gm/kg lean mass) after the first and second meal in HTG and NTG type II diabetics and controls.

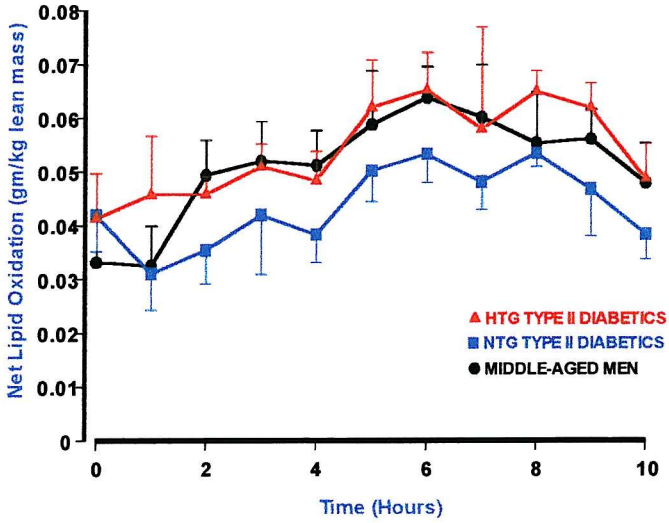
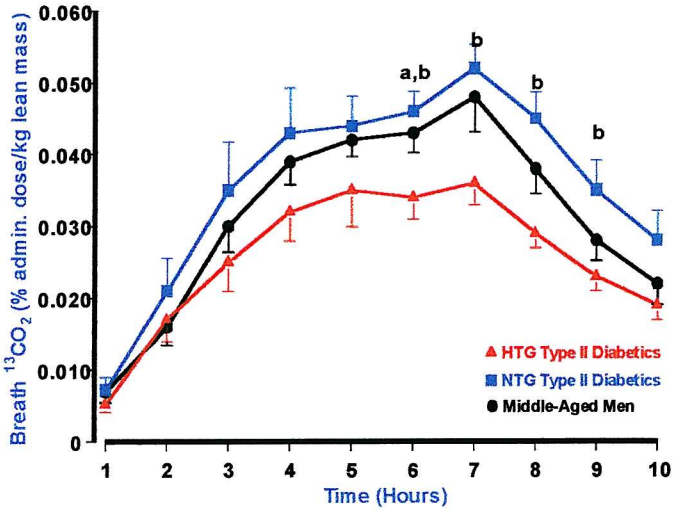


Figure 6.10 Time courses of changes in breath $^{13}CO_2$ excretion (% administered dose) after the first and second meal in HTG and NTG type II diabetics and controls.



a, HTG *v* controls; b, HTG *v* NTG. Lower case letters shows that the differences between the groups were statistically significant ($p<0.05$)

Figure 6.11 Scatter plots for various variables in the study. Green squares, HTG Type II diabetics; Blue squares, NTG type II diabetics; Red squares, controls.

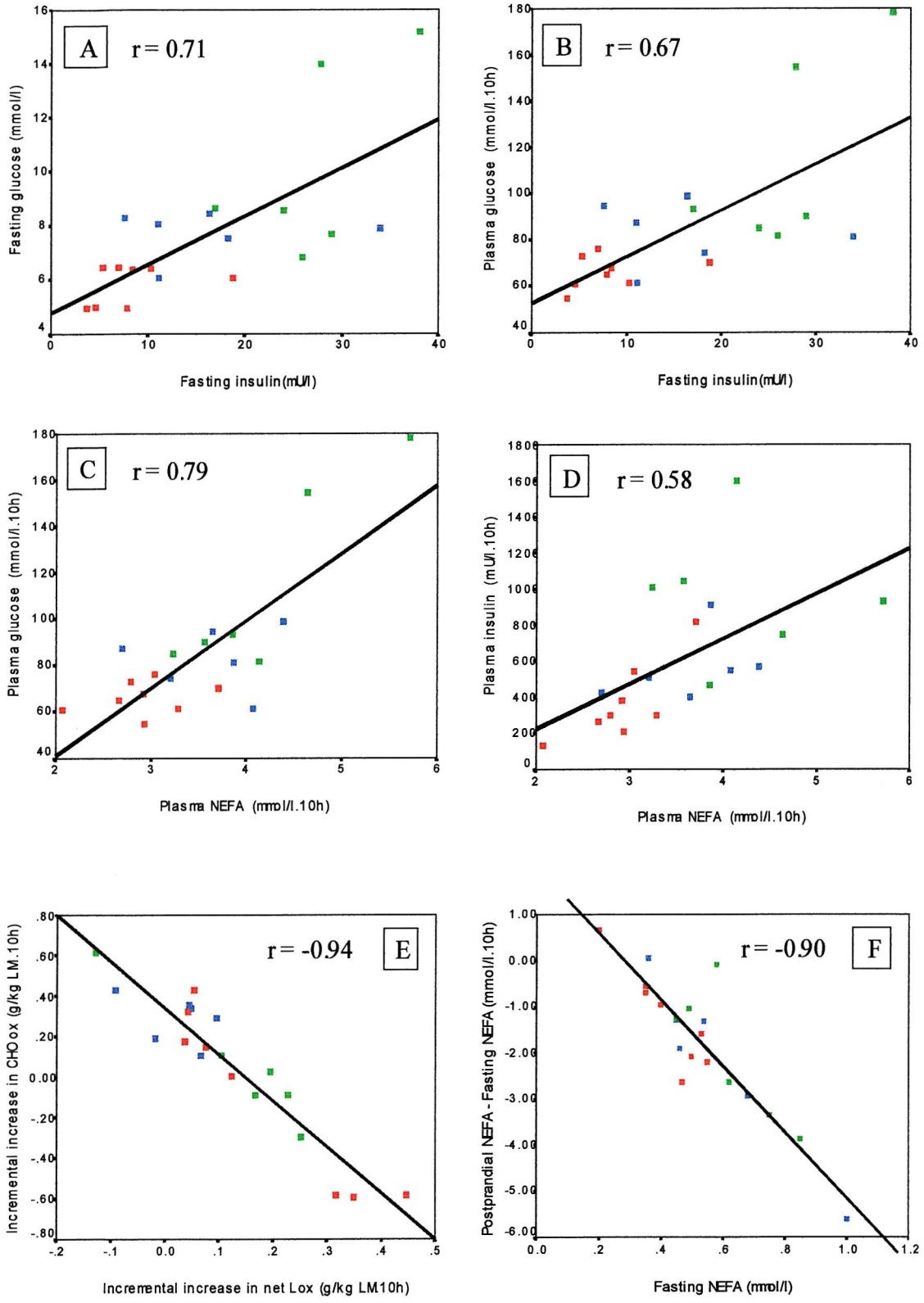


Figure 6.11 continued....

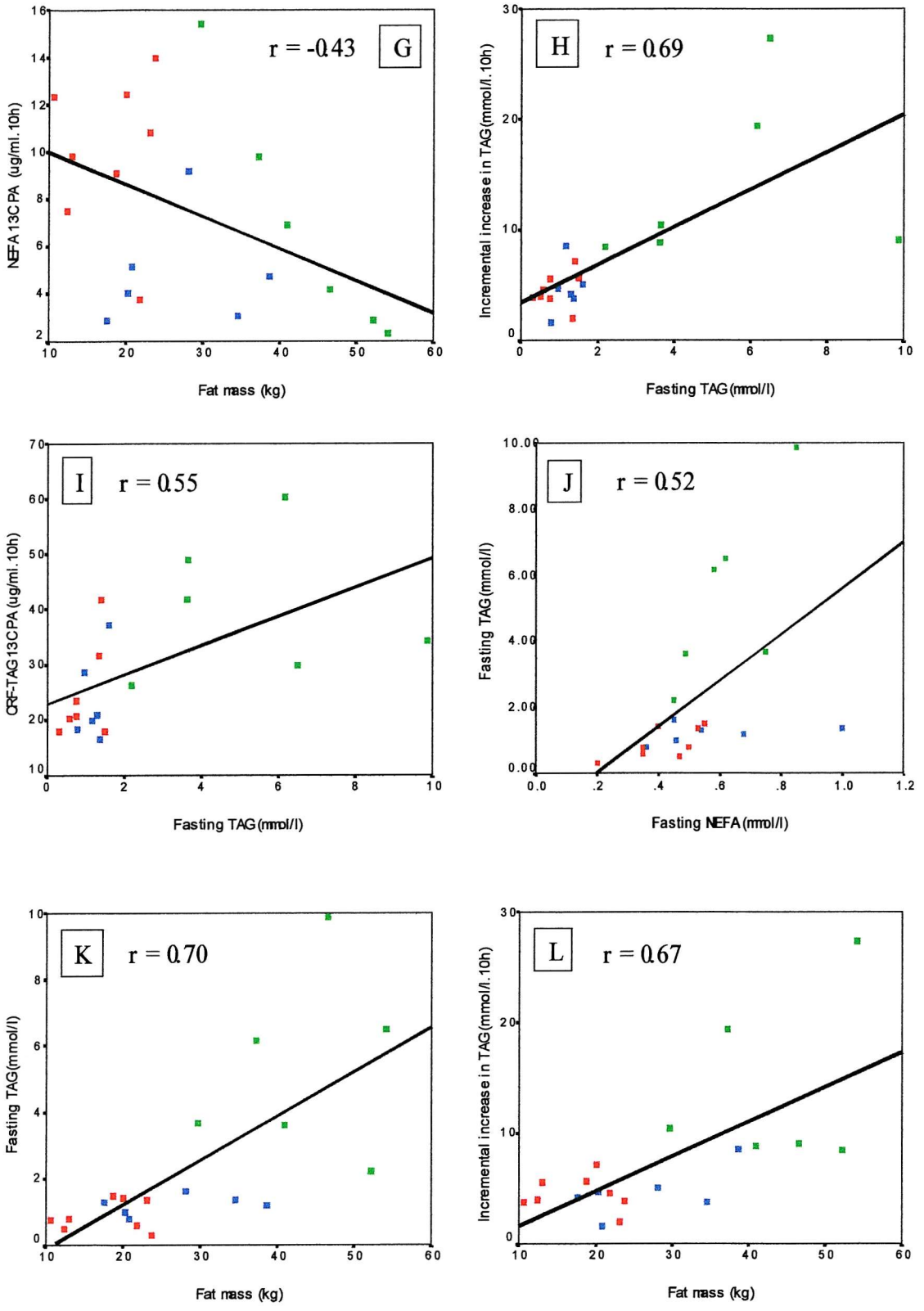


Figure 6.11 Continued....

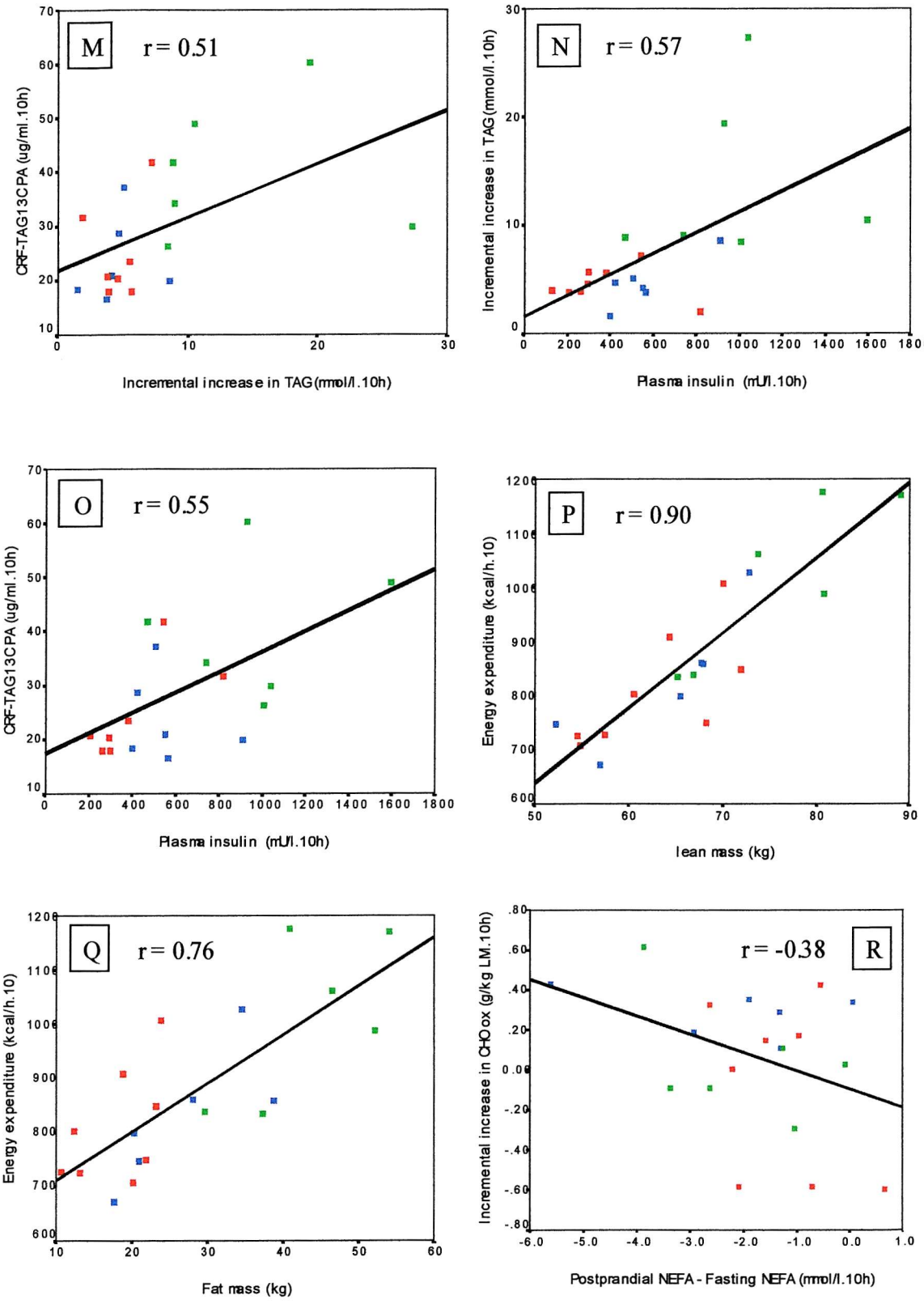
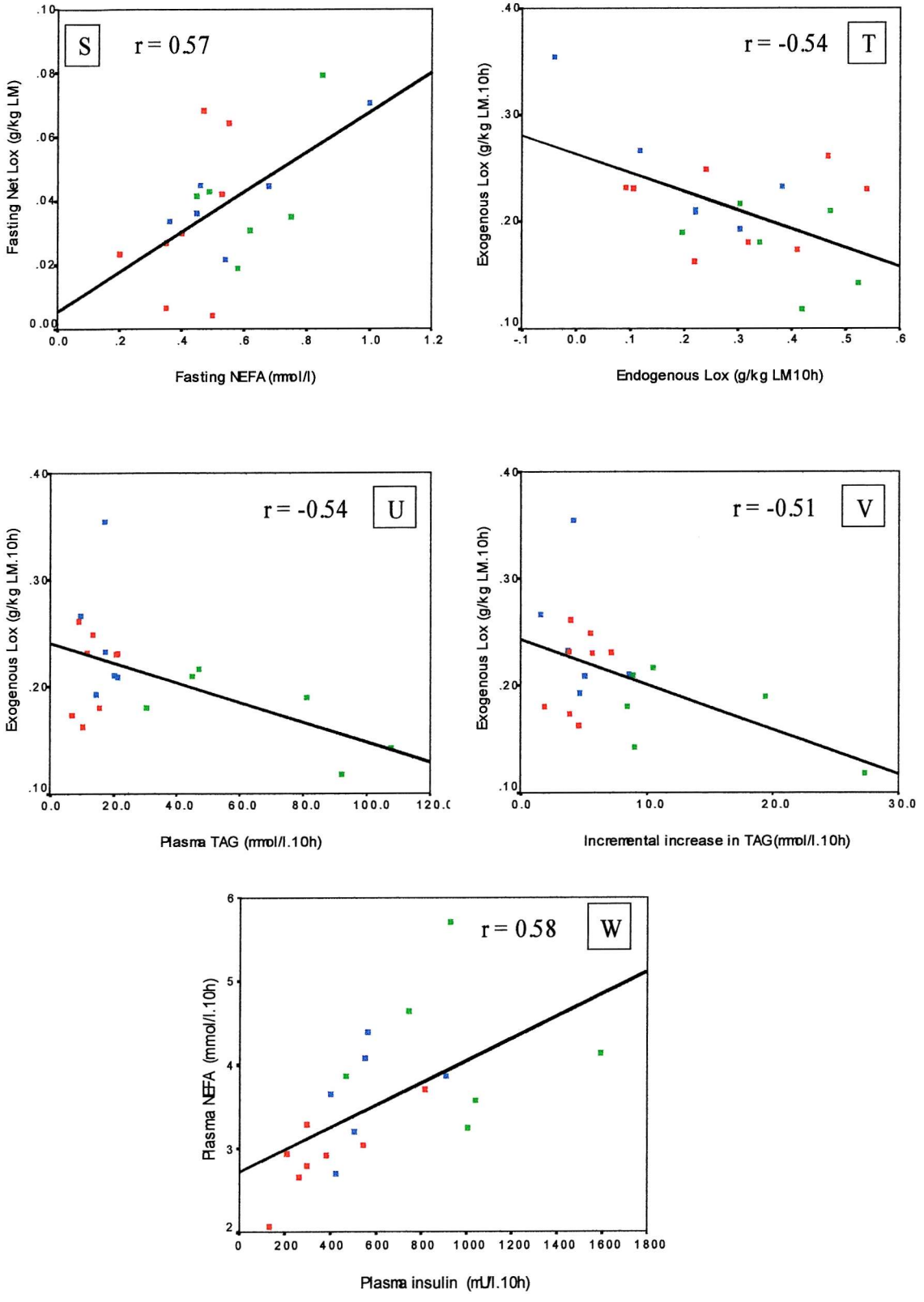


Figure 6.11 Continued....



6.5 Discussion

The present study describes for the first time postprandial lipid metabolism in diabetics by tracing the metabolism of exogenous lipid [(1,1,1 $^{13}C_3$) tripalmitin] through different pools of the body following the intake of two standard test meals (in sequence) while controlling for evening meal and physical activity. The fat content of the test meals represented more typical amounts of fat as used in normal daily life. In this study, particular attention was given to the (a) postprandial lipid metabolism in circulation and (b) postprandial energy expenditure and substrate oxidation.

The major finding of the present study was that the presence of fasting hypertriglyceridaemia (a marker for decreased metabolic competence for clearing lipids) in type II diabetes was associated with abnormalities in postprandial lipid metabolism such as a) decreased postprandial clearance of exogenous TAG from circulation b) decreased exogenous lipid oxidation and c) decreased appearance of exogenous fatty acids in NEFA fraction. The other major finding was that in the absence of fasting hypertriglyceridaemia as observed in NTG diabetics (marker for normal metabolic competence for clearing lipids relative to controls), insulin resistance did not result in decreased clearance of lipids from circulation and decreased exogenous lipid oxidation compared to middle-aged men. The explanation for this observation could be the fat load used in the test meal (36 gm), which was not sufficient enough to challenge the metabolic competence of NTG diabetics to clear lipids from circulation. The other postprandial abnormalities in diabetics included blunted and delayed suppression of NEFA and higher insulin responses.

6.5.1 Fasting and postprandial responses of plasma glucose, insulin, NEFA, TAG and ^{13}C palmitic acid in chylomicron TAG and free fatty acid fraction

6.5.1.1 Plasma glucose

Elevated fasting glucose concentrations in both diabetic groups, specifically in HTG diabetics, suggest that these subjects had poor diabetic control. Furthermore, elevated postprandial glucose concentrations in both diabetic groups, specifically in HTG diabetics than control, suggest impaired insulin mediated glucose disposal in diabetics. A significant positive correlation of fasting insulin with fasting glucose ($r=0.70$, $p=0.001$; Figure 6.11 A) and postprandial glucose AUC ($r=0.67$, $p=0.001$; Figure 6.11B) suggest that part of impaired glucose disposal was due to insulin resistance. Previous studies have also shown higher postprandial glucose and insulin concentrations in HTG diabetics

versus controls (Curtin et al, 1996; Taggart et al, 1997) and in HTG and NTG diabetics versus controls (Cooper et al, 1996).

