School of Public Health

Effect of dietary fats and metabolic determinants on chylomicron metabolism

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Declaration

To the best of my knowledge and belief this thesis titled "Effect of dietary fats and metabolic determinants on chylomicron metabolism" contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Number HR97/2011 and HR 151/2013.

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29th August 2016

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Abstract

Atherosclerosis is the underlying process of coronary heart disease, the leading cause of death in many developed countries including Australia. It is a chronic and multifactorial disease. There is convincing evidence that has established the link between atherosclerosis and postprandial hyperlipidaemia. The postprandial metabolism of dietary lipids can extend up to 8-10 h depending on dose. As breaks between meals are typically less than this, the efficiency of the metabolic machinery for processing these lipids becomes an important determinant of the exposure of arterial walls to pro-atherogenic lipoproteins.

The central paradigm of the current atherosclerosis model is the retention of remnant lipoproteins (chylomicrons and hepatic very low density lipoproteins (VLDL)) in the subendothelial space of arterial walls. Sustained concentrations of circulating remnant lipoproteins selectively bind to the subendothelial matrix and providing they are small enough will be retained in the subendothelial space. There is substantial evidence from cell lines, animal and human studies supporting the role of chylomicron particles on the development of atherosclerosis. Indeed the presence of apo B-48, a measure of chylomicron particle number, in the atherosclerotic tissue has been reported.

Chylomicrons are intestinally-derived lipoproteins that transport the absorbed dietary lipids to tissues expressing lipoprotein lipase (LPL). They are continuously secreted in various sizes depending on the fasting or postprandial state, triglyceride availability and apolipoprotein (apo) B-48 production rate. In the postprandial state, chylomicron particles tend to be larger in size to accommodate the increased flux of substrate absorbed (dietary triglyceride, cholesterol, phospholipids and fat soluble vitamins) instead of increasing the particle number. This highly efficient response is affected mainly by dietary fat ingestion (quantity and type of dietary fat). The dietary fatty acid composition has been recognised to modulate triglyceride-rich lipoprotein (TRL) response through their effect on lipid partitioning, chylomicron production and removal, and accessory factors.

Studies examining the effect of dietary fat on postprandial lipid metabolism do not directly measure the concentration of chylomicron particles in dense lipoprotein fractions. Rather, chylomicron lipid metabolism is mostly assessed by examining chylomicron particle number (apo B-48 or retinyl esters) in plasma or even indirectly as the concentration of triglyceride in less dense lipoprotein fractions (where small-sized pro-atherogenic remnants are not found). Moreover since the susceptibility to develop atherosclerosis differs individually either due to genetics or environmental factors, it is possible that there is an

interactive effect between dietary fats, genetic/metabolic determinants and chylomicron remnant metabolism.

In the fasting state, triglyceride, LDL cholesterol and HDL cholesterol concentrations are generally utilised as markers for atherogenic risk. However we propose that these markers may not completely describe atherogenic risk. Different types of hyperlipidaemia exert different atherogenic risk despite having high triglyceride concentration. Similarly, HDL cholesterol does not represent cholesterol content in the remnant lipoproteins. Instead, non-HDL cholesterol captures the cholesterol content of remnant lipoproteins. Given the atherogenic evidence of remnants is abundant, studies investigating the association between chylomicron/apo B-48 and lipid parameters are limited and specific to the Japanese population.

This thesis explores the association of apo B-48 concentration with metabolic and anthropometric parameters (Chapter 2), the atherogenic risk of hypertriglyceridaemia from the perspective of an involvement of remnant lipoproteins (explained in Chapter 3) and the acute effect of dietary fatty acid composition and metabolic determinants on postprandial chylomicron metabolism (explained in Chapter 4).

The results of this study (Chapter 2) confirm previous evidence that fasting apo B-48 concentration is strongly associated with triglyceride concentration. HDL cholesterol concentration was not associated with apo B-48 following gender and age adjustment. In subjects with MetS, who have higher basal apo B-48 concentration, the association and the contribution of triglyceride and non-HDL cholesterol on the variability of apo B-48 concentration were increased compared to the non-MetS subjects. However these measures give no indication of chylomicron remnant homeostasis, which are our primary concern given the evidence of their atherogenicity.

In Chapter 3, we demonstrated that normocholesterolemic subjects with hypertriglyceridemia exhibited an accumulation of small-sized chylomicron particles in fasting plasma. We suggest that this accumulation is due to a defect in catabolism and/or clearance of those particles rather than as a result of increased chylomicron production. The insulin resistant state of the hypertriglyceridaemic subjects in this study may contribute to the defective catabolism and uptake of remnants by the liver. Our findings further substantiate the role of chylomicron remnants in mechanisms leading to the increased risk of CVD in hypertriglyceridemic subjects.

Contrary to the conventional thought, the results of Chapter 4 showed that in normotriglyceridaemic subjects, the majority of chylomicron particles in the fasting state and their postprandial response are observed within the potentially pro-atherogenic Sf <20

fraction. This suggests that following dietary fat ingestion, a substantial proportion of circulating chylomicrons can directly enter the arterial walls.

We also observed that the magnitude of postprandial hyper-remnantaemia is dependent both on the nature of dietary fatty acids ingested and genetic determinants. We also identified a subset of subjects in this group who despite being normotriglyceridemic in the fasting state exhibit elevated postprandial triglyceride (hyper-responders (HR)). The HR exhibited a substantial accumulation of chylomicron remnants following consumption of a palm oil rich meal but that this feature was not observed in the normo-responders. We postulate that differential expression of key apolipoproteins (i.e. apo C-II, apo C-III, apo E) or a modest hydrolytic defect in muscle tissue may contribute to this differential response. The classical markers of plasma lipid homeostasis give no valuable insight into chylomicron metabolism, remnant homeostasis and the atherogenic risk.

The findings presented in this thesis provide more insight into the potential mechanisms of increased atherogenic risk in individuals with impaired triglyceridaemia and the interactive effect of the type of dietary fatty acid composition and genotype/metabolic determinants on chylomicron remnant response. Triglyceride concentration, LDL cholesterol and HDL cholesterol are not suitable surrogate markers for chylomicron remnant metabolism and atherogenicity. Our findings reiterate the importance of assessing remnant lipoprotein concentration.

List of Publications Included

This thesis contains two scientific articles published in peer-reviewed scientific journals and one manuscript submitted. Statements of contribution by co-authors and copyright declarations are provided in Appendix A.

1. Irawati D, Mamo JCL, Dhaliwal SS, Soares MJ, Slivkoff-Clark KM, James AP (2016). Plasma triglyceride and an inverse association with high-density lipoprotein cholesterol are poor surrogate markers of pro-atherogenic chylomicron remnant homeostasis in subjects with the metabolic syndrome. *Lipids in Health and Disease*, In press.

2. Irawati D, Mamo JCL, Soares MJ, Slivkoff-Clark KM, James AP (2015). Hypertriglyceridemic subjects exhibit an accumulation of small dense chylomicron particles in the fasting state. *Atherosclerosis,* 243(1), 236-241.

3. Irawati D, Mamo JCL, Soares MJ, Slivkoff-Clark KM, James AP. Dietary fat and genetic determinants of plasma chylomicron remnant homeostasis in normolipidemic subjects: Insight into atherogenic risk (2017). *British Journal of Nutrition*, in press.

Introduction and Structure of Thesis

Background

Coronary heart disease, the most common form of CVD, is the leading cause of death in Australia in 2011 (AIHW 2014). The increase in the prevalence of coronary heart disease has put a considerable strain on the public health system (financial and functional). Imbalanced diets play an important role in this problem. This includes consumption of high fat content food. To prevent or treat coronary artery disease, dietary guidelines since 1960s have emphasised a reduction in the consumption of food rich in saturated fat (Page, Allen et al. 1961). The quantity and the quality of dietary fats have been an ongoing issue in cardiovascular health.

In the well developed countries and those who have experienced an economic surge, access to food is not an issue. Consequently humans spend most part of the day in a postprandial state. Since lipid metabolism takes longer than carbohydrate and proteins, this places a greater burden on the metabolic machinery of the human body and poses more atherogenic lipoprotein exposure on the vascular walls. In 1979, Zilversmit introduced the idea that atherogenesis was indeed as a postprandial phenomenon (Zilversmit 1979), and since then the metabolism of triglyceride-rich lipoprotein (TRL) has been investigated extensively.

Chylomicrons are lipoprotein particles originating from the intestine. They carry the absorbed dietary lipids and enter the circulation compartment via the thoracic duct. Nascent chylomicrons are secreted in various sizes depending on the lipidation. In the circulation, chylomicron particles undergo lipolysis by LPL and exchange apolipoprotein contents and substrate (triglyceride and cholesteryl ester) with HDL particles. Following lipolysis, chylomicrons shed their lipid content leaving smaller remnants. Chylomicron remnants with their remaining lipid content then enter the liver for clearance. Remnants will be removed by hepatocytes predominantly through receptor-mediated pathways: LDL receptor, LDL receptor related proteins (LRP) after acquiring additional apo E, and heparan sulphate proteoglycans (HSPG) mediated by apo E, LPL and hepatic lipase (HL) (Fujioka and Ishikawa 2009). When their removals were delayed, a build-up of cholesterol-rich remnants would ensue.

The current atherogenesis model describes that the initiator of atherogenesis is subendothelial retention of cholesterol rich lipoproteins (Williams and Tabas 1995; Williams and Tabas 2005). Endothelium is a simple squamous epithelium lining the inner part of

cardiovascular system with a very tight connection between cells (Simionescu and Simionescu 1991). It separates two fluid compartments, plasma and interstitial fluid. The luminal side of endothelial membrane is continuously being exposed to circulating cells and plasma containing macromolecules, solutes and water.

Lipoprotein particles are transported across the artery walls via plasmalemmal vesicles by a highly active transport system (Simionescu and Simionescu 1991; Dallinga-Thie, Kroon et al. 2016). Only lipoproteins sized less than 70 nm in diameter, Sf 12 – 60 fraction or less may enter the artery walls (Stender and Zilversmit 1981; Shaikh, Wootton et al. 1991; Simionescu and Simionescu 1991; Zilversmit 1995). The entrance and retention of lipoprotein particles in the subendothelial tissue depends on the sustained lipoprotein plasma concentration, particle size and the selectivity of lipoprotein to bind with subendothelial matrix. A line of evidence has demonstrated the ability of chylomicron remnant entering and being trapped in subendothelial tissue (Mamo 1995; Proctor and Mamo 1996; Fujioka, Cooper et al. 1998; Proctor, Vine et al. 2002). Once chylomicron remnants are trapped in the subendothelial matrix, they can induce monocyte activation and the formation of lipid-laden macrophages (Botham and Wheeler-Jones 2013). Indeed apo B-48, a measure of chylomicron particle number, has been detected in atherosclerotic tissue in animal model and humans (Proctor and Mamo 1996; Pal, Semorine et al. 2003; Proctor and Mamo 2003; Nakano, Nakajima et al. 2008).

Despite that chylomicron remnant atherogenicity are evident, increased triglyceride and low HDL cholesterol concentrations are still utilised as markers for atherogenicity. Studies investigating the association between apo B-48 and other lipid parameters are limited and the subjects are specific to the Japanese population.

Atherosclerosis is a chronic condition and a multifactorial disease. Thus it requires a certain amount of time from retention of cholesterol-rich lipoprotein in the subendothelial matrix to develop atheroma lesion. Findings from an autopsy study on children and young adults with the cause of death non-cardiac suggested that subendhothelial retention has started as diffuse intimal thickening and occurs long before it become pathologic (Nakashima, Fujii et al. 2007; Tabas, Williams et al. 2007).

Dietary fatty acid composition has been recognised to implicate chylomicron metabolism at chylomicron assembly, hydrolysis, and clearance (Williams 1998; Williams, Bateman et al. 2004). However studies examining chylomicron response in the dense fraction are very limited as most of those studies measured chylomicron/triglyceride/retinyl esters in the lipid-rich fraction, a fraction consisted of large-sized lipoproteins which are less likely to penetrate the intimal membrane. Since the susceptibility to develop

atherosclerosis differs individually either due to genetics or environmental factors (Tabas, Williams et al. 2007), it is possible that dietary fats exert differential effect.

The observational statements which have provided the foundation for this PhD proposal are that:

1) CVD is the leading cause of death in Australia;
2) Exaggerated postprandial lipemia contributes to the development of atherogenesis;
3) Apo B-48 containing remnants can penetrate and be retained in the subendothelial matrix;
4) The findings of the effect of dietary fatty acid on postprandial remnants are limited and inconsistent;
5) The susceptibility to develop exaggerated remnant metabolism varies between individuals.

Hence the above arguments lead to the following hypothesis.

Hypothesis

Hypothesis 1: Apo B-48 concentration and their size distribution are associated with metabolic and anthropometric parameters in fasting.

Hypothesis 2: Dietary fats differentially affect chylomicron remnant metabolism in otherwise healthy normolipidaemic subjects.

Objectives

Objective 1: To explore the associations of apo B-48 concentration with metabolic and anthropometric parameters.

Objective 2: To examine the abundance and size distribution of chylomicron particles in individuals with and without hypertriglyceridaemia

Objective 3: To investigate the acute effect of dietary fatty acid composition and metabolic determinants on chylomicron metabolism in normotriglyceridaemic subjects.

Chapter Structure

Chapter 1: Literature review

This chapter provides a comprehensive review of the evidence to date on the role of chylomicrons and their putative role in postprandial dyslipidaemia leading to atherosclerosis, specifically the postprandial response in the triglyceride-deplete fraction. The literature review established the foundation of my hypothesis and objectives.

Chapter 2: The association of fasting chylomicron concentration with a range of anthropometric and lipid parameters: A cross-sectional study

Thesis objective addressed in this chapter:

Objective 1: To explore the associations of apo B-48 concentration with metabolic and anthropometric parameters.

Content of this chapter is covered by the manuscript:

Irawati D, Mamo JCL, Dhaliwal SS, Soares MJ, Slivkoff-Clark KM, James AP (2016). Plasma triglyceride and an inverse association with high-density lipoprotein cholesterol are poor surrogate markers of pro-atherogenic chylomicron remnant homeostasis in subjects with the metabolic syndrome. *Lipids in health and Disease*, In press.

Chapter 3: Chylomicron size distribution in subjects with and without hypertriglyceridaemia

Thesis objective addressed in this chapter:

Objective 2: To examine the abundance and size distribution of chylomicron particles in individuals with and without hypertriglyceridaemia

Content of this chapter is covered by the article:

Irawati D, Mamo JCL, Soares MJ, Slivkoff-Clark KM, James AP (2015). Hypertriglyceridemic subjects exhibit an accumulation of small dense chylomicron particles in the fasting state. *Atherosclerosis,* 243(1), 236-241.

Chapter 4: The effect of dietary fatty acid composition on plasma abundance of pro-atherogenic chylomicron remnants in postprandial lipaemia

Thesis objective addressed in this chapter:

Objective 3: To investigate the acute effect of dietary fatty acid composition and metabolic determinants on chylomicron metabolism in normotriglyceridaemic subjects.

Content of this chapter is covered by the article:

Irawati D, Mamo JCL, Soares MJ, Slivkoff-Clark KM, James AP. Dietary fat and genetic determinants of plasma chylomicron remnant homeostasis in normolipidemic subjects: Insight into atherogenic risk (2017). *British Journal of Nutrition*, In press.

Chapter 5: Discussion, limitation and future studies

This chapter provides a comprehensive discussion of chapter 2, chapter 3 and chapter 4, the implication of the findings, limitation and future direction.

Abbreviations

ABCA	ATP binding cassette transporter
ABCG	ATP binding cassette subfamily G
Apo	Apolipoprotein
AUC	Area under curve
BMI	Body mass index
CD	Cluster of differentiation
CETP	Cholesteryl ester transfer protein
COP	Coat protein complex
CRP	C-reactive protein
CVD	Cardiovascular disease
DGAT	Diacylglycerol acyltransferase
ELISA	enzyme-linked immunoabsorbent assays
FABP	Fatty acid binding protein
FATP	Fatty acid transport protein
GLP	Glucagon-like peptide
HDL	High density lipoprotein
HL	Hepatic lipase
HOMA-IR	Homeostasis model of insulin resistance
HSPG	Heparan sulphate proteoglycan
HTG	Hypertriglyceridaemic
IAUC	Incremental area under curve
IDL	Intermediate density lipoprotein
IL	Interleukin
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LMF	Lipase maturation factor
Lp(a)	Lipoprotein(a)
LPL	Lipoprotein lipase
LRP	LDL receptor related proteins

MAG	Monoacylglycerol
MCP	Monocyte chemo-attractant protein
MGAT	Monoacylglycerol acyltransferase
MetS	Metabolic syndrome
mRNA	Messenger ribonucleic acid
MUFA	Mono-unsaturated fatty acid
MTP	Microsomal transfer protein
NCEP-ATP	National Cholesterol Education Program Adult Treatment Panel
NEFA	Non-esterified fatty acid
NPC1L1	Niemann Pick C1-like1
NTG	Normotriglyceridaemic
PCSK9	Proprotein convertase subtilisin/kexin type 9
PCTV	Pre-chylomicron transport vesicles
PUFA	Poly-unsaturated fatty acid
RLP	Remnant-like protein
SAD	Sagittal abdominal diameter
Sar1b	Secretion associated Ras related GTPase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SREBP	Sterol regulatory element binding proteins
SE	Standard error
Sf	Svedberg flotation rate
SFA	Saturated fatty acid
SULF2	Sulfate glucosamine 6-O-endosulfatase-2
TRL	Triglyceride-rich lipoprotein
VAMP	vesicle-associated membrane protein
VLDL	Very low density lipoprotein
WHR	Waist hip ratio

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CHAPTER 1

Chapter 1: Literature review

1.1 Introduction

Increasing rates of obesity and the growing risk for MetS and CVD necessitate a greater understanding of the mechanisms involved in the development of atherosclerosis. This chapter presents a detailed review of the evidence to date on the role of chylomicron in atherosclerosis in relation to dietary fats particularly postprandial response in the dense fraction. This chapter outlines the potential mechanism of chylomicron remnant accumulation and the possibility of differential effect of dietary fats in different metabolic phenotype. This chapter also points out the importance of measuring chylomicron (apo B-48) concentration as a potential surrogate marker for atherogenicity.

1.2 Chylomicron structure

Chylomicrons are the largest circulating lipoprotein particles. Previously it was thought that chylomicrons were only present in circulation as large lipid rich particles and that they were mostly secreted following consumption of lipid containing meals. However there is now evidence that chylomicrons are continuously secreted in various sizes depending on the fasting or postprandial state, triglyceride availability (type and amount) and apolipoprotein (apo) B-48 production rate (Guo, Kohen Avramoglu, & Adeli, 2005; Hayashi et al., 1990; Zheng, Ikewaki, Walsh, & Sacks, 2006). Chylomicrons are spherical in shape containing: triacylglycerol (85-92%), phospholipids (6-12%), cholesterol (1-3%) and proteins (1-2%) (Frayn, 2009; Hussain et al., 1996). The molecules within lipoprotein particles are bound by non-covalent forces like other emulsion particles (Mills, Lane, & Weech, 1984).

Chylomicron particles consist of a core of hydrophobic materials which is surrounded by a hydrophilic monolayer envelope. The core contains of triglyceride and cholesteryl esters whereas the envelope is composed of phospholipids, free cholesterol and a mixture of specific proteins (i.e. apo B-48, apo A, apo C and apo E). These apolipoproteins are important for the structural integrity, functionality, scavenger receptor binding for lipoprotein particles and to act as cofactors or inhibitors for lipase activity and removal. The structural components of chylomicrons (apolipoprotein and substrate composition) influence lipolysis and remnant removal from the circulation.

Apo B-48 is the main apolipoprotein in chylomicrons synthesised by the enterocyte of small intestine, it does not interchange with other lipoproteins, and hence is a marker of chylomicron particles (Dash, Xiao, Morgantini, & Lewis, 2015). It serves as a structural protein essential for chylomicron assembly. In humans, apo B-48 is a translational product of the apo B gene located in chromosome 2 in human genome (Huang, Miller, Bruns, & Breslow, 1986; Hussain et al., 1996; Kane, Hardman, & Paulus, 1980). It is formed by post-transcriptional editing of apo B mRNA with a premature stop due to the conversion of codon 2153 from CAA (coding for glutamine) to UAA (a stop signal) by cytidine deaminase in the ribosomes (Powell et al., 1987). The conversion of C to U is mediated by a heterodimeric complex of apobec-1, an RNA-specific cytidine deaminase highly expressed in the small intestine in humans (Anant & Davidson, 2001; Giannoni et al., 1995; Hadjiagapiou, Giannoni, Funahashi, Skarosi, & Davidson, 1994) (Figure 1). This leads to the translation of a truncated apo B containing 48% of mature apo B primary sequence. Consequently apo B-48 does not contain the C-terminal portion of apo B which affects its interaction with hepatic uptake receptors (Marcel et al., 1987).

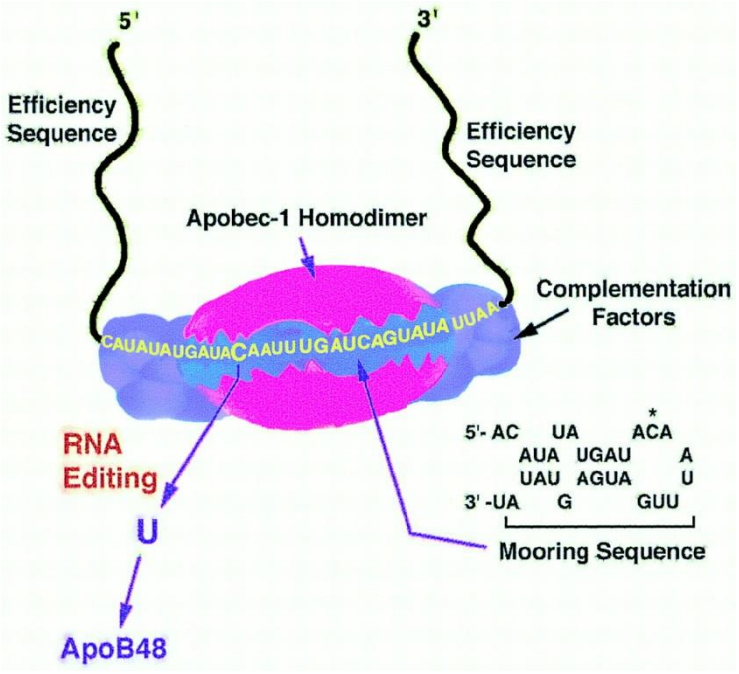

Figure 1. Apo B-48 as a result of m-RNA editing of apo B. Apo B-48 is produced from the C to U editing of nuclear apo B transcript. This reaction requires an 11-nucleotide cassette (UGAUCAGUAUA) known as mooring sequence, an AU-rich context and the presence of distal efficiency element 5' and 3' of the edited C (asterisked). It is proposed that the region flanks the edited C forming a stem loop structure. The apo B m-RNA editing complex

comprises of apobec-1 homodimer and auxiliary proteins known as complementation factors.

Source: Adaptation from Figure 1, (Davidson & Shelness, 2000) (permission not required)

1.3 Chylomicron metabolism

Chylomicrons are lipoproteins that transport dietary fat including lipids and fat soluble vitamins absorbed in the enterocytes. The process by which chylomicrons deliver these substrates from enterocytes to target tissues is a multistep and complex process.

Briefly chylomicrons are assembled in the enterocytes which involved synthesis of apo B-48 and lipidation of the primordial particles mediated by microsomal transfer protein (MTP), an endoplasmic reticulum-associated chaperone with the main role transferring neutral lipids (triglyceride, phospholipids and cholesterol ester) to the nascent apo B containing lipoproteins. Nascent chylomicrons are then secreted into the intestinal lymph and move towards the thoracic lymph duct. Once they enter plasma, chylomicrons interact with other lipoprotein particles exchanging apolipoproteins and undergo remodelling. Together with hepatic derived VLDL, chylomicrons constitute triglyceride-rich lipoproteins (TRL). Their triglyceride contents are transferred to the target tissues facilitated by lipoprotein lipase (LPL), an enzyme responsible for hydrolysing triglyceride. Following shedding their lipid contents, eventually chylomicrons shrink and become chylomicron remnants rich in cholesteryl ester. Remnants are then removed by hepatocytes predominantly through a receptor-mediated pathway. The details of the process are explained below.

1.3.1 Chylomicron assembly

The current model of chylomicron assembly indicates the presence of apo B-48 and MTP as obligate requirements. This process consists of apo B-48 synthesis and sequential addition of triglyceride to the primordial particle by the action of MTP (Hussain, 2000; C. M. Williams, Bateman, Jackson, & Yaqoob, 2004) as shown in Figure 2 and Figure 3.