The impaired insulin mediated glucose disposal could be at the level of a) decreased transport and uptake of glucose by muscle, and/or, b) decreased oxidative and non-oxidative disposal of glucose inside the cell. Furthermore, increased hepatic glucose output due to increased gluconeogenesis might also result in postprandial hyperglycaemia in diabetics. Since the CHO oxidation in diabetics was similar or even higher than control group, it appears that glucose uptake and oxidation does not appear to play a major role in postprandial hyperglycaemia in diabetics. However, lower non-oxidative glucose disposal (e.g. glycogen synthesis, lactate formation) and higher hepatic glucose output might play a role in hyperglycaemia. There is evidence that insulin resistance is a consequence of a reduced glucose deposition as glycogen in muscle (Beck-Nielsen *et al.,* 1992). Although in this study muscle glycogen synthesis or glycogen synthase activity were not measured, a low rate of skeletal muscle glycogen synthesis and impaired insulin-stimulated activation of glycogen synthase is expected in diabetic subjects of this study. This is confirmed by previous studies of Shulman *et al.* (1990) and Thorburn *et al.* (1990). Recently, Nikoulina *et al.,* (1997) have also suggested that the metabolic abnormalities of hyperglycaemia and hyperinsulinaemia contribute to the impairments of glycogen synthase in type II diabetes. The minimal glycaemic response in controls after the first meal and not after the second meal suggests that there was increased non-oxidative glucose disposal (i-e increased glycogen synthesis) when the glycogen stores were depleted after longer fasting period. However in type II diabetics, higher postprandial glycaemic response after first meal suggests decreased glycogen synthesis even after longer fasting period. Alternatively or in addition, a strong relationship of postprandial NEFA AUC with glucose AUC (r=0.79, p=0.001) and insulin AUC (r=0.58, p=0.01) suggests that higher NEFA concentrations in the insulin resistant state might have produced hyperglycaemia by increasing glucose output (Figure 6.11 C & D). Since, NEFA provide energy for gluconeogenesis, thus increasing hepatic glucose output (Foley, 1992). Results of one other study also supports this idea that impaired suppression of plasma NEFA levels after glucose ingestion lead to higher rate of hepatic glucose output and systemic glucose delivery (Kruszynska *et al.,* 1997).

Muscle TAG metabolism could also play an important role in non-oxidative glucose disposal. A trend of lower incremental increase in net Lox in diabetics versus

controls and a significant negative correlation between incremental increase in net Lox and incremental increase in CHO oxidation (r=-0.94, p=0.001; Figure 6.11 E) suggests an abnormality in lipid metabolism in diabetics might be related to non-oxidative glucose disposal as the glucose oxidation was not affected in type II diabetics. Recently a study also found a significant negative relationship between muscle TAG content and non-oxidative glucose disposal in humans (Pan *et al.,* 1997). Although the mechanisms responsible for this relationship are unclear, they may be central to an understanding of the link between lipid metabolism and insulin resistance.

6.5.1.2 Plasma insulin

In this study, insulin resistance was not determined directly, rather relative concentrations of fasting insulin were used for demonstrating insulin resistance. It has been previously shown that in subjects with normal and abnormal glucose tolerance, fasting insulin concentration is the best marker of insulin resistance as determined by whole body glucose uptake using euglycaemic hyperinsulinaemic clamp technique (Laakso, 1993). The observation of higher fasting and postprandial insulin responses in both diabetic groups, specifically HTG diabetics, suggests increased insulin resistance in these groups than controls. Furthermore higher fasting insulin concentrations in HTG diabetics suggest that they were more insulin resistant than NTG type II diabetics. The exact mechanism of hyperinsulinaemia are not known but it could be due to higher insulin production by pancreatic beta cells or decreased clearance by liver.

Insulin resistance in two diabetic groups (specifically in HTG diabetics) is further evident from higher fasting NEFA concentration and blunted and lesser suppression of NEFA in the postprandial state. A significant positive relationship between postprandial insulin AUC and postprandial NEFA AUC (r=0.58, p=0.01; Figure 6.11 W) also suggests that part of lack of suppression of NEFA was due to insulin resistance. It is not clear that whether higher NEFA concentrations causes insulin resistance or are a result of insulin resistance. Some studies have shown that plasma NEFA concentrations are largely determined by the action of insulin to suppress adipocyte lipolysis by suppression hormone sensitive lipase (Weiland *et al.,* 1980; Yki-Jarvinen & Taskinen, 1988) and promoting re-esterification of NEFA (Coppack *et al.,* 1992). While other studies have shown that increased plasma NEFA produce peripheral insulin resistance in diabetic subjects (Boden & Chen, 1995) and can also stimulate acute insulin secretion (Felber & Vanotti, 1994). Recently it has been found in dogs that NEFA can impair hepatic insulin

extraction in vivo at high and low insulin levels, an effect that may contribute to peripheral hyperinsulinaemia of obesity (Wiesenthal *et al.,* 1999). It is also suggested that free fatty acids or products of free fatty acids may decrease the number of surface receptors by increasing the rate of insulin receptor internalisation, decreasing the rate of receptor recycling, or both. The mechanisms responsible for insulin resistance remains to be determined, however, the relationship between hyperinsulinaemia and high NEFA concentrations suggests that they are related to each other.

6.5.1.3 Plasma NEFA and ^{13}C PA in NEFA fraction

Both diabetic groups had higher postprandial NEFA AUC but the results were only significantly different between HTG diabetics and controls. Previous studies have shown a defect in insulin suppression of NEFA in type II diabetes (Groop *et al.,* 1989). Insulin plays an important role in the suppression of NEFA in postprandial state. The mechanisms by which insulin lowers plasma NEFA levels include inhibition of TAG hydrolysis in the adipose cell to FFA and glycerol (Havel, 1965) and together with glucose, promotion of re-esterification of FFA in to TAG in adipose cells (Wolfe & Peters, 1987). In the present study, higher NEFA concentrations in postprandial state might be due to a) decreased adipocyte suppression of NEFA and/or b) decrease entrapment of NEFA after hydrolysis by LPL. The blunted and lesser suppression of NEFA alongside the lesser increase in ^{13}C PA in NEFA fraction suggest that higher postprandial NEFA in diabetics (specifically HTG diabetics) was due to blunted and lesser suppression of NEFA rather than poor entrapment of NEFA after LPL hydrolysis. Furthermore, the strong negative correlation between incremental increase in NEFA AUC and basal NEFA ($r=-0.90$; $p=.001$; Figure 6.11F) also suggest that higher NEFA AUC in diabetics was due to slower suppression of higher basal NEFA concentrations rather than poor entrapment of NEFA. On the other hand, sharp and higher suppression of NEFA along with higher ^{13}C PA in NEFA fraction of controls than diabetics suggest that the postprandial increase in plasma NEFA in controls might be mainly due to lack of entrapment after LPL hydrolysis. Since the diabetics had more fat mass than controls, a negative correlation between fat mass and NEFA ^{13}C PA ($r= -0.43$, $p=0.01$; Figure 6.11G) suggest that part of the lack of entrapment in controls might have occurred at the level adipose tissue.

In non-obese controls of this study the decreased uptake of exogenous fatty acids might be due to relatively small volume of adipose tissue (especially metabolically active visceral adipose tissue), which might have resulted in decreased re-esterification and

uptake of exogenous fatty acids. On the other hand in obese diabetic subjects the increased uptake of exogenous fatty acids might be due to large adipose tissue (especially metabolically active visceral adipose tissue), which might have resulted in increased re-esterification and uptake of exogenous fatty acids. Moreover, in the postprandial state since diabetics were losing more NEFA from adipose tissue due to lack of suppression as compared to controls, the greater ^{13}C NEFA uptake and re-esterification in type II diabetics might represent a mechanism to rebuild the continually exhausting stores of TAG from adipose tissue. A recent stable isotope study has also showed that the appearance of ^{13}C oleic acid in non-esterified fatty acid fraction was dramatically reduced in obese than the control (Binnert *et al,*. 1998). They have also found a strong negative correlation between fat mass and ^{13}C oleic acid in NEFA fraction (-0.84). As discussed in results section 6.4.1.6, data of ^{13}C NEFA concentrations should be interpreted with caution because the PA peaks detected by GC-IRMS were very small due to little concentration of PA in NEFA fraction, especially after the postprandial suppression of NEFA concentrations. Very small peaks can give erroneous results in terms of isotopic enrichments and substrate concentrations. Further studies are needed to resolve this issue by extracting NEFA from larger volumes of plasma (such as 2 ml or more) from lean and obese (with abdominal fat patterning) non-diabetics and type II diabetics respectively.

6.5.1.4 Plasma TAG and CRF-TAG ^{13}C PA

HTG diabetics had significantly higher fasting TAG than NTG diabetics and controls. Fasting TAG, which reflect the size of VLDL pool, is known to be a strong determinant of postprandial TAG increment in normal subjects (Cohn *et al.,* 1988b), obese patients (Lewis *et al.,* 1990) and type II diabetics (Syvanne *et al.,* 1994). In the present study a significant positive correlation of fasting TAG with postprandial TAG increment ($r=0.69$, $p=0.001$; Figure 6.11H) and CRF-TAG ^{13}C PA ($r=0.55$, $p=0.02$; Figure 6.11 I) also suggest the contribution of VLDL in the postprandial lipaemia. A significant positive correlation between fasting NEFA and fasting TAG ($r=0.52$, $p=0.01$; Figure 6.11J) and a significant positive correlation of fat mass with fasting TAG ($r=0.70$, $p=0.001$; Figure 6.11K) and incremental increase in TAG ($r=0.67$, $p=0.001$; Figure 6.11L) suggest that part of the source of TAG might be VLDL. However, this relationship only explains a part of variation in fasting plasma TAG to be due to fasting NEFA which serves as a source of fatty acids for VLDL TAG synthesis. This raises the question that whether the fasting TAG in the insulin resistant state represents the size of the pool of VLDL and/ or VLDL

remnants or both VLDL and chylomicron and their remnant and to what extent the source of fat in fasting VLDL is from exogenous origin? Owing to discussion in chapter 5 (section 5.5.1.4), it is suggested that fasting TAG might reflect both VLDL and chylomicron and their remnants. Previous studies have shown that HTG type II diabetic patients and non diabetic HTG patients had increased amounts of fasting apoB-48 (Karpe *et al.,* 1993a; Curtin *et al,* 1996; Taggart *et al,* 1997; Karpe *et al.,* 1999). Recently, it has been shown that after an oral fat challenge the fasting apo B48 concentration accurately reflects postprandial lipaemia in normal and hyperlipidaemic subjects ($r=0.91$, $p<0.001$; Smith *et al,* 1999). Further stable isotopically labelled fat studies are needed to confirm the presence of chylomicron and their remnants in the fasting state by giving labelled fat with the regular meals for 24 hours and then measuring CRF-TAG ^{13}C PA in the morning after 12-h fasting.

Postprandial TAG AUC was also significantly greater in HTG diabetics than NTG diabetics and controls. These results of higher postprandial TAG concentrations in HTG diabetics than NTG diabetics and controls are also confirmed by previous studies (Lewis *et al., 1991*; Tan *et al., 1995*; Cooper *et al., 1996*). Similarly other studies have shown higher postprandial plasma TAG concentrations especially at the later time points in non-diabetic HTG subjects versus NTG controls (Karpe et al., 1999). Since plasma TAG does not gives the relative contribution of endogenous and exogenous fat in circulation, in the present study ^{13}C labelled TAG was used to trace the exogenous TAG in circulation. The tracer fatty acid data showed that CRF-TAG ^{13}C PA was significantly greater in HTG diabetics than NTG diabetics and controls. Furthermore, a significant positive correlation between incremental increase in TAG and CRF-TAG ^{13}C PA ($r=0.51$, $p<0.01$; Figure 6.11 M) suggests that part of the increase in postprandial TG concentration was due to CRF-TAG. Previous studies using apo B48 as the marker of chylomicrons have also shown higher concentrations of either postprandial apo B-48 in diabetic subjects (Cohn *et al.,* 1993; Taggart et al, 1997) or both apo B-48 and apoB-100 in type II diabetics (Curtin *et al.,* 1996).

Higher postprandial CRF-TAG ^{13}C PA responses in HTG diabetics can be due to a) delayed absorption of TAG b) increased production and release of chylomicron in to circulation c) decreased clearance of chylomicron TAG at the level of LPL and chylomicron remnants at the level of hepatocytes. The sharp increase in CRF-TAG tracer fatty acids during the initial postprandial period in HTG diabetics suggests that delayed

absorption or delayed gastric emptying in the HTG diabetic patients was not a factor in higher postprandial lipaemia in this group. Although the peak fatty acid responses in CRF-TAG were higher in HTG diabetics but the differences between the three groups were not statistically significant. The trend of higher postprandial tracer fatty acid response in HTG diabetics might represent an enhanced formation and secretion of chylomicrons in HTG diabetics. Although the mechanisms for this are not clear, it has been proposed that HTG diabetics may package newly absorbed fat more efficiently than those with normal fasting TG concentrations (Le *et al.,* 1991). Although the mechanisms relating the role of insulin in modulating the synthesis and secretion of intestinal lipoproteins are not known, lack of control of insulin on MTP in insulin resistant state might play an important role. Alternatively, in the insulin resistant state, slower clearance and then further entrance of pre-formed chylomicrons along with newly synthesised chylomicrons after every meal might prolong the presence of chylomicrons and their remnants in the fasting state. Hence further entry of exogenous fat after a meal might result in higher peak responses in HTG subjects. Since in this study a labelled TAG was not given with the evening meal prior to the study day, the possibility of higher labelled fat in the CRF-TAG due to previously ingested labelled fat is excluded.

The decline of tracer fatty acid in CRF-TAG in the later part of postprandial phase was significantly slower in HTG diabetics than NTG diabetics and controls. This suggests that higher postprandial lipaemia in HTG diabetics could be explained to some extent by slower clearance of exogenous lipoproteins and is confirmed by other studies (Lewis *et al.,* 1991; Cooper *et al.,* 1996). Slower clearance of apoB48 particles has also been confirmed in non-diabetic hypertriglyceridaemic patients than controls (Karpe et al., 1999). A slower clearance of exogenous fat might be due to a) insulin resistance related decreased LPL activity b) greater competition between chylomicron and chylomicron remnants with VLDL for their TAG lipolysis by LPL and c) remnant particle removal by liver, which is a saturable process (Berr, 1992). LPL is an insulin dependent enzyme the activity of which is decreased in obesity (Eckel, 1987), type II diabetes (Nikkila,1984; Eckel, 1989; Pollare *et al.,* 1991) and in insulin resistant subjects (Chen *et al.,* 1994; Knudsen *et al.,* 1995). Since HTG diabetics were obese and more insulin resistant, the decreased activity of LPL might explain the slower clearance of CRF-TAG ^{13}C PA in this group. Furthermore, a correlation of postprandial insulin AUC with incremental increase in TAG AUC ($r=0.57$, $p=0.01$) and CRF-TAG ^{13}C PA ($r=0.55$, $p=0.01$) suggest that

slower clearance in HTG diabetics might be a consequence of insulin resistance related decreased activation of LPL activity (Figure 6.11 N & O respectively).

In the insulin resistant state a greater competition between chylomicrons and VLDL for their TAG removal might also be responsible for slower CRF-TAG ^{13}C PA removal in HTG diabetics. The antilipolytic effect of a mixed meal is reduced in obesity (Roust & Jensen, 1993) and insulin resistance (Frayn, 1993). It has been shown that intra-abdominal adipocyte have a higher sensitivity to the stimulation of lipid mobilisation processes, and that the antilipolytic effect of insulin on these cells is less than on the subcutaneous adipocytes. Hence, failure to suppress both the supply of NEFA from adipose tissue to liver after a meal and VLDL synthesis and release in the insulin resistant state results in sustained VLDL production in the postprandial state (Malmstrom *et al.*, 1997a; Malmstrom *et al.*, 1997b). Furthermore, NEFA which have not been trapped after the hydrolysis by LPL might also result in greater VLDL production after their uptake by liver in insulin resistant state. Since HTG diabetics were both obese and insulin resistant the overproduction of VLDL along with decreased activation of LPL in the postprandial period might result in greater competition between endogenous and exogenous TRL for the same lipolytic pathway. Furthermore a positive correlation between postprandial insulin AUC and NEFA AUC ($r=0.58$, $p=0.01$; Figure 6.11 D) suggest that lack of suppression of NEFA might have resulted in greater VLDL production thus leading to greater competition with chylomicrons for their TAG to be removed by LPL. Previous studies have also suggested that rate of supply of NEFA to the liver is the major factor controlling hepatic TAG secretion (Kissebah *et al.*, 1974; Byrne *et al.*, 1991). Furthermore, the slope of fall in NEFA levels in response to insulin infusion explained 66% of the variation in the TAG and apoB concentration (Yki-Jarvinen & Taskinen, 1988). Hence in the insulin resistant state decreased suppression of NEFA after every meal during a day might result in higher VLDL production and secretion and greater competition with chylomicrons for their TAG to be removed by LPL.

Although chylomicron remnants were not determined in this study, impaired hepatic uptake of chylomicron remnants might also indirectly cause accumulation of intestinally derived particles especially during the late hours of the postprandial state, as chylomicron remnant removal appears to be the rate-limiting step of postprandial fat clearance in humans (Berr, 1992). Studies in rats suggest that chylomicron and VLDL remnants compete for the same removal process (Cooper *et al.*, 1982). Finally, the

decreased removal of chylomicron remnants could also be due to primary removal defect at the level of hepatocyte receptor in HTG diabetics.

In summary it is suggested that delayed clearance of CRF-TAG ^{13}C PA in HTG diabetics might be related to impaired lipolytic processing of chylomicrons, and greater competition between chylomicron and VLDL for their TAG removal or ineffective removal of remnant particles by hepatic receptors.

6.5.2 Energy expenditure and substrate oxidation

6.5.2.1 Energy Expenditure and Thermic Effect of Food

Postprandial resting energy expenditure AUC (uncorrected for lean mass) was significantly higher in HTG diabetics. Recently it has also been shown that 24-h energy expenditure and sleeping metabolic rate were significantly higher in subjects with impaired glucose tolerance compared to those with normal glucose tolerance (Weyer *et al.,* 1999). Since the HTG diabetics had higher lean mass, the strong relationship between EE AUC and lean mass (r=0.90, p=0.001; Figure 6.11P) suggest that lean mass is the best predictor of EE. This observation is confirmed by a recent study in 916 non-diabetic subjects (Weyer *et al.,* 1999), which showed that lean mass is the single best predictor of EE. The relationship between fat mass and EE AUC was also strong (r=0.76, p=0.001; Figure 6.11Q) suggesting another important determinant of energy expenditure. This observation is also confirmed by previous reports (Toubro *et al.,* 1996).

Since the lean mass greatly varied between the three groups, energy expenditure was expressed as per kg lean mass. There were no differences in EE/kg lean mass between the three groups, however, HTG diabetics showed a trend towards higher EE/ kg lean mass..

Similarly there were no differences in incremental increase in energy expenditure/kg lean mass or thermic effect of food (TEF) between the three groups, however, NTG diabetics showed a trend towards lower TEF. Previous studies have shown that in insulin resistant state with glucose intolerance, the TEF is blunted (Golay *et al.,* 1983; Gougeon, 1996). Since the storage cost of glucose to glycogen is higher thus the blunted thermogenic response might be due to decreased glycogen synthesis. For example, the direct conversion of glucose to glycogen requires 2 mole of ATP for each mole of glucose stored. In this study, diabetics had higher trend of incremental increase in CHO oxidation than controls. This suggests that the trend of lower TEF in diabetics might be

due to alterations in non-oxidative pathways of glucose disposal such as decreased glycogen synthesis. Several other studies have found that insulin resistance induced by NEFA has a greater impact on the pathway of insulin stimulated glucose storage (Nuutila.*et al.,* 1992; Kelly *et al.,* 1993; Boden *et al.,* 1994). Furthermore, modest glycaemic responses in controls after first meal (after 12 hour of fasting) and not after the second meal (after 6 hour of fasting) suggest increase glycogen synthesis after longer fasting period to rebuild exhausted glycogen stores. In contrast to controls, higher glycaemic responses both after first and second meals in diabetics suggest decreased glycogen synthesis and decreased TEF. However, increased hepatic glucose output and decreased uptake cannot be ruled out for higher glycaemic responses in diabetics. Finally, trend towards lower incremental increase in net Lox in diabetics along with a positive correlation between TEF and incremental net Lox ($r=0.38$, $p=0.10$) suggest that lower incremental lipid oxidation in diabetics might also be partly responsible for the observed decreased TEF. In conclusion, decreased TEF in diabetics could be related to their decreased glycogen synthesis and decreased incremental increase in net Lox.