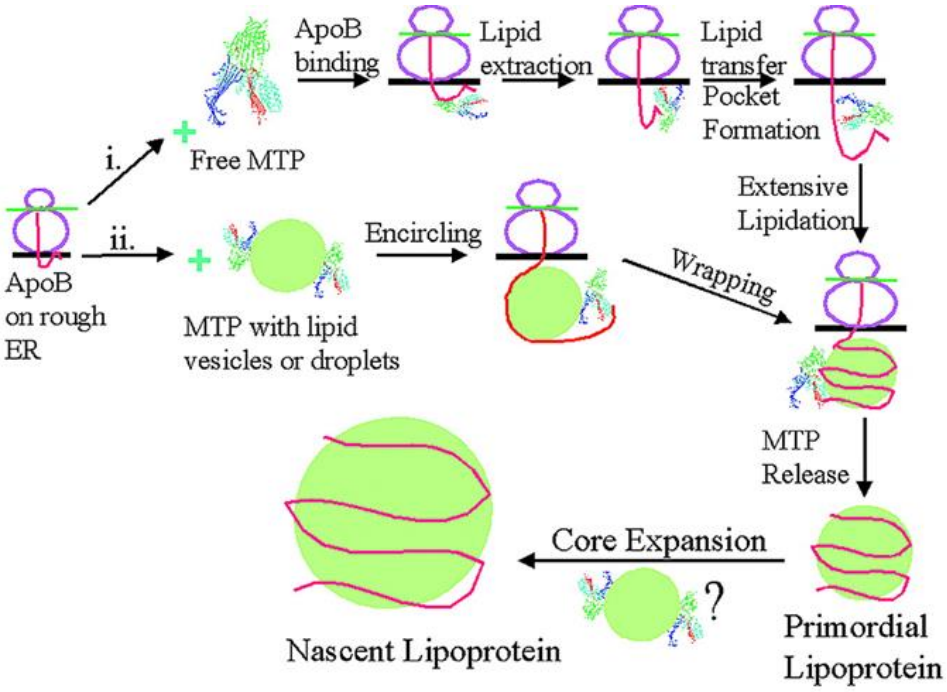

Figure 2. Apo B-MTP binding in lipoprotein assembly. The image shows the possible pathways of lipoprotein lipidation mediated by MTP. i) Free MTP binds to apo B. This process involves lipid extraction in endoplasmic reticulum membrane and pocket formation of apo B which serves as lipid nucleation site. ii) MTP associated with lipid droplets bind to apo B. Apo B would encircle and wrap around the MTP-lipid droplet complex resulting in an efficient lipidation.

Source: Adaptation from Figure 2B, (Hussain, Shi, & Dreizen, 2003)(permission not required)

During translation apo B is translocated across the rough endoplasmic reticulum membrane into the lumen of endoplasmic reticulum (Fisher & Ginsberg, 2002; Pariyarath et al., 2001). As shown in Figure 2, in the presence of triglyceride, the NH_2 terminus of apo B interacts with MTP, a soluble heterodimer of 97 kDa catalytic subunit and protein disulphide isomerase, producing an optimal folding of apo B. MTP binds to a donor membrane and extracts triglyceride molecules. The MTP containing the bound lipid then detaches from the membrane and shuttles the triglyceride molecules from endoplasmic reticulum membrane to apo B-48-containing lipoprotein resulting in the formation of a primordial chylomicron (Atzel & Wetterau, 1993; Dash et al., 2015).

Apo B-48 is synthesised constitutively however the size of chylomicron will depend on the amount of lipid transported. Unlipidated apo B-48 is misfolded and degraded via an endoplasmic reticulum associated degradation pathway, autophagy (Fisher & Williams, 2008) or proteosomal pathway (Fisher & Ginsberg, 2002; Fisher et al., 2001). However

inadequately lipidated intestinal apo B is less susceptible to degradation than hepatic apo B. Enterocytes are able to secrete apo B-48 containing lipoproteins as lipid-poor HDL-sized particles in the absence of dietary fat availability or storing apo B-48 in apical pool to be used when more lipid is available (C. Xiao, Hsieh, Adeli, & Lewis, 2011).

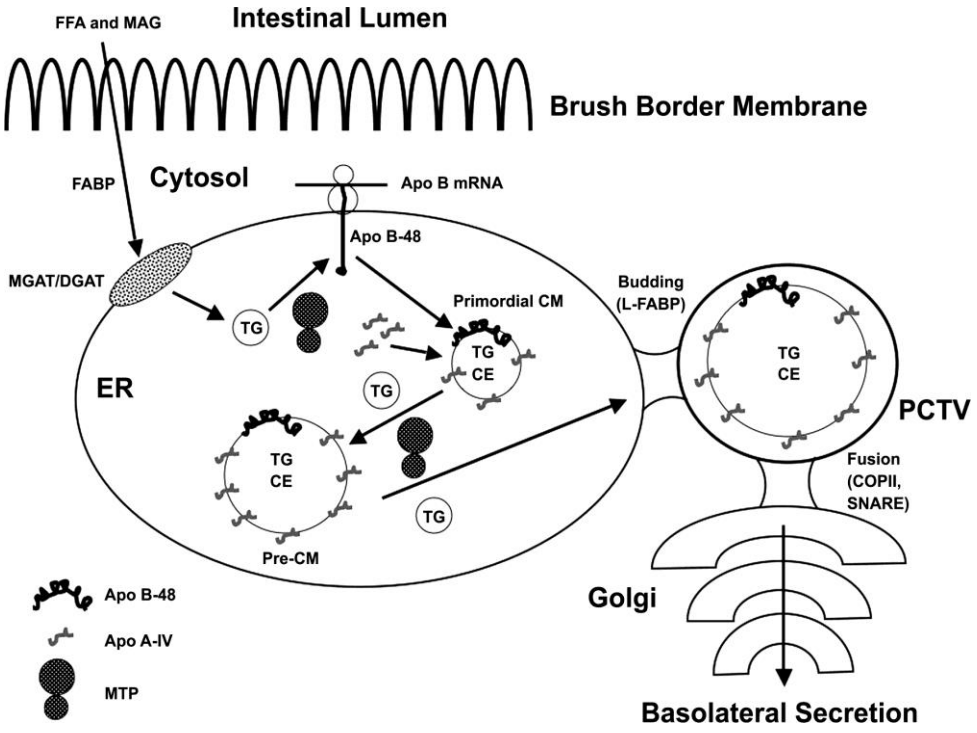

Figure 3. Chylomicron assembly.

Source: Adaptation from Figure 1, (Black, 2007) (permission not required)

In the second step of chylomicron assembly, facilitated by MTP, the primordial chylomicron continues to undergo lipidation by the fusion of endoluminal lipid droplets with primordial chylomicrons in the endoplasmic reticulum (Figure 3). The lipid droplets are composed of neutral lipids (triglyceride and cholesteryl ester) in the core surrounded by monolayer of phospholipids. The triglyceride is formed from dietary fatty acid and monoacylglycerol (MAG) mediated by monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT) respectively (Yen, Stone, Koliwad, Harris, & Farese Jr, 2008). The triglyceride products of DGAT reaction are extracted from endoplasmic reticulum membrane by MTP and being packed into the cores of cytosolic lipid droplets (Atzel & Wetterau, 1993; Yen et al., 2008). These lipid droplets incorporate with primordial chylomicrons allowing them to undergo progressive lipid engorgement.

Apo A-IV is added at this stage to maintain surface stability (Lu et al., 2006) (Figure 3). Apo A-IV is mainly synthesised in the enterocytes (Pan & Hussain, 2012). It has been suggested that apo A-IV stimulates pre-chylomicrons to grow larger by stabilising the expanded lipid/aqueous interface during lipoprotein assembly (Lu et al., 2006) and hence implicates apo A-IV incorporation into primordial chylomicrons at an early stage (F. Wang et al., 2015).

As shown in Figure 3, once the lipidation is complete, the nascent pre-chylomicrons are transported for secretion through the Golgi apparatus in the form of pre-chylomicron transport vesicles (PCTV). It consists of coat protein complex (COP) II, apo B-48, CD36, FABP-1 and vesicle-associated membrane protein-7 (VAMP7) (Mansbach & Siddiqi, 2010; Shahzad Siddiqi et al., 2010). The synthesis of PCTV and its budding from endoplasmic reticulum is initiated by FABP-1, a part of cytosolic protein complex consisting of Sar1b, Sec13 and small valosin-containing protein/p97 interactive protein (Dash et al., 2015). The FABP-1 complex are then released from endoplasmic reticulum after Sar1b underwent phosphorylation mediated by protein kinase C-zeta (S. Siddiqi & Mansbach Ii, 2012). Then they form a vesicular complex budding from endoplasmic reticulum which fuses with the cis-Golgi membrane by the interaction of VAMP7 with the Golgi membrane proteins (S. A. Siddiqi et al., 2006).

At this stage, pre-chylomicrons undergo maturation and receive apo A-I. Apo A-I is synthesised in enterocytes (Rachmilewitz, Albers, Saunders, & Fainaru, 1978). Suppression of apo A-I did not lead to a reduction in the release of triglyceride from enterocytes indicating that apo A-I may not be required in chylomicron lipidation (Jaschke et al., 2012). Therefore it is suggested that the attachment of apo A-I to chylomicrons occurs after chylomicron lipidation. Eventually chylomicrons will be secreted as a mature, nascent chylomicron particles into the pericellular space adjacent to lymphatic fenestrae and then enter the circulation at the thoracic duct (Abumrad & Davidson, 2012).

1.3.2 Chylomicron lipolysis and clearance

Once chylomicrons enter plasma, apo A-I dissociates rapidly (Vigne & Havel, 1981) and they further receive apo C, in particular apo C-II which is important for their lipolysis (Patsch, 1987). Apo C-II is mainly synthesised in the liver and to some extent in enterocytes. It is found bound to chylomicrons, hepatic origin TRL, LDL and HDL. The C-terminal helix of apo C-II is important for interaction with LPL and is required for maximal lipolysis of TRL (Kei,

Filippatos, Tsimihodimos, & Elisaf, 2012). Apo C-II guides chylomicrons to the active site of LPL in the surface of endothelial cells (Kei et al., 2012; LaRosa, Levy, Herbert, Lux, & Fredrickson, 1970; Zdunek et al., 2003). The interaction between apo C-II, LPL and TRL involves changes in lipoprotein surface pressure and apo C-II conformational structure (Meyers, Larsson, Olivecrona, & Small, 2015). The interaction between chylomicrons, LPL and apo C-II requires the presence of apo A-IV. The absence of apo A-IV has been reported to delay chylomicron hydrolysis. As a result, chylomicrons from apo A-IV deficient mice were found to be larger in size and were cleared more slowly from circulation (Kohan et al., 2012). The mechanism behind this could be that the loss of the ability of apo A-IV to modulate apo C-II, an activator of LPL (Goldberg, Scheraldi, Yacoub, Saxena, & Bisgaier, 1990).

LPL hydrolyses the triglyceride content of the bound chylomicrons resulting in the release of fatty acid. The fatty acids are then taken up by CD36 located on the plasma membrane of endothelial cells mainly in adipose and muscle tissue (Figure 4). LPL is a member of a triglyceride gene family consisting of 2 distinct domains, the amino-terminus domain and the smaller carboxyl-terminus domain (H. Wang & Eckel, 2009). The amino-terminus domain contains the catalytic triad which is important for lipolysis. The carboxyl-terminal domain contains the heparin-binding domain, possibly an important feature for binding lipoprotein. It is synthesised in adipocytes and striated myocytes and the synthesis is regulated by fasting and postprandial states. Following synthesis, LPL is matured by LMF1 and then secreted into the subendothelial space. LPL then binds to HSPG of the endothelial cells membrane and trancytosed to the luminal membrane of endothelial cells. Once LPL reaches the luminal surface, LPL is anchored by GPIHBP1 probably in lipid rafts together with syndecan 1 and a fatty acid transporter CD-36 (Geesje M. Dallinga-Thie et al., 2010; K. J. Williams, 2008).

LPL activity is influenced by the presence of apo C-III. As chylomicrons pass through circulation, they pick apo C-III from HDL or VLDL particles (Cohn, Marcoux, & Davignon, 1999; C. Xiao et al., 2011). Apo C-III inhibits LPL activity by interfering the binding of LPL to lipids (Julve, Martín-Campos, Escolà-Gil, & Blanco-Vaca, 2016).

After hydrolysis by LPL, the size of chylomicrons reduces. Phospholipids are transferred to HDL particles and unesterified cholesterols move from the core to the surface of the particles (Changting Xiao & Lewis, 2012). At this stage, they become chylomicron remnants with a high cholesteryl ester level, lysophosphatidylcholine, apo B-48 and apo E. The presence of apo E in chylomicron remnants is important as it mediates

hepatic recognition of remnant lipoproteins and their uptake (Blum, 1982). Remnant particles become smaller in size and denser. Chylomicron remnants continue to circulate until they are removed from the circulation primarily by the liver.

Apolipoprotein transfer from HDL to chylomicrons can be altered by the overexpression apo A-II. Apo A-II is mainly produced in the liver and to a smaller extent in the intestine. It has been demonstrated that overexpression of human apo A-II in mice induces postprandial hypertriglyceridemia (Boisfer et al., 1999). This overexpression altered the exchange of other apolipoproteins from HDL (i.e. apo C-II, apo C-III and apo E) which may affect chylomicron clearance.

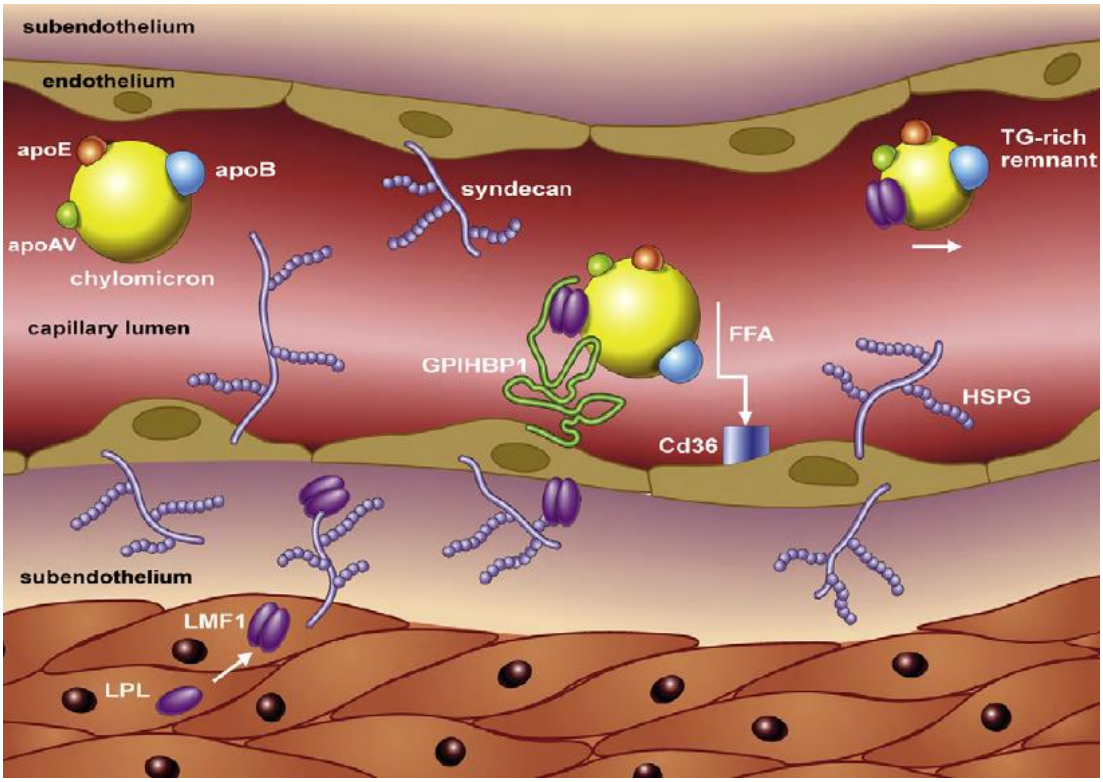

Figure 4. Hydrolysis of TRL. The image shows the sequential lipolytic stages from secretion of LPL in parenchymal cells (adipocytes or myocytes) to the binding of LPL anchored by GPHIHBP1 to chylomicron particles in the luminal membrane of endothelial cells.

Source: Adaptation from Figure 4, Dallinga-Thie et al 2010) (Licence number: 3934120991374).

After entering the liver compartment, chylomicron remnants flow into the liver sinusoids. To enter the space of Disse, chylomicron remnants must pass through the fenestrated endothelium in the liver. These fenestrae serve as a gate function for filtering lipoproteins. The circulating remnants are removed in the liver through several pathways

(Cooper, 1997). The first pathway is direct receptor-mediated uptake remnants. Facilitated by apo E, remnants bind to the heparan sulphate proteoglycans located on hepatocyte surface and then undergo internalisation mediated by a receptor-mediated pathway: low density lipoprotein (LDL) receptor and LDL receptor related proteins (LRP) (Fujioka & Ishikawa, 2009). LDL receptors likely accommodate the removal small remnants from the circulation whereas large remnants are likely to be cleared by the liver involving HSPG (Windler et al., 1996). The removal of remnants via LDL receptor can be categorized as either low capacity ($\leq 5x10^4$ binding sites per hepatocyte) or high affinity leading to rapid endocytosis (Stanford et al., 2009) with $t_{1/2}$ of about 10 minutes (M. S. Brown & Goldstein, 1986; K. J. Williams, 2008). Animal studies comparing wild type versus LDLR deficient livers *in vivo* have shown that in LDLR null livers, despite being rapidly cleared from the circulation by the liver, remnant lipoproteins stayed in the space of Disse longer (minimal 20 minutes), a contrast feature compared to the wild type livers. In the wild type livers, no remnant lipoproteins were found in the space of Disse. Instead the remnants promptly undergo endocytosis through LDL receptor pathway (Mortimer, Beveridge, Martins, & Redgrave, 1995). In addition to LDL receptor mediated clearance, LRP is also importance in chylomicron remnant clearance. Chylomicrons can be cleared through LRP1 via its interaction with apo E (Lillis, Van Duyn, Murphy-Ullrich, & Strickland, 2008). The binding of remnant lipoprotein and apo E to hepatic receptor is altered by apo C-III leading to the reduced remnant clearance via receptor mediated endocytosis (Sacks, 2015).

Remnant lipoproteins are also removed through sequestration mediated by syndecan1 HSPG by interacting with LPL, hepatic lipase and apo E bound to remnant particles HSPG (Cooper, 1997; K. J. Williams & Chen, 2010). This pathway has been suggested to remove large remnants (Nakajima et al., 2014; Windler et al., 1996). The syndecan pathway is categorized as high capacity ($>10^6$ lipoprotein binding sites per hepatocyte) and medium affinity leading to slower rate of endocytosis with $t_{1/2}$ of about 1 hour (Stanford et al., 2009; K. J. Williams, 2008). Apo A-V affects the removal of chylomicron from the circulation by a mechanism involving complex interactions between apo A-V and LPL, proteoglycans and GPIHBP1 (Nilsson, Heeren, Olivecrona, & Merkel, 2011).

The ability to clear chylomicron remnants via a number of pathways indicates the importance of remnant clearance mechanism in maintaining lipoprotein homeostasis and that those pathways cannot completely replace each other.

1.3.3 Chylomicron regulation

As mentioned previously, chylomicrons are produced continuously by enterocytes irrespective of the fasting or postprandial state (Guo et al., 2005). In the postprandial state, chylomicron particles tend to be larger in size to accommodate the increased flux of substrate absorbed (dietary triglyceride, cholesterol, phospholipids and fat soluble vitamins). The increase in chylomicron number is relatively small (Hayashi et al., 1990). This highly efficient response is affected mainly by dietary fat ingestion however other factors such as paracrine and systemic factors also hold a crucial role in all stages of chylomicron metabolism. This section will discuss some factors regulating chylomicron metabolism that is associated with the study objectives.

1.3.3.1 NEFA

Circulating NEFA has been shown to affect chylomicron production. A kinetic study examining the effect of a short term elevation in plasma NEFA on human intestinal and hepatic TRL production revealed a 69% and 39% increase in the rate of production of apo B-48 and apo B-100 containing TRL but no effect was observed on the catabolic rate (Duez et al., 2008). Similar findings were also observed in an animal study (Lewis et al., 2004). Elevated NEFA concentration, as a result of overproduction of hepatic NEFA and a decreased NEFA sequestration capacity of adipose tissue, is commonly observed in various metabolic states such as insulin resistance and type-2 diabetes (Lewis, 1997; Lewis, Carpentier, Adeli, & Giacca, 2002). However the mechanism by which elevated NEFA impacts chylomicron production remains debatable. It is proposed that increased NEFA concentration may promote lipoprotein production through increased pool size of NEFA /triglyceride in the enterocyte due to elevated substrate availability (Changting Xiao & Lewis, 2012), stabilization of intracellular apo B and decreased degradation (Lewis et al., 2004). Tracer studies conducted in pancreatectomised dogs on fat-free diets have suggested that the circulating non-dietary NEFA enter enterocytes, undergo esterification and are secreted into the circulation as chylomicrons (Steiner, Poapst, & Davidson, 1975).

1.3.3.2 Insulin

1.3.3.2.1 Effect of insulin on chylomicron production

Substantial evidence has demonstrated the inhibitory effect of insulin on chylomicron production in conditions where an intact insulin signalling pathway is maintained. Studies

using human foetus jejunal explants have demonstrated an inhibition of the secretion of apo B-48 containing lipoproteins following insulin exposure (Levy et al., 1996). This finding is supported by fasting and postprandial in vivo studies showing that during acute hyperinsulinemia induced by the euglycemic-hyperinsulinemic clamp chylomicron production was suppressed (Harbis et al., 2001; Pavlic, Xiao, Szeto, Patterson, & Lewis, 2010). The mechanism by which chylomicron production is suppressed by insulin remains to be completely elucidated. It has been suggested that insulin exerts its effects at the co- or post-translational level of apo B as insulin did not affect apo B mRNA level (Levy et al., 1996). Under hyperinsulinemic, euglycaemic clamp conditions Pavlic et al (2010) reported a reduced circulating NEFA concentration suggesting that insulin may also act partly through the inhibition of the lipolysis of stored triglyceride in adipose and extra-hepatic tissues (Pavlic et al., 2010). However it is likely that insulin also affects apo B-48 production of other means since suppression of NEFA release by Intralipid and heparin infusion altered the rate of apo B-48 production.

In insulin resistant conditions such as type-2 diabetes and metabolic syndrome, the insulin-mediated inhibition of chylomicron production is blunted resulting in an overproduction of chylomicron particles (Federico, Naples, Taylor, & Adeli, 2006; Nogueira et al., 2012). Kinetic studies in humans have demonstrated an increased rate of chylomicron production in type-2 diabetes (Hogue et al., 2007) and in insulin resistance (Duez et al., 2006). It has been proposed that in the insulin resistant state, protein tyrosine phosphatase-1B, extracellular signal-related kinase and sterol regulatory element-binding protein-1c pathway, and MTP gene transcription are activated, leading to increased stability of intracellular apo B-48 and de novo lipogenesis (Au, Kung, & Lin, 2003; Federico et al., 2006; Haidari et al., 2002). It was also found that NPC1L1 (a regulator of cholesterol absorption) and MTP expression were increased in duodenal biopsies from type-2 diabetes patients. This was thought to be due to a down-regulation of the cholesterol homeostasis regulators ABCG5 and ABCG8 which send cholesterol back into the intestinal lumen, increasing substrate availability (triglyceride and cholesterol) for chylomicron assembly (Lally, Tan, Owens, & Tomkin, 2006). Insulin also plays a role in the routing of pre-chylomicrons from the endoplasmic reticulum to the golgi apparatus. Insulin resistant mice exhibit alteration in pre-chylomicron routing from endoplasmic reticulum to the Golgi due to over-expression of Sar1b (Levy et al., 2014).

1.3.3.2.2 Effect of insulin on lipolysis

Once entering the plasma compartment, chylomicrons undergo lipolysis mediated by LPL. Insulin acts to increase LPL activity by a number of mechanisms (Sadur & Eckel, 1982). Insulin directly increases LPL activity via its stimulation of the transcription of the LPL gene. Hence in subjects with insulin resistance this leads to a diminished LPL activity and consequently delayed clearance from circulation (Maheux, Azhar, Kern, Chen, & Reaven, 1997). In addition subjects with type-2 diabetes have been found to have a lower adipose LPL activity both in fasting and postprandial states (Annuzzi et al., 2008). Insulin also modulates LPL activity through inhibition of apo C-III expression which would consequently result in increased LPL activity (Altomonte et al., 2004).

1.3.3.2.3 Effect of insulin on chylomicron clearance

Insulin affects the rate of chylomicron clearance in the liver. Insulin promotes LDLR gene expression via activation of the sterol regulatory element binding proteins (SREBP) 1 signal transduction pathway (Streicher et al., 1996). Insulin may also affects LDLR expression via upregulation of PCSK9, a key regulator of hepatic LDLR degradation (Ferri & Ruscica, 2016). Insulin induced upregulation of PCSK9 through activation of SREBP-1c transcription factor. Indeed subjects with insulin resistance exhibit decreased LDLR expression which would result in a delayed clearance of chylomicron remnants. Insulin also plays a role in the clearance of chylomicron remnants via modulation of HSPG structure. In subjects with insulin resistance, over-expression of hepatic SULF2 results in an increased degradation of matrix HSPG (Chen et al., 2009). Taken together these findings suggest that in the insulin resistant state, a general increase in chylomicron concentration in circulation possibly with a build-up of cholesterol-rich chylomicron would be found.

1.3.3.3 Dietary fat

Both the amount and type of fat consumed affect the postprandial chylomicron response. Consumption of increasing amounts of fat results in a proportional increase in the postprandial triglyceride and chylomicron response (Cohen, Noakes, & Benade, 1988; Dubois et al., 1998; Anthony P. James & John C. Mamo, 2012). However consumption of low amounts of fat (<15 g) do not result in an increased postprandial triglyceride or chylomicron concentration suggesting that the basal rate of chylomicron production and lipolysis is sufficient to avoid exaggerated postprandial lipaemia following low dose of fat

consumption (Dubois et al., 1998). Triglyceride clearance capacity was able to proportionally balance the fat assembled following fat load of 30 – 50 g fat. However when given a very high dose of fat load (80 g or more), exaggerated postprandial hypertriglyceridaemia ensues but not in dose-dependent pattern (Denis Lairon, 2008).

Chylomicron production in the enterocytes has the ability to accommodate a wide range of amounts of dietary fat by increased particle size or an increase in particle number secreted, or both. Consumption of a low fat dose (<14 g) in healthy males resulted in no significant increase in postprandial apo B-48 response (Anthony P. James & John C. Mamo, 2012). It seems that at low dose fat loading, the chylomicrons may respond by increasing their particle size instead of their particle number and the fat load is accommodated by the basal rate of chylomicron secretion and/or efficient lipolysis and clearance (Hayashi et al., 1990). In contrast, following consumption of a moderate fat dose (>19 g) increased postprandial chylomicron concentration ensued. This increased postprandial chylomicronaemia may result from an increased chylomicron secretion or delayed lipolysis and clearance.

Although dietary lipids are primarily packaged into chylomicron particles in the postprandial state, recent evidence suggests that a portion of these are stored as cytosolic lipid droplets within the enterocyte. These may represent a source of triglyceride for chylomicron production during the interprandial phase (Beilstein, Carrière, Leturque, & Demignot, 2016).

A number of studies have compared the effect of different dietary fatty acid composition on postprandial triglyceride, apo B-48 and/or chylomicron responses. The fatty acid chain length and presence of double bonds affects their metabolic pathway and thus the extent of postprandial triglyceride or chylomicron response. Short and medium chain triglycerides are completely hydrolysed to fatty acids and glycerol by pancreatic lipase. They are mainly absorbed as free fatty acids and only a very small portion as mono-diacylglycerols (Bach & Babayan, 1982). Fatty acids with shorter chain length (less than 12-14 carbons) are not good substrates for esterification due to the absence of specific medium-chain acyl-CoA synthase (Frayn, 2009) and they are not easily bound to fatty acid binding protein (Bach & Babayan, 1982). Therefore they are rapidly absorbed into circulation via the hepatic portal vein as NEFA bound to albumin and are transported to the liver to be oxidised. Thus fat challenges using meals rich in short fatty acid or medium chain fatty acid generally results in a lower postprandial triglyceride response (Mekki, Charbonnier, Borel, Leonardi, & et al., 2002). A kinetic study showed no significant change

in apo B-48 pool size and no effect on TRL apo B-48 production rate and clearance following coconut based MCT oil consumption (Tremblay et al., 2014).

The effect of long chain fatty acids (saturated, monounsaturated and polyunsaturated fatty acid) on the postprandial lipid response is much more complex. There are several proposed mechanisms involved in modulation of postprandial lipid response by different types of fatty acids. Animal and in vitro studies have reported that the rate of absorption and preference for incorporation of dietary fatty acids into triglyceride or other lipids in enterocytes modulates the chylomicron postprandial response. Long chain SFA are poorly absorbed and therefore will influence triglyceride availability for the chylomicron assembly (Kalogeris & Story, 1992). Thus it is expected that they would be retained in the enterocytes longer and/or released as part of smaller sized chylomicron particles. In addition to chain length, gastrointestinal lipid handling is also influenced by the degree of saturation. Stearic acid is substantially less absorbed than palmitic acid, oleic acid or linoleic acid (A. E. Jones, Stolinski, Smith, Murphy, & Wootton, 1999) (P. J. H. Jones, Pencharz, & Clandinin, 1985). The differences in absorption could be attributed by difference in affinity to bind fatty acid binding proteins or difference in lipid partitioning in the enterocytes. An in vitro study using the Caco-2 intestinal cell line demonstrated that cells incubated with palmitic acid secreted more dense lipoproteins than those incubated with linoleic acid (Van Greevenbroek, Voorhout, Erkelens, Van Meer, & De Bruin, 1995). A greater proportion of linoleic acid was incorporated into triglyceride than palmitic acid. However inconsistent findings were observed in human studies. Compared to palmitate and oleate, linoleate is incorporated more into phospholipids and cholesteryl ester (Hodson, McQuaid, Karpe, Frayn, & Fielding, 2009). Among SFA, myristate is preferentially partitioned into triglyceride (>95%) whereas a greater portion of palmitate (18%) and stearate (33%) are partitioned into phospholipids (Hughes et al., 1996).

Dietary fatty acid composition has also been recognised to affect chylomicron metabolism through modulation of their lipolytic rate and clearance. Chylomicrons containing PUFA are converted to chylomicron remnants more rapidly than those containing SFA or MUFA. This could be due to different lipolytic rate by LPL and different preferential hepatic uptake by HL with the higher preference for PUFA-enriched chylomicrons (Kathleen M. Botham, Avella, Cantafora, & Bravo, 1997; Bravo et al., 1995; Rahman, Avella, & Botham, 2000). Animal and in-vitro studies showed a greater LPL hydrolysis of short chain unsaturated monoacyl glycerol and triglyceride (Groot, de Boer, Haddeman, Houtsmuller, & Hülsmann, 1988; Hulsmann, Oerlemans, & Jansen, 1980). The

mechanism of increased adipose LPL activity following PUFA challenge could be through activation of PPAR γ induced by linoleic acid and arachidonic acid serving as PPAR γ agonists (Oyekan, 2011; Schoonjans et al., 1996). In humans, n-3 PUFA reduces postprandial triglyceride and chylomicron response by directly increasing LPL activity during fed state and enhanced chylomicron clearance partly through reducing VLDL secretion (Park & Harris, 2003). The findings of increased LPL activity are inconsistent for MUFA (Bergouignan, Momken, Schoeller, Simon, & Blanc, 2009). Dietary n-3 and n-6 PUFA could also modulate uptake via upregulation of hepatic LDLR expression (Spady & Woollett, 1990). Dietary fats have also been found to modify apolipoprotein composition and thus may affect fatty acid handling. Meals containing SFA induce a greater amount of apo E and apo C-III in triglyceride-rich lipoproteins than meals rich in unsaturated fatty acids (K. G. Jackson, Wolstencroft, Bateman, Yaqoob, & Williams, 2005).

The ability of enterocytes to absorb a wide range quantity of dietary fats by shifting the size of chylomicron released from small/VLDL-like in the interprandial state to large chylomicrons is a result of metabolic adaptation of the enterocyte to the lipid influx from the diet (Buttet et al., 2014). When challenged with a fatty meal, chylomicrons tend to increase their size instead of increasing their particle numbers indicating the presence of this lipid driven metabolic adaptation (Cartwright & Higgins, 1999; Karpe, Olivecrona, Hamsten, & Hultin, 1997). In situ, an isolated segment of jejenum from mice fed a high fat diet (40% weight/weight) for 6 weeks was able to absorb a greater amount of fatty acids and induce higher mitotic index and mRNA expression of genes involved in lipid uptake (FATP4, FAT/CD-36), intracellular trafficking (intestinal and liver fatty acid binding proteins/ I-FABP and L-FABP) and lipoprotein synthesis (MTP, apo A-IV) (Petit et al., 2007). LPL regulators (apo C-II and apo C-III) were also modulated to maintain efficient triglyceride clearance.

Consumption of isocaloric diets rich in SFA, n-6 PUFA and n-3 PUFA for 25 days in normolipidemic subjects showed that the postprandial chylomicron response to a single oral fat challenge was greater in SFA diet followed by n-6 PUFA and n-3 PUFA (M. S. Weintraub, Zechner, Brown, Eisenberg, & Breslow, 1988). In that study, chylomicrons containing n-6 PUFA and n-3 PUFA produced during each acute postprandial test were hydrolysed faster than the SFA-containing chylomicrons whereas no effect of chronic diets was observed on lipolysis. It is suggested that chronic diets are more likely to affect the catabolic machinery. Recently, a 12-week supplementation with n-3 fatty acid ethyl esters (46% eicosapentaenoic acid and 38% doxosahexaenoic acid) in obese individuals on

hypocaloric diet results in a decreased baseline and postprandial triglyceride and apo B-48 response (A. T. Y. Wong, Chan, Barrett, Adams, & Watts, 2014). The n-3 fatty acid ethyl ester supplementation reduces basal apo B-48 secretion rate but not during the postprandial state.

In summary many factors affect chylomicron metabolism. Circulating NEFA modulates chylomicron production whereas insulin reduces chylomicron production and maintains efficient chylomicron hydrolysis and uptake. Dietary fats affect chylomicron metabolism at almost every stage (absorption, intestinal lipid partitioning, lipolysis and removal).

1.4 Chylomicron and hepatic lipoprotein interactions in postabsorptive and postprandial state

To maintain a balanced steady state of lipid homeostasis between the postabsorptive and postprandial states, intestinal and hepatic derived TRL exhibit many dynamic and well-coordinated interactions. In the postabsorptive state, lipid absorption in the intestine practically ceases however the production and release of lipid-poor apo B-48 and hepatic derived lipoproteins continue (C. Xiao et al., 2011). The presence of chylomicrons in the circulation during the fasting state has been reported previously (Campos, Khoo, & Sacks, 2005; A. P. James & J. C. Mamo, 2012; Karpe, Bell, Bjorkegren, & Hamsten, 1995; Karpe & Hamsten, 1994; Mamo, Smith et al., 1998; Schaefer, Jenkins, & Bryan Brewer Jr, 1978). It is suggested that the source of lipid in the intestine is derived from: 1) NEFA and glycerol imported from the circulation; 2) NEFA generated from de novo lipogenesis in enterocytes (Federico et al., 2006; Haidari et al., 2002); and 3) release of triglyceride from cytosolic lipid droplets in enterocytes (Beilstein et al., 2016; Shojaee-Moradie, Ma, Lou, Hovorka, & Umpleby, 2013; C. Xiao et al., 2011).

During fasting, endogenous triglyceride production maintains the continuity of substrate supply for extrahepatic tissues. Approximately 20 – 30 g/day endogenous triglyceride is produced through VLDL in normolipidemic males (M. Adiels et al., 2006; Cohn, Patterson, Uffelman, Davignon, & Steiner, 2004), a much lower concentration than is typically transported from the intestine (around 100 g/day with almost complete absorption) in postprandial state (Gades & Stern, 2005; C. Xiao et al., 2011). The source of lipid in VLDL during the fasting state is mostly derived from the circulating NEFA released from adipose tissue lipolysis (70 – 80%) (Barrows & Parks, 2006).

Food ingestion initiates the transition from fasted to fed states. The chylomicron response to a meal starts even before ingested. Tasting, but not swallowing, the meal has been reported to trigger an increased release of chylomicrons suggesting a taste-gut-brain axis involvement in preparing the digestive systems including enterocyte synthesis and the capacity of chylomicrons for the incoming lipid load (Buttet et al., 2014; Mattes, 2009). Following dietary absorption, chylomicron secretion capacity increases preferentially through lipid enrichment and enlargement of their particle size rather than increasing the particle number (Cohn, McNamara, Cohn, Ordovas, & Schaefer, 1988). Approximately 82% of the increased postprandial triglyceride concentration is contributed by the apo B-48 TRL-derived triglyceride (Cohn et al., 1993). Despite this substantial contribution, the increase in apo B lipoprotein postprandially is mainly contributed by apo B-100 and not apo B-48 lipoproteins due to competition for hydrolisis in which apo B-48 lipoproteins are more preferred (Brunzell, Hazzard, Porte Jr, & Bierman, 1973; Karpe et al., 1995). The lipid-rich chylomicrons in the circulation are hydrolysed by insulin-stimulated LPL and eventually become enriched in cholesteryl ester. The majority of the triglyceride content in chylomicrons is taken up by extrahepatic tissues expressing LPL. During hydrolysis, a portion of NEFA escapes from cellular uptake and enters the plasma pool. This NEFA spill over may be utilised for the synthesis of chylomicron and VLDL or stored as lipid droplets in the enterocyte and liver respectively.

The interaction between chylomicrons and hepatic VLDL in maintaining lipid homeostasis in both postabsorptive and postprandial states is also mediated by gut hormones. Immediately after meal ingestion, L and K cells located in the distal intestine are stimulated resulting in GLP-1 secretion. GLP-1 then induces β cells in pancreas to secrete insulin. In the postprandial state, the increased insulin concentration down-regulates adipose tissue lipolysis, and NEFA flux to the liver and intestine. Postprandial hyperinsulinemia may also suppress the production of nascent chylomicron and VLDL particles in order to minimise competition for lipolysis and removal between chylomicron and VLDL particles (C. Xiao et al., 2011).

In summary, to ensure the continuity of lipid transport in the circulation in the postabsorptive, postprandial and the transition between these states, the interaction between chylomicrons and hepatic VLDL involves a range of signals including nutrient, neural and hormonal. These signals implicate chylomicron and hepatic VLDL metabolism at various stages: lipoprotein assembly, transport, storage, redistribution and clearance. In

conditions where accumulation of chylomicron remnants occurs, this efficient interaction is disrupted resulting in a greater exposure of cholesterol rich remnants to the vascular wall.

1.5 Cholesterol-rich chylomicrons

The increase in circulating lipoprotein particle number in the postprandial state is mostly due to an increase in apo B-100 containing lipoproteins with apo B-48 containing lipoproteins only contributing 20% of the increased particle number (Karpe et al., 1995; Schneeman, Kotite, Todd, & Havel, 1993). Despite there being a lower number of chylomicron particles in circulation, each chylomicron remnant has been reported to contain 63,900 cholesterol molecules whereas one LDL particle contains only 1,771 cholesterol molecules, a 40 times more than LDL which may explain their atherogenicity (C. J. Fielding, 1992).

The source of cholesterol in chylomicrons can be from diet and/or from endogenous lipid exchange with cholesterol-rich lipoproteins (HDL and LDL). Both chylomicron and HDL lipoproteins play an important role in the transport of dietary cholesterol from the intestine (Hussain, 2014; Iqbal & Hussain, 2005). Cholesterol esters are mostly carried by chylomicrons and this process is mediated by MTP whereas free cholesterol is carried by HDL via ABCA1. The contribution of cholesterol carrying capacity is greater in chylomicrons than in HDL apo A-1 lipoproteins. Intestinal MTP-ablated mice exhibit reduced cholesterol absorption by around 92% and enterocytes secretion by 70% (Iqbal, Parks, & Hussain, 2013). Ablation of intestinal ABCA1 in mice resulted in 28% and 30% decrease in cholesterol absorption and secretion respectively. Combined deficiency of MTP and ABCA1 decreased cholesterol secretion by 80%.

The other source of cholesterol in chylomicrons is from endogenous lipid exchange in reverse cholesterol transport. Reverse cholesterol transport is the movement of cholesterol from peripheral cells to the liver via plasma compartment (C J Fielding & Fielding, 1995; Frayn, 2009). Cholesterol from peripheral cells is transported to the liver by apo A-I particles via ABCA1 or by more mature spherical HDL particles via ABCG1. For efficient cholesterol uptake, within HDL particle free cholesterol is esterified into cholesteryl ester by LCAT clearing more space for additional free cholesterol uptake on the HDL surface (Annema & Tietge, 2012). In the plasma compartment, this cholesterol is transported in a reverse pathway heading back to the liver. In humans and a number of species, CETP, a hydrophobic glycoprotein, provides a shunt between the forward and the

reverse cholesterol transport pathways by facilitating neutral lipid (cholesteryl ester, triglyceride, retinyl ester and a small portion of phospholipid) transfer between triglyceride-rich (chylomicron and hepatic derived) lipoproteins and cholesterol-rich lipoproteins (LDL and HDL particles) (Tall, 1993). CETP can promote net transfer of cholesteryl ester made by LCAT with or without an equimolar exchange of triglyceride. In individuals with normal CETP activity, the major route of cholesterol uptake by the liver is through chylomicron remnants, hepatic VLDL remnants and LDL (Tall, 1993). This lipid transfer has been suggested to maintain intact HDL homeostasis by balancing the augmentation of HDL mass (driven by LPL and LCAT) with a reduction in HDL mass by diverting their cholesteryl ester content to triglyceride-rich lipoproteins facilitated by CETP and HL. In contrast, in conditions where CETP activity increased (such as in high fat consumption and in insulin resistance), the transfer rate of cholesteryl ester from HDL to TRL is augmented resulting in a high influx of cholesterol to the liver. The excessive deposition of cholesterol in the liver eventually down-regulates LDLR expression. As a result, cholesterol-rich remnants will build up in the circulation.

1.6 The role of chylomicrons in atherogenesis

The theory that atherosclerosis is a postprandial phenomenon has attracted a lot of attention since the finding of cholesterol-rich chylomicron remnants accumulated in tunica intima of animal models (D. B. P. D. Zilversmit, 1979). Since then numerous studies have attempted to elucidate the potential mechanism for this accumulation. Atherosclerosis arises from the accumulation of fatty deposits in the arterial wall. According to the Response-to-Retention model of atherogenesis, the initial stage involves the retention of apo B lipoproteins (chylomicron remnants, VLDL remnants, and LDL) on subendothelial extracellular matrix (K. J. Williams & Tabas, 1995; K. J. Williams & Tabas, 2005). The retained lipoproteins undergo modification (i.e. aggregation, oxidation) and induce a maladaptive inflammatory response involving: activation of endothelial cells, monocytes entering subendothelial tissue and endocytosis of the retained lipoproteins by monocyte-derived macrophages resulting in the formation of cholesterol-laden foam cells (Tabas, Williams, & Borén, 2007).

1.6.1 Subendothelial accumulation of atherogenic remnants

The entry of lipoproteins into subendothelial tissue depends on the concentration of lipoproteins accumulated in the circulation and the lipoprotein properties (size, charge and endothelial permeability/ability to bind to proteoglycan) (Tabas et al., 2007). In the circulation, lipoproteins dynamically flux into and out of the arterial wall by transcytosis, a transport system using a specialised clathrin-coated plasmalemmal vesicles (M. Simionescu & Simionescu, 1991). The size of this vesicle is 100 nm in diameter and thus can only transport lipoproteins and macromolecules less than 70 nm in diameter. Therefore large lipoproteins such as chylomicrons and hepatic VLDL cannot cross the endothelium but their remnants can (D. B. Zilversmit, 1995).

Chylomicron remnants can penetrate the artery walls as effectively as LDL. The rate of intimal clearance and fractional loss of remnant sized lipoproteins (Sf 12 – 60) was observed to be similar to that of LDL sized lipoproteins in an in vivo study of subjects undergoing elective carotid endarterectomy (Shaikh et al., 1991). This finding suggests that remnants may contribute to the accumulation of lipid in subendothelial matrix.

It is estimated that approximately 2500 transport vesicles leave the endothelial plasma membrane per minute (G. M. Dallinga-Thie, Kroon, Borén, & Chapman, 2016). Thus the selectivity of lipoproteins to enter and be retained within subendothelial tissue depends on the extent which they interact with the subendothelial matrix. An important component of the subendothelial matrix related to lipoprotein retention is proteoglycan. It has been suggested that the retention of lipoproteins is due to the interaction between positively charged apo B lipoproteins and negatively charged sulphate groups of proteoglycan sugar (Tabas et al., 2007). Apo B-48 containing lipoproteins bind to proteoglycan at site B-Ib of apo B-48 (Flood et al., 2002). Evidence supporting the selectivity of chylomicrons retained within subendothelial tissue also comes from a study using an atherosclerosis-prone animal model showed a greater arterial retention of apo B-48 containing lipoprotein than apo B-100 containing lipoproteins (Proctor, Vine, & Mamo, 2002). The causal mechanism between lipoproteins, proteoglycans and early atherogenesis has been demonstrated in an animal study showing that mice expressing proteoglycan-binding-defective LDL develop less atherosclerotic lesions than the wild-type mice LDL (Skalen et al., 2002).

An additional factor that may promote lipoprotein retention is the presence of LPL in the arterial wall. LPL can promote lipoprotein retention by its non-enzymatic function in

bridging lipoprotein-matrix (Proctor et al., 2002). Large TRLs (apo B-48 and apo B-100 containing TRLs) are hydrolysed by LPL and their size would shrink reaching to the size where they are able to penetrate the subendothelial tissue. This explains why patients with LPL deficiency do not develop atherosclerosis (Mamo, 1995). LPL may also be an anti-atherogenic molecule through LPL-mediated lipolysis and lipoprotein removal from the circulation.

1.6.2 Chylomicron remnants, monocyte/macrophage activation and foam cell formation

Once remnants in the subendothelial tissue, they interact with monocytes resulting in intracellular lipid accumulation of monocytes and induction of inflammatory response which would activate monocytes. Monocytes can be activated through accumulation of cytosolic lipid droplets (Bentley et al., 2011), upregulation of apo B-48 receptor mRNA expression (Bermudez et al., 2012; Varela et al., 2011) and LRP1 (Gower et al., 2011), increase expression of leukocyte activation markers (CD11b) (Alipour et al., 2008), upregulation of a number of markers related to monocyte adhesion (Gower et al., 2011; Kawakami, Tanaka, Nakajima, Shimokado, & Yoshida, 2002), increased production of reactive oxygen species (Bentley et al., 2011) and modulation of migratory and chemotaxis markers (IL-8, MCP1) (Bentley et al., 2011). These mechanisms will result in the adherence of monocytes to the endothelium.

The presence of chylomicron remnants in the subendothelial tissue may induce a substantial increase in cytoplasmic lipid droplets in animal and human macrophage cell line studies (Batt et al., 2004; Fujioka, Cooper, & Fong, 1998; Napolitano, Rivabene, Avella, Botham, & Bravo, 2001; Yu & Mamo, 2000). Chylomicron remnants are also able to interact directly with macrophages to trigger foam cell formation (Fujioka et al., 1998; Mamo, 1995; Proctor & Mamo, 1996; Proctor et al., 2002). Accumulation of lipid engorged macrophage foam cells forms fatty streak, the hallmark of atherosclerosis. Several mechanism of uptake chylomicron remnants by macrophages have been reported: receptor-mediated LDLR and LRP1 apo E-dependent (Bejta et al., 2007), apo B48R apo B-dependent (M. L. Brown et al., 2000), scavenger receptor CD36 (Bejta et al., 2007), non-receptor mediated pathway via LPL (K. M. Botham & Wheeler-Jones, 2013; Palmer et al., 2005) and phagocytosis (Bejta et al., 2007; Elsegood & Mamo, 2006; Mamo, Elsegood, Gennat, & Yu, 1996). Indeed the presence of chylomicron apo B-48 in atherosclerotic tissue together with apo B-100 in

human atherosclerotic plaque has been reported (Nakano et al., 2008; Pal, Semorine, Watts, & Mamo, 2003).

1.7 Chylomicron and postprandial lipaemia

In subjects consuming three or four meals and snacks per day, triglyceride concentrations increase by 70% over the course of 24-hours with triglyceride reaching the fasting level only in the early morning (3:00 – 6:00 AM) (C. M. Williams, 1997). The repeated consumption of lipid-rich meals throughout the day and the fact that their complete metabolism may take up to 8-10 hours (Frayn, 2009) indicates that human body is exposed to circulating dietary lipids throughout the day.

1.7.1 Postprandial lipaemia: Definition and diagnosis

Postprandial lipaemia is a normal physiological condition where TRL and remnants are increased postprandially. During the postprandial state, the main change in lipid parameters in the blood is hypertriglyceridaemia. Postprandial hyperglycemia may occur, however this happens in the earlier postprandial period (Kolovou, Anagnostopoulou, Daskalopoulou, Mikhailidis, & Cokkinos, 2005). In normal postprandial lipaemia, the increase in triglyceride and TRL concentration are transitory and generally will last from 6 – 8 hours after fatty meal ingestion (D. C. Chan, Pang, Romic, & Watts, 2013). It is suggested that the mechanism of postprandial hypertriglyceridaemia is a result of competition between intestinal and hepatically derived TRL (chylomicrons and hepatic VLDL) for lipolysis and clearance.

The importance of measuring lipid profile postprandially emerges after prospective studies have shown that nonfasting triglyceride concentration predicts the cardiovascular events (myocardial infarction, ischemic heart disease) (Bansal et al., 2007; B. G. Nordestgaard, Benn, Schnohr, & Tybjærg-Hansen, 2007). Epidemiological studies have shown that the risk of a cardiovascular event increase when the non-fasting triglyceride concentration is 165 mg/dL in Japan (Teramoto et al., 2013) and 175 mg/dL in Europe (Børge G. Nordestgaard et al., 2016). Bansal et al (2007) reported that higher nonfasting triglyceride concentration was associated with an increased risk of future cardiovascular events (nonfatal myocardial infarctions, ischemic strokes, coronary vascularisation, cardiovascular mortality) (Bansal et al., 2007). This association was independent of the baseline cardiac risk factors, other lipid levels (total and HDL cholesterol) and insulin

resistance. In contrast, the association between fasting triglyceride concentration and cardiovascular event weakened after adjustment for these factors. Nonfasting triglyceride concentration collected 2 – 4 hours after the last meal showed the strongest association with the occurrence of cardiovascular events. Similarly, Nordestgaard et al (2007) also reported that increased nonfasting triglyceride was associated with increased risk of myocardial infarction, ischemic heart disease and mortality (B. G. Nordestgaard et al., 2007). They also demonstrated that with increased nonfasting triglyceride, remnant cholesterol concentration also increased. In response to normal food intake, the change of lipid, lipoproteins and apolipoprotein profiles were minimal, therefore the use nonfasting triglyceride level to predict cardiovascular disease risk would be more preferable since it simplifies the clinical care of the patients (Langsted, Freiberg, & Nordestgaard, 2008).