6.5.2.2 CHO oxidation

In the present study, no statistically significant differences were observed in basal CHO oxidation in two diabetic groups versus controls. These results are supported by previous studies which demonstrated normal rates of glucose oxidation in postabsorptive state in obese type II diabetics (Boden *et al.,* 1983) and obese NTG and HTG type II diabetics (Widen *et al.,* 1992). However, studies in obese diabetic Pima Indians, found a significant decrease in basal glucose oxidation (Ravussin *et al.,* 1983; Bogardus *et al.,* 1984a). The exact reasons for differences in the results of these studies are not known, however, differences in study populations and severity of diabetes might explain these differences. In the present study and in Boden *et al.* (1983) study the diabetics were obese Caucasians whereas in Bogardus *et al.* (1984a) study the patients were obese Pima Indians.

HTG and NTG diabetics showed a trend towards higher postprandial CHO oxidation than controls, however, the differences between the three groups were not statistically significant. Contrary to these results a number of previous studies have found reduced glucose oxidation in type II diabetics (Thorburn *et al.,* 1990; Golay *et al.,* 1998). Although the underlying mechanisms of reduced glucose oxidation or normal or increased glucose oxidation are not known, but number of plausible mechanisms exists. Randle *et al.*

(1988) postulated that inhibition of pyruvate dehydrogenase (PDH) by elevated NEFA is a major factor leading to oxidative defect in diabetes. It has also been speculated that reduced glucose oxidation in type II diabetes is merely a consequence of reduced glucose transport in to the cells with less glucose available to traverse the oxidative pathway (Thorburn *et al.,* 1990). With this scenario, the oxidative path way would function normally when given an adequate supply of glucose substrate. An alternate explanation is the presence of intrinsic defect in the oxidative pathway, which is not a consequence of either reduced glucose transport or elevated fatty acid oxidation. Further studies are needed to explore the underlying mechanisms responsible for reduced glucose oxidation in type II diabetics.

In the present study trend of higher CHO oxidation in diabetics leads to the assumption that insulin resistance was not pronounced at the level of glucose uptake and oxidation as observed in the above mentioned studies. Rather it appears that hyperglycemia or hyperinsulinaemia might have stimulated glucose uptake in diabetics. This is supported by previous studies showing that hyperglycaemia *per se*, by mass action effect, enhances tissue glucose uptake and augment the rate of insulin-mediated glucose metabolism (DeFronzo *et al.,* 1983; Bogardus *et al.,* 1984b). Furthermore, in normal subjects hyperglycemia has been claimed to stimulate glucose oxidation over a wide range of plasma glucose and plasma insulin levels (Thiebaud *et al.,* 1982; Walters *et al.,* 1992). Hence, normal or trend of higher rates of CHO oxidation in diabetics of present study suggest that the defect was not at the level of glucose oxidation rather it might be at the level of non-oxidative glucose disposal. Previous studies have shown defects in both oxidative and non-oxidative glucose metabolism in type II diabetics (Felber *et al.,* 1987; Golay *et al.,* 1988) and suggested that non-oxidative pathways were more affected (Felber *et al.,* 1987; Golay *et al.,* 1988). Although lactate levels were not measured in the present study, elevated lactate levels would suggest reduced glycogen synthesis due to reduced glycogen synthase activity in the skeletal muscle with more glucose entering in to glycolytic pathway to lactate.

In the present study incremental increase in net Lox was strongly and inversely correlated to incremental increase in incremental CHO oxidation in the postprandial state ($r= -0.94$, $p=0.001$; Figure 6.11 E). Similarly there was a trend towards relationship between incremental increase in postprandial NEFA and incremental increase in CHO oxidation ($r= -0.38$, $p=0.09$; Figure 6.11 R). Since the diabetics showed a trend towards

normal or higher CHO oxidation and a trend towards lower net Lox, these relationships suggest that rate of lipid oxidation is an important determinant of CHO oxidation and that the decline in lipid oxidation, is in part, responsible for the rise in CHO oxidation. It has been previously reported that in type II diabetics muscle NEFA utilisation is suppressed, determining an abnormally low rate of lipid oxidation with increased glucose oxidation (Kelley & Simoneau, 1994). The results of the present study are also comparable to Golay *et al.,* (1988), who found that both in control and lean type II diabetics, the normal insulin mediated decline in lipid oxidation was responsible for the rise in glucose oxidation. In agreement with these studies, Sidossis & Wolfe (1996) propose that glucose availability and oxidation can act to inhabit lipid oxidation, a circumstance that could favour accentuation of fatty acid esterification to TAG with in muscle. Recently, Pan *et al.* (1997) suggested that muscle lipid content increases with insulin resistance. He further found that muscle TAG bore a stronger relation to insulin resistant related glucose storage than to glucose oxidation. Hence, these results of normal and increased CHO oxidation in diabetics along with decreased incremental increase in net Lox again suggests that insulin resistance in diabetics might be more pronounced at the level of non-oxidative glucose disposal and lipid oxidation.

6.5.2.3 Lipid oxidation

This study shows for the first time that HTG diabetics have defective exogenous lipid metabolism. On the other hand, HTG and NTG diabetics showed a trend towards higher basal net Lox/kg lean mass than controls. A positive correlation between fasting net Lox/ kg lean mass and fasting plasma NEFA (r=0.57, p=0.001; Figure 6.11 R) was also observed. This suggests that plasma NEFA in the basal state serve as an important source for basal net Lox.

HTG diabetics showed a trend towards higher postprandial net Lox/kg lean mass and endogenous Lox than NTG diabetics and controls. On the other hand, breath $^{13}CO_2$/kg lean mass was lower in HTG diabetics than NTG diabetics and controls. This suggests that the trend towards higher net Lox in HTG diabetics might be due their higher endogenous Lox rather than exogenous Lox. A negative correlation between endogenous Lox and exogenous Lox (r= -0.54, p=0.01; Figure 6.11T) further suggests that HTG diabetics oxidised less exogenous fat than NTG diabetics and controls. Since, both HTG and NTG diabetics showed a trend towards lower incremental increase in net Lox/kg lean mass than controls, this suggests that the trend towards increased net Lox in HTG diabetics might be

due to their trend towards higher basal net Lox. Furthermore, a negative correlation between incremental net Lox and incremental CHO oxidation suggest that part of decreased increase in incremental net Lox net in HTG diabetics could be due to higher CHO oxidation. Higher postprandial TAG and CRF-TAG ^{13}C PA in HTG diabetics along with negative correlation between exogenous lipid oxidation and postprandial TAG AUC ($r= -0.54$, $p=0.01$; Figure 6.11U) and incremental increase in TAG AUC ($r= -0.51$, $p=0.02$; Figure 6.11V) suggest that decreased clearance of plasma TAG in HTG diabetics might have also contributed in decreased exogenous lipid oxidation in HTG diabetics. Previous studies have also demonstrated that during postprandial state type II diabetics have reduced rates of lipid oxidation (Kelly & Simoneau, 1994). The mechanisms for reduced postprandial Lox in diabetics are not clear. However, it has been suggested that hyperglycemia in type II diabetics could be associated with impaired FFA oxidation in muscle.

6.6 Summary

The results of this study suggested that the presence of fasting hypertriglyceridaemia (a marker for decreased metabolic competence for clearing lipids) resulted in a) decreased postprandial clearance of exogenous TAG from circulation b) decreased exogenous lipid oxidation and c) decreased appearance of exogenous fatty acids in NEFA fraction. Furthermore, in the absence of fasting hypertriglyceridaemia (marker for normal metabolic competence for clearing lipids) as observed in NTG type II diabetics, the diabetes or insulin resistance *per se* did not result in further decreased clearance of lipids from circulation and further decreased exogenous lipid oxidation. Finally, ^{13}C NEFA data of this study suggested that the clearance of exogenous fatty acids from circulation increases with the increase in metabolically active abdominal adipose tissue depot. However further confirmation is needed in this respect by extracting ^{13}C PA from larger volumes of NEFA fractions in lean and obese individuals with abdominal fat patterning. Since the HTG diabetics had significantly higher fat mass and fasting triglyceride, it was not clear that whether the higher fat mass or the higher fasting TAG resulted in defective postprandial lipid metabolism in this group.

CHAPTER 7

General Discussion

7.1 Introduction

The central hypothesis for this thesis was that as the age increases levels of lipids in the circulation also increases depending upon the metabolic competence of an individual to clear lipids from circulation, the types and amounts of lipids and CHO within the diet and the level of physical activity. The levels of lipids in circulation will then determine the level of insulin resistance, the risk of developing obesity, type II diabetes and atherosclerosis (Figure 1.1). Since the concept of "metabolic competence of clearing lipids from circulation" and role of insulin/insulin resistance in postprandial lipid metabolism form the central basis of discussions in the following sections of this chapter, the reader is referred to sections 1.1 and 2.2 for the background information. Due to limitations of time and resources it was not possible to address all the components of the hypothesis including how increased levels of lipids result in insulin resistance (2.4.3) and how insulin resistance increases the risk of cardiovascular diseases (section 2.4.5). Rather, the main objective of this thesis was to focus on the central part of hypothesis namely, how exogenous lipid is metabolised in healthy individuals and those with various degrees of insulin resistance in a setting where the amount and type of lipid and CHO in test meal and level of physical activity are controlled. The specific objectives of the thesis are outlined in section 1.2.

The first principal finding of this thesis was that the presence of increasing fasting TAG (a marker for decreased metabolic competence for clearing lipids) resulted in a) decreased postprandial clearance of exogenous TAG from the circulation b) decreased exogenous lipid oxidation and c) decreased appearance of exogenous fatty acids in NEFA fraction. The second principal finding was that in the absence of fasting hypertriglyceridaemia as observed in NTG diabetics (marker for normal metabolic

competence for clearing lipids), diabetes or insulin resistance *per se* did not result in further decreased clearance of lipids from circulation and further decreased exogenous lipid oxidation as compared to controls. Both of these findings are discussed in detail in following sections. Whilst the observation of decreased clearance of lipids in fasting hypertriglyceridaemia have been suggested by the previous studies, this is the first occasion in which direct evidence (using novel stable isotope tracer methodology) of altered exogenous lipid clearance and oxidation has been demonstrated.

The main objective of this chapter is to integrate and discuss the principal findings of these present studies in the light of the central hypothesis and what is generally understood / held to be true from the relevant current literature. The chapter is divided into seven main sections. Section 7.2 discusses the studies which demonstrates the precision of GC-C-IRMS measurements of isotopic enrichments and tracer fatty acid concentrations and precision in lipid extraction procedures. It also discusses the studies demonstrating the repeatability of postprandial lipid metabolism. The main objective of this section is to demonstrate that any differences observed in postprandial lipid metabolism between groups were not compounded by analytical precision or within-subject variability. Section 7.3 and 7.4 describes age-related changes and effect of diabetes on postprandial lipid metabolism. These sections also identifies future studies which are needed to better characterise the underlying mechanisms responsible for impaired postprandial lipid metabolism in these conditions. Since insulin plays an important role in postprandial lipid metabolism, section 7.5 discusses the relationships between postprandial lipid metabolism and plasma insulin concentrations. Section 7.6 presents the models for postprandial lipid metabolism in healthy men and insulin resistant men and discusses that whether the studies of this thesis support or refute these models. In contrast to previously published models of postprandial lipid metabolism, these models include additional information in terms of clearance of exogenous lipid from circulation, appearance of exogenous fatty acids in NEFA fraction and exogenous lipid oxidation. This section also discusses the mechanisms by which insulin resistance increases the risk of developing cardiovascular disease. Section 7.7 discusses study design considerations for future studies in order to answer the questions which could not be answered by this thesis work. The final section describes the summary and conclusions drawn from this thesis work and recommendation for future studies for identifying the mechanisms responsible for decreased clearance of TAG from circulation and for the management of hypertriglyceridaemia.

7.2 Validation of stable isotope tracer methodology in terms of precision and repeatability of postprandial lipid metabolism.

Gas Chromatography-Combustion-Isotope Ration Mass Spectrometry (GC-C-IRMS; Europa Scientific Ltd., Crew, UK) is a relatively new commercial system, which can measure the extent to which the isotopic enrichments of ^{13}C is increased over baseline/background levels (natural abundance) in blood and stool samples. As discussed in section 4.1, the precision of stable isotope tracer methodology has been addressed by others (Jones, 1996) for the measurements of stable isotopic enrichments in breath, stool and urine (20/20 IRMS with G/S/L interface) but not for the measurements made using GC-C-IRMS. This is the first occasion when a new method was developed and validated for characterising exogenous lipid handling in the postprandial state. In this respect, the first of the two validation studies described the validity of precision of lipid extraction procedures and measurements of stable isotopic enrichments and concentrations in blood samples made through GC-C-IRMS system. Postprandial lipid metabolism has been extensively studied during the recent years, however, there are no publications which precisely described the repeatability of postprandial lipid metabolism as observed for the same subject on two identical conditions in which strict adherence to experimental conditions were applied. Hence, the second study addressed the issue of repeatability of postprandial lipid metabolism. Based on the results of these validation studies a new standardised protocol was developed for characterising exogenous lipid handling in the postprandial state (described in chapter 4) and then applied in subsequent studies described in this thesis.

The results of the validation study suggest that GC-C-IRMS system can precisely measure stable isotopic enrichment and ^{13}C palmitic acid concentrations in chylomicron-rich fraction TAG with a coefficient of variances (%) of 0.96 and 3.68 respectively (sections 4.5.1 and 4.5.2). Furthermore, this study also demonstrated the precision of lipid extraction procedures with low coefficient of variances (1.24%) for stable isotopic enrichment ($^{0}/_{00}$) and ^{13}C palmitic acid concentration (8.09%) in chylomicron-rich fraction TAG (μg/ml plasma) (sections 4.5.1 and 4.5.2). The between-sample variability can be further reduced by careful sample derivatization, good chromatography and appropriate baseline correction of the chromatograms. In the light of these results it could be said with confidence that the within group and between group variability in the subsequent studies of this thesis would be minimal (due to factors such as precision of the instrument and

lipid extractions procedures) as the similar protocol was used for these studies.

The results of repeatability study demonstrated that the postprandial responses of plasma TAG, NEFA and ^{13}C palmitic acid concentration in CRF-TAG were highly repeatable for the group as a whole as the differences between the trials were small (1.1%, 4.5%, and 1.1% respectively; Table 4.4). On the other hand, postprandial glucose responses were less repeatable as evident from relatively large differences between the trials (8%; Table 4.4 and section 4.9.2). This relatively poor repeatability of plasma glucose concentrations would not affect the interpretation of plasma glucose concentrations in the diabetic group as these subjects were recruited with established diabetes and their glucose concentrations were well above the current diagnostic criteria (fasting glucose levels > 6.1 mmol/l). On the other hand, poor repeatability of plasma glucose concentrations could falsely identify subjects (in young men and middle-aged men) as normal or impaired glucose tolerant. One way of identifying subjects correctly with normal/abnormal fasting glucose levels could be by checking fasting blood glucose levels on more than 2 occasions, however, this was impracticable. Generally, the results of this study suggested that if the preceding diet and level of physical activity were controlled the intra-individual variability between two identical trials can be reduced. The practical implication of the repeatability of lipid responses as observed in the present study is that reliable postprandial tolerance tests can be conducted in clinical settings after the intake of standard test meal (while controlling at least the previous evening meal and physical activity) in order to identify individuals with decreased metabolic competence of clearing lipids from circulation.

The results of this methodological study also suggested that ^{13}C excretion in stools was repeatable and the absorption of ^{13}C palmitic acid was almost complete (~99%), as only 1% of the administered label was recovered in the stools. This is discussed in section 4.9.1. It appeared that presenting the tracer as a TAG incorporated into an emulsion resulted in minimal loss in stool. The implication of these results was that the determination of ^{13}C excretion in stools was not be required for the correction for ^{13}C label excreted in breath CO_2 (as % absorbed dose) in the subsequent studies of this thesis.

Finally, the repeatability study demonstrated that energy expenditure (kcal/kg LM) and substrate oxidation (CHO oxidation, gm/kg LM; net lipid oxidation, gm/kg LM; breath $^{13}CO_2$, % AD/kg LM and exogenous lipid oxidation, gm/kg LM) were also more repeatable as the differences between the two trials were small (1.5%, 3%, 10%, 3%, and

4.2% respectively; Table 4.5). On the other hand, incremental increase in net Lox (gm/kg LM), endogenous lipid oxidation (gm/kg LM) and thermic effect of food (kcal/kg LM) were less repeatable as the differences between the two trials were large (69%, 20% and 28% respectively). This is discussed in section 4.9.4

The high repeatability of postprandial lipid metabolism suggests that the metabolic competence of an individual for clearing lipids from circulation is intrinsic in nature. By controlling the preceding diet, level of physical activity, type and amount of lipid and CHO in test meal, a fairly repeatable response in postprandial lipid metabolism can be achieved if examined on two different occasions. Hence by using the same level of control in preceding diet, level of physical activity, and type and amount of lipid and CHO in test meal, one can minimise the intra-individual variability in postprandial lipid responses in other groups of individual such as middle-aged men, diabetics etc.

7.3 Age-related changes in postprandial lipid metabolism

This is the first occasion that stable isotope tracer methodology has been used in conjunction with indirect calorimetry to study age-related changes in postprandial lipid metabolism. The present study showed decreased clearance of exogenous TAG (CRF-TAG ^{13}C PA) from the circulation in middle-aged men compared to that observed in young controls (section 5.5.1.4). The observation of decreased or slower clearance of exogenous TAG from circulation in increased age group is also supported by previous studies using retinyl esters as a marker of exogenous TAG metabolism. The present study further provided insight into postprandial lipid metabolism by showing higher appearance/retention of exogenous fatty acids within the plasma NEFA fraction (NEFA ^{13}C PA; section 5.5.1.3) and decreased exogenous lipid oxidation (breath $^{13}CO_2$; section 5.5.2.3) in the older men. Since increasing age is related with increased risk of developing obesity, type II diabetes and CVD, this study supports the view that disturbed postprandial lipid metabolism associated with increasing age might also be related to increased risk of developing these diseases. Since the underlying mechanisms that are responsible for this apparent impairment in postprandial lipid metabolism with increasing age remains unclear, further studies are required. Possible stable isotope studies which might be able to provide a clear picture of mechanisms responsible for impaired postprandial lipid metabolism in the setting of increased age include:

Young men		Middle-aged men				
		Study 1	**Study 2**	**Study 3**	**Study 4**	**Study 5**
Fasting TAG	Normal	Matched*	High**	Matched	Matched	Matched
Total Fat Mass (FM)	Normal	Matched	Matched	High	Matched	Matched
Abdominal FM	Normal	Matched	Matched	Matched	High	Matched
Fasting glucose	Normal	Matched	Matched	Matched	Matched	High
Fasting Insulin	Normal	Matched	Matched	Matched	Matched	High

*, values matched to young men; **, values significantly higher than young men

- Study 1 would test the hypothesis that the impaired postprandial lipid metabolism in middle-aged men is due to age *per se,* whilst controlling for fasting TAG, total fat mass, abdominal fat mass, fasting glucose and fasting insulin.
- Study 2 would test the hypothesis that the impaired postprandial lipid metabolism in middle-aged men is primarily due to age associated increase in fasting TAG, whilst controlling for total fat mass, abdominal fat mass, fasting glucose and fasting insulin.
- Study 3 would test the hypothesis that the impaired postprandial lipid metabolism in middle-aged men is primarily due to age associated increase in total fat mass, whilst controlling for altered fasting TAG, abdominal fat mass, fasting glucose and fasting insulin.
- Study 4 would test the hypothesis that the impaired postprandial lipid metabolism in middle-aged men is primarily due to age associated increase in abdominal fat mass, whilst controlling for fasting TAG, total fat mass, fasting glucose and fasting insulin. Whilst theoretically desirable, it is unlikely to find subjects with increased abdominal fat but matched for total fat mass.
- Study 5 would test the hypothesis that the impaired postprandial lipid metabolism in middle-aged men is primarily due to age associated increase in insulin resistance whilst controlling for fasting TAG, total fat mass, and abdominal fat mass.