Currently there is still no established method for diagnosing impaired postprandial lipaemia. To assess the TRL postprandial response, standard methods measuring the response of plasma triglyceride, retinyl esters, remnant-like particles or apo B-48 after ingesting an oral fat load following an overnight fast can be applied (D. C. Chan et al., 2013). Postprandial responses are generally analysed as the total area under the curve (AUC), indicating the total lipid response to a fat load, and the incremental AUC (IAUC), representing the change in lipid response beyond baseline levels. Apo B-48 is considered as the most suitable marker as it is a non-exchangeable marker for the quantity of circulating chylomicron particles/remnants (Smith, Watts, Dane-Stewart, & Mamo, 1999). It is recommended that the lipid content of the test meal should be between 40 – 60 g, an amount that can stimulate lipid metabolism without overloading the system, and include protein and carbohydrate (minimal 50 – 100 g) to provoke adequate insulin response (D. Lairon, Lopez-Miranda, & Williams, 2007).

1.7.2 Exaggerated postprandial lipaemia

In postprandial hypertriglyceridaemia, the magnitude and the duration of the TRL response is exaggerated resulting in accumulation of TRL and their remnants in the circulation. It has been suggested that the clearance capacity of remnants is the main determinant of the accumulation of chylomicrons and chylomicron remnants and the magnitude of postprandial lipaemia (Martin Adiels et al., 2012). A decreased capacity of clearance pathways could be due to decreased hepatic receptor activity, defect on the ligand of the receptor (i.e. apo A-V, apo E, apo C-III) and impaired LPL activity (D. C. Chan et al., 2013).

The greater the magnitude and the duration of postprandial lipaemia, the greater the exposure of vascular wall to cholesterol-rich remnants.

1.7.2.1 Obesity and insulin resistance

Previous studies have demonstrated that overweight/obese subjects had raised fasting concentration of chylomicron particles (Couillard et al., 2002; Annette T. Y. Wong, Chan, Pang, Watts, & Barrett, 2014) suggesting impaired TRL metabolism. A recent prospective cohort study in children, whom at least one of the parents was classified as obese or large waist, demonstrated that over 2-year period, greater changes in central adipocity was associated with apo B-48 compared to the classic lipid markers (Wilke et al., 2016). For every increase of 1 kg/m^2 in central obesity, there were approximately 14 times increase in apo B-48 concentration in children with lower baseline central fat mass index compared to those with higher baseline.

Increased adiposity is associated with an increased influx of NEFA to the liver which will stimulate hepatic VLDL production and gluconeogenesis. An increased in-flux of NEFA entering the liver also impairs the hepatic extraction of insulin leading to hepatic insulin resistance. Insulin resistance increases de novo lipogenesis in the liver and the amount of fatty acids and triglyceride entering the liver due to a reduction in its normal antilipolytic effect in adipose and extra-hepatic tissues (Pavlic et al., 2010). LDLR expression is also altered resulting in impaired catabolism of apo B containing lipoproteins (D. C. Chan et al., 2013). Insulin resistance also affects intestinal TRL metabolism via overproduction of apo B-48 lipoprotein (Duez et al., 2006). Results from a kinetic study in obese individuals has revealed that exaggerated postprandial lipaemia in visceral obesity is associated with overproduction and disrupted catabolism of apo B-48 containing lipoproteins (Annette T. Y. Wong et al., 2014).

1.7.2.2 Metabolic syndrome

Metabolic syndrome (MetS) is a cluster of metabolic abnormalities characterised by dysglycemia, raised blood pressure, elevated triglyceride concentration, low high density lipoprotein (HDL) cholesterol level and obesity (central adiposity) (Alberti et al., 2009). It has been reported that individuals with MetS exhibit an exaggerated postprandial triglyceride response (Kolovou, Anagnostopoulou, Pavlidis et al., 2005). The magnitude of the postprandial triglyceride response has been found to increase with increasing number

of MetS components that an individual possesses (K. G. Jackson et al., 2012). Men who had less than 3 components of MetS exhibited lower incremental triglyceride response to a sequential meal challenge than those with 3 or more MetS components.

The pathophysiology of MetS is largely attributed to insulin resistance (Eckel, Grundy, & Zimmet, 2005). Increased supply of hepatic lipid substrate and delayed clearance of TRL and remnants has been suggested as the mechanism of MetS (Dick C. Chan, Barrett, & Watts, 2004). The production rate of both chylomicron and VLDL triglyceride increased (Annette T. Y. Wong et al., 2014). Lipolysis and clearance of TRL or remnants were delayed, which might be due to decreased LPL activity, hepatic remnant receptor (LDLR) activity and the synthesis of HSPG mediated by insulin resistance (D. C. Chan, Watts, Barrett, Mamo, & Redgrave, 2002; D. C. Chan, Watts, Redgrave, Mori, & Barrett, 2002; Mamo et al., 2001). MetS is also associated with the increased synthesis of apo C-III possibly due to decreased expression of hepatic PPAR α has also associated with MetS (Shachter, 2001). Apo C-III inhibits lipolysis and hepatic clearance.

1.8 Evidence to date: Metabolic determinants of chylomicron accumulation

1.8.1 Introduction

According to the Response-to-Retention theory of atherogenesis, retention of remnants (chylomicrons and hepatic VLDL) in subendothelial tissue serves as the initiator for atherogenesis (K. J. Williams & Tabas, 1995; K. J. Williams & Tabas, 2005). Evidence has shown that fasting serum apolipoprotein B-48 concentration, a measure of chylomicron particle number, is significantly associated with the existence of atherosclerosis and coronary artery disease (Alipour et al., 2012; Masuda et al., 2012). Previous studies have demonstrated that overweight / obese subjects exhibiting impaired lipid metabolism also had raised fasting concentration of chylomicron particles (Couillard et al., 2002; Annette T. Y. Wong et al., 2014). Higher fasting chylomicron concentration was also found in subjects with MetS (Kinoshita et al., 2009). Those metabolic phenotypes are at risk of CVD indicating the possibility of some commonalities in terms of metabolic defects affecting both chylomicron metabolism and the other parameters. In the next section we will discuss evidence to date of studies investigating the association between chylomicron and other metabolic parameters.

1.8.2 Metabolic determinants

A limited number of studies have investigated the association of apo B-48 concentration with other lipid or metabolic parameters (Table 1.1) (Hanada et al., 2012; Kinoshita et al., 2009; Sakai et al., 2003; Sato et al., 2009). In these studies the concentration of chylomicron particles, as measured by apo B-48, was strongly associated with triglyceride concentration. In the fasting state, plasma triglyceride concentration is mainly contributed by triglyceride carried by VLDL and their remnants. It is interesting that despite apo B-48 being much lower in concentration than apo B-100 in the fasting state, apo B-48 was still significantly associated with triglyceride suggesting a common metabolic defect leading to accumulation of these triglyceride-rich lipoproteins.

Apo B-48 level in normolipidaemic and hyperlipidaemic subjects has been elegantly reported by Sakai et al. (2003). They investigated the fasting concentration of apo B-48 in normolipidemic (n=335) and hyperlipidemic Japanese (n=253) (Sakai et al., 2003). They reported that apo B-48 concentration strongly correlated with triglyceride concentration (r=0.45) but not with total cholesterol. The distribution of fasting apo B-48 concentration in the nomolipidaemic subjects in this study was not normally distributed suggesting that among these subjects, there was a group of subjects exhibiting hyperchylomicronaemia despite having normal lipid levels. The increased fasting apo B-48 indicates impaired removal of chylomicron/chylomicron remnants from the circulation (Sakai et al., 2003). In the hyperlipidaemic subjects, the distribution of apo B-48 varied widely with a very high concentration of apo B-48 in subjects with type I (LPL deficiency), III (familial dysbetalipoproteinemia) and V hyperlipidaemia (total cholesterol concentration ≥220 mg/dl and triglyceride concentration ≥500 mg/dl). Despite this subjects with type I, III and V hyperlipidaemia exhibited increased fasting triglyceride, the atherogenicity risk differed. Type III hyperlipidaemia is more susceptible to develop atherosclerosis due to the increased number of circulating small particles whereas type I and V hyperlipidaemia exhibited increased number of apo B-48-containing lipoprotein and triglyceride enrichment in chylomicron particles.

In addition to triglyceride, chylomicrons also hold a crucial role in transporting dietary cholesterol together with HDL pathway and the involvement of chylomicrons in reverse cholesterol transport. HDL cholesterol and LDL cholesterol are generally accepted as markers for higher CVD risk. However, findings from previous studies consistently showed weak or no association between apo B-48 with cholesterol profile (total cholesterol, LDL cholesterol and HDL cholesterol). Kinoshita et al (2009) reported that the

association between apo B-48 level and cholesterol profile was weak (r between apo B-48-total cholesterol 0.24 in men and 0.2 in women and r between apo B-48-HDL cholesterol -0.18 in men and -0.15 in women) (Kinoshita et al., 2009). Sato et al (2009) found no significant association between apo B-48 and total cholesterol, LDL and HDL cholesterol concentration. Instead they reported a strong correlation between apo B-48 and triglyceride concentration (r=0.791), RLP-triglyceride (r=0.745), RLP-cholesterol (r=0.768) and remnant lipoprotein cholesterol (r=0.811). (Sato et al., 2009).

Based on the findings of the above-mentioned studies and the atherogenicity evidence of chylomicrons, the utilisation of triglyceride and HDL cholesterol concentration as surrogate markers for a higher risk of CVD may need to be revisited. HDL cholesterol does not represent cholesterol content in the chylomicron remnants and hepatic derived remnants. Instead those cholesterol portions are captured when non-HDL cholesterol or remnant cholesterol concentration is measured. To the best of our knowledge, association between apo B-48 and non-HDL cholesterol or remnant cholesterol concentration has not been explored yet. Similarly, despite having high triglyceride concentration, the atherogenic risk of different type of hyperlipidaemia differs. This underscores the importance to evaluate the utilisation of increased triglyceride concentration as a marker for atherogenicity.

The association between apo B-48 concentration with other parameters has been reported. Kinoshita et al (2009) demonstrated that HOMA-IR level and BMI significantly associated with apo B-48 concentration (Kinoshita et al., 2009). In contrast Hanada et al (2012) found no significant contribution of BMI to the variability of apo B-48 concentration (Hanada et al., 2012). Considering that these studies were conducted in the Japanese population, therefore it is important to also demonstrate whether this observation holds in other populations. Ethnic variability implicates the body fat percentage and muscularity and thus may affect lipid utilisation leading to modification of risk of metabolic disease (Deurenberg, Deurenberg-Yap, & Guricci, 2002; Dulloo, Jacquet, Solinas, Montani, & Schutz, 2010; Norgan, 1994).

1.9 Evidence to date: The effect of dietary fatty acid composition on chylomicron size distribution and postprandial lipemia

1.9.1 Introduction

Atherosclerosis is a multifactorial disease. It is widely accepted that in addition to genetics and environment, dietary composition plays an important role in particular the quantity and type of dietary fat present. Type of dietary fatty acid composition has been recognised to modulate TRL response through their effect on lipid partitioning, chylomicron production and removal, and accessory factors (i.e. insulin). After being absorbed in the enterocytes and released into the circulation, nascent chylomicrons carrying dietary triglyceride and cholesterol are hydrolysed by LPL in extrahepatic capillary beds. This process removes a portion of their lipid content resulting in a reduction in their particle size. In the circulation, chylomicrons also acquired other apolipoproteins including apo E to prepare them for removal from the circulation by the liver. Smaller, partially hydrolysed remnants have been suggested to be more atherogenic than the larger TRL (Evans et al., 1993; Ji et al., 1993; Krauss, 1998). One technique to detect and quantify small sized remnants is by separating remnant lipoproteins according to their density (Cohn et al., 1999). The next sections will discuss methods to assess chylomicron size distribution and evidence to date from human studies investigating the effect of dietary fats on lipoprotein fraction response in postprandial lipaemia.

1.9.2 Methods to assess chylomicron size distribution

In previous studies, investigation on chylomicron during postprandial state was mostly performed in the triglyceride-rich lipoprotein fraction (ρ <1.006 g/mL) by using either density gradient or sequential ultracentrifugation to separate lipoproteins based on differences in density. Since lipoprotein substances have a relatively low density, they will float after following ultracentrifugation in a solvent of greater density. By overlaying plasma with a known density solution and performing ultracentrifugation lipoprotein particles with density less than the density solution will float to the top and can be isolated (Mills et al., 1984).

Classically circulating chylomicrons are commonly identified by using density (ρ) < 1.006 g/mL (Mills et al., 1984; Su, Nzekwu, Cabezas, Redgrave, & Proctor, 2009). To produce various fractions of TRL, speed and time of ultracentrifugation are modified resulting lipoprotein fraction of Sf >400 (mostly contains nascent chylomicrons), Sf 20 – 400 (mostly

VLDL), Sf 12 – 20 (mostly IDL) and Sf 0 – 12 (mostly LDL) (K. G. Jackson & Williams, 2004). However, the use of TRL subfractionation is hampered by the findings that only small-sized chylomicrons can enter the arterial wall therefore determination of chylomicrons in ρ <1.006 g/mL may not capture remnant particles at ρ >1.006 g/mL (Figure 5).

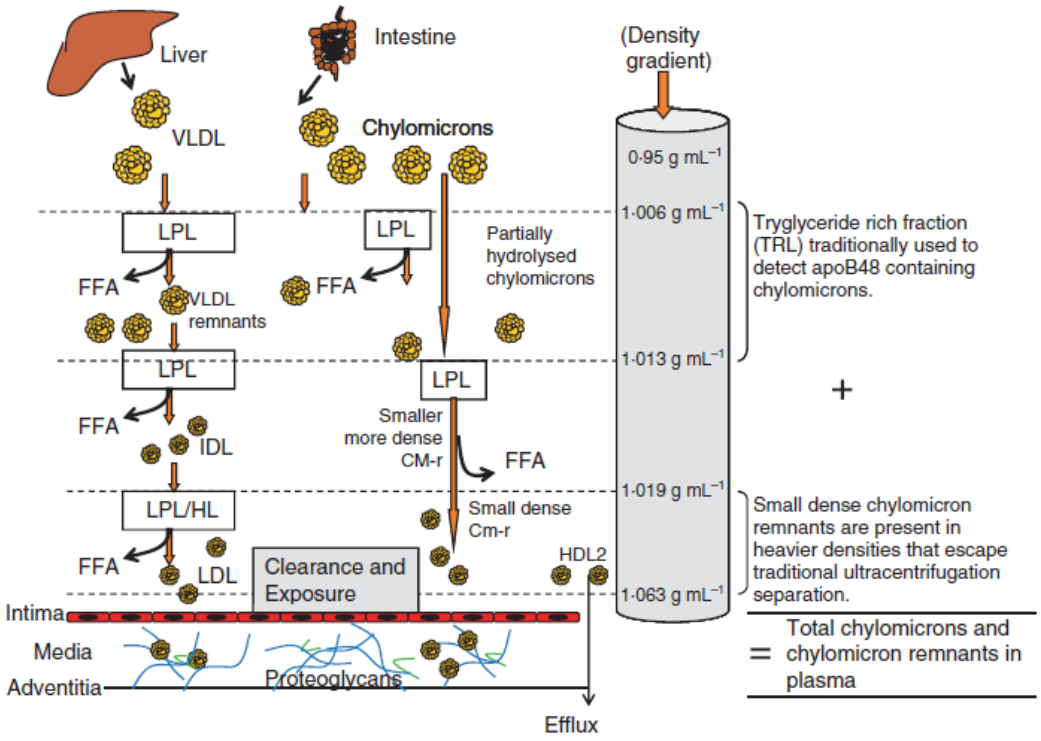

Figure 5. Density distribution of chylomicrons and VLDL in plasma compartment. This figure shows the size distribution of chylomicrons and VLDL from secretion to clearance/vascular exposure. Measuring chylomicrons in TRL fraction may not capture the small dense chylomicron remnants.

Source: Adaptation from Figure 2, Su et al (2009) (Licence number: 3937601209723)

To detect the presence of chylomicrons, there are several direct (sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and enzyme-linked immunoabsorbent assays (ELISA)) and indirect methods (retinyl esters). The approach used in the direct methods is by detecting the presence apo B-48, a unique and non-transferable apolipoprotein contained chylomicron particle (Phillips et al., 1997). SDS-PAGE, a combination of staining and densitometric scanning, is the most widely method used for detecting chylomicron. However the use of this method may be limited by the multi-step process with no general protocol. Moreover the inter-assay variability between studies are large (Su et al., 2009). Immunoblotting and ELISA were developed since monoclonal and polyclonal antiserum to apo B-48 were found (Lorec et al., 2000; Uchida,

Kurano, & Ito, 1998). Immunoblotting has been widely accepted as one of the most sensitive and accurate method in quantifying apo B-48 however the impracticality (multi-step and time consuming) to apply the method on large cohort may limit its usage (Su et al., 2009). ELISA is introduced to generate a high throughput approach. The original procedure of ELISA is a competitive ELISA developed by Lorec et al (2000). In this procedure, the samples are pre-incubated with appropriate detergent and the plate is coated with apo B-48 antiserum. Concentration of apo B-48 is inversely proportional to the amount of apo B-48 antiserum bound on the plate (Lorec et al., 2000). Sakai et al (2003) developed sandwich ELISA. In this method, monoclonal anti apo B-48 antiserum is coated onto the wells of ELISA plate. Following incubation of diluted samples and washing, antigen (apo B-48) binds to the antibody. After the antigens are immobilized, a second monoclonal antibody linked to enzyme binds to the immobilized antigen (biotin-conjugated anti-apo B-48 antibody and peroxidise-conjugated streptavidin). The final step is adding substrate which is converted by the enzyme into a coloured product (Figure 6). Apo B-48 concentration is directly proportional to the amount of apo B-48 bound to the monoclonal antiserum (Sakai et al., 2003). The advantage of ELISA method is the high specificity in detecting apo B-48 and the feasibility to use in large samples. However the influence of storage and preparation of the samples on the exposure of immunogenic sites on apo B-48 need to be considered (K. G. Jackson & Williams, 2004).

In this thesis, a combination of sequential flotation ultracentrifugation and sandwich ELISA developed by Sakai et al (2003) (Sakai et al., 2003) was performed. With this method, the distribution of apo B-48 in lipoprotein fraction can be quantified and, by extension, may capture the apo B-48 in the pro-atherogenic fraction.

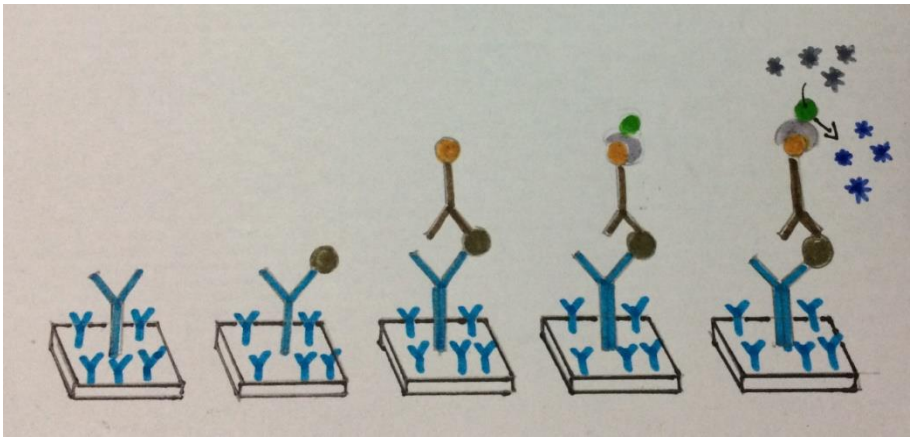

Figure 6. Schematic figure of apo B-48 sandwich ELISA. **Y**: antigen; : antigen; : biotin-conjugated anti-apo B-48 antibody; : peroxidise-conjugated streptavidin; *: substrate.

Source: Adaptation from http://www.elisa-antibody.com/ELISA-Introduction/ELISA-Principle.

1.9.3 Postprandial chylomicron response

The acute and chronic effects of dietary fatty acid composition on TRL postprandial response has been investigated in a number of studies. These are shown in Table 1.2. All these studies compared triglyceride and/or lipoprotein responses to an oral fat challenge of differing fatty acid challenge. The changes in triglyceride, apo B-48 or retinyl ester concentration in a number of lipoprotein fractions ranging from Sf >1000 to Sf 0 – 20 fraction or ρ (density) <1.006 g/mL to ρ =0.019 g/mL were reported in those studies.

In the less dense fraction (Sf >400 or ρ <1.006 g/mL), it is consistently shown that meals rich in MUFA induced increased postprandial response of either triglyceride, apo B-48 or retinyl esters whereas inconsistent findings (greater, similar or smaller compared to MUFA) were observed in SFA and PUFA meal challenge. Acute meal studies in humans and Caco-2-cells, a human colon carcinoma cell line, have shown to induce larger particles size and numbers following exposure/ingestion of oleic acid (K. G. Jackson, D. M. Robertson, B. A. Fielding, K. N. Frayn, & C. M. Williams, 2002a; Van Greevenbroek, Van Meer, Erkelens, & De Bruin, 1996; C. M. Williams et al., 2004). The production of larger particle size and numbers could be due to the modulation of apo B mRNA editing enzyme or MTP expression or activity (Van Greevenbroek, Robertus-Teunissen, Erkelens, & De Bruin, 1998; Van Greevenbroek et al., 1996).

The meal effect on lipoproteins in the VLDL size and density (Sf 60 – 400 and Sf 20 – 60 fractions) was inconsistent. A number of studies have reported increased postprandial triglyceride and/or apo B-48 concentration in VLDL fraction following MUFA and SFA challenge and a decrease postprandial triglyceride response when challenged with PUFA meal however others reported no significant postprandial response between the meals observed (K. G. Jackson, M. D. Robertson, B. A. Fielding, K. N. Frayn, & C. M. Williams, 2002b; Mekki et al., 2002; Mero et al., 1998). The different findings may be contributed to differences in the dose of fat, type and fats used as the comparators, meal size, meal base, the presence of other nutrients and habitual diets used in those studies (Denis Lairon, 2008).

Of particular interest is the postprandial response in the dense fraction (Sf <20). Given the atherogenicity evidence of chylomicron remnants, only a number of studies examined apo B-48 postprandial response in IDL fraction or denser (de Bruin, Brouwer, van

Linde-Sibenius Trip, Jansen, & Erkelens, 1993; Demacker, Reijnen, Katan, Stuyt, & Stalenhoef, 1991; Lozano et al., 2012; Mero et al., 1998), a lipoprotein fraction collected by ultracentrifugation with density of 1.006< ρ <1.019 g/mL or Sf 12 – 20 (Cohn et al., 1999). PUFA meal induced a 43% lower apo B-48 IAUC in the dense fraction in ρ <0.019 g/mL compared to the SFA meal (Demacker et al., 1991), while other reported no significant difference in apo B-48 response in the dense fraction (Mero et al., 1998). De Bruin et al. (1993) demonstrated that the percent increase in postprandial apo B-48 remnant concentration (ρ = 1.006 – 1.019 g/mL) at 2 and 4 hours after the test meal was higher in MUFA meal than in PUFA meal in normolipidemic suggesting remnants rich in PUFA were removed faster from the circulation (de Bruin et al., 1993). The low response of apo B-48 in the dense fraction following PUFA meal could be due to the increase in lipolytic rate by LPL and preferential hepatic uptake by HL of PUFA-enriched chylomicrons (Kathleen M. Botham et al., 1997; Bravo et al., 1995; Rahman et al., 2000). The lower postprandial triglyceride response following PUFA meal can be due to repression of apo C-III gene and induction of LPL expression mediated by PPAR (Kliewer et al., 1997; Staels et al., 1998).

A greater postprandial apo B-48 response in the dense fraction following a test meal challenge has been reported to associate with the existence of CVD (Karpe, Steiner, Uffelman, Olivecrona, & Hamsten, 1994; M. Weintraub et al., 1996). Karpe et al. (1994) reported a strong correlation between postprandial apo B-48 response in Sf 20 – 60 and atherosclerosis progression score following an oral fat tolerance test (Karpe et al., 1994). Weintraub et al (1996) reported a greater retinyl palmitate response in the non-chylomicron fraction (Sf <1000) (M. Weintraub et al., 1996).

Exaggerated postprandial lipaemia also occurs in obesity, type-2 diabetes and metabolic syndrome. Individuals with visceral obesity exhibited a higher secretion rate and a lower catabolic rate of apo B-48 (Annette T. Y. Wong et al., 2014) leading to an exaggerated medium and small TRL postprandial response (Couillard et al., 1998, 1999). The effect of dietary fatty acid composition on postprandial chylomicron response in the dense fraction has been reported by Lozano et al. (2012). They investigated the postprandial response between normolipidemic subjects with high BMI and low BMI following olive oil meal, butter meal and walnut meal (Lozano et al., 2012). They reported that the high BMI group exhibited higher IAUC triglyceride both in large (ρ <1.006 g/mL) and small TRL fraction (ρ =1.006 – 1.019 g/mL) compared to the low BMI group. Olive oil consumption induced a lower apo B-48 IAUC in the small TRL fraction irrespective of the BMI status. This finding was contradictory with the finding by de Bruin et al. (1993)

demonstrating increased postprandial apo B-48 remnant concentrations following a MUFA-rich meal than that of PUFA-rich meal in normolipidemic men (de Bruin et al., 1993). It is possible that genetic variation, such as apo E polymorphism, may play a role in the differences in apo B-48 response (Bergeron & Havel, 1996; Ooi, Watts, Ng, & Barrett, 2015).