7.4 Postprandial lipid metabolism in HTG and NTG type II diabetes mellitus

This is the first occasion in which stable isotope tracer methodology was used in conjunction with indirect calorimetry to study postprandial lipid metabolism in type II diabetes. This study showed decreased clearance of exogenous TAG (CRF-TAG ^{13}C PA) from circulation in HTG type II diabetics than NTG type II diabetics and controls (section 6.5.1.4). This study also showed that diabetes *per se* (as indicated by higher insulin, glucose and NEFA concentrations in NTG type II diabetics than controls) was not related to disturbed postprandial lipid metabolism (6.5.1.4). These observation of decreased or slower clearance of exogenous TAG from circulation in HTG type II diabetics *v* NTG type II diabetics and controls is also supported by previous studies using retinyl esters as a marker of exogenous TAG metabolism or using apo B48 as a marker of particles carrying exogenous TAG in circulation (6.5.1.4). The present study provided further insight into postprandial lipid metabolism by showing a decreased appearance of exogenous fatty acids in plasma NEFA fraction (NEFA ^{13}C PA; section 6.5.1.3) in HTG and NTG type II diabetics and decreased exogenous lipid oxidation (breath $^{13}CO_2$; section 6.5.2.3) in HTG type II diabetics. Since type II diabetes is associated with increased risk of CVD, this study supports the view that disturbed postprandial lipid metabolism associated with HTG type II diabetes might be related to increased risk of developing CVD. Since the underlying mechanisms responsible for disturbed postprandial lipid metabolism in HTG type II diabetics remains unclear, further studies are needed. Possible stable isotope studies which might be able to provide a clear picture of mechanisms responsible for defective postprandial lipid metabolism in type II diabetes include:

	Controls	Type II diabetics			
		Study 1	Study 2	Study 3	Study 4
Fasting TAG	Normal	Matched*	High**	Matched	Matched
Total Fat Mass (FM)	Normal	Matched	Matched	High	Matched
Abdominal FM	Normal	Matched	Matched	Matched	High
Fasting glucose	Normal	High	High	High	High
Fasting Insulin	Normal	High	High	High	High

*, values matched to controls; **, values significantly higher than controls

- Study 1 would test the hypothesis that the impaired postprandial lipid metabolism in type II diabetics is due to diabetes *per se,* whilst controlling for fasting TAG, total fat mass and abdominal fat mass.
- Study 2 would test the hypothesis that the impaired postprandial lipid metabolism in type II diabetics is primarily due to increase in fasting TAG, whilst controlling for total fat mass and abdominal fat mass.
- Study 3 would test the hypothesis that the impaired postprandial lipid metabolism in type II diabetics is primarily due to increase in total fat mass, whilst controlling for fasting TAG and abdominal fat mass.
- Study 4 would test the hypothesis that the impaired postprandial lipid metabolism in type II diabetics is primarily due to increase in abdominal fat mass, whilst controlling for fasting TAG and total fat mass. Whilst theoretically desirable, it is unlikely to find subjects with increased abdominal fat but matched for total fat mass.

7.5 Fasting and postprandial responses of plasma glucose, insulin, NEFA, TAG and ^{13}C palmitic acid in chylomicron TAG and free fatty acid fraction

In the light of current review literature and central hypothesis for this thesis the relationship of insulin with above blood parameters is discussed here. Since it has been previously shown that in subjects with normal and abnormal glucose tolerance, fasting insulin concentration was the best marker of insulin resistance as determined by whole body glucose uptake using euglycaemic hyperinsulinaemic clamp technique (Laakso, 1993). Hence, in the present study hyperinsulinaemia was considered as a surrogate marker of insulin resistance.

Does hyperinsulinaemia affects fasting and postprandial plasma glucose levels?

Taking together the results of the four studies suggests that hyperinsulinaemia does affect plasma glucose levels, however, it should be noted that in contrast to diabetic group, glucose homeostasis was better maintained in the postprandial state in middle-aged men and young men (Figures 7.1 and 7.2). Furthermore, a significant positive correlation of fasting insulin with fasting glucose ($r=0.76$, $p=0.001$) and postprandial glucose ($r=0.74$, $p=0.001$) as well as a positive relationship between postprandial insulin AUC and postprandial glucose AUC ($r=0.50$, $p=0.01$) suggest that as the insulin concentrations increases, plasma glucose levels also increases not only in the fasting state but also in the

postprandial state (Appendix 8.8, Figure 7.7 A, B, and C respectively). The relationship between postprandial insulin concentrations and glucose concentrations are not very strong because hyperinsulinaemia was maintaining glucose levels near to baseline in young men and middle-aged men but not in diabetics. Furthermore, greater inter-individual variability in postprandial plasma glucose and insulin responses in NTG and HTG diabetics has further affected the strength of the relationship.

Is there any evidence from previous studies that insulin resistance is related to increased plasma glucose levels? There is no argument on the effect of insulin resistance on glucose utilisation in diabetes because insulin resistance is a characteristic feature of type II diabetes (DeFronzo, 1988). There is also evidence that glucose utilisation declines with increasing age (Rosenthal *et al.,* 1982; Rowe *et al.,* 1983; Pagano *et al.,* 1984). In type II diabetics, it has been suggested that the metabolic abnormalities of hyperglycaemia and hyperinsulinaemia contribute to the impairments of glycogen synthase (Nikoulina *et al.*, 1997). Hence, in diabetics the decline in glucose utilisation is mainly attributed to reduced glucose deposition as glycogen in muscle, due to low rate of skeletal muscle glycogen synthesis and impaired insulin-stimulated activation of glycogen synthase in diabetic subjects (Shulman *et al.,* 1990; Thorburn *et al.,* 1990). Furthermore, since NEFA provide energy for gluconeogenesis, impaired suppression of plasma NEFA levels after glucose ingestion lead to higher rate of hepatic glucose output (Foley, 1992) and systemic glucose delivery (Kruszynska *et al.,* 1997).

Does insulin resistance result in increased lipid mobilisation?

Taken together the results of the four studies suggests that hyperinsulinaemia is associated with increased lipid mobilisation. Postprandial suppression of NEFA was highest in young men and lowest in HTG diabetics (Figure 7.3). Furthermore, as the fasting and postprandial insulin responses were lowest in young men and highest in HTG diabetics, a significant positive relationship between fasting NEFA and fasting insulin concentrations ($r=0.64$, $p=0.001$) as well as between postprandial insulin AUC and postprandial NEFA AUC ($r=0.69$, $p=0.001$), suggest that insulin resistance results in decreased suppression of NEFA from adipocytes especially in the postprandial state (Appendix 8.8 & Figure 7.7 D & E respectively). A positive correlation between a) fat mass and fasting insulin concentrations ($r=0.83$, $p=0.001$), b) fat mass and postprandial insulin AUC ($r=0.72$, $p=0.0001$), and c) fat mass and incremental increase in insulin AUC ($r=0.65$, $p=0.001$) further suggests that this apparent lack of suppressive effect of insulin

in adipose tissue resulted in greater lipid mobilisation both in the fasting and postprandial states (Appendix 8.8 & Figure 7.7 F, G & H respectively).

Is there any evidence from previous studies that insulin resistance results in increased lipid mobilisation? Many studies have shown that plasma NEFA concentrations are largely determined by the action of insulin to suppress adipocyte TAG lipolysis by suppression hormone sensitive lipase (Weiland *et al.,* 1980; Yki-Jarvinen & Taskinen, 1988) and promoting re-esterification of NEFA in to TAG in adipose tissue (Coppack *et al.,* 1992). Increased lipid mobilisation occurs in obesity and insulin resistance state because the antilipolytic effect of a mixed meal is reduced in obesity (Roust & Jensen, 1993) and insulin resistance (Frayn, 1993). Moreover the antilipolytic effect of insulin is less on intra-abdominal fat cells than subcutaneous and thus resulting in less suppression of NEFA and more lipid mobilisation from intra-abdominal cells in postprandial state.

Does insulin resistance results in increased postprandial plasma TAG and CRF-TAG ^{13}C PA concentrations?

Taken together the results of the four studies suggests that insulin resistance does result in increased postprandial plasma TAG and CRF-TAG ^{13}C PA concentrations especially in HTG type II diabetics, and at the later time points, and after second meal (Figure 7.4 and 7.5). It should be noted that, although NTG diabetics were more insulin resistant as apparent from their fasting and postprandial insulin, glucose and NEFA concentrations than middle aged men, their plasma TAG and CRF-TAG ^{13}C PA concentrations however were comparable to that seen in middle-aged men. The explanation for this observation could be the lipid load used in the test meal (36 gm) was insufficient enough to challenge the metabolic competence of NTG diabetics to clear lipids from circulation. A previous study in NTG diabetics has shown higher postprandial concentrations of both plasma TAG and chylomicron TAG (Reznik *et al.,* 1996) after giving a high fat meal containing 60 g fat/m^2 body surface area, consisting 67% calories as fat, 14% as CHO and 19% as protein.

A significant positive correlation between fasting insulin and fasting TAG ($r=0.73$, $p=0.001$; Appendix 8.8 & Figure 7.7 I), and between CRF-TAG ^{13}C PA AUC and fasting TAG ($r=0.58$, $p=.002$; Figure 7.7J) suggest that as the insulin resistance increases lipid clearance from circulation decreases thus leading to higher fasting TAG concentrations. Similarly, positive correlations between postprandial insulin AUC and postprandial TAG

AUC (r=0.62, p=0.001; Appendix 8.8 & Figure 7.7K), suggest that as the insulin resistance increases, postprandial TAG concentrations (chylomicron and VLDL) also increases. Finally, positive relationships between a) the incremental increase in insulin AUC and incremental increase in TAG AUC (r=0.54, p=0.002), and b) postprandial insulin AUC and CRF-TAG ^{13}C PA AUC (r=0.60, p=0.001; Table 7.1& Figure 7.7 L & M respectively) further suggest that as insulin resistance increases postprandial concentrations of exogenous TAG also increases. The reason for the relatively weak relationships between postprandial insulin and TAG (including CRF-TAG ^{13}C PA) concentrations is discussed in the previous paragraph. Furthermore, the additional explanation could be the greater within group variability in metabolic competence for clearing lipids from circulation.

What is the evidence from other studies that insulin resistance affects fasting and postprandial lipid metabolism? Studies have shown that increased triglyceride and low HDL cholesterol concentrations within plasma are associated with insulin resistance in type II diabetics (Chaiken *et al.,* 1991; Widen *et al.,* 1992). Insulin resistance is also present in non-diabetic subjects with hypertriglyceridaemia (Yki-Jarvinen & Taskinen, 1988; McKane *et al.,* 1990). Furthermore, even in young individuals there is an association between fasting plasma insulin and fasting plasma triglyceride (Jiang *et al.,* 1995; Raitakari *et al.,* 1995; Bonora *et al.,* 1996).

How does insulin affect postprandial lipid metabolism? Insulin affects most probably postprandial lipid metabolism through its effect on a) lipoprotein lipase activity and b) hepatic VLDL secretion. LPL is an insulin-dependent enzyme, the activity of which is decreased in obesity (Eckel, 1987), NIDDM (Nikkila,1984; Eckel, 1989; Pollare *et al.,* 1991) and in insulin resistant subjects (Chen *et al.,* 1994; Knudsen *et al.,* 1995). In obesity and other states of insulin resistance, LPL loses its responsiveness to insulin in adipose tissue (Eckel, 1987). Coppack *et al.,* (1992) have shown that the action of LPL across subcutaneous adipose tissue depot increases postprandially in normal subjects, whereas this increase is blunted or absent in obese subjects. Furthermore, the importance of lipolysis by LPL is well documented in type I hyperlipoproteinaemia characterised by a massive accumulation of chylomicrons in plasma due to deficiency in LPL activity (Brunzell, 1995). Overexpression of the LPL in mice results in rapid clearance of chylomicrons and a decrease in VLDL levels (Liu, 1994). Studies have shown a correlation between the activity of LPL in the tissue and its uptake of fatty acids from

chylomicron (Jeppesen *et al.,* 1995a; Eckel *et al.,* 1995; Potts *et al.,* 1995). Hence activation of LPL is an important determinant in the clearance of TAG from circulation.

Insulin acutely suppresses the hepatic secretion of VLDL-TAG (Durrington *et al.,* 1982; Bartlett *et al.,* 1988). It has also been suggested that rate of supply of NEFA to the liver is the major factor controlling hepatic TAG secretion (Byrne *et al.,* 1991, Kissebah *et al.,* 1974). Hence, failure to suppress both the supply of NEFA from adipose tissue to liver after a meal as well as VLDL synthesis and release in the insulin resistant state results in sustained VLDL production in the postprandial state (Malmstrom *et al.,* 1997). It has been suggested that chylomicrons and VLDL particles not only compete for lipolysis by LPL (Brunzell *et al.,* 1973) but also their remnants compete for the same removal process (Cooper *et al.,* 1982). Hence, in the insulin resistant state decreased suppression of NEFA from adipose tissue and lack of inhibition of VLDL production from liver will result in increased TAG concentrations due to competition between chylomicrons and VLDL at the site of LPL and their remnants at the site of liver.

Does insulin resistance results in decreased exogenous lipid oxidation?

Taken together the results of the four studies suggests that insulin resistance results in decreased exogenous lipid oxidation (Figure 7.6). A negative correlation between postprandial insulin concentrations and exogenous lipid oxidation ($r= -0.37$, $p=0.06$) suggests that insulin resistance does contribute to the reduction in exogenous lipid oxidation (Appendix 8.8 & Figure 7.7N). However, it should be noted that this relationships is not very strong. As discussed in the previous section, the explanation for this observation could be that the lipid load used in the test meal (36 gm) was not sufficient to challenge the metabolic competence of NTG diabetics to clear lipids from circulation, hence failing to result in further decreased exogenous lipid oxidation than middle-aged men. Large inter-individual variability in exogenous lipid oxidation in NTG diabetics and higher exogenous lipid oxidation after the second meal may have further weakened the relationship.

Does insulin resistance affect the appearance of exogenous fatty acids in circulation?

Taken together the results of the four studies suggests that hyperinsulinaemia does not affect the appearance of exogenous fatty acids in circulation. No relationship was found between hyperinsulinaemia and appearance of exogenous fatty acids in circulation. However, a negative association was found between fat mass and appearance of fatty acids

in circulation (r= -0.45, p=0.01; Figure 7.7 O). As discussed in results section 6.4.1.6, the data regarding ^{13}C NEFA concentrations should be interpreted with caution because the PA peaks detected by GC-C-IRMS were very small due to very low concentration of PA in the NEFA fraction, especially after the postprandial suppression of NEFA concentrations. Very small peaks can give erroneous results in terms of isotopic enrichments and substrate concentrations.

7.6 So what are the proposed models for lipid metabolism in healthy men and insulin resistant men?

Model for lipid metabolism in healthy men

In healthy men with a given level of physical activity, if the metabolic competence for clearing lipids from circulation match the amounts of lipid and CHO intake, the increase in postprandial lipid, glucose and insulin concentrations will be within the normal range. The net effect of normal lipid concentrations in circulation will be normal insulin sensitivity, which will lead to normal clearance of lipids from circulation and normal exogenous lipid oxidation. The mechanisms by which normal insulin sensitivity results in further increased clearance of lipids from circulation will include: a) rapid suppression of release of NEFA from adipose tissue (which otherwise serve as a substrate for VLDL TAG synthesis in liver), both by inhibition of hormone sensitive lipase and stimulation of re-esterification of NEFA in to TAG, b) rapid inhibition of formation and release of VLDL from the liver (VLDL TAG is not needed in circulation in the postprandial state as chylomicrons are bringing loads of TAG), hence resulting in decreased concentration of VLDL in circulation, and c) activation or stimulation of adipose tissue LPL, hence resulting in increased removal of TAG-rich lipoproteins from circulation. The net result of these insulin-stimulated co-ordinated changes in lipid metabolism will be increased clearance of TAG form the circulation, which will lead to normal levels of lipid, glucose and insulin in the fasting state (Figure 7.8).

The results of healthy young men study supports this model in terms of normal fasting and postprandial lipid, glucose, and insulin concentrations, and exogenous lipid oxidation. This model is also supported by other studies in literature in terms of normal fasting and postprandial lipid, glucose and insulin concentrations. However, studies have not yet been conducted other than this to support decreased exogenous fatty acids in circulation and normal exogenous lipid oxidation as proposed in this model. Moreover, the

higher appearance of exogenous fatty acids in circulation does not support this model. As discussed in results section 6.4.1.6, data of ^{13}C NEFA concentrations should be interpreted with caution. If however, the appearance of exogenous fatty acids represents a real phenomenon in young men, then it might be due to decreased uptake by adipose tissue. The volume of adipose tissue (especially in the abdomen, which is more metabolically active) is small in healthy young men compared to that found in obese subjects thus resulting in decreased re-esterification of the increasingly large amounts of incoming fatty acids. Further studies are needed in young healthy men to resolve this issue by titrating NEFA from larger volumes of plasma (such as 2 ml instead of 0.5 ml of plasma in this case) and giving them different levels of oral lipid loads (less than 37.5g as in this study) to determine the level of metabolic competence at which large amounts of exogenous fatty acids do not appear in the plasma.

How may the above model of lipid metabolism be altered in men with insulin resistance?

In insulin resistant men with a given level of physical activity, if the metabolic competence for clearing lipids from circulation does not match the consumed amount of lipids and CHO, there will be a greater increase in postprandial lipid, glucose and insulin concentrations than that seen in younger men. The net effect of higher lipid concentrations in the circulation will further exaggerate insulin resistance, which will lead in turn to reduced clearance of lipids from the circulation and reduced exogenous lipid oxidation. The mechanisms by which insulin resistance may achieve these effects include: a) reduced suppression of release of NEFA from adipose tissue (which will then serve as a substrate for VLDL TAG synthesis in liver) both by reduced inhibition of hormone sensitive lipase and decreased stimulation of fatty acid re-esterification, b) reduced inhibition of formation and release of VLDL from the liver thus resulting in increased concentration of VLDL in circulation, and c) decreased activation or stimulation of adipose tissue LPL thus resulting in decreased removal of TAG-rich lipoproteins from circulation. The net result will be a situation in the postprandial state as chylomicron and VLDL compete with each other not only for lipolysis by LPL (which is also not well activated) and there remnants compete for there removal at the site of liver. This will result in decreased clearance of TAG form circulation, which will lead to higher levels of lipid, glucose and insulin in the fasting state (see figure 7.9).

The results of the study on middle-aged men supports this model in terms of higher

fasting and postprandial lipid, glucose and insulin concentrations and lower entrapment of exogenous fatty acids and lower exogenous lipid oxidation. This model is also supported by other studies in literature in terms of higher fasting and postprandial lipid, glucose and insulin concentrations in aged compared to young men. However, studies have not yet been conducted other than those presented here to support decreased entrapment of exogenous fatty acids in circulation and decreased exogenous lipid oxidation in middle-aged men. As discussed in section 6.4.1.6, data of ^{13}C NEFA concentrations should be interpreted with caution because the PA peaks detected by GC-C-IRMS were very small due to little concentration of PA in NEFA fraction, especially after the postprandial suppression of NEFA concentrations.

The results of the study on NTG diabetics supports this model in terms of further higher fasting and postprandial NEFA, glucose and insulin concentrations. But it does not support the model in terms of ever greater postprandial TAG concentrations and ever lower rates of exogenous lipid oxidation. It also does not support this model in terms of further lower entrapment of exogenous fatty acids. Other studies in literature also supports higher fasting and postprandial NEFA, glucose and insulin concentrations. As discussed in postprandial TAG and exogenous lipid oxidation sections, the explanation for the observation of failing to further increase in TAG concentrations and decrease exogenous lipid oxidation with increased insulin resistance could be the lipid load used in the test meal (36 gm). This lipid load might not be sufficient enough to challenge the metabolic competence of NTG diabetics to clear lipids from circulation thus failing to result in further increase in TAG concentrations and further decrease in exogenous lipid oxidation. A previous study in NTG diabetics has shown higher postprandial concentrations of both plasma TAG and chylomicron TAG (Reznik *et al.*, 1996) after giving a fatty meal containing 60 g fat/m^2 body surface area, consisting 67% calories as lipid, 14% as CHO and 19% as protein. In other words, although NTG diabetics were more insulin resistant as apparent from their fasting and postprandial insulin, glucose and NEFA concentrations than middle aged men, their metabolic competence of clearing lipids from circulation was similar to that as to middle-aged men thus resulting in comparable fasting and postprandial TAG concentrations and exogenous lipid oxidation. Further studies are needed to test if increasing the lipid load more than 50 or 100 gm result in decreased clearance of lipid from circulation and decreased exogenous lipid oxidation in NTG diabetics. On the other hand, studies have not yet been conducted other than those presented here to support

increased entrapment of exogenous fatty acids from circulation in NTG diabetics. As discussed in the results section (6.4.1.6), data of ^{13}C NEFA concentrations should be interpreted with caution. However, if increased uptake of exogenous fatty acids represents a real phenomenon in NTG diabetics, then it might be due to an increased uptake by adipose tissue especially abdominal adipose tissue which is more metabolically active. Since the NTG diabetics had higher fat mass and BMI (probably higher abdominal fat mass than middle-aged men and young men) and were losing more NEFA from adipose tissue due to lack of suppression due to insulin resistance, the greater ^{13}C NEFA uptake and re-esterification in the NTG diabetics might represent a mechanism to rebuild the continually exhausting stores of TAG from metabolically active adipose tissue.

The results of the study on the HTG diabetic subjects supports this model in terms of further elevations in fasting and postprandial lipid, glucose and insulin concentrations and ever lower exogenous lipid oxidation. It does not support the model in terms of further lower entrapment of exogenous fatty acids rather it showed increased entrapment of exogenous fatty acids. Other studies in literature also supports higher fasting and postprandial lipid, glucose and insulin concentrations HTG diabetics. Similarly, studies have not yet been conducted other than this to support further decreased entrapment of exogenous fatty acids in circulation in HTG diabetics. However, a previous stable isotope study in NTG obese subjects (Binnert *et al,*. 1998) has showed that the appearance of ^{13}C oleic acid in non-esterified fatty acid fraction was dramatically reduced in obese than the control. They have also found a strong negative correlation between fat mass and ^{13}C oleic acid in NEFA fraction ($r = -0.84$). As discussed in the previous section, if increased uptake of exogenous fatty acids represents a real phenomenon in HTG diabetics, then it might be due to increased uptake by adipose tissue especially abdominal adipose tissue which is more metabolically active. Further studies are needed in both lean and obese diabetics to see if increased fat especially abdominal fat mass is associated with increased entrapment of exogenous fatty acids. Increased precision (confidence of measurement) would be achieved by extracting ^{13}C PA in NEFA from larger volumes of plasma (such as 2 ml instead of 1 ml of plasma in this case).

What is the evidence that insulin resistance/hyperinsulinaemia is associated with increased risk cardiovascular disease?

Many studies have shown that high insulin levels are associated with cardiovascular disease (Ducimetiere *et al.,* 1980; Reaven, 1988; Despres *et al.,* 1996). For example, insulin resistant type II diabetics had more atherogenic cardiovascular risk profiles (such as high TAG, preponderance of small dense LDL and low HDL cholesterol) than insulin-sensitive type II diabetics (Haffner *et al.,* 1999). In type II diabetic subjects, increased fasting insulin concentrations (a commonly used surrogate for insulin resistance) predicted the development of coronary heart disease in the Finnish Study (Ronnemaa *et al.,* 1991).

How is higher concentration of TAG in the circulation related to increased risk of developing CVD?

Higher concentration and prolonged circulation of triglyceride-rich lipoproteins increases the process of neutral transfer of lipids with the subsequent enrichment of HDL and LDL with triglycerides and enrichment of triglyceride-rich lipoproteins and their remnants with cholesterol esters. This reaction is catalysed by cholesteryl-ester transfer protein (Tall, 1986; Tall, 1993). Several studies testify that the transfer of cholesteryl esters from HDL to the triglyceride-rich lipoproteins is enhanced during postprandial state (Esinberg, 1984). The TAG in HDL and LDL is further hydrolysed preferentially by hepatic lipase (because of the absence of apo C II in these particles) resulting in small dense LDL and HDL particles. Individuals with triglycerides in the range of 0.5-1.5 mmol/l have predominantly large LDL, whereas those with levels of TAG above 1.5 mmol/l have an increasing propensity to generate small dense LDL. The best evidence of a threshold of 1.5 mmol/l is seen in pregnancy (Sattar *et al.,* 1997).

Cholesterol-enriched, triglyceride-rich lipoprotein remnants are atherogenic as they are able to promote deposition of cholesterol in macrophages (Proctor & Mamo, 1996), which result in the formation of atherosclerotic plaque. Furthermore, small dense LDL particles are more susceptible to oxidation (Chait *et al.,* 1993). Oxidatively modified LDL results in increased uptake by macrophage scavanger receptor (Henriksen *et al.,* 1983), thus result in the formation of cholesterol loaded foam cell which is a prominent feature of atherosclerotic plaque (Gown *et al.,* 1986). Finally low HDL compromises reverse cholesterol transport. The correction of dyslipidaemia by triglyceride lowering drugs such

as fibrates result in the reduction in the progression of atherosclerosis and in the incidence of coronary events as observed in fibrate-based BECAIT (Ericsson *et al.,* 1996) and LOCAT (Frick *et al.,* 1997) studies despite little or no change in total LDL levels. Taken together, the results of these studies suggest that disturbed postprandial lipid metabolism is related to increased risk of atherosclerosis.

7.7 Study design considerations in future studies

This section discusses the need of future studies in order to answer the questions which could not be answered by the this thesis work.

Fasting TAG: does it represent the size of the pool of VLDL and/ or VLDL remnants or both VLDL and chylomicron and their remnant?

Fasting TAG, which is traditionally thought to reflect the size of VLDL pool, is known to be a strong determinant of postprandial TAG increment in normal subjects (Cohn *et al.,* 1988b), obese patients (Lewis *et al.,* 1990) and type II diabetics (Syvanne *et al.,* 1994). However, it is not clear due to methodological constraints whether the fasting TAG represents the size of the pool of VLDL and/ or VLDL remnants or both VLDL and chylomicron and their remnants? Furthermore, to what extent is the source of TAG in fasting VLDL derived from recently consumed exogenous lipid? Since young men had the lowest fasting TAG concentrations as well as increased clearance of CRF-TAG ^{13}C PA and lower appearance of CRF-TAG ^{13}C PA after second unlabelled meal than middle-aged men and diabetics. It is therefore proposed that in the insulin resistance states, slower clearance of exogenous TAG along with further appearance of previously eaten TAG after every meal might lead to the presence of exogenous TAG in the late fasting state in the form of chylomicron and chylomicron remnants. Further stable isotopically labelled lipid studies in conjunction with apo B100 and apo B48 determination are needed to confirm the presence of chylomicron and their remnants in the fasting state by giving labelled TAG with the regular meals for 24 hours and then measuring CRF-TAG ^{13}C PA in the morning after 12-h fasting.

Decreased clearance of postprandial TAG: does it represent decreased clearance of CRF-TAG or VLDL TAG or both CRF-TAG and VLDL TAG ?

Studies using retinyl esters and apo B as markers of CRF-TAG suggests that higher concentrations of TAG over the later part of the postprandial period represents decreased clearance of CRF-TAG (Krasinski *et al.,* 1990a; Lewis *et al.,* 1991; Cooper *et*

al., 1996; Borrel *et al.,* 1998; Karpe et al., 1999). As discussed in section 1.1, concern has been raised about retinyl palmitate for delay in absorption as compared to triacylglycerol and apo B48 and its transference to other lipoproteins such as LDL and VLDL. Hence increased concentrations of retinyl esters at the later part of postprandial period may be misleading. Similarly the primary disadvantage for apo B48 is that it represents the particles carrying TAG but not the amount of TAG carried by these particles. Hence higher or lower concentrations of apo B48 may be misleading because few TAG-rich apo B particles (decreased apo B concentration) may contain more TAG than many TAG-poor particles (increased apo B concentration). The studies presented in this thesis also suggests that decreased clearance of TAG is due to decreased clearance of CRF-TAG as indicated by decreased removal of ^{13}C PA from the TAG in circulation. This interpretation was also supported by the observation of decreased appearance of ^{13}C in breath. Since ^{13}C palmitic acid concentration was not directly determined in VLDL particles, the present studies do not shed light on the clearance of VLDL TAG. Stable isotope tracer studies should be extended to VLDL metabolism in conjunction with apo B100 and apo B48 determination in order to obtain a more complete understanding of the clearance of TAG-rich lipoproteins. However the inclusion of VLDL metabolism in tracer studies may not provide extra information due to 1) the presence of chylomicron remnants within the prepared VLDL fraction as there is no satisfactory method to separate chylomicron remnants because of their size and density as VLDL particles have 2) the presence of fatty acids derived from adipose tissue in VLDL TAG especially in an increasingly insulin resistance state. An alternate approach to determine the clearance of VLDL TAG could be to infuse intralipid particles of VLDL size labelled with tracer TAG. However this method would again not be able to differentiate the metabolism of VLDL and chylomicron remnants unless apo B100 or apo B48 was also incorporated in intralipid particles.

Sources of NEFA in circulation in postprandial state: exogenous or endogenous in origin?

Findings of the present studies support the view that the source of postprandial NEFA could either be derived predominantly from exogenous or endogenous sources depending upon the levels of insulin resistance and percentage of abdominal fat. In young and middle-aged men of normal body weight, the source of NEFA in circulation during the postprandial period might be predominantly derived from newly arrived exogenous fatty acids resulting from relatively poor uptake by a small volume of adipose tissue

(especially the more metabolically active abdominal tissue). On the other hand, in obese diabetic subjects the principal source of NEFA might be derived from endogenous fatty acids from adipose tissue due to decreased suppression of NEFA release from adipose tissue and the increased uptake of exogenous fatty acids by large volume of adipose tissue (especially the more metabolically active adipose tissue). A recent stable isotope study (using comparable approach to that presented here) has also showed that the appearance of ^{13}C oleic acid in the NEFA fraction of plasma was dramatically reduced in obese than the control (Binnert *et al,*. 1998). They have also found a strong negative correlation between fat mass and ^{13}C oleic acid in NEFA fraction (r = -0.84). Since abdominal obesity was not determined in the subjects of this thesis, further stable isotope studies are needed to get a clear picture of source of NEFA in circulation by extracting NEFA from larger volumes of plasma (such as 2 ml or more) from lean and obese (with abdominal fat patterning) healthy young and middle-aged men and type II diabetics respectively. Further studies in lean healthy young subjects can also be conducted to examine if decreasing the lipid load less than 36 g (as used in thesis studies) results in increased uptake of exogenous NEFA.

Challenging the metabolic competence of clearing lipids from circulation: how much lipid load is needed in the test meal or in the preceding meal before fasting?

Some studies have shown that high lipid load in test meal results in higher TAG responses in NTG diabetics (Reznik et al, 1996), while others have shown no effect (Lewis et al, 1991; Cooper et al, 1996;). The NTG diabetics in this thesis showed comparable plasma TAG and CRF-TAG ^{13}C PA concentrations to middle-aged men, although they were more insulin resistant as apparent from their fasting and postprandial insulin, glucose and NEFA concentrations than the middle aged men. The explanation for this observation could be the lipid content of either preceding evening meal (22 g) or lipid content of study day test meal (36 gm) or both were not sufficient to challenge the metabolic competence of NTG diabetics to clear lipids from circulation thus resulting in normal fasting and postprandial TAG levels. Future stable isotope studies in NTG diabetics could be conducted in order to examine the effect of lipid load on the fasting and postprandial TAG by increasing lipid load (> 40 g) either in the preceding evening meal or both preceding evening meal and study day test meal.

Is plasma insulin a true marker of insulin resistance in the postprandial state?

It is generally agreed that fasting plasma insulin concentrations represents a good marker of insulin resistance (Laakso, 1993). However, it has been demonstrated that the increased insulin activity, seen in patients with hyperinsulinaemia, may be due to proinsulin and other insulin like molecules (Nagi et al, 1990). Hence insulin measured by radioimmunoassay might overestimate insulin measurements as it does not specifically measure insulin but also cross reacts with proinsulin. Furthermore a more complete characterisation of the insulin response can be obtained by measuring c-peptide which is excreted into the circulation with equimolar amounts of insulin. Owing to the above discussion it is suggested that in the future stable isotope studies involving insulin resistance states, true insulin resistance may be measured either by insulin-glucose clamps or by C-peptide concentrations.

7.8 Summary/Conclusion

The results of validation study demonstrated precision in lipid extraction procedures and in the measurement of ^{13}C palmitic acid enrichment and concentrations by GC-C-IRMS system. The variability between samples can be further reduced by careful sample derivatization, good chromatography and appropriate baseline correction. The observation of high enrichment values in chylomicron-rich fraction TAG implies that less ^{13}C PA label might be used in future tracer studies

The results of the repeatability study suggested that except for plasma glucose the postprandial responses of plasma TAG, NEFA, ^{13}C palmitic acid in CRF-TAG and breath $^{13}CO_2$ were repeatable as the differences between two trials were small. It appeared that if the preceding diet and physical activity are controlled the variability between two identical trials can be reduced. The practical implication of the repeatability of lipid responses as observed in the present study is that reliable fasting and postprandial lipid tolerance tests can be conducted in clinical settings after the intake of standard test meal (while controlling at least the previous evening meal and physical activity) in order to identify individuals with reduced metabolic competence for clearing lipids from circulation. Furthermore, ^{13}C excretion in stools was minimal (1% of the administered label was recovered in the stools) and repeatable as the differences observed between two identical trials were small. The implication of these results was that the determination of ^{13}C excretion in stools was not be required for the correction for ^{13}C label excreted in breath CO_2 (as % absorbed dose) in the subsequent studies of this thesis

Taken together, the results of studies of young men, middle-aged men, NTG and HTG type II diabetics suggest that the presence of increasing fasting TAG (a marker for decreased metabolic competence for clearing lipids) resulted in a) decreased postprandial clearance of exogenous TAG from circulation b) decreased exogenous lipid oxidation and c) decreased appearance of exogenous fatty acids in NEFA fraction. Furthermore, in the absence of fasting hypertriglyceridaemia (a marker for normal metabolic competence for clearing lipids) as observed in NTG type II diabetics, the diabetes or insulin resistance *per se* did not result in further decreased clearance of lipids from circulation and further decreased exogenous lipid oxidation. Finally, ^{13}C NEFA data of these studies suggested that the clearance of exogenous fatty acids from circulation increases with the increase in the size of metabolically active abdominal adipose tissue mass. However further

confirmation is needed in this respect by extracting ^{13}C PA from larger volumes of NEFA fractions in lean and obese individuals with abdominal fat patterning.

Since the exact mechanisms of decreased clearance of exogenous TAG from circulation and decreased exogenous lipid oxidation are still unclear, future tracer studies are needed to test the hypothesis that the impaired exogenous TAG clearance is due to:

A) Increased competition between CRF-TAG and VLDL-TAG for TAG clearance by LPL by examining postprandial lipid metabolism in HTG subjects before and after the removal of VLDL particles from fasting plasma by plasmapheresis technique (Lupein et al, 1976) **OR** by examining the postprandial lipid metabolism in HTG subjects before after decreasing the CRF-TAG in circulation by Bilio Pancreatic Diversion Surgery (Flanchbaum & Choban, 1998) or by pancreatic lipase inhibitors (Weibel et al, 1987).

B) Increased VLDL production due to increased adipose tissue mass by examining postprandial lipid metabolism in HTG subjects before and after the surgical removal of adipose tissue or by reducing adipose tissue mass by chronic caloric restriction.

C) Decreased LPL activity by examining postprandial lipid metabolism before and after the intravenous infusion of heparin, which results in the release of LPL from its catalytic sites into the circulation.

Future studies are also needed to examine the effects of insulin and glucose concentrations on exogenous lipid clearance by setting a lipaemic clamp, while increasing or decreasing the glucose and insulin concentrations respectively. Moreover, studies are also needed to examine the effects of lipid infusions on glucose homeostasis and CHO oxidation by setting a euglycaemic hyperinsulinaemic clamp, while increasing bolus infusions of intralipid or previously harvested ^{13}C labelled chylomicron rich fraction.

In the studies of this thesis, since hypertriglyceridaemia was associated with decreased clearance of exogenous TAG from circulation and decreased exogenous lipid oxidation, future diet and drug therapies should be targeted at the level of gut to decrease the absorption of exogenous lipid thus resulting in decreased competition between VLDL and chylomicrons (due to decreased chylomicrons in circulation) at the level of their TAG hydrolysis by LPL and remnant clearance by hepatic receptors. This will not only improve fasting and postprandial lipaemia but also improve glycaemia and insulin sensitivity because of decreased lipid in circulation and weight loss. Further prospective studies are

also needed to observe the effects of improved insulin sensitivity (by lowering exogenous lipid in circulation) on the regression of atherosclerosis and major coronary events, especially in obesity and type II diabetes.

Figure 7.1 Time courses of changes in plasma glucose concentrations (mmol/l) after first and second meal in young men, middle-aged men, NTG and HTG type II diabetics.

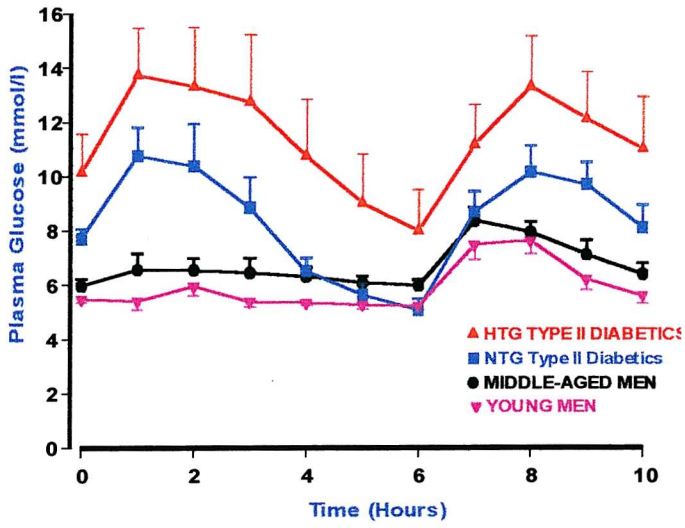


Figure 7.2 Time courses of changes in plasma insulin concentrations (mU/l) after first and second meal in young men, middle-aged men, NTG and HTG type II diabetics.

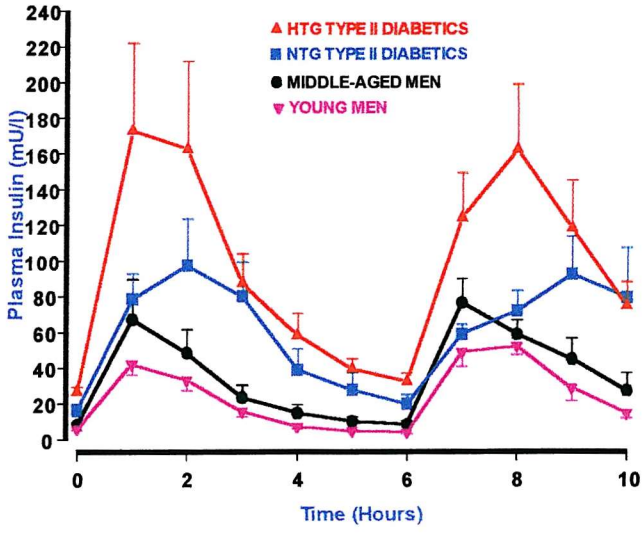


Figure 7.3 Time courses of changes in plasma NEFA concentrations (mmol/l) after first and second meal in young men, middle-aged men, NTG and HTG type II diabetics.