Dai et al. (2009) reported that the average triglyceride concentration during postprandial state in Sf 20 – 60 fraction was lower in equidominant meal (a meal with equal proportion of SFA, MUFA and PUFA) and PUFA meal than the MUFA meal indicating that by increasing PUFA ratio may attenuate the atherogenic lipoprotein profile in patients with type 2 diabetes (Dai et al., 2009). In that study, they did not performed apo B-48 determination. The consumption of n-6 PUFA in overweight/obese men was shown to increase the fractional catabolic rate of hepatic lipoprotein remnants (Van Schalkwijk et al., 2014).

Based on the findings in the above-mentioned studies, it appears that dietary fats may exert a differential effect on apo B-48/triglyceride response in the dense fraction and this interaction is implicated by metabolic phenotype. However the evidence are limited and the findings are inconsistent.

1.10 Concluding remarks

A large body of evidence including epidemiological, clinical, animal and cell studies verifies the atherogenicity of chylomicron/chyomicron remnants. However despite this, there is a dearth of studies investigating the metabolic determinants of elevated chylomicron concentrations. Furthermore given the variability in lipid metabolism and body fat distribution in subjects of differing ethnic background it is important to investigate these metabolic determinants in different population groups. In addition, although a number of studies have investigated the effects of dietary fatty acid composition on postprandial lipaemia and atherosclerosis risk, the lack of information about the combined effects of dietary fatty acid composition and exaggerated postprandial lipaemia on chylomicron response in the dense fraction supports the basis of this thesis.

Table 1.1 Human studies examining the association between apo B-48 and a range of metabolic parameters

Authors	Study details	Apo B-48 measured by	Results and comments
(Sakai et al., 2003)	Study: cross-sectional and postprandial Aim: to evaluate the metabolism of chylomicron and chylomicron remnants in normolipidemic and hyperlipidemic subjects. Subjects: 335 normolipidemic Japanese (281 men and 54 women, age 47±7 years) and 239 hyperlipidemic subjects stratified according to type of hyperlipidemia (type I, IIa, IIb, III, IV and V) Primary outcome: concentration of apo B-48 in each group using a novel ELISA system.	Sandwich ELISA	Ao B-48 concentration in normolipidemic subjects: 5.2 ± 3.8 μg/mL. Very high level of apo B-48 level in type I, III and V (7 – 18 higher). Strong association between apo B-48 and TAG (r =0.45). No association between apo B-48 and total cholesterol.
(Kinoshita et al., 2009)	Study: cross-sectional Aim: to evaluate the association between chylomicron remnant (measured as apo B-48) and MetS and to investigate factors regulating apo B-48 levels in fasting plasma. Subjects: 1349 rural Japanese, 528 men, 821 women,	Sandwich ELISA	Strong association between apo B-48 and TAG (r=0.53 in men and r=0.48 in women). Strong association between apo B-48 and HOMA-IR (r=0.51 in men and r=0.43 in women). Weak to moderate correlation between apo B-

Authors	Study details	Apo B-48 measured by	Results and comments
	63±13 years. Primary outcome: relationship of apo B-48 with serum lipids and MetS.		48 and cholesterol profiles. Higher apo B-48 level in subjects with MetS. No information on lipid lowering medication exclusion criteria.
(Hanada et al., 2012)	Study: cross-sectional Aim: to evaluate the performance of chemiluminescent enzyme immunoassay (CLEIA) and examine correlations between serum apoB-48 level and other lipid parameters. Subjects: 6 controls and 273 clinical samples, Japanese. Primary outcome: performance of CLEIA and correlation between apo B-48 and lipid parameters.	CLEIA	High correlation between apo B-48 measured with CLEIA and ELISA. Strong correlation between apo B-48 and RLP chol. Higher apo B-48 level in subjects with MetS.
(Sato et al., 2009)	Study: postprandial study Aim: to characterise lipid profiles in fasting state in order to detect postprandial lipemia.	CLEIA	HTG group has higher concentration of TAG, RemL chol, RLP TAG, RLP C and apo B-48.

Authors	Study details	Apo B-48 measured by	Results and comments
	Subjects:24 normolipidemic subjects, age 22±2 years, male/female: 11/13. Primary outcome: TAG, remnant chol and TAG, apo B-48 concentration fasting and postprandial. HTG group: TAG >150 mg/dL at 2 hour.		

Table 1.2 Effect of different fatty acid composition on postprandial TAG and lipoprotein responses

Authors	Subjects and study design	Lipoprotein fraction	Type and Fats used	Fatty acid composition	Fat	Findings
(Dai et al., 2009)	T2DM subjects	Sf>400, Sf 60-400,	Liquid -butter	Meals: -Equadominant meal	53g	Sf> 400 [TAG] ↓ equidominant vs MUFA. Sf 60 – 400 [TAG] ↓ equidominant vs MUFA.

Authors	Subjects and study design	Lipoprotein fraction	Type and Fats used	Fatty acid composition	Fat	Findings
		Sf 20-60, Sf 12-20	-sunflower oil -olive oil -lard	(SFA:MUFA:PUFA=1:1:1) -MUFA diet (1:1.7:1.2) -PUFA diet (1:1.7:2.3)		Sf 20-60 [TAG] ↓ Equidominant and PUFA vs MUFA. No apo B48 measurement
(K. G. Jackson et al., 2005)	Normolipidemic Crossover design	Sf>400, Sf 60-400, Sf 20-60	Mixed meal -Palm oil -cocoa butter -safflower oil -olive oil	Meals: -mixture Palm oil (29 g)+ cocoa butter (21 g)(26.1 g SFA:19.5 g MUFA: 3.2 g PUFA) /50 g -Safflower oil (7.8 g: 8.2 g: 31.8 g) -Olive oil (7.1 g: 34.9 g: 5.9 g)	50g	Sf > 400 AUC and IAUC [TAG] ↓PUFA vs MUFA and SFA. Sf>400 & Sf 60-400 AUC and IAUC [apo B-48] ↑ MUFA meal.
(K. G. Jackson et al., 2002a)	Normolipidemic Crossover design	Sf>400	Mixed meal -Palm oil -cocoa butter	Test oils: -palm oil (50% SFA:40% MUFA: 10% n-6 PUFA:0% n-3 PUFA)% by wt -safflower oil (11%:15%:74%:0%)	40 g	↑ [apo B-48] at 300 min in olive oil vs other oil meals. ↓response for the ratio of TAG to apo B-48 and less marked postprandial change with

Authors	Subjects and study design	Lipoprotein fraction	Type and Fats used	Fatty acid composition	Fat	Findings
			-safflower oil -olive oil Breakfast and lunch	-fish/safflower oil (19%:20%:39%:22%) -olive oil (17%:72%:11%:0%)		time after olive oil than other oil meals. Meal effect for olive oil after the second meal
(K. G. Jackson et al., 2002b)	Normolipidemic Crossover design	Sf>400, Sf 60-400, Sf 20-60	Mixed meal -palm oil -safflower oil -fish oil -olive oil	Test oils: -palm oil (50% SFA:40% MUFA: 10% n-6 PUFA:0% n-3 PUFA)% by wt -safflower oil (11%:15%:74%:0%) -fish/safflower oil (19%:20%:39%:22%) -olive oil (17%:72%:11%:0%)	40g	↑ Sf 60 – 400 [TAG] and [apo B48] (300 min) vs Sf >400. ↑ Sf 60-400 IAUC apo B48 in palm oil, safflower oil and olive oil vs in Sf >400. ↑ Sf >400 IAUC apo B48 in olive oil vs any of other dietary oils.
(Mekki et al., 2002)	Young normolipidemic Crossover design	Sf>400, Sf 60-400, Sf 20-60	Mixed meal -butter -olive oil	Meals: -Butter (53.7% SFA:26.8% MUFA: 19.5% PUFA)% by wt, 100 mg of chol -Olive oil (24.6%: 51.9%:21.0%), no	40g	Sf> 400 [TAG] ↓ on butter meal. ↑Sf 60 – 400 & Sf 20-60 [TAG] on butter meal.

Authors	Subjects and study design	Lipoprotein fraction	Type and Fats used	Fatty acid composition	Fat	Findings
			-sunflower oil	chol -Sunflower oil (25.5%:27.7%:48.2%), no chol -No fat (47.6%:27.1%:24.4%)		No measurement of apo B-48 concentration
(Mero et al., 1998)	Middle age non obese men Crossover design	Sf>400 Sf 60-400 Sf 20-60 Sf 12-20 Sf 0-12	Mixed meal Soy bean oil, the other not mentioned	Test meals: -mixed meals (64.5% SFA: 30.4% MUFA: 5.1% PUFA) -soybean oil (15.1%:25.9%: 59.0%) -cream (68.5%: 27.6%: 3.9%)	63 g	↔ IAUC TAG between three meals in all fractions. ↔ IAUC apo B48 between 3 FTTs in all fractions.
(Callow, Summers, Bradshaw, & Frayn, 2002)	Normolipidemic Crossover design	d= 1.020-1.063 g/ml	Mixed meal -sunflower oil - sunflower margarine	Meals: -PUFA rich meal (12.6% SFA:33.9% MUFA: 53.5% PUFA) % by weight -SFA meal (65.7%:28.3%:6.0%)	60.8 g 60.1 g 5.2 g	↔ PP TAG and CM between meals No measurement of apo B-48 concentration

Authors	Subjects and study design	Lipoprotein fraction	Type and Fats used	Fatty acid composition	Fat	Findings
			- butter - double cream	-Low fat meal (37.9%:26.9%:35.2%)		
(Pedersen, Marckmann, & Sandström, 1999)	Normolipidemic Crossover design	-d<1.006 ; chylomicron -d<1.006 of cthe infranatant (chylomicron remnant and VLDL) -bottom fraction (LDL + HDL)	Mixed meal -rapeseed oil - sunflower oil - palm oil	-rapeseed oil (7% SFA: 63% MUFA: 30% PUFA) % by mol -sunflower oil (11.1%:21.4%:67.5%) - palm oil (39.2%:46.8%:14.1%)	17.4g breakfast 63.6 g for lunch	↔ in lipoprotein and apolipoprotein responses after 3 types of meals were consumed. No apo B48 measurement
(Sauvant et al., 2003)	Normolipidemic Crossover design	Sf>1000	Mixed meal -butter	Meals: -Butter (66.6% SFA) by weight	40g	↑chylomicron retinyl palmitate/oleate and stearate concentration whatever the test meals were.

Authors	Subjects and study design	Lipoprotein fraction	Type and Fats used	Fatty acid composition	Fat	Findings
			-Olive oil -Sunflower oil	-Olive oil (75.5% MUFA) -Sunflower oil (67% PUFA) -Fat free meal (1.3 g fat)		↑ retinyl palmitate AUC for LCFA (olive and sunflower). Peaks: 3 hours for fat free meal, 2-3 hours for butter, and 4 hours for olive and sunflower oil. No apo B48 measurement
(de Bruin et al., 1993)	Normolipidemic men Crossover design	-Sf>1000 -Sf<1000 -Sf 4 - 400 (1.006<d<1.019)	Liquid -soybean oil -olive oil	Meals: -Soybean oil emulsion (20% wt:vol) -Olive oil emulsion (17% Olive oil, 3% soybean oil) -cream (in other study)	25 g/m^2 (total dose: 49±3 g fat)	↑AUC and peak [RP] in olive oil emulsion (Sf>1000 fraction) vs soybean Earlier RP peak in 1.006<d<1.019 in olive oil emulsion ↑ [apo B-48] at 2 and 4 hours after olive oil fat loads in 1.006<d<1.019 remnant fraction
(Thomsen et al., 1999)	Normolipidemic	-Sf>1000	Mixed meal -unsalted	Meals: -Control meal (energy-free soup plus	80 g	↑TAG IAUC butter meal vs olive oil meal in chylomicron fraction and non-chylomicron

Authors	Subjects and study design	Lipoprotein fraction	Type and Fats used	Fatty acid composition	Fat	Findings
	Crossover design	-Sf≤1000	butter -olive oil	50g carbohydrate as white bread) -Butter meal (control meal + 100 g butter / 72% SFA) -Olive oil meal (control meal + 80 g olive oil / 74% MUFA)		fraction ↑[RP] butter meal vs others in the CM-rich fraction No apo B48 measurement
(Thomsen, Storm, Holst, & Hermansen, 2003)	Type 2 diabetes subjects Crossover design	-Sf>1000 -Sf≤1000	Mixed meal -unsalted butter -olive oil	Meals: -Control meal (energy-free soup plus 50g carbohydrate as white bread) -Butter meal (control meal + 100 g butter / 72% SFA) -Olive oil meal (control meal + 80 g olive oil / 74% MUFA)	80 g	↑TAG IAUC butter meal vs olive oil and control meals. ↔ TAG IAUC between olive oil and butter meal in chylomicron rich and chylomicron poor fractions. No apo B48 measurement
(Sakr et al.,	Normolipidemic	-Sf>400	Liquid	Test meals:	60 g	↔ IAUC for TAG vegetable oils in chylomicron rich fraction and chylomicron

Authors	Subjects and study design	Lipoprotein fraction	Type and Fats used	Fatty acid composition	Fat	Findings
1997)	Crossover design	-Sf 20-400	-sunflower oil -oleic sunflower oil -soya bean oil -grapeseed oil -beef tallow	-sunflower oil meal (12.4% SFA: 20.3% MUFA: 67.3% PUFA) % by wt -oleic sunflower oil meal (8.8%:73.1%:18.3%) -mixed oil (11.3%: 39.0%: 49.7%), containing 420 g/kg sunflower oil, 150 g/kg soyabean oil and 50 g/kg grapeseed oil -beef tallow (49.7%: 45.1%: 5.2%)		poor fraction ↓ IAUC TAG after beef tallow vs other meals in chylomicron rich fraction At 4 hour after the ingestion, chylomicron diameter larger to smaller: Sunflower oil, mixed oil, oleic sunflower oil and beef tallow; ↔ at after 8 hours No apo B48 measurement
(Tholstrup, Sandström, Bysted, & Hølmer, 2001)	Young normolipidemic men Crossover design	-chylomicron Sf>400 -VLDL and chylomicron remnants d<1.006	Mixed meal -Tristearin -Tripalmitin -Trimyristin - high oleic acid	Test meals: -high in palmitic and myristic acids -high in palmitic acid -high in stearic acid -high in oleic acid	41-47% (1g/kg BW)	↔ [TAG] between fractions at 4 h. LCSFA stearic and palmitic acids resulted in ↓in plasma total and chylomicron TAG after 4 h than did the other test fats and the return to post-absorptive values was slower.

Authors	Subjects and study design	Lipoprotein fraction	Type and Fats used	Fatty acid composition	Fat	Findings
		kg/L -LDL and HDL d>1.006 kg/L.	sunflower oil - high linoleic sunflower oil -Trans 18:1 (hydrogenation of high oleic acid sunflower oil)	-high in linolenic acid -high in trans fatty acid See table 1 in the article		No apo B48 measurement
(Zampelas et al., 1995) (Kim G. Jackson et al., 1999)	Young normolipidemic men	d<1.006 g/ml	Mixed food -coconut oil -palm oil -olive oil -sunflower oil	Test meals: -low MUFA meal (17.2% SFA: 12% MUFA: 5% PUFA)% by energy -moderate MUFA (12.3%: 17.1%: 4.6%) -high MUFA (5.4%: 24.1%: 4.6%)	40 g	↔ IAUC TAG between three meals. A biphasic TAG response was observed in the high MUFA meal with TAG peaks at 180 and 360 minutes.

Authors	Subjects and study design	Lipoprotein fraction	Type and Fats used	Fatty acid composition	Fat	Findings
(Roche, Zampelas, Jackson, Williams, & Gibney, 1998)	Young normolipidemic men	d<1.006 g/ml	Mixed meal Not mentioned	Test meals: -low MUFA diet (12% en MUFA) (20g SFA: 14 g MUFA: 5.7 g PUFA) /40 g -moderate MUFA diet (17% MUFA)(14.6g: 20 g: 5.4 g) -high MUFA diet (24% MUFA)(6.3 g: 28.3 g: 5.4 g)	40 g	↔ AUC, maximum postprandial plasma concentrations and time to maximum postprandial plasma TAG and apo B48 concentration between the three test meals. ↔ [Apo B48]between meals in TRL and TPR fractions
(Demacker et al., 1991)	Young normolipidemic Crossover design	d< 1.019 g/ml	-butter fat -sunflower oil -margarine (n-6 PUFA)	Test oil: -SFA meal (21% SFA:12% MUFA: 3% PUFA) -PUFA meal (10%: 9%: 18%)	90 g /day	↓AUC apo B-48 d<1.019 PUFA meal vs the SFA meal ↔ AUC TAG d<1.019 PUFA meal vs the SFA meal
(Zampelas et al., 1998)	Young normolipidemic men	d<1.006 g/ml	Mixed meal -coconut oil -Palm oil	Test meals: -SFA meal (oil blend= coconut oil 30 g/100 g: palm oil 30 g/ 100 g: olive oil	40 g	↔AUC TAG for both meals between Southern and Northern Europeans ↔AUC apo B48 between meals in Southern

Authors	Subjects and study design	Lipoprotein fraction	Type and Fats used	Fatty acid composition	Fat	Findings
			-Olive oil -Sunflower oil	30 g/ 100 g: sunflower oil 10 g/ 100 g) -MUFA meal (olive oil 100 g/100 g)		and Northern Europeans
(Karupaiah, Tan, Chinna, & Sundram, 2011)	Young normolipidemic Crossover design	d<1.006 g/ml	Mixed meal -coconut oil -corn oil -cocoa butter Palm olein	Test meal: -lauric + myristic acid (LM) -palmitic acid (POL) -stearic acid (STE)	31%	↑AUC TAG STE vs POL and LM No apo B48 measurement
(Lozano et al., 2012)	High and low BMI young men Crossover design	-large TRL (chylomicron fraction of TRL/ d<1.006) -small TRL (non-chylomicron	-extra virgin olive oil -butter -walnuts	Meals: - Olive oil meal (SFA:MUFA:PUFA =22% : 38%: 4% and 0.7% a-linolenic acid) - butter meal (35%:22%:4% and 0.7%) - walnuts meal (20%: 24%:16% and 4%)	60%	↑ I AUC for small TRL-TG in HW subjects in butter or walnut meals There are BMI effect and diet effect on small TRL-TG. Meal effect on large and small TRL apo B48.

Authors	Subjects and study design	Lipoprotein fraction	Type and Fats used	Fatty acid composition	Fat	Findings
		/ d<1.019)				

CHAPTER 2

Chapter 2

The association of fasting chylomicron concentration with a range of anthropometric and lipid parameters: A cross-sectional study

Content of this chapter is covered by the manuscript:

Irawati D, Mamo JCL, Dhaliwal SS, Soares MJ, Slivkoff-Clark KM, James AP (2016). Plasma triglyceride and an inverse association with high-density lipoprotein cholesterol are poor surrogate markers of pro-atherogenic chylomicron remnant homeostasis in subjects with the metabolic syndrome. *Lipid and Health Disease*, In press.

Thesis objective addressed in this chapter:

Objective 1: To explore the associations of apo B-48 concentration with metabolic and anthropometric parameters

2.1 Synopsis

2.1.1 Background

Triglyceride level has been widely accepted as an independent risk factor for CVD (Hokanson & Austin, 1996). However it has been the subject of debate whether plasma triglyceride or the lipoprotein particles independently contributed to the higher risk of CVD. In the fasting state, there is a switch of function in delivering triglyceride to LPL expressing tissues from mainly intestinal TRL (chylomicrons) in the postprandial state to hepatic TRL. Thus circulating triglyceride concentration during fasting is attributed mainly from hepatic TRLs. In fasting, the production of chylomicron in enterocytes still continue however with practically no dietary fat absorbed, the released chylomicrons would be triglyceride-poor (C. Xiao et al., 2011).

There is growing evidence that fasting apo B-48 concentration, a unique marker of chylomicron particle number, is associated with the existence of atherosclerosis and coronary artery disease (Alipour, Valdivielso et al. 2012; Masuda, Sugimoto et al. 2012; Tomkin and Owens 2012). The finding of apo B-48 particles in rabbit and human atherosclerotic tissue (Nakano et al., 2008; Pal et al., 2003; Proctor & Mamo, 2003) indicates that post-hydrolysed chylomicron remnants are an important key player in the development of atherosclerosis. To enter and be retained in the subendothelial tissue, lipoproteins need to be small in size and selectively bind to the subendothelial matrix. A line of evidence has demonstrated that apo B-48 particles from circulation was detected in the dense lipoprotein fraction (Campos et al., 2005; Karpe, 2012), these particles have the ability to penetrate the arterial wall to preferentially become trapped within the subendothelial space (Mamo and Wheeler 1994; Proctor and Mamo 1996), and induce inflammation (Yu and Mamo 2000; Batt, Avella et al. 2004).

Elevated plasma triglyceride is generally utilized as an indicator for exaggerated postprandial lipaemia. In combination with low HDL cholesterol concentration, triglyceride serves as a marker for dyslipidaemia and thus cardiovascular risk. However plasma triglyceride concentration principally reflects the lipolytic cascade instead of an indication of post-hydrolysed atherogenic remnants accumulation. Indeed association between triglyceride and apo B-48 concentration has been reported (Kinoshita et al., 2009; Sakai et al., 2003; Sato et al., 2009) but these findings may be specific to a Japanese population.

The primary objective of this study was to explore the association between plasma apo B-48 concentration with metabolic and anthropometric parameters in Australian population. To investigate whether the association was reliant on an altered phenotype of MetS instead of the individual cardiometabolic parameters, we grouped the subjects based

on the presence of MetS. This also allowed the evaluation of whether the associations hold in individuals with MetS (Dekker et al., 2005; Galassi, Reynolds, & He, 2006; J. Wang et al., 2007). Previous studies have reported a greater fasting concentration of apo B-48 in individuals with MetS (Hanada et al., 2012; Kinoshita et al., 2009; Masuda et al., 2014).

2.1.2 Methods in brief

Two hundred and fifteen Australian Caucasian participants were randomly selected from a larger cross-sectional study conducted at Curtin University in 2011. Participants receiving lipid lowering medication were excluded. This study has been approved by Curtin University Human Research Ethics Committee (HR97/2011). The primary outcome of this study is the association between apo B-48 concentration with a range of measures of metabolic and anthropometric parameters. MetS criteria was based on Alberti et al (2009) (Alberti et al., 2009).

2.1.3 Results in brief

Apo B-48 concentration was strongly correlated with triglyceride concentration (manuscript Table 3). Moderate association between apo B-48 with non-HDL cholesterol was observed. Apo B-48 was inversely correlated with HDL cholesterol concentration. Whereas apo B-48 was weakly associated with total cholesterol and a range of anthropometric parameters (waist circumference and WHR). We also found that apo B-48 concentration was not associated with either insulin concentration or HOMA-IR level. When we adjusted for age and gender, the contribution of those parameters on the variability of apo B-48 concentration was low (Table 2.1 in the Supplementary result section). Subjects with MetS exhibited an enhanced association of apo B-48 with triglyceride, total, non-HDL and LDL cholesterol than those without MetS (manuscript Table 4).

2.1.4 Discussion and conclusion in brief

The findings of this study confirm previous reports that fasting apo B-48 concentration is strongly associated with triglyceride concentration. In subjects with MetS, who have higher basal apo B-48 concentration, the slope of the association of Z scores for apo B-48 with triglyceride and non-HDL cholesterol and the coefficient of variation were increased compared to the non-MetS subjects. However these measures give no indication of chylomicron remnant homeostasis, which are our primary concern given the evidence of their atherogenicity. The reduced HDL cholesterol concentration observed in MetS was not associated with the increase in apo B-48 concentration, suggesting that factors

independent of chylomicron metabolism such as insulin resistance are possible (Rashid, Watanabe, Sakaue, & Lewis, 2003).

Elevated triglyceride concentration in MetS suggests a lipolytic alteration that is augmented by insulin resistance (Annuzzi et al., 2008). Altered lipolysis leads to an increased residency time for chylomicrons and VLDL in the circulation and thus favours increased exchange of lipid between lipoproteins to result in accumulation of cholesterol-rich remnants (Charles & Kane, 2012; Mann, Yen, Grant, & Bihain, 1991).

Our findings suggest the importance of assessing TRL remnants. Defect in the metabolism and clearance of triglyceride rich lipoproteins are likely contributing to increased cardiovascular risk.

2.2 Manuscript

Lipids in Health and Disease

Plasma triglyceride and an inverse association with high-density lipoprotein cholesterol are poor surrogate markers of pro-atherogenic chylomicron remnant homeostasis in subjects with the metabolic syndrome.

--Manuscript Draft--

Manuscript Number:	LHAD-D-16-00229R1
Full Title:	Plasma triglyceride and an inverse association with high-density lipoprotein cholesterol are poor surrogate markers of pro-atherogenic chylomicron remnant homeostasis in subjects with the metabolic syndrome.
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Abstract:	Aim: Subjects with metabolic syndrome (MetS) exhibit impaired lipoprotein metabolism and have an increased risk of cardiovascular disease. Although the risk is attributed primarily to the risk associated with individual components, it is also likely affected by other associated metabolic defects. Remnants of postprandial lipoproteins show potent atherogenicity in cell and animal models of insulin resistance and in pre-diabetic subjects with postprandial dyslipidemia. However, few studies have considered regulation of chylomicron remnant homeostasis in MetS per se. This study measured the plasma concentration in Caucasian men and women of small dense chylomicrons following fasting and explored associations with metabolic and anthropometric measures. Methods: A total of 215 Australian Caucasian participants (median age 62 years) were investigated. Of them, 40 participants were classified as having MetS. Apolipoprotein (apo) B-48, an exclusive marker of chylomicrons, metabolic markers and anthropometric measures were determined following an overnight fast. Results: The fasting apo B-48 concentration was 40% higher in subjects with MetS than those without MetS. In all subjects, triglyceride (r=0.445, P<0.0005), non-HDL cholesterol (r=0.28, P<0.0005) and HDL cholesterol concentration (r=-0.272, P<0.0005) were weakly associated with apo B-48 concentration. In subjects with MetS, the association of apo B-48 with triglyceride and non-HDL cholesterol was enhanced, but neither were robust markers of elevated apo B-48 in MetS (r= 0.618 and r= 0.595 respectively). There was no association between apo B-48 and HDL cholesterol in subjects with MetS. Conclusion: This study demonstrates a substantial accumulation of pro-atherogenic remnants in subjects with MetS. We have shown that in a Caucasian cohort, the fasting plasma concentration of triglyceride or HDL/non-HDL cholesterol serves as poor surrogate markers of atherogenic chylomicron remnants. These findings suggest that subjects with MetS exhibit a chronic defect in chylomicron metabolism that is likely to contribute to their increased CV risk.
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Response to Reviewers:	Response to reviewers: Reviewer 1 We thank reviewer 1 for their review and kind words. Minor revisions: 1. Page 2, line 70: "aberration/s" should be "aberrations". This has now been corrected as requested. 2. Table 1: "Systolic" and "Diastolic" would be better to "Systolic BP" and "Diastolic BP". This has now been corrected as requested. 3. Page 8, line 303: "Mets" should be "MetS" in the same manner as before. This has now been corrected as requested. Kind regards Tony James

Plasma triglyceride and an inverse association with high density lipoprotein cholesterol are poor surrogate markers of pro-atherogenic chylomicron remnant homeostasis in subjects with the metabolic syndrome.

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Abstract

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Methods: A total of 215 Australian Caucasian participants (median age 62 years) were investigated. Of them, 40 participants were classified as having MetS. Apolipoprotein (apo) B-48, an exclusive marker of chylomicrons, metabolic markers and anthropometric measures were determined following an overnight fast.

Results: The fasting apo B-48 concentration was 40% higher in subjects with MetS than those without MetS. In all subjects, triglyceride ($r=0.445$, $P<0.0005$), non-HDL cholesterol ($r=0.28$, $P<0.0005$) and HDL cholesterol concentration ($r=-0.272$, $P<0.0005$) were weakly associated with apo B-48 concentration. In subjects with MetS, the association of apo B-48 with triglyceride and non-HDL cholesterol was enhanced, but neither were robust markers of elevated apo B-48 in MetS ($r= 0.618$ and $r= 0.595$ respectively). There was no association between apo B-48 and HDL cholesterol in subjects with MetS.

Conclusion: This study demonstrates a substantial accumulation of pro-atherogenic remnants in subjects with MetS. We have shown that in a Caucasian cohort, the fasting plasma concentration of triglyceride or HDL/non-HDL cholesterol serves as poor surrogate markers of atherogenic chylomicron remnants. These findings suggest that subjects with MetS exhibit a chronic defect in chylomicron metabolism that is likely to contribute to their increased CV risk.

Keywords: metabolic syndrome, chylomicron, apolipoprotein B-48.

Introduction

It is recognised that patients with metabolic syndrome (MetS) are at risk developing CVD [1-3]. Increased plasma triglyceride and decreased HDL cholesterol are important components of MetS syndrome, however subjects with MetS are otherwise typically normocholesterolemic.

A substantial body of evidence supports the contention that hydrolysed remnants of postprandial chylomicrons are highly atherogenic [4, 5]. Apo B-48 concentration, a unique marker of chylomicron particles, is associated with atherosclerosis and coronary artery disease [4, 6, 7]. The presence of apo B-48 containing particles in rabbit and human atherosclerotic tissue [8-10] suggests that post-hydrolyzed chylomicron remnants are an important risk for development of atheroma. It was previously thought that chylomicrons are synthesized and released by the intestine only after dietary fat ingestion and that they are primarily found in lipid-rich lipoprotein fractions. However, we and others have reported the presence of chylomicron particles in more dense, lipid-poor lipoprotein fractions in the absence of dietary fat availability [11-13]. Small sized (remnant) chylomicron particles

are able to penetrate the arterial wall and get trapped within the subendothelial space [14, 15]. This can cause lipid accumulation in macrophages, a hallmark of early atherogenesis, without prior chylomicron remnant oxidative modification (Yu and Mamo 2000; Batt, Avella et al. 2004).

Subjects with MetS have been found to have greater fasting concentrations of apo B-48 [16-18]. However these observations have been made in a Japanese population and hence have been grouped using the Japanese Metabolic Syndrome criteria [7, 16, 18-20]. It is therefore important to examine the association between apo B-48 and metabolic parameters in other populations.

The metabolic aberrations leading to an accumulation of chylomicron remnants in MetS are unclear, but studies in animal models and insulin resistant subjects suggest exaggerated rates of constituent biosynthesis as a consequence of intestinal hypertrophy [21]; decreased lipolysis because of reduced expression of endothelial lipoprotein lipase [22] and thereafter, lower rates of receptor mediated endocytosis post-hydrolysis contribute that collectively results in increased vascular exposure.

Plasma triglyceride is commonly utilized as a marker of postprandial lipaemia and indeed fasting levels have been reported to correlate with fasting apo B-48 concentration [16, 19, 20]. However, plasma triglyceride principally reflects the lipolytic cascade and is by extension less indicative of accumulation of post-hydrolyzed atherogenic remnants. Greater plasma abundance of triglyceride is commonly associated with reduced plasma concentration of HDL cholesterol. The latter reflects principally, decreased shedding of surface phospholipids and cholesterol from triglyceride rich lipoproteins during lipolysis and genesis of nascent discoidal HDL lipoproteins. In MetS, heightened plasma triglyceride and/or low HDL cholesterol may be associated with an accumulation in plasma of atherogenic chylomicron remnants, however neither measure are equivocal surrogate markers of remnant homeostasis per se.

Apo B-48 is produced exclusively by absorptive cells of the small intestine and is an obligatory moiety of nascent chylomicrons. In normolipidemic subjects with coronary artery disease and in subjects with familial hypercholesterolemia, apo B-48 is markedly elevated despite otherwise normal levels of plasma triglyceride [23, 24]. The plasma abundance of small dense chylomicrons in Caucasian subjects with MetS has not been previously reported. Moreover, altered chylomicron homeostasis in MetS may be associated with one or more of the MetS components, however this has not been directly considered. This study reports on associations of fasting apo B-48 with metabolic and anthropometric measures in Caucasian men and women.

Methods

Subjects

Two hundred and fifteen Australians with European ancestry were sampled randomly from a larger cross sectional study investigating the relationship between serum vitamin D, bioactive calcium, parathyroid hormone homeostasis and cognitive performance conducted at our institute in 2011 [25]. An additional exclusion criteria was used for the present study to exclude subjects on lipid lowering medication. MetS criteria was based on the new harmonising criteria of Alberti et al (2009) [26]. All participants have given written informed consent prior participating in the study. The study has been

approved by the Curtin University Human Research Ethics Committee (HR97/2011) and was performed in accordance to Declaration of Helsinki.

Anthropometric measurements

After having fasted for at least 8 hours, prior to fasting blood collection, a range of anthropometric measurements were performed. Body weight was measured after voiding, with participants wearing limited clothing on a digital balance and recorded to the nearest 100 g. Standing height was measured using a portable stadiometer fixed to the wall, without shoes and recorded to the nearest 0.1 cm. Waist circumference and ratio was measured and calculated as described by Norton and Olds (2000) [27]. Sagittal abdominal diameter (SAD) was measured by using an abdominal calliper while the participants were in a standing position. The SAD was measured as the distance between the largest point of abdomen and the back at the end of normal expiration. Blood pressure will be assessed using a semi-automatic (arm cuff) blood pressure monitor (Omron, Vernon Hills, IL) in supine position. All measurements were done by a certified anthropometrist.

Lipid, insulin and glucose assays

Fasting serum samples were collected by low-speed centrifugation of serum separator Vacutainer™ tubes (Becton Dickinson, Franklin Lakes, NJ, USA) that were left to clot for 30 minutes. All samples were then either analysed immediately or stored at -80°C prior to analysis. Fasting serum triglyceride, cholesterol and glucose were determined by Pathwest Laboratories using their routine automated procedures on an Architect c1600 analyser. Serum triglyceride, total cholesterol, HDL cholesterol and glucose concentration were measured by using specific enzyme-based colorimetric reagents (Abbott Diagnostics, Abbott Laboratories, Abbott Park, USA; CV <2%). LDL cholesterol was estimated by using a modified version of Friedewald formula [28]. Non-HDL cholesterol concentration was calculated as the difference between serum total cholesterol and HDL cholesterol concentration. Insulin level was determined by using the Mercodia insulin ELISA kit (Mercodia AB, Uppsala, Sweden) according to the manufacturer's instructions. Insulin resistance was assessed by calculation of HOMA-IR score [29].

Apo B-48 determination

Chylomicron concentration was measured by determining apo B-48 concentration. Apo B-48 is the major structural protein for chylomicron assembly and it is not interchangeable [30]. Apo B-48 concentration was measured by using a commercial sandwich ELISA method using a monoclonal antibody raised against the C-terminal region of apo B-48 (Shibayagi Human apo B-48 ELISA Kit, Ishihara, Shibukawa, Japan) according to the manufacturer's instructions. The details of the validation method refers to Kinoshita et al [31].

Statistical analysis

All data were analysed using SPSS version 21 (SPSS Inc., Chicago, USA). The results are all presented in the tables as mean values ± standard error (SE) for normally distributed data and median (25^{th} and 75^{th} interquartile range) if the data was skewed. Data that were not normally distributed were natural log transformed when necessary. Statistical significance was assessed using Independent-samples T test, Mann-Whitney test (between 2 groups) and ANOVA (3 groups or more). Post-hoc comparison between each group was assessed using Bonferroni test. To assess the significant difference in apo B-48 between the MetS and no-MetS group, GLM univariate were used. Spearman correlations were

computed to assess the association between parameters. The strength of association between apo B-48 and a range of anthropometric and metabolic determinants adjusted for age and gender were calculated by using Z score derived regression coefficient between apo B-48 and other variables. Regression coefficient denotes the standard deviation change in apoB-48, as a result of 1 standard deviation change in the variable. A p value < 0.05 was considered significant.

Results

Subject characteristics

The subject characteristics are shown in Table 1. The median age of the whole subjects was 62 years. Subjects with MetS were older and had higher anthropometric measures related to overweight (BMI, waist circumference, WHR and SAD) and were more likely to be dyslipidemic (high triglyceride and/or low HDL cholesterol). Subjects with MetS exhibited a higher non-HDL cholesterol concentration than those without MetS, but no significant differences in total cholesterol or LDL cholesterol concentration were observed in the MetS group. They were also more insulin resistant than those without MetS group.

Apo B-48, anthropometric and lipid parameters in MetS

The median serum apo B-48 concentration for the whole group was 4.8 μg/mL (3.4 – 7.9). Subjects with MetS exhibited a 40% greater apo B-48 concentration than those without MetS (Table 1; P<0.01). The difference between groups remained significant following adjustment for age and gender (Table 2). In subjects not classified as having MetS, the concentration of apo B-48 did not appear to be affected by the presence of 0, 1 or 2 MetS components.

The association between fasting apo B-48 and the range of outcome measures was examined in all subjects and also after grouping based on the presence of MetS. When examined on the whole group, apo B-48 concentration was found to correlate with triglyceride (r=0.445, p<0.0005), non-HDL (r=0.28, p<0.0005) and HDL cholesterol concentrations (r=-0.272, p<0.0005) (Table 3). Weak correlations were observed between apo B-48 concentration both LDL cholesterol (r=0.191, p=0.005) and total cholesterol (r=0.14, p=0.042). Apo B-48 concentration was also weakly associated with waist circumference and WHR (r=0.162, P=0.017 and r=0.178, P=0.009). However no association was observed between apo B-48 and either insulin concentration, or HOMA-IR level in all subjects.

When grouped based on MetS status, subjects with MetS exhibited an enhanced association of apo B-48 with triglyceride, total- (r=0.618, p<0.0005), non-HDL (r=0.595, p<0.0005) and LDL cholesterol (r=0.576, p<0.0005) than those without MetS (Table 2). However a negative association with HDL cholesterol concentration and apo B-48 was only observed in subjects who did not exhibit MetS. Waist circumference was inversely correlated with apo B-48 in the MetS group.

Since apo B-48 concentration was associated with age, we then assessed the association with correcting for age and gender (Table 4). We observed a minor enhancement in the association between apo B-48 and triglyceride concentration in both groups. The magnitude of association between apo B-48 and several cholesterol profiles (total cholesterol, non-HDL and LDL cholesterol) was greater in the MetS group compared to the no MetS group. However the contribution of those variables on the variability of apo B-48 was less than 40%. We also observed that in the MetS group, apo B-48 was inversely associated with BMI and insulin. To assess the contribution of the lipid variables

strongly associated with apo B-48 concentration, we combined the triglyceride and non-HDL cholesterol in a model adjusted for age and gender. The contribution of these variables to apo B-48 variability was 23.3% (R^2=0.233) in subjects without MetS and 45.1% (R^2=0.451) in those with MetS.

Discussion

In this study, we have examined the importance of chylomicron remnants, as measured by apo B-48, in subjects with and without MetS. We report that subjects with MetS had approximately 40% higher fasting concentration of apo B-48 than those without MetS. The association between the fasting concentration of chylomicron remnants and a range of anthropometric and lipid measures was also examined in subjects with and without MetS. In the whole group apo B-48 concentration correlated with fasting triglyceride concentration and cholesterol profile. In subjects with MetS these correlations increased in strength but as only 45% of the variability in apo B-48 was predicted by the fasting concentration of triglyceride and non-HDL cholesterol, we conclude that these are not robust markers of chylomicron remnant homeostasis. This conclusion suggests that there are a significant proportion of subjects with MetS that have high apo B-48, but normal triglyceride and cholesterol profile; and hence the importance of apo B-48 determination in subjects with MetS.

Our observation that subjects with MetS exhibit a 40% higher fasting concentration of apo B-48 compared to those without suggests an impairment in chylomicron metabolism in these subjects. Our findings confirm and extend those previously reported in a Japanese population where it was reported that subjects with MetS had on average a 34% or 42% higher fasting apo B-48 concentration (in males and females respectively) compared to control subjects without MetS [16]. Interestingly we also observed that elevated apo B-48 concentration was only observed in subjects who exhibited three or greater MetS components (and hence exhibited MetS) suggesting that impaired chylomicron metabolism is reliant on the altered phenotype of MetS rather than simply being a consequence of its individual parameters. Chylomicron metabolism may be impaired in subjects with MetS at the level of production, lipolysis and/or clearance. Increased production of apo B-48 particles via elevated availability of substrate in enterocytes (i.e. due to increased expression of NPC1L1), increased MTP activity, decreased LPL activity and reduced LDL receptor and LDL receptor protein activity have been proposed as a possible mechanism of the altered chylomicron metabolism [32, 33]. In subjects with MetS, hepatic overproduction of VLDL has been reported [34, 35]. Alternatively, exaggerated secretion of chylomicron particles in MetS may be indicative of intestinal hypertrophy, however this cannot be determined in this cross sectional study.

In subjects without MetS, there was a relatively weak correlation of triglyceride with apo B-48, contrasting the result observed in the MetS group. Since triglyceride concentration in plasma is mainly contributed by the triglyceride associated with VLDL particles, the association between apo B-48 and triglyceride concentration suggests a common metabolic defect that is more pronounced in those with MetS, leading to accumulation of these triglyceride-rich lipoproteins. In order to standardise for the magnitude of these parameters we also examined the association between the Z scores of apo B-48 and anthropometric and lipid/metabolic determinants. This allowed us to compare the slope of these relationships when standardised for one standard deviation change in each parameter, and also to correct for age and gender. However the slope of the association of Z-scores for most of these parameters was remarkably low, and although triglyceride had the highest slope for association with apo B-48 in the no MetS group, it had a low coefficient of variation suggesting that

triglyceride is a poor surrogate marker of chylomicron remnant homeostasis. In subjects with MetS, the slope of the association of Z scores for apo B-48 with triglyceride and non-HDL cholesterol and the coefficient of variation were increased, nonetheless these measures were still not predictive of chylomicron remnant homeostasis.

In subjects with MetS the elevated triglyceride concentration is suggestive of a lipolytic defect. Although not directly measured in this study, subjects with MetS may have decreased expression of LPL as a consequence of insulin resistance [22]. A defect in lipolysis would be expected to promote an increased residency time for TRLs including chylomicron particles and favour increased lipid exchange promote accumulation of TRL remnants [36, 37]. In the plasma compartment, remodelling of lipoproteins involves the exchange of cholesteryl ester and triglyceride between triglyceride-rich (chylomicron and hepatic derived) lipoproteins and cholesterol-rich lipoproteins (LDL and HDL particles). Although we have observed an increased circulating concentration of chylomicron remnants in subjects with MetS this change was not associated with a decrease in HDL cholesterol concentrations. It seems that other factors independent to chylomicron metabolism such as HDL particle instability due to insulin resistance may implicate the low concentration of HDL cholesterol in MetS [38].

Clearance of chylomicron remnants post hydrolysis is a highly efficient process that occurs principally via internalization by LDL receptor and LDL receptor-related protein 1 after remnants were bound to the HSPG of the surface of hepatocytes facilitated by apo E [39]. Insulin resistance has been reported to associate with diminished remnant clearance by impairing the HSPG structure via modulation of hepatic SULF2 expression [40] and by altering LDL receptor via PCSK9 [41]. If accumulation of chylomicron remnants in MetS occurs principally as a consequence of impaired lipoprotein binding and/or depressed receptor expression, then by extension one would anticipate a strong association with LDL cholesterol. The findings show that in subjects with MetS, the association of LDL cholesterol with apo B-48 was strengthened in comparison to control subjects with presumably adequate expression of apo B/E receptor. The findings suggest that decreased expression may have contributed to an accumulation of chylomicron remnants in MetS subjects. However as only one-third of the variability in apo B-48 can be explained by LDL cholesterol, this does not support the contention that LDL cholesterol serves as a good surrogate marker of pro-atherogenic remnant homeostasis in plasma, and that MetS is not associated with a major defect in high affinity clearance pathways. The latter may be indicative of the findings that chylomicron remnants require greater clusters for apo B/E receptors on the plasma membrane for internalization, whereas LDL particles interact with singular receptors. A modest reduction in receptor expression will therefore have a greater effect on clearance of chylomicron remnants in comparison to LDL particles. By extension, LDL cholesterol would be a poor predictor of remnant homeostasis, particularly if receptor expression is attenuated.

Non HDL cholesterol is defined as the cholesterol associated with LDL, VLDL remnants, Lp(a), and chylomicron remnants and as these represent all the lipoproteins currently believed to contribute to atheroma development [42] this measure has been suggested to be included in routine lipid profiles. In the present study, in addition to triglyceride, non-HDL cholesterol concentration was also the major lipid contributor to the variation in apo B-48 concentration in subjects with MetS as shown by the regression analysis. Our observation of a strong association between concentrations of apo B-48 and non-HDL cholesterol in subjects with MetS is indicative of the importance chylomicron remnants to the variability of this measure, however nonetheless, as with triglyceride concentrations,

there still remains a significant proportion of individuals who exhibit increased chylomicronemia in the absence of elevated triglyceride or non-HDL cholesterol concentrations. Furthermore the concentration of HDL cholesterol was not associated with apo B-48 in either group after adjustment for age and gender. These findings clearly demonstrate that HDL cholesterol is not a suitable surrogate to consider chylomicron remnant homeostasis in Caucasian subjects with MetS.

Substantial evidence has indicated that an altered chylomicron metabolism might relate to insulin resistance [43, 44]. Our results show no significant associations between apo B-48 and either insulin concentrations or HOMA-IR score, however after adjustment for age and gender there was a moderate association between insulin and concentrations, but only in the MetS group. Interestingly Kinoshita et al (2009) reported a strong association between apo B-48 and HOMA-IR score in both males and females [16]. However differences in the ethnicity and subject characteristics may explain this discrepancy. Interestingly we found that in individuals with MetS, the association between apo B-48 concentration and some anthropometric measures was inversely correlated. This could be due to the diminished LPL activity in adipose tissue caused by insulin resistance [45]. It seems that apo B-48 concentration in circulation is associated with the inability to store fat in adipose tissue in individual with MetS. However this needs to be clarified in a larger sample size.

The results from this study have extended the findings from previous studies [16, 19, 20] that have reported the association between apo B-48 and a range of lipid and anthropometric measures in Japanese population. Our observation that apo B-48 concentration correlated with triglyceride and non-HDL cholesterol in subjects with MetS may partly explain the increased atherogenic risk in MetS, however the importance of an increased concentration of apo B-48 for atherogenic risk in this group remains. The present study has several limitations. Firstly, the cross-sectional nature of the design limits the causative interpretation between variables. Second, the older age range of our subjects may not represent the variation of apo B-48 level in general population. Third, the relatively small number of subjects with MetS may overestimate the association.

Conclusion

In summary, our investigation confirms previous reports that fasting apo B-48 concentration is strongly associated with triglyceride concentration. Although we observed that this association was stronger in subjects with MetS there still remains a significant proportion of the variability in apo B-48 that is not predicted by components of MetS. These observations are suggestive of the importance of assessing TRL remnants, in particular chylomicron remnants in subjects with MetS. These findings suggest that subjects with MetS exhibit a lipoprotein metabolic defect the manifestation of which affects the metabolism and clearance of triglyceride-rich lipoproteins likely contributing to increased cardiovascular risk.

Abbreviations

Apo, apolipoprotein; BMI, body mass index; CETP, cholesteryl ester transfer protein; CRP, C-reactive protein; CVD, cardiovascular disease; HDL, high density lipoprotein; HL, hepatic lipase; HOMA-IR, homeostasis model of insulin resistance; HSPG, heparan sulphate proteoglycan; LDL, low density lipoprotein; Lp(a), lipoprotein(a); LPL, lipoprotein lipase; MetS, Metabolic syndrome; MTP, microsomal transfer protein; NPC1L1, Niemann-Pick C1-like 1; PCSK9, proprotein convertase subtilisin/kexin type 9; SAD, sagittal abdominal diameter; SE, standard error; SULF2, sulfate glucosamine 6-O-

endosulfatase-2; TRL, triglyceride-rich lipoprotein; VLDL, very low density lipoprotein; WHR, waist hip ratio.

Declarations

Ethics approval and consent to participate

All participants have given and signed written informed consent prior participating in the study. The study has been approved by the Curtin University Human Research Ethics Committee (HR97/2011) and was performed in accordance to Declaration of Helsinki.

Availability of data and material

The raw data will not be shared as participant's did not consent to this.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and researched the literature: DI, AJ and JM. Performed the experiments: DI and AJ. Analysed the data: DI assisted by SD, AJ, JM and MS. Wrote the manuscript: DI, AJ and JM. Reviewed and critically revised the manuscript for important intellectual content: JM, MS, KC, SD and AJ. All authors approved the final version of the manuscript.

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Table 1. Subject characteristics

Characteristics	Total (n=215)	No-MetS (n=175)	MetS (n=40)
Gender (Male/Female)	68/147	46/129	22/18
Age (years)	62 (56 – 69)	62 (56 – 68)	66 (59 – 71)*
BMI (kg/m^2)	26.3 ± 0.3	25.5 ± 0.3	29.9 ± 0.6**
Waist circumference (cm)	87.1 ± 0.9	84.1 ± 1	100.3 ± 1.4**

WHR	0.83 ± 0.01	0.81 ± 0.01	0.92 ± 0.01**
SAD (cm)	24.7 ± 0.3	23.6 ± 0.3	29.3 ± 0.5**
Systolic BP (mmHg)	141 ± 1	139 ± 2	150 ± 3**
Diastolic BP (mmHg)	83 ± 1	82 ± 1	85 ± 2
Triglyceride (mmol/L)	1.1 ± 0.03	1 ± 0.03	1.7 ± 0.1**
Total chol (mmol/L)	5.4 ± 0.1	5.4 ± 0.1	5.5 ± 0.2
LDL chol (mmol/L)	3.3 ± 0.1	3.3 ± 0.1	3.5 ± 0.1
HDL chol (mmol/L)	1.5 ± 0.03	1.6 ± 0.03	1.2 ± 0.03**
Non-HDL chol (mmol/L)	3.9 ± 0.1	3.8 ± 0.1	4.3 ± 0.2**
Glucose (mmol/L)	5.1 (4.8 – 5.4)	5.1 (4.8 – 5.3)	5.6 (5.3 – 5.9)**
Insulin (mIU/L)	6 ± 0.2	5.4 ± 0.2	8.8 ± 0.5**
HOMA-IR	1.4 ± 0.1	1.2 ± 0.04	2.2 ± 0.1**
CRP (µg/mL)	2.9 ± 0.2	2.6 ± 0.2	4.3 ± 0.4**
ApoB-48 (µg/mL)	4.8 (3.4 – 7.9)	4.6 (3.3 – 7.5)	6.8 (3.9 – 11.4)**

Metabolic parameters		Number of subjects	
BMI ≥ 25 kg/m2	129 (60%)	91 (52%)	38 (95%)
Large waist	104 (48%)	68 (39%)	36 (90%)
Triglyceride ≥ 1.7 mmol/L	31 (14%)	6 (3%)	25 (63%)
Low HDL chol	30 (14%)	10 (5%)	20 (50%)
Hypertension	147 (69%)	111 (63%)	36 (92%)
Fasting glucose ≥ 5.6 mmol/L	32 (15%)	11 (6%)	21 (53%)

Mean ± SE for normally distributed variables or median (25^{th} and 75^{th} interquartile range) for non-normally distributed variables; *p < 0.05, **p < 0.01; MetS compared to no-MetS group. P values between groups were based on independent t-test and Mann-Whitney test for data that is normally and non-normally distributed data respectively.