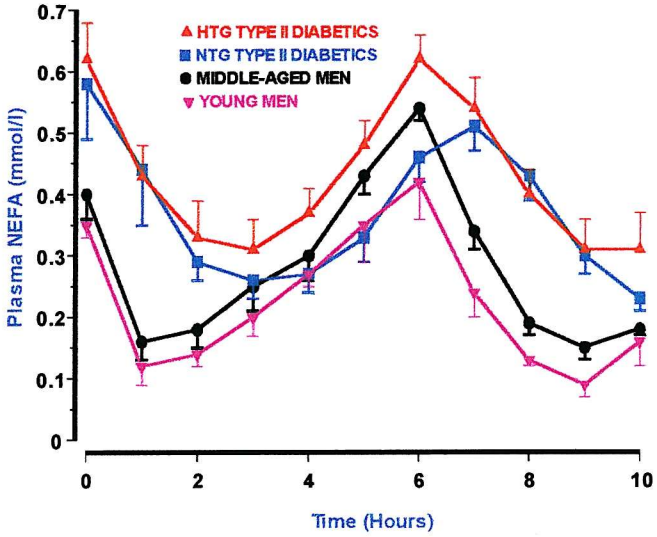


Figure 7.4 Time courses of changes in plasma TAG concentrations (mmol/l) after first and second meal in young men, middle-aged men, NTG and HTG type II diabetics.

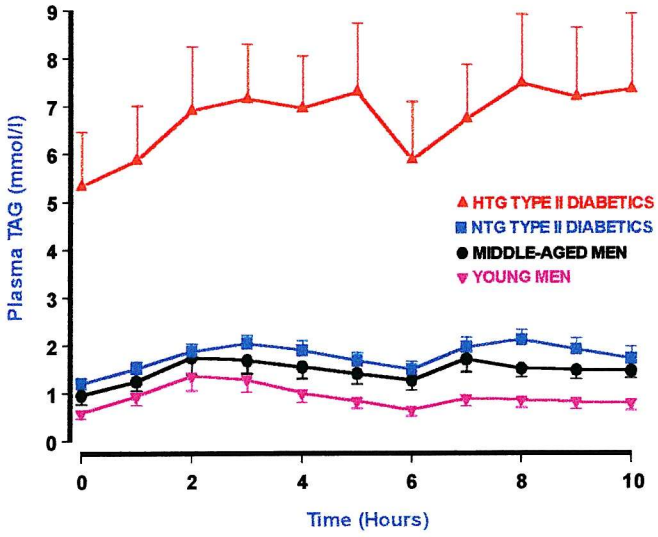


Figure 7.5 Time courses of changes in CRF-TAG ^{13}C PA concentrations (μg/ml) after first and second meal in young men, middle-aged men, NTG and HTG type II diabetics.

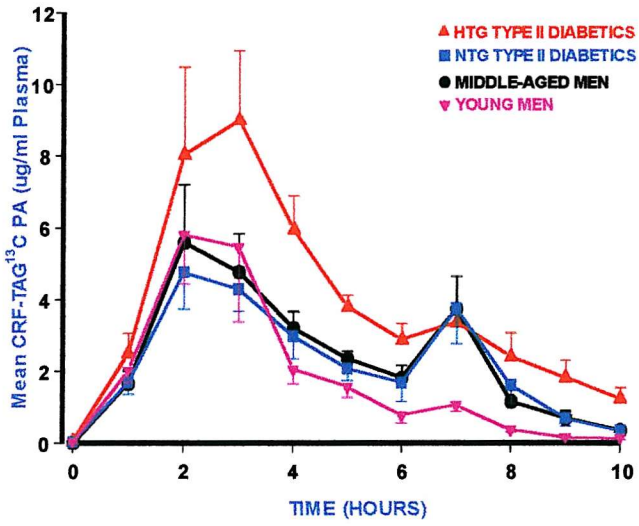


Figure 7.6 Time courses of changes in breath $^{13}CO_2$ excretion (% administered dose) after first and second meal in young men, middle-aged men, NTG and HTG type II diabetics.

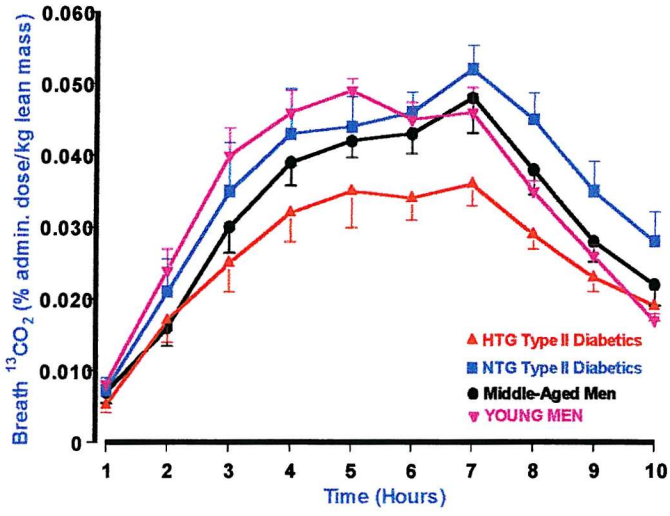


Figure 7.7 Scatter plots for various variables in the study . Blue squares, HTG diabetics; Pink squares, NTG diabetics; Green squares, middle-aged men; Red squares, young men.

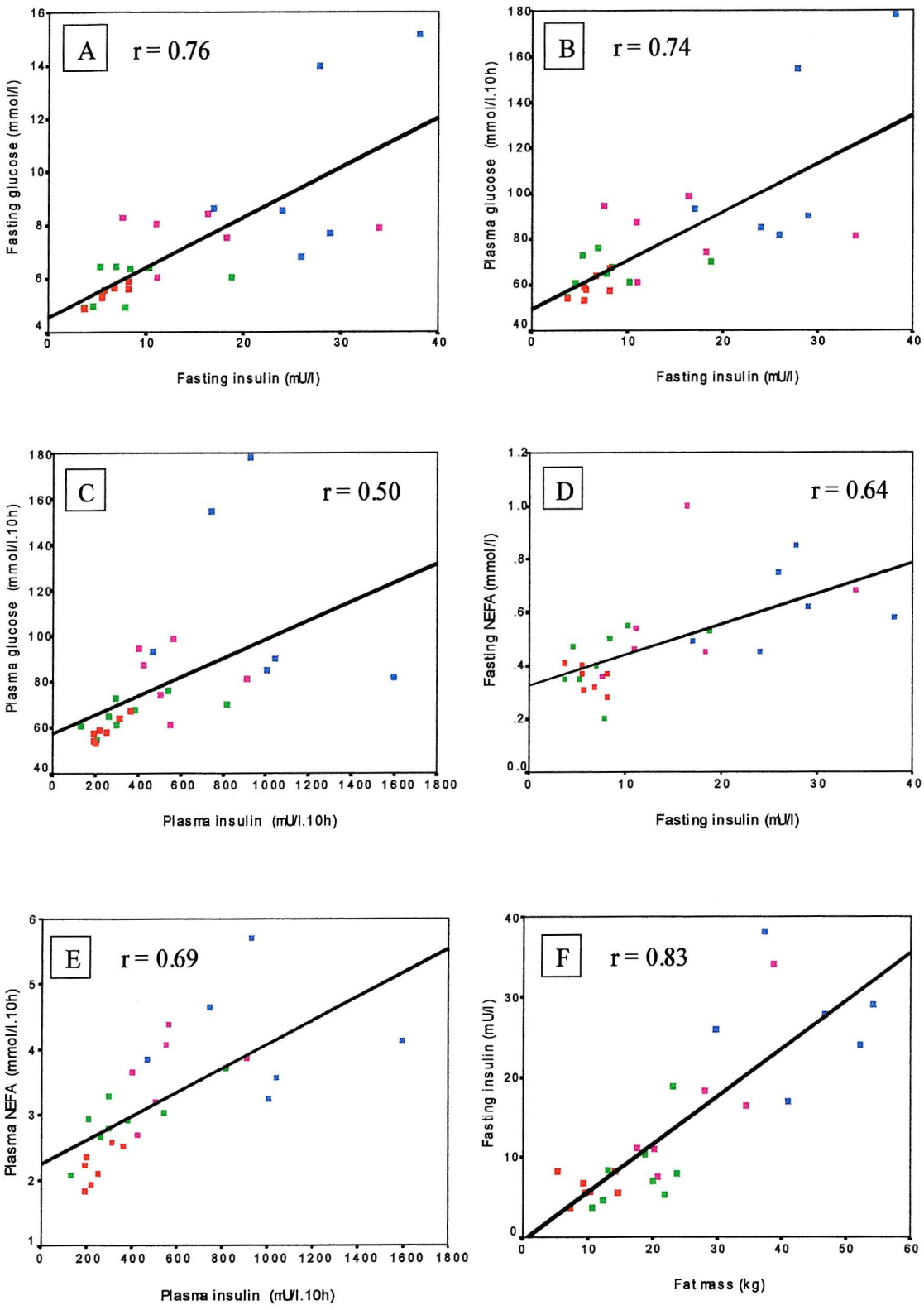


Figure 7.7 continued....

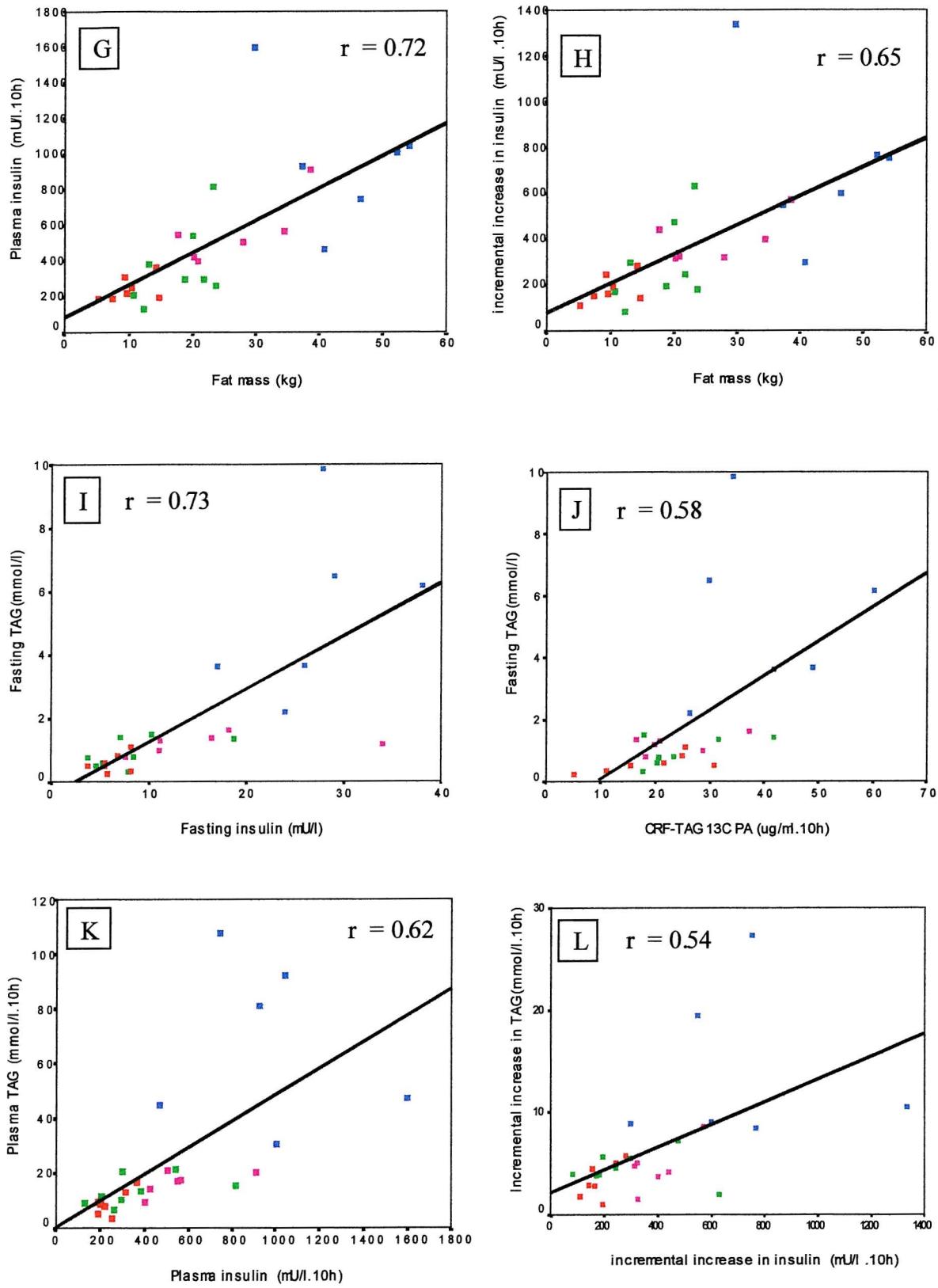


Figure 7.7 continued....

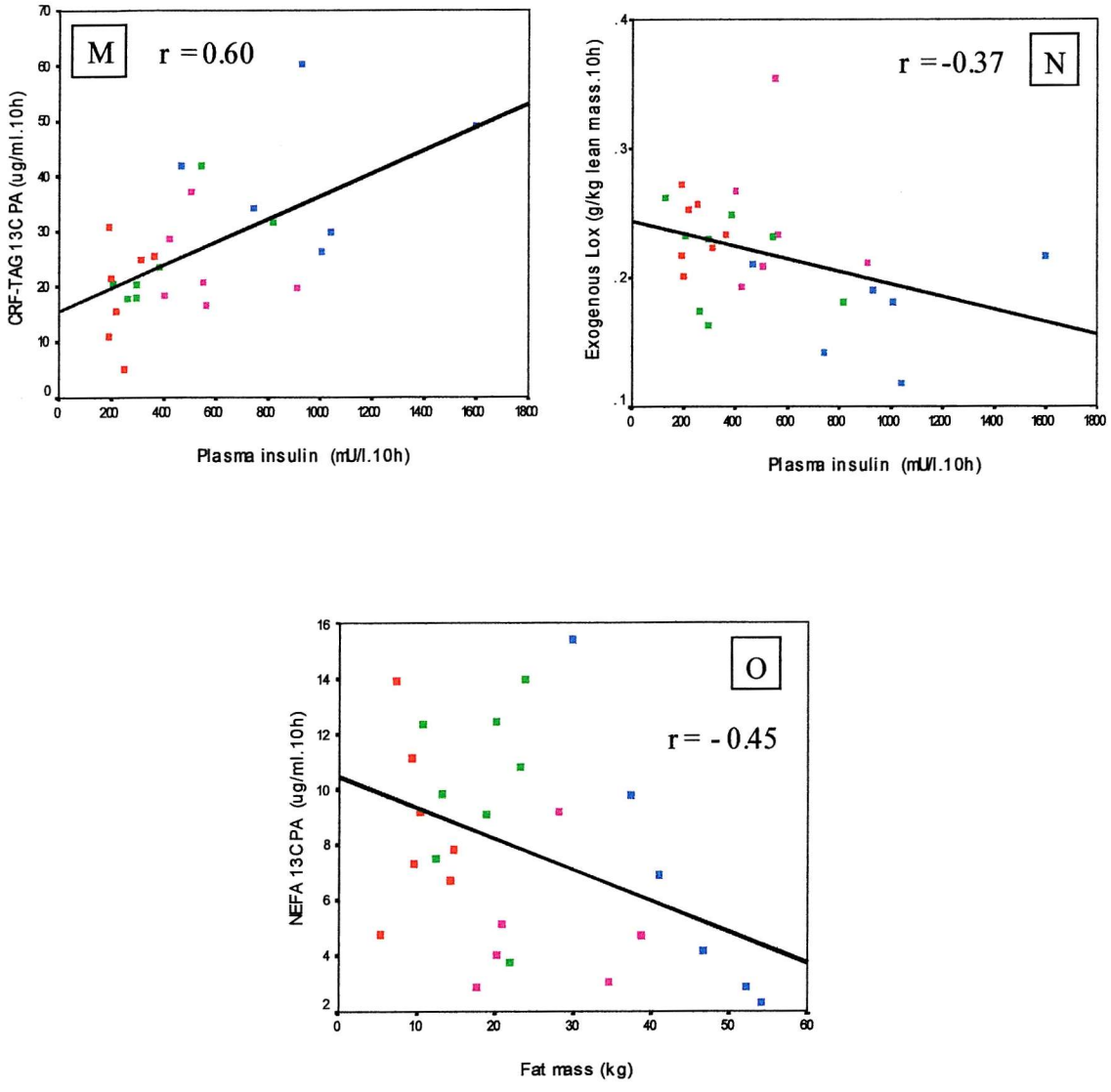


Figure 7.8. Model for lipid metabolism in healthy young men

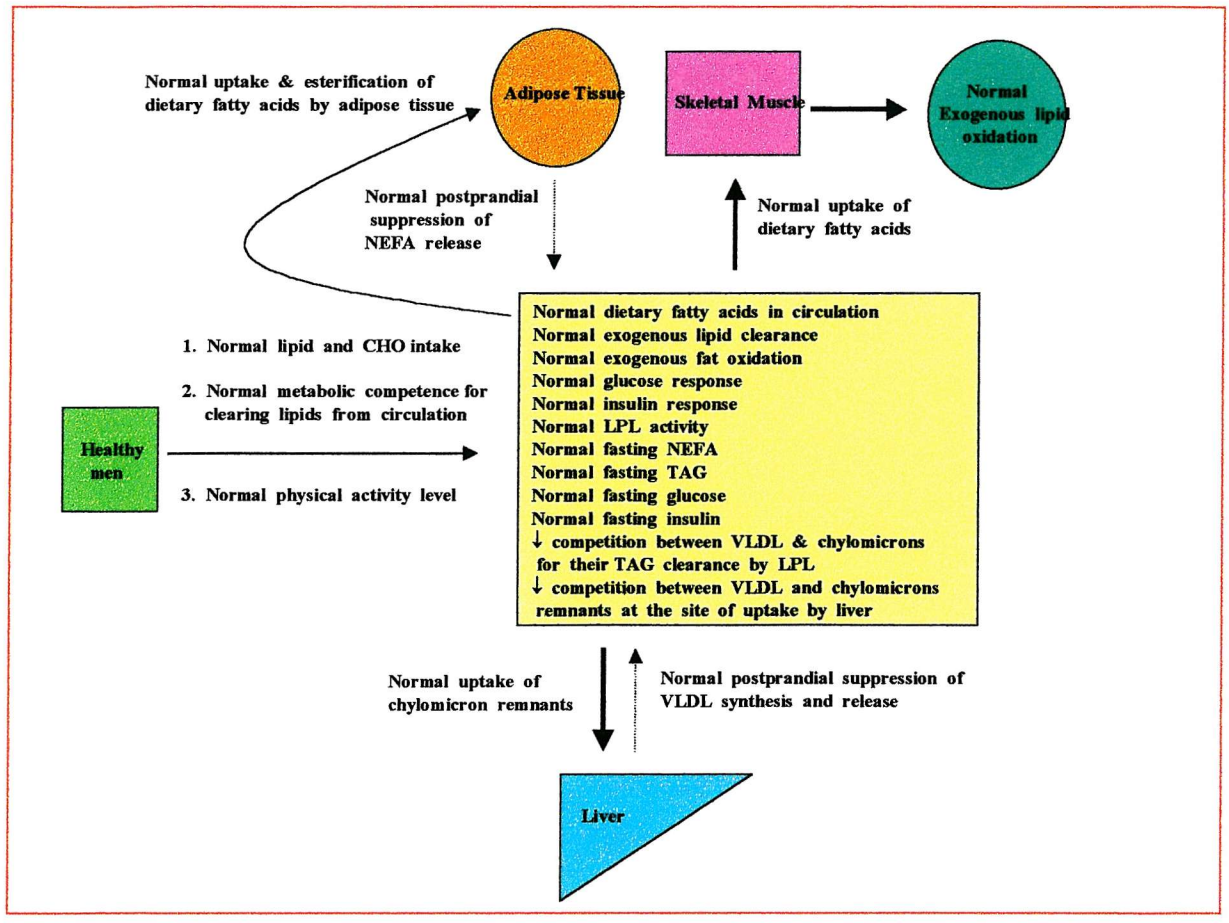
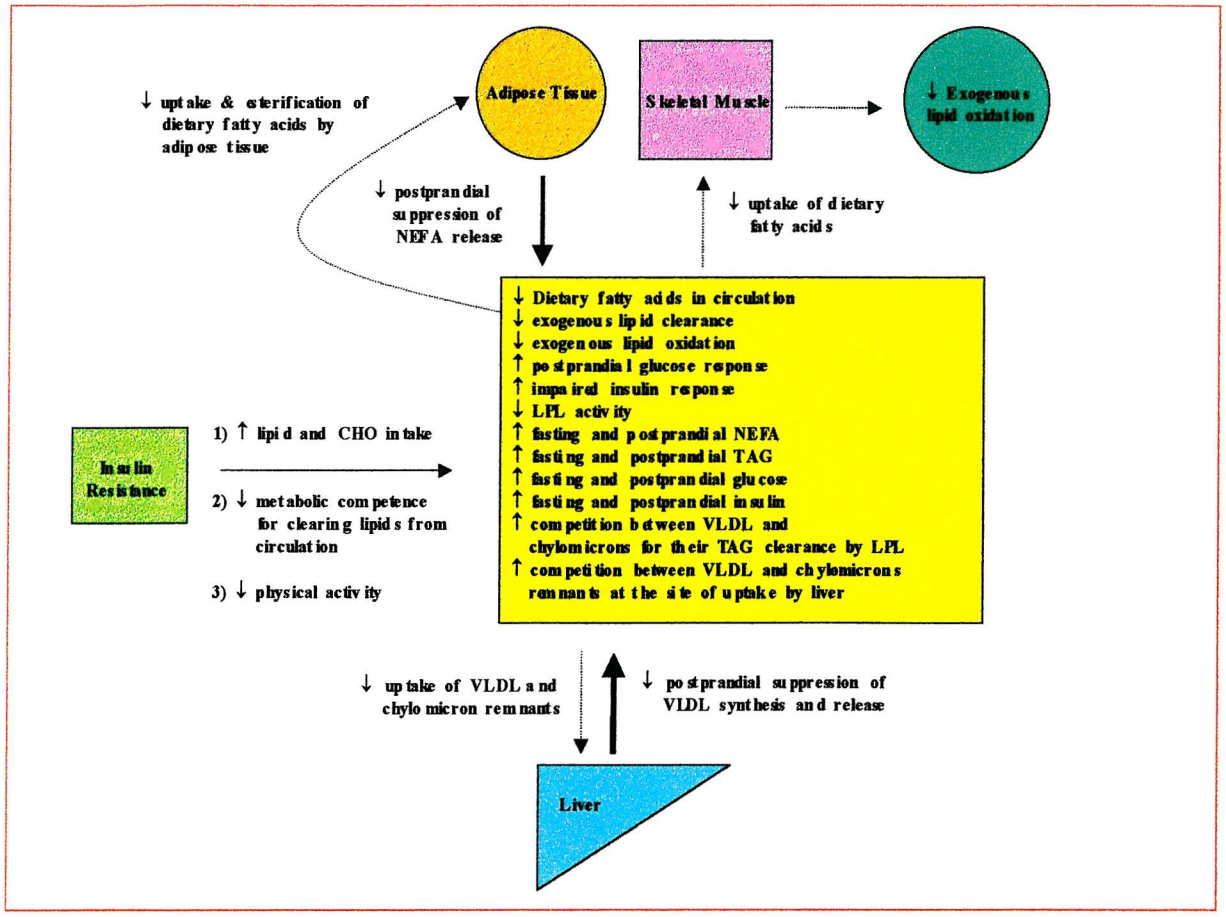