Table 2. Concentration of apo B-48 according to MetS and in different cumulative number of MetS components

Presence of MetS	N	Geometric mean (95% CI) (µg/mL)
No-Mets	175	4.7 (4.3 – 5.2)
MetS	40	6.6 (5.4 – 8.0)
		P=0.001[a]
MetS components		
0	37	4.7 (3.8 – 5.9)[b]
1	69	4.7 (4 – 5.6) [b]
2	69	4.7 (4.1 – 5.3) [b]
≥3	40	6.6 (5.4 – 8) [c]
		P=0.004[a]

[a] p value after adjusting for age and gender; different letters (b and c) indicate significant differences.

Table 3. Non-parametric correlations between anthropometric and lipid/metabolic determinants with Apo B-48 (without adjustment)

Variables	Correlation coefficient (p value)		
	Total (n=215)	No Mets (n=175)	MetS (N=40)
Triglyceride*	**0.445** ***(p<0.0005)***	**0.362** ***(p<0.0005)***	**0.618** ***(p<0.0005)***
HDL cholesterol*	**-0.272** ***(p<0.0005)***	**-0.278** ***(p<0.0005)***	0.254 *(p=0.113)*

Age	**-0.230** ***(p=0.001)***	**-0.262** ***(p<0.0005)***	**-0.32** ***(p=0.044)***
Non-HDL cholesterol	**0.28** ***(p<0.0005)***	**0.157** ***(p=0.038)***	**0.595** ***(p<0.0005)***
WHR	**0.178** ***(p=0.009)***	0.147 *(p=0.053)*	-0.26 *(p=0.105)*
Waist circumference*	**0.162** ***(p=0.017)***	0.123 *(p=0.106)*	**-0.317** ***(p=0.046)***
Systolic*	-0.053 *(p=0.441)*	-0.106 *(p=0.163)*	-0.026 *(p=0.875)*
LDL cholesterol	**0.191** ***(p=0.005)***	0.094 *(p=0.214)*	**0.533** ***(p<0.0005)***
BMI	0.105 *(p=0.123)*	0.078 *(p=0.308)*	-0.27 *(p=0.092)*
CRP	-0.006 *(p=0.929)*	-0.073 *(p=0.335)*	-0.005 *(p=0.977)*
Insulin	0.113 *(p=0.1)*	0.069 *(p=0.365)*	-0.158 *(p=0.331)*
Glucose*	-0.001 *(p=0.984)*	-0.067 *(p=0.381)*	-0.222 *(p=0.168)*
SAD	0.118 *(p=0.089)*	0.06 *(p=0.439)*	-0.193 *(p=0.247)*
HOMA-IR	0.108 *(p=0.113)*	0.058 *(p=0.446)*	-0.201 *(p=0.214)*
Diastolic*	0.024 *(p=0.729)*	0.023 *(p=0.761)*	-0.066 *(p=0.691)*
Cholesterol	**0.139** ***(p=0.042)***	0.021 *(p=0.78)*	**0.576** ***(p<0.0005)***

The variables were presented in order based on the strength of association (strong to weak) in the no MetS group. Cells represent Spearman's Rank correlation (p-values). Significant correlations are in bold font. *Variable use to determine the presence of MetS.

Table 4. The association between Z score of apo B-48 and anthropometric and lipid/metabolic determinants, after adjustment for age and gender

Variables	Regression coefficient based on Z-scores	
	No Mets	MetS
Triglyceride*	**0.534** ***(p<0.0005, R^2=0.233)***	**0.634** ***(p=0.001, R^2=0.436)***
Non-HDL cholesterol	**0.204** ***(p=0.007, R^2=0.135)***	**0.546** ***(p=0.008, R^2=0.378)***
Glucose*	-0.152 *(p=0.208, R^2=0.105)*	-0.057 *(p=0.668, R^2=0.245)*
LDL cholesterol	**0.149** ***(p=0.047, R^2=0.118)***	**0.458** ***(p=0.039, R^2=0.327)***
Cholesterol	0.144 *(p=0.059, R^2=0.116)*	**0.547** ***(p=0.009, R^2=0.374)***
HDL cholesterol*	-0.132 *(p=0.082, R^2=0.113)*	0.154 *(p=0.693, R^2=0.244)*
Waist circumference*	0.077 *(p=0.336, R^2=0.102)*	-0.453 *(p=0.092, R^2=0.299)*
WHR	0.073 *(p=0.453, R^2=0.1)*	0.077 *(p=0.791, R^2=0.242)*
Systolic*	-0.064 *(p=0.371, R^2=0.101)*	0.06 *(p=0.782, R^2=0.278)*
SAD	0.058 *(p=0.468, R^2=0.097)*	-0.420 *(p=0.092, R^2=0.317)*
BMI	0.053 *(p=0.443, R^2=0.1)*	**-0.603** ***(p=0.002, R^2=0.422)***
CRP	-0.039 *(p=0.57, R^2=0.099)*	-0.153 *(p=0.375, R^2=0.257)*
Insulin	0.014 *(p=0.896, R^2=0.097)*	**-0.428** ***(p=0.049, R^2=0.319)***
Diastolic*	0.013 *(p=0.846, R^2=0.097)*	-0.165 *(p=0.377, R^2=0.293)*
HOMA-IR	-0.005 *(p=0.972, R^2=0.097)*	-0.429 *(p=0.069, R^2=0.308)*

Regression coefficient represents the standard deviation change in apoB-48, as a result of 1 standard deviation change in the variable.The variables were presented in orders based on the strength of association (strong to weak) in the no MetS group. Cells represent: slope estimate (p-value for estimate, R^2 for model after adjustment). Significant associations are in bold font. *Variable use to determine the presence of MetS.

Supplementary result

Table 2.1 The association between Z score of apo B-48 and anthropometric and lipid/metabolic determinants, after adjustment for age and sex

Variables	Total
Triglyceride*	**0.58** ***(p<0.0005, R^2=0.304)***
Non-HDL cholesterol	**0.353** ***(p<0.0005, R^2=0.167)***
Glucose*	0.009 *(p=0.911, R^2=0.058)*
LDL cholesterol	**0.269** ***(p<0.0005, R^2=0.117)***
Cholesterol	**0.275** ***(p<0.0005, R^2=0.118)***
HDL cholesterol*	**-0.232** ***(p=0.002, R^2=0.1)***
Waist circumference*	**0.17** ***(p=0.023, R^2=0.08)***
WHR	**0.242** ***(p=0.007, R^2=0.09)***
Systolic*	-0.034 *(p=0.641, R^2=0.058)*
SAD	0.142 *(p=0.053, R^2=0.073)*
BMI	0.082 *(p=0.211, R^2=0.064)*
CRP	0.038 *(p=0.556, R^2=0.059)*
Insulin	0.083 *(p=0.362, R^2=0.061)*
Diastolic*	0.001 *(p=0.997, R^2=0.057)*
HOMA-IR	0.094 *(p=0.391, R^2=0.061)*

Regression coefficient represents the standard deviation change in apoB-48, as a result of 1 standard deviation change in the variable. Cells represent: slope estimate (p-value for estimate, R^2 for model after adjustment). Significant associations are in bold font. *Variable use to determine the presence of MetS.

CHAPTER 3

Chapter 3

Chylomicron size distribution in subjects with and without hypertriglyceridemia

Content of this chapter is covered by the article:

Irawati D, Mamo JCL, Soares MJ, Slivkoff-Clark KM, James AP (2015). Hypertriglyceridemic subjects exhibit an accumulation of small dense chylomicron particles in the fasting state. *Atherosclerosis,* 243(1), 236-241.

Thesis objective addressed in this chapter:

Objective 2: To examine the abundance and size distribution of chylomicron particles in individuals with and without hypertriglyceridemia

3.1 Synopsis

3.1.1 Background

In the previous chapter, we reported a strong association between fasting apo B-48 and triglyceride concentration (see chapter 2) and concluded that the mechanisms of increased CVD risk induced by fasting hypertriglyceridemia remain unresolved. It is suggested that the increased triglyceride level is associated with elevated cholesterol-rich remnant lipoproteins, of which one of the contributors is the cholesterol-rich chylomicron remnants.

A high fasting concentration of apo B-48 in the circulation is associated with the prevalence of coronary artery disease (Alipour et al., 2012; Masuda et al., 2012). According to the Response-to-Retention theory, atherogenesis is initiated by trapping of cholesterol-rich lipoproteins in the subendothelial matrix (K. J. Williams & Tabas, 1995; K. J. Williams & Tabas, 2005). This suggests that particle size and binding selectivity are critical in remnant delivery and retention in the intima. The ability of chylomicron remnants to enter intimal tissue (Mamo, Proctor, & Smith, 1998), bind artery wall proteoglycans (Flood et al., 2002; Proctor et al., 2002) and induce foam cell formation through lipid enrichment in macrophages is well reported (Napolitano et al., 2001; Proctor & Mamo, 1996; Proctor et al., 2002; van Lenten et al., 1985). Furthermore, apo B-48 has been found in human atherosclerotic plaque (Nakano et al., 2008; Pal et al., 2003).

Conventionally, the quantification of chylomicrons is performed in TRL fraction (Sf >400). This fraction consists of the large lipoproteins rather than the small, dense remnants that have a greater potential to deliver cholesterol to the subendothelial matrix and contribute to atherosclerotic plaque. This chapter investigates the concentration and size distribution of chylomicron in subjects with and without hypertriglyceridemia.

3.1.2 Methods in brief

Twelve hypertriglyceridemic and twelve age and gender matched normotriglyceridemic subjects were sampled from a larger cross-sectional study performed at Curtin University in 2011. The subjects were selected randomly by an external statistician. The criteria for hypertriglyceridemia followed the NCEP-ATP III criteria (fasting triglyceride ≥ 1.7 mmol/L). The primary outcome of this study was the apo B-48 concentration in the following lipoprotein fractions: Sf >400, Sf 20-400 and Sf <20. Lipoprotein fractionation is detailed in the Supplementary Method section within this chapter. Ethics was approved by Curtin University Human Research Ethics Committee (HR97/2011).

3.1.3 Results in brief

Hypertriglyceridemic subjects exhibited a two-fold higher concentration of serum apo B-48-containing lipoproteins compared to those with a normal triglyceride concentration (manuscript Figure 1A). In both groups the majority of apo B-48 was present in the Sf <20 fraction but subjects with hypertriglyceridemia had a greater accumulation of these dense particles. The apo B-48 concentrations of the less dense fractions (Sf >400 and Sf 20-400) were also higher in the hypertriglyceridemic subjects.

In subjects with a higher serum triglyceride concentration the triglyceride particles were distributed evenly in each fraction (manuscript Figure 2A). Conversely in normotriglyceridemic subjects the majority of triglyceride particles were in the dense Sf <20 fraction. The estimated lipoprotein size as indicated by triglyceride to apo B ratio was greater in the less dense fraction (Sf >400 and Sf 20-400) in subjects with hypertriglyceridemia (manuscript Figure 3). No difference in serum cholesterol and NEFA concentration between groups was observed.

3.1.4 Discussion and conclusion in brief

Normocholesterolemic hypertriglyceridemic subjects in our study exhibited an accumulation of small-sized chylomicron particles in fasting plasma. We suggest that this accumulation is due to defective catabolism and/or clearance of those particles rather than as a result of increased chylomicron production. Further, we postulate that insulin resistance of the hypertriglyceridemic subjects in this study contributed to the defective catabolism and uptake of remnants by the liver. Insulin resistance triggers hepatic expression of SULF2 that leads to augmented HSPG de-sulfation in the liver (Chen et al., 2009; K. J. Williams & Chen, 2010) which is a suggested mechanism that could account for the higher accumulation of dense apo-B48-containing particles.

Even though the fasting triglyceride concentration and the triglyceride to apo B ratio in the less dense fraction were higher in hypertriglyceridemic subjects, there was no difference in serum NEFA concentration between the groups. Chylomicrons secretion from enterocytes relies on the availability of an intracellular lipid supply and insufficient lipidation of primordial particles will result in degradation of apo B-48. In fasting, primordial chylomicrons are lipidated from intracellular lipid supply which may derived from NEFA taken up from the circulation (Shojaee-Moradie et al., 2013). Since the hypertriglyceridemic subjects had a similar fasting NEFA concentration to normotriglyceridemic subjects, this supports our suggestion that the alteration in chylomicron metabolism was not at secretion, but more likely to be at the lipolysis or remnant clearance stages.

Our findings further substantiate the role of chylomicron remnants in mechanisms leading to CVD in hypertriglyceridemic subjects, in particular defective lipoprotein clearance that results in particle accumulation.

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Hypertriglyceridemic subjects exhibit an accumulation of small dense chylomicron particles in the fasting state

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ABSTRACT

Aim: Normocholesterolemic subjects with elevated fasting plasma triglycerides are at increased risk of atherosclerosis through mechanisms that are not yet delineated. We hypothesized that elevated plasma triglyceride is associated with increased vascular exposure to pro-atherogenic lipoprotein remnants. To test this hypothesis, the abundance, and size distribution of chylomicron particles were determined in individuals with and without hypertriglyceridemia.

Methods: Twelve hypertriglyceridemic subjects (HTG group, triglyceride concentration ≥1.7 mmol/L) and twelve normotriglyceridemic subjects (NTG group) matched for age and gender were studied. The distribution of chylomicron particles was assessed by determining the fasting concentration of apo B-48 in serum lipoprotein fractions with Svedberg flotation rates of (Sf) > 400, Sf 20–400 and Sf < 20.

Results: The total concentration of apo B-48 in subjects with HTG was almost twice that observed in NTG controls with ~80% of the increase residing in the Sf < 20 fraction (HTG: 8.7 ± 1.0 μg/mL vs NTG: 5.0 ± 0.6 μg/mL; P = 0.016). Significantly greater concentrations of apo B-48 were also observed in the less dense Sf 20–400 (HTG: 1.1 ± 0.2 μg/mL vs NTG: 0.4 ± 0.07 μg/mL; P < 0.001) and the Sf > 400 (HTG: 1.1 ± 0.3 μg/mL vs NTG: 0.3 ± 0.04 μg/mL; P < 0.001) fractions. An accumulation of triglyceride was also observed across all lipoprotein fractions in HTG subjects compared to NTG (Sf 400 & Sf 20–400: P < 0.001 and Sf < 20: P = 0.013).

Conclusion: Normocholesterolemic, moderately hypertriglyceridemic subjects are at increased atherogenic risk due to greater apo B-48 concentration in the small, dense lipoprotein fraction.

1. Introduction

The presence of fasting hypertriglyceridemia is associated with an increased risk of cardiovascular disease but the mechanism/s for this association have not been completely elucidated [1]. One possibility is that high triglyceride levels are a marker for elevated cholesterol-rich remnant lipoproteins which, once taken up into the intima, may lead to atherosclerotic plaques. One of the components of cholesterol-rich remnant lipoproteins includes chylomicron remnant particles. There is strong evidence demonstrating that fasting serum apo B-48 concentration, a measure of chylomicron particle number, is significantly associated with the existence of atherosclerosis and coronary artery disease [2,3]. Studies investigating human atherosclerotic plaque have shown that apo B-48 containing lipoproteins were found in atherosclerotic tissue together with apo B-100 [4,5].

Circulating chylomicron particles exist in a range of particle sizes, and hence density, depending on the extent of associated lipid [6,7]. The less dense, lipid rich particles would be those typically isolated in a Sf > 400 lipoprotein fraction. Following lipolysis by lipoprotein lipase (LPL) smaller and denser particles are formed, eventually resulting in the production of chylomicron remnants. Lipid depleted remnant particles are taken up by the

Abbreviations: Apo, apolipoprotein; BMI, body mass index; CETP, cholesteryl ester transfer protein; CRP, C-reactive protein; HDL, high density lipoprotein; HOMA-IR, homeostasis model of insulin resistance; HTG, hypertriglyceridemic; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LPL, lipoprotein lipase; NCEP-ATP, National Cholesterol Education Program Adult Treatment Panel; NEFA, non-esterified fatty acid; NTG, normotriglyceridemic; SULF2, heparan sulfate glucosamine-6-O-endosulfatase-2; TRL, triglyceride rich lipoprotein; Sf, Svedberg flotation rate; VLDL, very low density lipoprotein.

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liver by high affinity processes, however prior to clearance there is the potential for these particles to penetrate the artery wall. Chylomicron remnants can penetrate the artery walls as effectively as LDL [8] and importantly carry 5 to 20 times as much cholesterol per particle compared to LDL [9]. Furthermore chylomicron and other remnant particles can be taken up by scavenger receptors on macrophages that are present in the sub-endothelial space and result in an accumulation of triglyceride and cholesterol within the macrophage and in doing so can trigger foam cell formation [10–13]. Incomplete efflux of lipoproteins, and the high affinity of chylomicron remnants to the arterial wall proteoglycans are likely to be the key steps in the initiation of atherosclerosis [12,14]. As the influx of lipoproteins into sub-endothelial tissue is continuous, remnant particle size is an important factor in arterial uptake [15,16].

Chylomicron particles are continuously secreted from intestinal epithelial cells even after extended periods of fasting. Indeed in free living subjects we have determined that constitutive biosynthesis of chylomicrons over a 14 h period contributed 75% of the total flux highlighting the importance of basal rate chylomicron production [17]. In viscerally obese subjects with hypertriglyceridemia we have reported increased fasting concentrations of chylomicron particles (apo B-48); an observation which may, at least in part, be due to an observed reduction in LDL-receptor expression on mononuclear cells in these subjects [18]. This defect would result in an accumulation of pro-atherogenic chylomicron remnant particles. Conventionally chylomicron particles have been measured in a chylomicron rich lipoprotein fraction (Sf > 400) however this does not give any insight into the levels of the small, more dense pro-atherogenic chylomicron remnants. Rather assessment of apo B-48 concentrations in different density lipoprotein fractions gives an indication of the size of chylomicron particles present and allows determination of whether there is a build-up of chylomicron remnant particles. In the present study, we have aimed to investigate whether part of the increased atherogenic risk associated with hypertriglyceridemia is due to an increased accumulation of denser pro-atherogenic remnant chylomicron particles.

2. Methods

2.1. Subjects

The participants (n = 24) in this study were sampled from a larger cross sectional study conducted at our institute in 2011 [19]. The inclusion and exclusion criteria were previously described [19] and the study has been approved by the Curtin University Human Research Ethics Committee (HR97/2011). Twelve hypertriglyceridemic subjects and twelve normotriglyceridemic age and gender matched subjects were selected. Hypertriglyceridemia was based on NCEP-ATP III criteria being triglyceride concentration ≥1.7 mmol/L [20]. The number of subjects required to demonstrate statistical significance in apo B-48 concentration in lipoprotein fractions was 11 in each group. This was calculated using the method described by Altman (1980) and was based on a power of 80%, $\alpha = 0.05$ and standardised difference 1.3 [21,22]. Participants were advised to have a low fat dinner meal prior the study day and to come to the clinical room after a 12-h overnight fast.

2.2. Anthropometric measurements

Following the overnight fast, weight, height and waist circumference were measured. Body weight was measured after voiding, with participants wearing light clothing on a digital balance and recorded to the nearest 100 g. Standing height was measured using a portable stadiometer, without shoes and recorded to the nearest 0.1 cm. Waist circumference was measured as described by Norton and Olds (2000) [23]. Blood pressure was assessed using an automatic (arm cuff) blood pressure monitor (Omron, Vernon Hills, IL) in supine position. All measurements were done by an experienced anthropometrist. Following this fasting venous blood samples were collected into serum separator Vacutainer™ tubes (Becton Dickinson, Franklin Lakes, NJ, USA). The samples were allowed to clot for 30 min and then underwent low-speed centrifugation. Serum was isolated and stored at −80 °C.

2.3. Lipoprotein fractionation

Lipoprotein fractions of Sf > 400 and Sf 20–400 were collected by sequential floatation ultracentrifugation of serum samples using a method based on that originally described by Havel et al. (1955) and modified from that described by James and Mamo (2012) [24,25]. Briefly, a salt solution with density of 1.006 g/mL as described in [24] was added to 1 mL serum in 5 mL thin walled tubes (Beckman Coulter, NSW Australia). Chylomicron rich fractions (Sf > 400) were collected following brief centrifugation at 40,000 rpm (30 min, 20 °C, AH-650 swing-out rotor (Thermo Scientific, Scoresby, Australia)) of serum overlayed with density solution (1.006 g/mL). The fraction was collected by aspirating approximately 1 mL of the top layer. The tube was then topped up with density solution (1.006 g/mL) and spun again under the same conditions for 20.5 h to float up the VLDL fraction (Sf 20–400). This lipoprotein fraction was also collected by careful aspiration (approximately 1 mL). The remaining infranatant was regarded as the IDL fraction (Sf < 20). The volume of all supernatants and infranatant collected were measured using a glass bore syringe (Hamilton, Nevada, USA). These volumes were used in the apo B-48, apo B-100, triglyceride, and cholesterol concentration calculation. Lipoprotein fractions were then divided into aliquots and immediately stored at −80 °C before further analysis.

2.4. Lipid, insulin and glucose assays

Fasting serum triglyceride, cholesterol, glucose and CRP were determined by Pathwest Laboratories using their routine automated procedures on an Architect c1600 analyser. Serum triglyceride, total cholesterol, HDL cholesterol and glucose concentration were measured by using specific enzyme-based colorimetric reagents (Abbott Diagnostics, Abbott Laboratories, Abbott Park, USA; CV <2%). LDL cholesterol was estimated by using a modified version of the Friedewald formula [26]. Triglyceride and cholesterol concentrations in the lipoprotein fractions were measured with an enzyme-based colorimetric reagent (Trace) supplied by Thermo Fisher Scientific Inc., Australia. Insulin level was determined by using the Mercodia insulin ELISA kit (Mercodia AB, Uppsala, Sweden; CV <5%) according to the manufacturer's instructions. NEFA concentration was measured by using the ACS-ACOD method (WAKO NEFA C, Osaka, Japan; CV <3%) according to the manufacturer's instructions. All samples were analyzed in duplicate and within a single batch. The level of insulin resistance was assessed by HOMA-IR [27].

2.5. Apo B-48 and apo B-100 determination

Chylomicron concentration was measured by determining apo B-48 concentration using a commercial sandwich ELISA method using a monoclonal antibody raised against the C-terminal region of apo B-48 (Shibayagi Human apo B-48 ELISA Kit, Ishihara, Shibukawa, Japan; CV <3%). This method has been validated [28]. The determination of apo B-48 in lipoprotein fractions (Sf > 400 and Sf 20–400) were analyzed according to the manufacturer's

instructions but with the following modified dilution factors. Samples from Sf > 400 were diluted 1 in 50 and samples from Sf 20–400 were 1 in 100. These dilutions were done to ensure that the apo B-48 concentrations in those fractions remained within the standard curve. The concentrations of apo B-48 determined in lipoprotein fractions were standardised by converting to an amount arising from 1 mL of serum. The concentration of apo B-48 in Sf < 20 was calculated from the difference between the concentration of apo B-48 in serum and in the less dense fractions (Sf > 400 and Sf 20–400). Apo B-100 concentration was measured by using a commercial sandwich ELISA method (Mabtech Human Apo B ELISA ^{PRO} Kit, Sweden; CV = 2%). The determination of apo B-100 in serum and lipoprotein fractions (Sf > 400 and Sf 20–400) were analyzed according to the manufacturer's instructions. Similar to the apo B-48 determination, we modified the dilution factors as follow: serum samples were diluted 1 in 8000, samples from Sf > 400 were 1 in 10 and samples from Sf 20–400 were 1 in 200. The number of triglyceride molecules per apo B particle (triglyceride to apo B ratio) was calculated by dividing the molarity of triglyceride by the molarity of apo B (apo B-48 and apo B-100 concentrations combined) in each lipoprotein fraction. The molarity of apo B-48 and apo B-100 was calculated by dividing the concentration of apolipoprotein in each fraction by their respective molecular mass (apo B-48 = 264 kD and apo B-100 = 549 kD).

2.6. Statistical analysis

All data were analyzed using SPSS version 21 (SPSS Inc., Chicago, USA). The results are presented in the tables as mean values ± standard error (SE) or median with 25th and 75th interquartile range. Distribution of apo B-48, triglyceride and cholesterol particles in lipoprotein fractions are presented as a percentage of the serum concentration. Datasets which had a skewed distribution were natural log transformed. Between group comparisons were performed by independent Student t-Test. The effect of significant parameters between groups on apo B-48 concentration in lipoprotein fractions was assessed using univariate general linear modeling with the fasting level of that parameter as a covariate. A p value < 0.05 was considered significant.