Figure 7.9 Model for lipid metabolism in insulin resistant men



CHAPTER 8

Appendices

Appendix 8.1A Prescribed Diet

Brief:

To provide a rotating 3 day prescribed diet fed at 1.5 x individuals measured BMR. The diet was fixed to provide 10 MJ of energy with additional energy requirements provided by an incremental component. Both the diet and incremental component (which can be used with the emulsion as the test meal) had a fixed composition which is typical of the present UK diet for adult (16-65 years) men [Gregory *et al* 1990].

Diet composition:

40% energy fat

45% energy carbohydrate - 58% starch
42% sugar

15% protein

Fatty acid composition:

~ 17.9% energy from SFA

~ 13.4% energy from MUFA

~ 0.84% energy from n-3 PUFA

~ 5.9% energy from n-6 PUFA

~ 2.37% energy from TUFA

PUFA:SFA ratio 0.40

Cholesterol 390 mg

Appendix 8.1B Sample calculations for incremental component requirement.

100g of incremental component = **1104 kJ**
which is comprised of **64g** white bread, **24g** ham **and 12g** clover margarine.

Subject's **BMR** = **7771 kJ**

Total Energy Expenditure **(1.5 X BMR)** = **11657 kJ**

Standard menu provides energy = **10176 kJ**

Extra energy required = 11657-10176 = **1481 kJ**

Grams of incremental component required = $\frac{\textbf{1481 X 100}}{\textbf{1104}}$

= **134 gm**

134 gm of required incremental component will provide 1481 kJ of extra energy required.

Which is comprised of **85.76 gm** (64%) of bread, **32 gm** (24%) of ham and **16 gm** (12%) of clover

Appendix. 8.2A Menu for study day

Breakfast:

Emulsion
Ham sandwich

Lunch:

Emulsion
Ham sandwich

Dinner:

Cheese and tomato pizza with chips
Lemon cheese cake

Appendix. 8.2B Test meal and emulsion composition

The quantity of test meal and emulsion was same for all individuals participating in different studies

Test Meal

100g *white bread*

38g *ham*

19g *clover margarine*

Emulsion

22g double cream

3.5g extra virgin olive oil

3g sunflower oil

12g casein

9g glucose

4.5g beet sugar

10g chocolate Nesquick

140g still mineral water

^{13}C labelled tripalmitin [10 mg/kg body weight for young men and middle-aged men. Average ^{13}C tripalmitin intake of middle-aged men (~ 780 mg) for obese type II diabetics]

200g orange juice

Test Meal and Emulsion composition:

	Emulsion	**Test Meal**	**Total**
Energy:	1735 kJ (415 kcal)	1512.5 kJ (362 kcal)	3247.5 (788 kcal)
Fat:	18.1g = 39.7% E	17.6g = 43.2% E	35.7g = 41.5% E
CHO:	49.5g = 45.3% E	40.8g = 43.2% E	90.3g = 44.3% E
Protein:	15.5g = 15.2% E	12.2g = 13.7% E	27.7g = 14.5% E
SFA:	13.3% E	18.4% E	16.0% E
MUFA:	13.5% E	14.4% E	14.0% E
n-3 PUFA:	0.9% E	0.2% E	0.5% E
n-6 PUFA:	4.7% E	4.8% E	4.8% E
TUFA:	2.4% E	1.5% E	2.0% E

E, energy

Appendix. 8.3A ^{13}C Enrichment values of foods consumed in study day meals.

Food item	^{13}C Delta Enrichment*
Yoghurt	-25.24
Ham sandwich	-26.21
Pizza and chips	-27.03
American fries	-27.19
Emulsion and Orange juice.	-25.16
Lemon cheese cake	-23.27

* Mean of three values

Appendix.8.3B ^{13}C enrichment values of breath CO_2 over the period of 10 hours after the consumption of test meal without ^{13}C labelled fatty acid in two healthy female subjects.

Time (hours)	*^{13}C enrichment of Breath CO_2*	
	subject 1	*subject 2*
Baseline	-25.46	-25.23
1	-25.19	-24.71
2	-24.84	-24.76
2	-24.74	-24.41
4	-24.68	-24.37
5	-25.04	-24.60
6	-24.94	-24.76
7	-24.73	-24.51
8	-24.47	-24.44
9	-24.40	-24.31
10	-24.68	-24.93

Appendix 8.4A Flow chart of procedures involved in the recovery of ^{13}C palmitic acid (PA) from chylomicron-rich fraction TAG and NEFA after the administration of ^{13}C tripalmitin dose in meals and calculation of 13C PA concentration.

Administration of ^{13}C palmitic acid dose with meals.

⇓

10 ml blood

⇓←——*centrifugation at 2500rpm for 15 minutes*

2.66 ml plasma

⇓←——*Density gradient ultracentrifugation at 40,000 rpm for 30 minutes at 15°C*

chylomicron fraction (0.5 to 1 ml plasma also used at this stage for NEFA extraction)

⇓←——*60 μg internal std. added (C17:0 TAG and C21:0 FA) (Folch lipid extraction)*

Chylomicron lipids and plasma lipids

⇓←——Thin Layer Chromatography

Chylomicron-TAG & plasma NEFA

⇓←——*Overnight treatment with 2% sulphuric acid in methanol*

Fatty acid methyl esters (FAME)

⇓←—— *60 μg external std. Added (C23:0 fatty acid methyl ester)*

1 μg FAME + external std. mixture injected in to GC-IRMS

⇓

- ^{13}C PA delta enrichments $$\delta\ ^{13}C\ ^{0}/_{00} = \frac{(^{13}C/^{12}C)_{Sample} - (^{13}C/^{12}C)_{Standard}}{(^{13}C/^{12}C)_{Standard}} \times 1000$$

- Area under the peak of Internal std. (C17:0, C21:0)
- Area under the peak of External std. (C23:0)
- Area under the peak of PA (C16:0)

Appendix 8.4B Equations involved in the calculation of ^{13}C palmitic acid (PA) concentration in chylomicron-rich fraction TAG and NEFA fraction

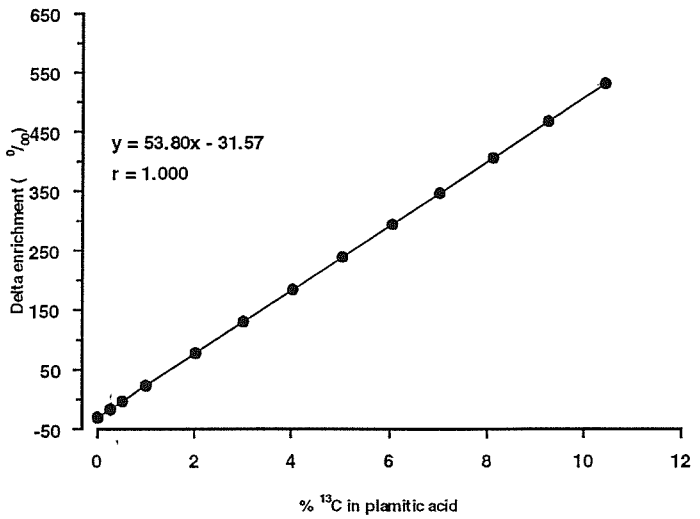
$$^{13}C \text{ palmitic acid } (\mu g/\text{fraction}) = \frac{\text{Concentration of total PA} * \%\ ^{13}C \text{ PA / Total PA}}{100}$$

Where,

$$\text{Concentration of total PA} = \frac{\text{Internal std. added} \times \text{area under peak of total PA}}{\text{area under the peak of internal std.}}$$

$$\%\ ^{13}C \text{ PA / Total PA} = \frac{^{13}C \text{ PA delta enrichment} + 31.57 \text{ (from std curve)}}{53.80}$$

Percentage of enriched palmitic acid per total palmitic acid in CRF-TAG fraction and NEFA fraction was calculated through a calibration curve constructed by injecting palmitic acid standards enriched with various percentages of ^{13}C palmitic acid in to GC-IRMS.. Palmitic acid was enriched with 0 to 10% excess [1-^{13}C] palmitic acid. The measured ^{13}C enrichment was expressed as $\delta\ ^{13}C\ ^{0}/_{00}$ referring to international standard (PDB). The standard curve was linear from 0 to 10% [1-^{13}C] palmitic acid , r = 1.000 with y= 53.80x - 31.57.



Appendix 8.4C Equations for calculation of ^{13}C excretion in stool

$$\% \text{ faecal excretion/24 hours} = \frac{IR * (\delta^{13}C_t - \delta^{13}C_{t=0})}{\text{mg } ^{13}C \text{ administered}} * \text{faecal carbon/24 hours (mmol)} * 100$$

Where,

$IR = (R_{PDB}/1000)*(\text{mol. wt. of PA / isotopic purity of } ^{13}C \text{ PA}* \text{ \# of C atoms labelled})*100$

$R_{PDB} = ^{13}C : ^{12}C$ ratio of PDB, reference material (0.0112372)

$$\text{Faecal C /24h} = \{\frac{\text{C prod (mg)}}{\text{Atomic wt C}} * [\text{total wet S (mg)} * \text{total dry S(mg)}]\} / \text{dry S combusted (mg)}$$
$$\text{wet stool for drying (mg)}$$

C = carbon

C_t = delta value of stool at time t following ^{13}C tripalmitin dose administration

$C_{t=0}$ = delta value of stool at baseline

C prod = carbon produced after dry stool combustion in IRMS

PA = palmitic acid

S = stool

mol = molecular

wt = weight

Appendix 8.4D Equations for calculation of ^{13}C excretion in breath

$$\% \text{ administered dose/h} = \frac{\text{mmol excess } ^{13}C/\text{mmmol } CO_2}{\text{mmol } ^{13}C \text{ administered}} * VCO_2 * 100$$

Where

$$\text{mmol excess } ^{13}C/\text{mmmol } CO_2 = (\delta^{13}C_t - \delta^{13}C_{t=0}) * R_{PDB} * 1/1000$$
(mmol excess ^{13}C/mmmol CO_2 is the breath enrichment obtained from IRMS)

$$\text{mmol } ^{13}C \text{ administered} = \frac{[^{13}C \text{ PA dose (mg)}]}{\text{mol. wt. of PA}} * [\text{IP of } ^{13}C \text{ PA} * \text{ \# of C atoms labelled}/100]$$

VCO_2 (mmol/hour) = VCO_2 ml/minute * 60/22.4

(VCO_2 (mmol/hour) measured by indirect calorimetry)

C_t = delta value of breath at time t following ^{13}C tripalmitin dose administration

$C_{t=0}$ = delta value of breath at baseline

IP = isotopic purity of labelled palmitic acid

Appendix 8.5. ANOVA Tables for chapter 6

(A)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Plasma glucose (mmol/l.10h)	Between Groups	7835.744	2	3917.872	6.723	.007
	Within Groups	9906.186	17	582.717		
	Total	17741.930	19			

(B)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Incremental increase in plasma glucose (mmol/l.10h)	Between Groups	131.344	2	65.672	1.368	.281
	Within Groups	816.289	17	48.017		
	Total	947.633	19			

(C)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Plasma insulin (mU/l.10h)	Between Groups	1238369	2	619184.5	8.683	.003
	Within Groups	1212265	17	71309.703		
	Total	2450634	19			

(D)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
incremental increase in insulin (mU/l .10h)	Between Groups	662186.3	2	331093.2	6.354	.009
	Within Groups	885766.3	17	52103.898		
	Total	1547953	19			

Appendix 8.5 continued

(E)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Plasma NEFA (mmol/l.10h)	Between Groups	5.627	2	2.814	6.482	.008
	Within Groups	7.379	17	.434		
	Total	13.007	19			

(F)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Postprandial NEFA - Fasting NEFA (mmol/l.10h)	Between Groups	3.470	2	1.735	.780	.474
	Within Groups	37.835	17	2.226		
	Total	41.305	19			

(G)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Plasma TAG (mmol/l.10h)	Between Groups	11540.503	2	5770.251	19.746	.000
	Within Groups	4967.920	17	292.231		
	Total	16508.423	19			

(H)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Incremental increase in TAG (mmol/l.10h)	Between Groups	364.927	2	182.463	9.002	.002
	Within Groups	344.563	17	20.268		
	Total	709.490	19			

Appendix 8.5 continued

(I)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CRF-TAG 13C PA (ug/ml.10h)	Between Groups	1054.155	2	527.077	5.278	.017
	Within Groups	1597.702	16	99.856		
	Total	2651.857	18			

(J)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
NEFA 13C PA (ug/ml.10h)	Between Groups	93.357	2	46.679	3.522	.053
	Within Groups	225.301	17	13.253		
	Total	318.658	19			

(K)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Energy expenditure (g/kg LM.10h)	Between Groups	.493	2	.247	.221	.804
	Within Groups	19.011	17	1.118		
	Total	19.504	19			

(L)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
incremental energy expenditure / leanmass	Between Groups	.292	2	.146	.678	.521
	Within Groups	3.657	17	.215		
	Total	3.949	19			

Appendix 8.5 continued

(M)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CHO oxidation (g/kg LM.10h)	Between Groups	.210	2	.105	1.780	.199
	Within Groups	1.005	17	5.914E-02		
	Total	1.216	19			

(N)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Incremental increase in CHO ox (g/kg LM.10h)	Between Groups	.476	2	.238	2.170	.145
	Within Groups	1.864	17	.110		
	Total	2.340	19			

(O)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Net Lox (g/kg LM.10h)	Between Groups	3.511E-02	2	1.755E-02	.953	.405
	Within Groups	.313	17	1.843E-02		
	Total	.348	19			

(P)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Incremental increase in net Lox (g/kg LM.10h)	Between Groups	8.600E-02	2	4.300E-02	2.369	.124
	Within Groups	.309	17	1.815E-02		
	Total	.395	19			

Appendix 8.5 continued

(Q)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
breath 13co2 AUC / mass	Between Groups	2.765E-02	2	1.382E-02	3.423	.056
	Within Groups	6.867E-02	17	4.039E-03		
	Total	9.632E-02	19			

(R)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Endogenous Lox (g/kg lean mass.10h)	Between Groups	9.205E-02	2	4.602E-02	2.123	.150
	Within Groups	.368	17	2.168E-02		
	Total	.461	19			

(S)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Exogenous Lox (g/kg lean mass.10h)	Between Groups	1.410E-02	2	7.048E-03	3.423	.056
	Within Groups	3.501E-02	17	2.059E-03		
	Total	4.910E-02	19			

Appendix 8.6 Pearson correlation coefficients for various variables in the study of chapter 5.

	Fat mass	Lean mass	Basal glucose	AUC gluc	Δ gluc	Basal insulin	AUC insulin	Δ insulin	Basal NEFA	AUC NEFA	Δ NEFA	Basal TAG	AUC TAG	Δ TAG	CRF-TAG 13C PA	NEFA 13C PA	Basal EE	AUC EE	Δ EE	Basal CHO ox	AUC CHO ox	Δ CHO ox	Basal net Lox	AUC net Lox	Δ net Lox	Breath 13CO2	Endo Lox	Exo Lox
Fat mass		0.45 p=0.09	0.44 p=0.10	0.68 ** p=0.00	0.46 p=0.09	0.51* p=0.05	0.58* p=0.02	0.57* p=0.02		0.72** p=0.00	0.37 p=0.17	0.45 p=0.09	0.44 p=0.10		0.35 p=0.22						-0.52* p=0.05	-0.71** p=0.00			0.45 p=0.09	-0.74** p=0.00	0.40 p=0.14	-0.74** p=0.00
Lean mass	0.45 p=0.09					0.37 p=0.17					0.43 p=0.11													0.37 p=0.18		-0.68** p=0.00	0.52* p=0.05	-0.68** p=0.00
Basal glucose	0.44 p=0.11			0.74** p=0.00		0.46 p=0.08	0.59* p=0.02	0.59* p=0.02	0.40 p=0.14	0.55* p=0.03		0.64* p=0.01	0.63* p=0.01	0.42 p=0.12		-0.32 p=0.24												
AUC glucose	0.68** p=0.00		0.73** p=0.00		0.55* p=0.03	0.44 p=0.10	0.70** p=0.00	0.73** p=0.00		0.57* p=0.03		0.52* p=0.05	0.57* p=0.03	0.51* p=0.05	0.52* p=0.05							-0.49 p=0.07	-0.32 p=0.25				-0.37 p=0.18		-0.37 p=0.18
Δ glucose	0.46 p=0.09			0.55* p=0.03					-0.39 p=0.16		0.50* p=0.05				0.45 p=0.11	0.33 p=0.22				0.34 p=0.21						-0.38 p=0.16		-0.38 p=0.16
Basal insulin	0.51* p=0.05	0.37 p=0.17	0.46 p=0.08	0.44 p=0.10			0.84** p=0.00	0.75** p=0.00	0.42 p=0.11	0.65* p=0.01		0.57* p=0.03	0.40 p=0.14															
AUC insulin	0.58* p=0.02		0.59* p=0.02	0.70** p=0.00		0.84** p=0.00		0-98** p=0.00	0.34 p=0.21	0.77** p=0.00		0.69** p=0.00	0.58* p=0.02		0.60* p=0.02											-0.37 p=0.18		-0.37 p=0.18

	Fat mass	Lean mass	Basal glucose	AUC gluc	Δ gluc	Basal insulin	AUC insulin	Δ insulin	Basal NEFA	AUC NEFA	Δ NEFA	Basal TAG	AUC TAG	Δ TAG	CRF-TAG 13C PA	NEFA 13C PA	Basal EE	AUC EE	Δ EE	Basal CHO ox	AUC CHO ox	Δ CHO ox	Basal net Lox	AUC net Lox	Δ net Lox	Breath 13CO2	Endo Lox	Exo Lox
Δ insulin	0.57* p=0.02		0.59* p=0.02	0.73** p=0.00	0.34 p=0.21	0.75** p=0.00	0.98** p=0.00			0.76** p=0.00		0.69** p=0.00	0.60* p=0.02		0.66* p=0.01			-0.35 p=0.19								-0.37 p=0.17		-0.37 p=0.17
Basal NEFA			0.40 p=0.14		-0.39 p=0.16	0.42 p=0.11	0.34 p=0.21			0.40 p=0.13	-0.84** p=0.00	0.53* p=0.04	0.44 p=0.10							-0.39 p=0.14	-0.53* p=0.04							
AUC NEFA	0.72** p=0.00		0.55* p=0.03	0.57* p=0.03		0.65* p=0.01	0.77** p=0.00	0.76* p=0.01	0.40 p=0.14			0.78** p=0.00	0.73** p=0.00	0.38 p=0.17	0.54* p=0.05	0.35 p=0.20	-0.33 p=0.22	-0.31 p=0.26			-0.47 p=0.08	-0.49 p=0.06			0.33 p=0.23	-0.53* p=0.04		-0.53* p=0.04
Δ NEFA	0.37 p=0.17	0.43 p=0.11			0.50* p=0.05				-0.84** p=0.00											0.49 p=0.07			-0.36 p=0.19			-0.52* p=0.04		-0.52* p=0.04
Basal TAG	0.45 p=0.09		0.64* p=0.01	0.52* p=0.05		0.57* p=0.03	0.69** p=0.00	0.69* p=0.01	0.53* p=0.01	0.78** p=0.00			0.96** p=0.00	0.57* p=0.03	0.65* p=0.01													
AUC TAG	0.44 p=0.10		0.63* p=0.01	0.57* p=0.03		0.40 p=0.14	0.58* p=0.02	0.60* p=0.02	0.44 p=0.09	0.73** p=0.00		0.96** p=0.00		0.77** p=0.00	0.71** p=0.00													
Δ TAG			0.42 p=0.12	0.51* p=0.05						0.38 p=0.17		0.57* p=0.02	0.77** p=0.00		0.65* p=0.01					0.34 p=0.12		-0.36 p=0.19						

CRF-TAG 13C PA	0.35 p=0.23			0.52* p=0.05	0.45 p=0.10		0.60* p=0.03	0.66* p=0.01		0.54* p=0.05		0.65* p=0.01	0.71** p=0.00	0.65* p=0.01		0.44 p=0.12						-0.30 p=0.30	-0.33 p=0.25		-0.33 p=0.25		-0.34 p=0.25
	Fat mass	**Lean mass**	**Basal glucose**	**AUC gluc**	**Δ gluc**	**Basal insulin**	**AUC insulin**	**Δ insulin**	**Basal NEFA**	**AUC NEFA**	**Δ NEFA**	**Basal TAG**	**AUC TAG**	**Δ TAG**	**CRF-TAG 13C PA**	**NEFA 13C PA**	**Basal EE**	**AUC EE**	**Δ EE**	**Basal CHO ox**	**AUC CHO ox**	**Δ CHO ox**	**Basal net Lox**	**AUC net Lox**	**Δ net Lox**	**Breath 13CO2**	**Endo Lox**	**Exo Lox**
NEFA 13C PA			-0.32 p=0.24		0.33 p=0.23					0.35 p=0.20					0.44 p=0.12				0.50* p=0.05									
Basal EE										-0.33 p=0.23								0.85** p=0.00	-0.36 p=0.19		0.37 p=0.18		0.36 p=0.19			0.36 p=0.19		0.36 p=0.19
AUC EE							-0.32 p=0.25	-0.35 p=0.19		-0.31 p=0.26					-0.31 p=0.28		0.85** p=0.00				0.36 p=0.19					0.48 p=0.07		0.48 p=0.07
Δ EE																0.50* p=0.05	-0.36 p=0.19							0.34 p=0.22				
Basal CHO ox				0.33 p=0.22	0.34 p=0.22				-0.39 p=0.15		0.49 p=0.07			0.34 p=0.22							0.38 p=0.16	-0.61* p=0.02	-0.87** p=0.00	-0.47 p=0.08	0.69** p=0.00	-0.38 p=0.17	-0.37 p=0.16	-0.38 p=0.17
AUC CHO ox	-0.52* p=0.05									-0.53* p=0.05	-0.47 p=0.08						0.37 p=0.18	0.36 p=0.19		0.38 p=0.16		0.50 p=0.06		-0.71** p=0.00	-0.31 p=0.26		-0.76** p=0.00	

	Fat mass	Lean mass	Basal glucose	AUC gluc	Δ gluc	Basal insulin	AUC insulin	Δ insulin	Basal NEFA	AUC NEFA	Δ NEFA	Basal TAG	AUC TAG	Δ TAG	CRF-TAG 13C PA	NEFA 13C PA	Basal EE	AUC EE	Δ EE	Basal CHO ox	AUC CHO ox	Δ CHO ox	Basal net Lox	AUC net Lox	Δ net Lox	Breath 13CO2	Endo Lox	Exo Lox
ΔCHO ox	-0.71** p=0.00		-0.32 p=0.26	-0.49 p=0.07	-0.32 p=0.24					-0.49 p=0.06				-0.36 p=0.19						-0.61* p=0.02	0.50 p=0.06		0.60* p=0.01		-0.90** p=0.00	0.59* p=0.02	-0.30 p=0.27	0.59* p=0.02
Basal net Lox				-0.32 p=0.25	-0.30 p=0.27						-0.36 p=0.19				-0.30 p=0.29		0.36 p=0.19			-0.87** p=0.00		0.60* p=0.02		0.61* p=0.02	-0.71** p=0.00	0.42 p=0.12	0.50* p=0.05	0.42 p=0.12
AUC net Lox		0.37 p=0.18													-0.32 p=0.25					-0.47 p=0.08	-0.71** p=0.00		0.61* p=0.02				0.97** p=0.00	
Δnet Lox	0.45 p=0.09									0.33 p=0.22									0.34 p=0.22	0.68** p=0.00		-0.90** p=0.00	-0.71** p=0.00			-0.48 p=0.07		-0.48 p=0.07
Breath 13CO2	-0.75** p=0.00	-0.68** p=0.00		-0.37 p=0.18	-0.38 p=0.16		-0.37 p=0.18	-0.37 p=0.18		-0.53* p=0.04	-0.52* p=0.04				-0.33 p=0.25		0.36 p=0.19	0.48 p=0.07		-0.38 p=0.16		0.59* p=0.02	0.42 p=0.12		-0.48 p=0.07			
Endo Lox	0.40 p=0.14	0.52* p=0.05																		-0.37 p=0.17	-0.76** p=0.00		0.50* p=0.05	0.97** p=0.00				
Exo Lox	-0.75** p=0.00	-0.68** p=0.00		-0.37 p=0.18	-0.38 p=0.16		-0.37 p=0.18	-0.37 p=0.18		-0.53* p=0.04	-0.52* p=0.04				-0.33 p=0.25		0.36 p=0.19	0.48 p=0.07		-0.38 p=0.16		0.59* p=0.02	0.42 p=0.12		-0.48 p=0.07			

AUC, area under the curve; gluc, glucose; Δ, incremental increase above the baseline levels ; NEFA, non-esterified fatty acids; TAG, triacylglycerol; CRF, chylomicron rich fraction; PA, palmitic acid; EE, energy expenditure; CHO, carbohydrate; Lox, lipid oxidation; endo, endogenous; exo, exogenous

Appendix 8.7 Pearson correlation coefficients for various variables in the study presented in the chapter 6

	Fat mass	Lean mass	Basal gluc	AUC gluc	Δ gluc	Basal insulin	AUC insulin	Δ insulin	Basal NEFA	AUC NEFA	Δ NEFA	Basal TAG	AUC TAG	Δ TAG	CRF-TAG 13C PA	NEFA 13C PA	Basal EE	AUC EE	Δ EE	Basal CHO ox	AUC CHO ox	Δ CHO ox	Basal net Lox	AUC net Lox	Δ net Lox	Breath 13CO2	Endo Lox	Exo Lox
Fat mass		0.84** p=0.00	0.60** p=0.00	0.56* p=0.00		0.61 ** p=0.00	0.63** p=0.00	0.54* p=0.01	0.46* p=0.04	0.51* p=0.01		0.70** p=0.00	0.73** p=0.00	0.67** p=0.00		-0.43* p=0.05										-0.61** p=0.00	0.39 p=0.08	-0.61** p=0.00
Lean mass	0.84** p=0.00					0.55* p=0.01	0.43 p=0.06	0.36 p=0.12				0.50* p=0.03	0.53* p=0.02	0.55* p=0.01		-0.34 p=0.14								0.43* p=0.05		-0.72** p=0.00	0.61** p=0.00	-0.72** p=0.00
Basal glucose	0.60** p=0.00			0.97** p=0.00	0.49* p=0.03	0.70** p=0.00	0.37 p=0.10		0.46* p=0.05	0.79** p=0.00		0.78** p=0.00	0.76** p=0.00	0.47* p=0.02	0.55* p=0.01											-0.35 p=0.12		-0.35 p=0.12
AUC glucose	0.56* p=0.01		0.97** p=0.00		0.66** p=0.00	0.68** p=0.00	0.38 p=0.10		0.45* p=0.04	0.79** p=0.00		0.78** p=0.00	0.77** p=0.00	0.50* p=0.01	0.59** p=0.00							0.31 p=0.16				-0.38 p=0.10		-0.38 p=0.10
Δ glucose			0.49* p=0.03	0.66** p=0.00						0.46* p=.05		0.45* p=0.04	0.47* p=0.04	0.40 p=0.08	0.49* p=0.05											-0.32 p=0.16		-0.32 p=0.16
Basal insulin	0.79** p=0.00	0.55* p=0.01	0.70** p=0.00	0.67** p=0.00			0.80** p=0.00	0.68** p=0.00	0.57* p=0.01	0.75** p=0.01		0.69** p=0.00	0.73** p=0.00	0.69* p=0.02	0.54* p=0.05											-0.44* p=0.05		-0.44* p=0.05
AUC insulin	0.63** p=0.01	0.43 p=0.06	0.37 p=0.11	0.38 p=0.11		0.80** p=0.00		0.98** p=0.00	0.52* p=0.01	0.58* p=0.01	-0.32 p=0.16	0.51* p=0.04	0.55* p=0.02	0.57* p=0.01	0.55* p=0.01											-0.31 p=0.18		-0.31 p=0.18

	Fat mass	Lean mass	Basal gluc	AUC gluc	Δ gluc	Basal insulin	AUC insulin	Δ insulin	Basal NEFA	AUC NEFA	Δ NEFA	Basal TAG	AUC TAG	Δ TAG	CRF-TAG 13C PA	NEFA 13C PA	Basal EE	AUC EE	Δ EE	Basal CHO ox	AUC CHO ox	Δ CHO ox	Basal net Lox	AUC net Lox	Δ net Lox	Breath 13CO2	Endo Lox	Exo Lox
Δ insulin	0.54* p=0.02	0.36 p=0.12				0.67** p=0.00	0.98** p=0.00		0.48* p=0.03	0.49* p=0.03	-0.33 p=0.15	0.47* p=0.05	0.46* p=0.05	0.49* p=0.03	0.50* p=0.03													
Basal NEFA	0.46* p=0.04		0.46* p=0.03	0.45* p=0.04		0.57p=0.08	0.52* p=0.02	0.48* p=0.03		0.63** p=0.00	-0.90** p=0.00	0.52* p=0.01	0.50* p=0.01				0.33 p=0.17		-0.38 p=0.10	-0.41 p=0.07		0.42 p=0.07	0.57** p=0.00	0.31 p=0.17	-0.50* p=0.03		0.30 p=0.20	
AUC NEFA	0.52* p=0.01		0.79** p=0.00	0.79** p=0.00	0.46* p=0.05	0.75** p=0.00	0.58* p=0.01	0.49* p=0.03	0.63** p=0.00			0.65** p=0.00	0.64** p=0.00	0.43 p=0.06	0.53* p=0.02						0.40 p=0.08							
Δ NEFA							-0.32 p=0.15	-0.33 p=0.15	-0.90** p=0.00							0.32 p=0.18	-0.31 p=0.17		0.42 p=0.07	0.53* p=0.02	0.32 p=0.16	-0.38 p=0.09	-0.65** p=0.00	-0.45* p=0.04	0.49* p=0.03		-0.41 p=0.07	
Basal TAG	0.70** p=0.00	0.50* p=0.02	0.78** p=0.00	0.78** p=0.00	0.45* p=0.03	0.69** p=0.00	0.51* p=0.02	0.47* p=0.04	0.52* p=0.01	0.65** p=0.00			0.98** p=0.00	0.69** p=0.00	0.55* p=0.02											-0.51* p=0.01	0.36 p=0.11	-0.51* p=0.01
AUC TAG	0.73** p=0.00	0.53* p=0.02	0.76** p=0.00	0.77** p=0.00	0.47* p=0.02	0.73** p=0.00	0.55* p=0.01	0.49* p=0.03	0.49* p=0.02	0.64** p=0.00		0.98** p=0.00		0.79** p=0.00	0.57** p=0.00											-0.54* p=0.01	0.35 p=0.13	-0.54* p=0.01
Δ TAG	0.67** p=0.00	0.55* p=0.01	0.47* p=0.04	0.50* p=0.01	0.40 p=0.08	0.69** p=0.00	0.57* p=0.01	0.47* p=0.04		0.43 p=0.06		0.69** p=0.00	0.79** p=0.00		0.51* p=0.01											-0.50* p=0.01		-0.50* p=0.01

CRF-TAG 13C PA			0.55* p=0.01	0.59* p=0.01	0.49* p=0.05	0.54* p=0.02	0.55* p=0.02	0.50* p=0.03		053* p=0.01		0.55* p=0.01	0.57* p=0.01	0.51* p=0.03													
	Fat mass	**Lean mass**	**Basal gluc**	**AUC gluc**	**Δ gluc**	**Basal insulin**	**AUC insulin**	**Δ insulin**	**Basal NEFA**	**AUC NEFA**	**Δ NEFA**	**Basal TAG**	**AUC TAG**	**Δ TAG**	**CRF-TAG 13C PA**	**NEFA 13C PA**	**Basal EE**	**AUC EE**	**Δ EE**	**Basal CHO ox**	**AUC CHO ox**	**Δ CHO ox**	**Basal net Lox**	**AUC net Lox**	**Δ net Lox**	**Breath 13CO2**	**Endo Lox**	**Exo Lox**
NEFA 13C PA	-0.43* p=0.05	-0.35 p=0.13																									
Basal EE									0.32 p=0.16									0.91** p=0.00					0.58* p=0.01	0.61* p=0.01			0.57* p=0.01	
AUC EE																	0.91** p=0.00						0.46* p=0.05	0.60* p=0.01			0.53* p=0.02	
Δ EE									-0.38 p=0.10		-0.42 p=0.07						-0.42 p=0.07						-0.39 p=0.09		0.38 p=0.10			
Basal CHO ox									-0.40 p=0.07		0.53* p=0.02										0.52* p=0.02	-0.79** p=0.00	-0.77** p=0.00	-0.33 p=0.15	0.77** p=0.00			
AUC CHO ox				0.32 p=0.17						0.40 p=0.08										0.52* p=0.02			-0.35 p=0.12	-0.59* p=0.01			-0.54* p=0.01	

	Fat mass	Lean mass	Basal gluc	AUC gluc	Δ gluc	Basal insulin	AUC insulin	Δ insulin	Basal NEFA	AUC NEFA	Δ NEFA	Basal TAG	AUC TAG	Δ TAG	CRF-TAG 13C PA	NEFA 13C PA	Basal EE	AUC EE	Δ EE	Basal CHO ox	AUC CHO ox	Δ CHO ox	Basal net Lox	AUC net Lox	Δ net Lox	Breath 13CO2	Endo Lox	Exo Lox
ΔCHO ox									0.42 p=0.07		-0.38 p=0.09									-0.79** p=0.00			0.64** p=0.00		-0.94** p=0.00			
Basal net Lox									0.57* p=0.01		-0.65** p=0.00						0.58* p=0.01	0.46* p=0.04	-0.39 p=0.09	-0.77** p=0.00	-0.35 p=0.13	0.64** p=0.00		0.70** p=0.00	-0.75** p=0.00		0.64** p=0.00	
AUC net Lox		0.43* p=0.05							0.32 p=0.17		-0..45* p=0.04						0.61** p=0.00	0.60* p=0.01		-0.34 p=0.15	-0.59* p=0.01		0.70** p=0.00				0.95** p=0.00	
Δnet Lox									-0.50* p=0.03		0.49* p=0.03								0.38 p=0.10	0.77** p=0.00		-0.94** p=0.00	-0.75** p=0.00					
Breath 13CO2	-0.61** p=0.00	-0.72** p=0.00	-0.35 p=0.08	-0.38 p=0.10	-0.32 p=0.16	-0.44* p=0.05	-0.31 p=0.18					-0.51* p=0.02	0-54* p=0.01	-0.51* p=0.02													-0.54* p=0.02	
Endo Lox	0.39 p=0.09	0.61** p=0.00									-0.41 p=0.07	0.36 p=0.11	0.35 p=0.13				0.57* p=0.01	0.53* p=0.02			-0.55* p=0.01		0.63** p=0.00	0.95** p=0.00		-0.54* p=0.01		-0.54* p=0.01
Exo Lox	-0.61** p=0.00	-0.72** p=0.00	-0.35 p=0.08	-0.38 p=0.10	-0.32 p=0.16	-0.44* p=0.05	-0.31 p=0.18					-0.51* p=0.02	0-54* p=0.01	-0.51* p=0.02													-0.54* p=0.01	

AUC, area under the curve; gluc, glucose; Δ, incremental increase above the baseline levels ; NEFA, non-esterified fatty acids; TAG, triacylglycerol; CRF, chylomicron rich fraction; PA, palmitic acid; EE, energy expenditure; CHO, carbohydrate; Lox, lipid oxidation; endo, endogenous; exo, exogenous

Appendix 8.8 Pearson correlation coefficients for various variables in combined data for all 4 groups such as young men, middle-aged men, NTG type II diabetics and HTG type II diabetics.

	Fat mass	Basal gluc	AUC gluc	Δ gluc	Basal insulin	AUC insulin	Δ insulin	Basal NEFA	AUC NEFA	Δ NEFA	Basal TAG	AUC TAG	Δ TAG	CRF-TAG 13C PA	Breath $^{13}CO_2$	Exo Lox
Basal insulin	0.83** p=0.00	0.76** p=0.00	0.74** p=0.00	0.38* p=0.05		0.84** p=0.00	0.73** p=0.00	0.64** p=0.00	0.79** p=0.01		0.73** p=0.00	0.76** p=0.00	0.72** p=0.00	0.57* p=0.03	-0.47* p=0.05	-0.47* p=0.15
AUC insulin	0.72** p=0.00	0.49* p=0.01	0.50* p=0.01	0.34 p=0.09	0.84** p=0.00		0.98** p=0.00	0.60** p=0.00	0.69** p=0.00	-0.33 p=0.10	0.59** p=0.00	0.62** p=0.00	0.62** p=0.00	0.60** p=0.00	-0.37 p=0.06	-0.37 p=0.06
Δ insulin	0.65** p=0.00	0.40* p=0.04	0.41* p=0.03		0.73** p=0.00	0.98** p=0.00		0.56** p=0.00	0.62** p=0.00	-0.33 p=0.09	0.55** p=0.00	0.57** p=0.00	0.54** p=0.00	0.56** p=0.00	-0.33 p=0.10	-0.33 p=0.10

Chapter 9

References

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