3. Results

3.1. Subject characteristics

The subject characteristics are shown in Table 1. There was no significant difference in age, BMI and waist circumference between NTG and HTG groups. The blood pressure was higher in the HTG group compared to those in NTG group (P < 0.05). Serum total cholesterol, HDL cholesterol, LDL cholesterol, NEFA, glucose and CRP concentration were not significantly different between groups. However, insulin concentration and HOMA-IR score were almost twice as high in the HTG group (P < 0.05).

3.2. Apo B-48 and apo B-100 concentrations in lipoprotein fractions

The concentration of chylomicron particles was greatest in the more dense Sf < 20 fraction in both groups with this fraction containing approximately 80% of the total apo B48. The remainder being essentially equally distributed between the less dense Sf 20–400 and Sf > 400 fractions. The total (serum) apo B-48 concentration was approximately two-fold greater in the HTG compared to the NTG group (10.9 ± 1.3 vs 5.7 ± 0.7 μg/mL; P = 0.002) (Fig. 1A). Although the concentration of apo B48 in each lipoprotein fraction was significantly higher in the HTG group compared to the NTG group (Sf > 400: 1.1 ± 0.3 vs 0.3 ± 0.04 μg/mL; P < 0.001; and Sf 20–400: 1.1 ± 0.2 vs 0.4 ± 0.07 μg/mL; P < 0.001) (Fig 1A), the majority of the elevated apo B48 in the HTG group resided in the Sf < 20 fraction (HTG: 8.7 ± 1.0 μg/mL vs NTG: 5.0 ± 0.6 μg/mL in NTG; P = 0.016). The HTG group also exhibited a greater concentration of apo B-100 compared to the NTG group in both serum (857.0 ± 30.8 vs 771.8 ± 26.5 μg/mL; P < 0.05) (Fig. 1B) and in the Sf 20–400 fraction (17.8 ± 1.2 vs 12.8 ± 1.3 μg/mL; P = 0.01) however concentrations in the other fractions were not significantly different between groups.

Univariate analysis was also performed to assess the contribution between the presence of hypertriglyceridemia and other significant parameters on apo B-48 concentration in lipoprotein fractions. HOMA-IR level significantly contributed to the concentration of apo B-48 in Sf > 400 (HOMA-IR: regression coefficient = 0.654, P = 0.037). The concentration of apo B-48 in all fractions was significantly greater in the HTG group compared to the NTG group after being corrected for HOMA-IR level.

Table 1
Subject characteristics between normotriglyceridemic and hypertriglyceridemic groups.

Parameter	NTG (n = 12)[a]	HTG (n = 12)[a]
Men/women	6/6	6/6
Age (years)	61.2 ± 2.7	61.8 ± 2.7
BMI (kg/m^2)	26.5 ± 0.9	28.2 ± 0.6
Waist circumference (cm)	90.4 ± 3.4	95.2 ± 3.1
Systolic blood pressure (mmHg)	132 ± 3	147 ± 4**
Diastolic blood pressure (mmHg)	80 ± 2	87 ± 2*
Triglyceride (mmol/L)	1.1 (1.0–1.3)	2.0 (1.8–2.8)**
NEFA (mmol/L)	0.5 ± 0.04	0.5 ± 0.04
Total cholesterol (mmol/L)	5.4 ± 0.3	5.8 ± 0.3
HDL cholesterol (mmol/L)	1.5 (1.2–1.9)	1.3 (1.0–1.4)
LDL cholesterol (mmol/L)	3.4 ± 0.2	3.4 ± 0.3
Glucose (mmol/L)	5.1 ± 0.1	5.5 ± 0.3
Insulin (mIU/L)	5.6 ± 0.6	8.7 ± 1.2*
HOMA-IR	1.2 (1.0–1.3)	1.9 (1.2–2.9)*
CRP (μg/mL)	1.7 (0.7–3.3)	2.9 (1.3–6.0)
On hypertension medication	1	2
On hyperglycemic medication	0	1

Significant differences between groups are indicated by *P < 0.05; **<0.01.
[a] Mean ± SE or median (25th and 75th interquartile range).

3.3. Triglyceride and cholesterol particles in lipoprotein fractions

The total (serum) concentration of triglyceride in the HTG group was approximately twice that observed in the NTG group (2.5 ± 0.3 vs 1.1 ± 0.1 mmol/L; P < 0.001) (Fig. 2A). This accumulation of triglyceride in the HTG group was evident in all lipoprotein fractions with each having a significantly greater concentration of triglyceride than that observed in the NTG group. However the extent of this difference was greater in the less dense fractions in which the triglyceride concentration in the HTG group was approximately 3–4 times that observed in the NTG group (Sf > 400: 0.8 ± 0.2 vs 0.2 ± 0.03 mmol/L, P < 0.001; and Sf 20–400: 0.8 ± 0.1 vs 0.3 ± 0.04 mmol/L, P < 0.001). Whereas the majority of the triglyceride in the NTG group was located in the more dense Sf < 20 fraction. When we calculated the ratio of triglyceride content to apo B particle in each fraction, it was observed that the ratio in the Sf > 400 fraction was ~4 times greater in the HTG group compared to the NTG group (P < 0.001) and in Sf 20–400 was two-fold greater (P < 0.001) (Fig. 3).

There was no difference in the total (serum) concentration of cholesterol between groups with by far the majority being located in the Sf < 20 fraction. The remainder of the cholesterol in both

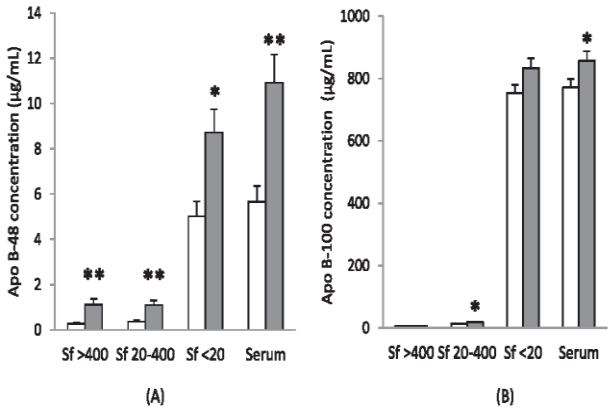

Fig. 1. Mean concentration of apo B-48 (A) and apo B-100 (B) in different lipoprotein fractions; □ represents NTG group; ■ represents HTG group. Significant differences between groups are indicated by *P < 0.05, **<0.01 compared to NTG group.

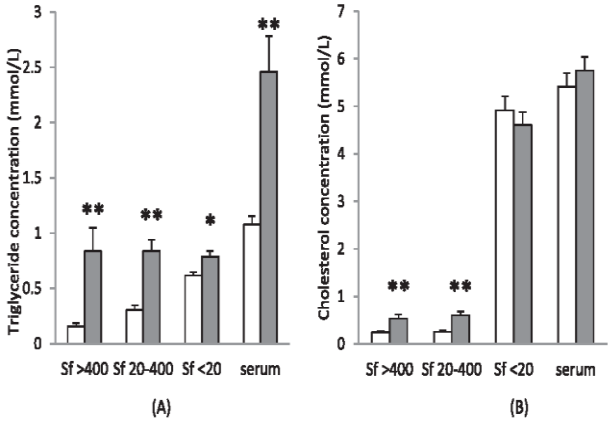

Fig. 2. Mean concentration of triglyceride (A) and cholesterol (B) in different lipoprotein fractions; □ represents NTG group; ■ represents HTG group. Significant differences between groups are indicated by *P < 0.05, **<0.01 compared to NTG group.

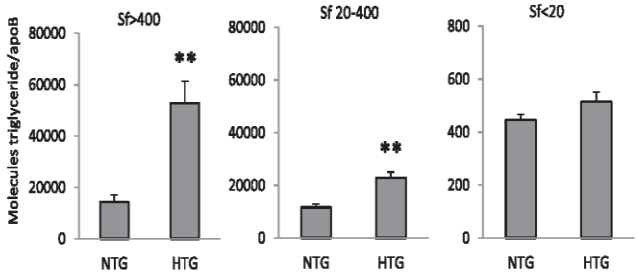

Fig. 3. Trigylceride contents per apo B particle in Sf > 400, Sf 20–400 and Sf < 20 fractions. Significant differences between groups are indicated by *P < 0.05, **<0.01 compared to NTG group.

groups being essentially equally distributed in the Sf 20–400 and Sf > 400 fraction with the HTG group exhibiting a small, but significantly higher concentration of cholesterol in the Sf > 400 and Sf 20–400 fractions than that observed in the NTG group (Sf > 400: 0.5 ± 0.09 vs 0.2 ± 0.02 mmol/L; P = 0.001 and Sf 20–400: 0.6 ± 0.08 vs 0.3 ± 0.03 mmol/L; P = 0.001) (Fig. 2B).

4. Discussion

We have investigated whether normocholesterolemic subjects with HTG also exhibit an increased circulating concentration of pro-atherogenic lipoprotein remnants. We assessed the size distribution of chylomicron particles in these subjects by measuring

the concentration of apo B48 in different density lipoprotein fractions.

Subjects with HTG were observed to have an elevated fasting concentration of chylomicron particles as measured by total apo B48 concentration in serum, compared to NTG controls. Interestingly the majority of this accumulation was observed in the more dense, atherogenic lipoprotein fraction Sf < 20. Regardless of the state of hypertriglyceridemia, at least in the fasting state, it appears that the majority of chylomicron particles in circulation are of a small (remnant) size contributing to the Sf < 20 lipoprotein fraction (Fig. 1A). A similar finding has been reported in normolipidemic subjects [29]. It is thought that the high proportion of chylomicron particles in the Sf < 20 fraction in the fasting state may be a result of the production of small dense chylomicron particles resulting from a lack of dietary lipid [7]. While this mechanism is likely to be occurring in both normo and HTG subjects, the greater accumulation of small dense chylomicron remnant particles in HTG subjects suggests that the catabolism and/or clearance of these particles is impaired.

In the present study, we also measured the distribution of triglyceride and cholesterol in each lipoprotein fraction. The extent of triglyceride enrichment was greater in the Sf > 400 and Sf 20–400 fractions in the HTG group (Fig. 2A). Higher cholesterol concentration in the less dense fractions (Sf > 400 and Sf 20–400) (Fig. 2B) were observed in the HTG group but despite this there was no difference in the total serum concentration of cholesterol in the HTG subjects compared to NTG controls. As the majority of cholesterol in circulation resides with LDL particles [30] it was not surprising that we observed approximately 80% of the cholesterol pool in the Sf < 20 fraction (Fig. 2B). However in the less dense fractions, which do not contain LDL particles, the HTG group exhibited a small but significantly higher concentration of cholesterol. This difference is likely associated with the observed higher concentration of chylomicron particles in these fractions and/or increased concentration of VLDL remnant particles which are typically observed in HTG subjects (Fig. 1A and B) [31]. Indeed the HTG group also exhibited a greater concentration of apo B-48 in both the Sf > 400 and Sf 20–400 lipoprotein fractions (Fig. 1A). Similar findings have been reported previously [21,32].

The higher triglyceride to apo B ratio in Sf > 400 and Sf 20–400 fractions observed in the HTG group (Fig. 3) indicating the presence of lipoproteins of larger particle size [33] is suggestive of a delayed lipolysis of the triglyceride rich lipoproteins [34]. In the fasting state chylomicron production rates are affected by the availability of lipid [35] with cytoplasmic stores of triglyceride in the enterocyte being a lipid source for chylomicron assembly [36]. However when lipid supply is limited, such as in the fasting state, intracellular degradation of apo B occurs suggesting that the increased concentration of apo B48 in these less dense fractions may result from an increased enterocytic storage pool of triglyceride [36], or alternatively a decreased lipolysis of the circulating chylomicron particles. In the fasting state circulating NEFA can be used as a lipid source for enterocytic lipoprotein synthesis [36] hence it is possible that our observation of an increased lipoprotein particle size in subjects with HTG result from an increased availability of NEFA in circulation. However we observed that there was no significant difference in serum NEFA concentration between groups (Table 1) and hence it is more likely that the increased particle size results from impaired lipolysis of triglyceride rich lipoproteins. Any defect in the lipolysis of triglyceride rich lipoproteins would lead to an increased time available for cholesteryl ester triglyceride exchange. The high level of plasma triglyceride may promote the distribution of cholesteryl esters among lipoproteins due to reduced rate of triglyceride hydrolysis or increased CETP activity [37,38]. This possibility is inferred from our findings in which the extent of triglyceride and cholesterol enrichment was much greater in the Sf > 400 and Sf 20–400 fractions in the HTG group (Fig. 2A and B). These results are in agreement with the findings from Sniderman et al. (2012), who observed that isolated VLDL fractions from subjects with hypertriglyceridemia were more cholesterol-enriched than the normotriglyceridemic controls [37]. The delayed lipolysis can be caused by insulin resistance, which was observed in the HTG group. Based on univariate analysis, the high concentration of apo B-48 in Sf > 400 in the HTG group is associated with insulin resistance. It has been reported that in insulin resistance, the ability of insulin to stimulate the transcription of LPL gene or maintain the cytoplasmic LPL mRNA concentration is impaired [39].

Despite our observation of an increased concentration of triglyceride rich chylomicron particles in the HTG group, the majority of the increased chylomicron concentration in these subjects was in the more dense Sf < 20 lipoprotein fraction. It is possible that this accumulation results from impaired clearance of these particles by the liver. Although from this cross sectional study, we cannot delineate the mechanism behind this accumulation, it has been suggested that insulin resistance induces hepatic overexpression of SULF2 leading to an accelerated de-sulfation of HSPG in the liver [40,41]. This would be expected to result in an impairment of the uptake of remnant lipoproteins by the liver and could explain the greater build-up of small dense chylomicron particles in the HTG group (Fig. 1A), and hence may contribute to the greater risk of developing atherosclerosis in subjects with HTG [42]. The elevated concentration of chylomicron particles in the Sf < 20 fraction in the HTG group may infer a larger net flux rate of small sized chylomicrons into the arterial wall [43,44]. As the efflux of chylomicron remnants from subendothelial space is not as rapid as LDL or other plasma lipoproteins [45], the observed accumulation of apo B-48 in the Sf < 20 fraction in the HTG group likely poses a greater atherogenic risk. As one chylomicron remnant is thought to contain approximately 25 fold more cholesterol molecules compared to an LDL particle [30], this accumulation may explain how a greater arterial cholesterol deposition could occur in normocholesterolemic HTG subjects.

We have extended the findings from previous studies [21,46,47] that have reported that the majority of chylomicron particles exist in lipoprotein fractions with Svedberg flotation rates of up to Sf 20–60 by examining the distribution of remnants within the more dense pro-atherogenic Sf < 20 fraction. Lipoproteins in Sf < 20 fraction are estimated to have a particle diameter of less than 30–55 nm [48], consistent with pro-atherogenic lipoprotein size phenotype [49]. Our observation that the majority of the elevation in chylomicron particles in subjects with HTG occurs in this fraction may explain, at least in part, the increased atherogenic risk observed in this group of subjects. However it should also be noted that the concentration of apo B48 in this fraction was determined by calculation (rather than direct measurement).

In conclusion, we report that normocholesterolemic subjects with hypertriglyceridemia exhibit an accumulation of small dense chylomicron particles, in the fasting state. We speculate that this accumulation may result from either a catabolic defect or an increased enterocytic production of small dense chylomicrons, or a combination of both. It is interesting that these defects were detected even in the fasting state when the circulating levels of chylomicron particles are low and less likely to saturate the lipolytic and catabolic pathways. It would be of further interest to extend these findings and examine the effect on chylomicron size distribution in these subjects during postprandial lipemia when there is greater competition for lipolytic and clearance pathways. Nonetheless our findings in fasting subjects are suggestive of a role of chylomicron remnants in cardiovascular disease risk in HTG subjects.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

DI conducted the study, analysed the data and wrote the manuscript. JM developed the study design and wrote the manuscript. MS assisted the data analysis and wrote the manuscript. KS wrote the manuscript. AJ conceived the idea of the study, developed the study design, assisted the data analysis and wrote the manuscript. The authors would like to thank Virginie Lam and Ryusuke Takechi for providing samples and technical support.

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3.3 Supplementary methods

Lipoprotein fractionation

To separate and isolate lipoprotein fractions of serum samples, a sequential flotation ultracentrifugation method was used (Havel, Eder, & Bragdon, 1955; Anthony P. James & John C. Mamo, 2012). This technique fractionates lipoproteins by overlaying serum with NaCl density solutions adjusted to the fraction to be isolated. The flotation rate at which the centrifuged lipoproteins float to the top of the tube are a function of mass and hydrated density.

The first step to estimating the time needed to float lipoprotein particles at certain flotation rate was to calculate 'k' value of the rotor in the desired spin (40,000 rpm), r_{min} and r_{max} (see in the AH-650 rotor guideline) by using the formula:

$$k = \frac{\ln rmax - \ln rmin}{3600\ \omega^2} \times 10^{13} \qquad \text{(equation 1)}$$

r_{max} for rotor AH-650 = 106.0 mm; r_{min}=62.8 mm; $\omega = 0.105 \times RPM$. So the k value for 40,000 rpm in AH-650 rotor is 82.3.

The second step was to calculate the value of F. It should be noted that the F value must relate to the conditions in the preparative tube instead of using the conventional scale which is defined by analytical ultracentrifugation at 26°C in NaCl solution of density 1.063 g/mL (Mills et al., 1984). The formula used was:

$$F = Sf\ \frac{(1.0063 - d) \times 1.0173}{(1.063 - d) \times \eta} \qquad \text{(equation 2)}$$

d represents the density of the lipoprotein particle and η represents the viscosity of a NaCl solution of density 1.006 g/mL at the temperature of the preparative ultracentrifugation.

The third step was to calculate time to run from the following formula:

$$t = \frac{k}{F}\ \text{(hours)} \qquad \text{(equation 3)}$$

or

$$\frac{ta}{tb} = \frac{ka}{kb} \qquad \text{(equation 4)}$$

Ta = time to run in rotor A; ka = k value in rotor A at specific spin

Tb = time to run in rotor B; kb = k value in rotor B at specific spin

Using the above equations, the time taken to float lipoprotein particles of density Sf >400 at 20°C in the AH-650 rotor was 30 minutes, whereas to float Sf 20 – 400 particles took 20.5 hours.

The preparation of density solutions followed the procedure developed by Lindgren (1975) (Lindgren, 1975). Briefly, to prepare a density solution with a density of 1.006 g/mL, 11.45 g of NaCl was added to 1000 g double deionised water. To this solution, 100 mg of Na_2EDTA and 1 mL of 1 M NaOH were added per litre of density solution. The final pH was 7 and the density of the solutions were confirmed prior use.

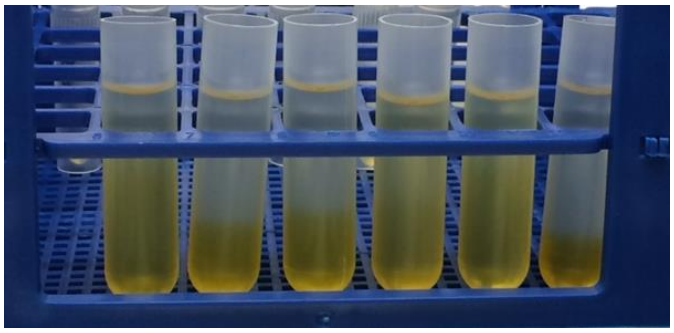

Figure 3.1 The plasma are overlayed with density solution.

Figure 3.2 Cloudy appearance in the top layer of the samples (plasma and density solution) (left) and the supernatants collected (right).

Ultracentrifugation was conducted using a Sorvall Ultraseries, rotor AH-650 at 40,000 rpm and 20°C. The procedure was as follows. One millilitre of plasma was overlayed with a density salt solution (1.006 g/mL) in 5 mL thin walled tubes (Thermo Scientific, Scoresby, Australia) until the liquid reached around 1 cm below the top (Figure 3.1). The ultracentrifuge tubes were placed into the buckets. The liquid was further added with the same density salt solution until it reached 2 mm below the top. The buckets and the lids

were weighed to ensure that the buckets were balanced. Subsequently the buckets were gently loaded into the rotor and the speed, temperature, time, and the type of rotor used for ultracentrifugation were set (Sorvall ultraseries, rotor AH 650).

To float the Sf >400 lipoproteins, the samples were spun at 40,000 rpm at 20°C for 30 minutes. After spinning, the Sf >400 lipoprotein fraction (approximately 1 mL) was aspirated and placed into sterile tubes. Figure 3.2 shows that when the concentration of lipoprotein Sf >400 is high, the top layer will have a cloudy appearance. The infranatants were then topped up with the density solution until the liquid reached 2 mm below the top of the tube. The buckets were spun again for 20.5 hours at the same speed and temperature. During this spinning, large and small VLDL-sized particles float to the top of the tube, leaving lipoproteins Sf <20 in the infranatants. A similar method of collection by aspiration was applied (as above). The volume of all lipoprotein fractions and infranatants were measured using a 1 mL glass bore syringe (Hamilton). These volumes were used to calculate the amount of triglyceride and cholesterol in each lipoprotein fraction. After this process the lipoprotein aliquots were stored at -80^{0}C for later analysis.

CHAPTER 4

Chapter 4

The effect of dietary fatty acid composition on plasma abundance of pro-atherogenic chylomicron remnants in postprandial hyperlipidemia

Content of this chapter is covered by the manuscript:

Irawati D, Mamo JCL, Soares MJ, Slivkoff-Clark KM, James AP. Dietary fat and physiologic determinants of plasma chylomicron remnant homeostasis in normolipidemic subjects: Insight into atherogenic risk.

Under consideration for publication.

Thesis objective addressed in this chapter:

Objective 3: To investigate the acute effect of dietary fatty acid composition and metabolic determinants on chylomicron metabolism in normotriglyceridemic subjects.

4.1 Synopsis

4.1.1 Background

The majority of humans spend most of their lives in the postprandial state. Following ingestion of dietary fats, there is an increase in triglyceride-rich chylomicrons however this elevation is temporary. Stimulated by insulin, LPL bound GPIHBP1 mediates the hydrolysis of triglyceride from TRL releasing NEFA to be taken up by the endothelial cells via CD 36 (Geesje M. Dallinga-Thie et al., 2010). Eventually chylomicrons and VLDL become smaller forming remnant lipoproteins which are removed from the circulation mainly via receptor-mediated pathway in the liver (Cooper, 1997).

The increase in postprandial triglyceride concentration reflects the net effect of dietary absorption in the intestine, chylomicron assembly and secretion by the enterocytes and lipolysis by LPL. However previous evidence has shown that it is the remnant lipoproteins that are associated with atherogenicity (Mamo, Proctor et al., 1998). Indeed in Chapter 3, we found that in the fasting state the majority of apo B-48 containing lipoproteins were present in the Sf <20 fraction (Irawati, Mamo, Soares, Slivkoff-Clark, & James, 2015). However we observed that the distribution of chylomicron concentration was skewed indicating genetic variability in chylomicron metabolism either in the basal production rates and/or clearance of chylomicrons.

A large body of evidence has reported the effect of dietary fatty acid on chylomicron remnant metabolism through modulation of chylomicron assembly (i.e. MTP activity, lipid droplet formation and stability), LPL preference and clearance rate (C. M. Williams, 1998; C. M. Williams et al., 2004). Amongst dietary fatty acids, SFA has been associated with atherogenicity through the poorly defined alteration in chylomicron metabolism, hypercholesterolemic effect and vascular inflammation (Esser, van Dijk, Oosterink, Müller, & Afman, 2013; Lamarche & Couture, 2015; Mensink, Zock, Kester, & Katan, 2003; Ruiz-Núñez, Dijck-Brouwer, & Muskiet, 2016). The type of dietary fats also influences the extent of postprandial lipemia (C. M. Williams, 1997, 1998; C. M. Williams et al., 2004), however most of the evidence examined the postprandial response in lipid-rich lipoprotein fractions, a rather than more dense fractions containing small-sized pro-atherogenic remnants.

In this chapter, we compared the chylomicron remnant postprandial response following meals rich in either long chain SFA, medium chain SFA and PUFA.

4.1.2 Methods in brief

In this cross-over designed study, healthy normotriglyceridaemic subjects were recruited to receive in random order an isoenergetic mixed meal containing 40 g of either: palm oil (PO) (SFA:MUFA:PUFA=60%:31%:9%), coconut oil (CO) (SFA:MUFA:PUFA=92%:8%:0%), or rice bran oil (RBO) (SFA:MUFA:PUFA=28%:40%:32%) on 3 occasions. The plasma distribution of chylomicron apo B-48 was determined in a triglyceride rich lipoprotein fraction (Sf >400), in partially hydrolysed remnants (Sf 20 – 400) and in the triglyceride deplete fraction (Sf <20). The detail of the lipoprotein fraction has been explained in Chapter 3 (Supplementary method).

The primary outcome of this study was the apo B-48 IAUC in subjects with exaggerated plasma triglyceride (≥1.7 mmol/L) 4 hours after consuming the dietary challenge rich in PO (hyper-responders/HR) compared to subjects with plasma triglyceride less than 1.7 mmol/L at the peak of the absorptive period (normal responders/NR). Ethics was approved by Curtin University Human Research Ethics Committee (HR 151/2013). The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN 12614000352606).

4.1.3 Results in brief

Twenty-six normotriglyceridemic subjects participated in the study. In fasting, around 74% of the chylomicrons was in the Sf <20 fraction (apo B-48 in plasma: 8.3 ± 1.2 μg/mL, in Sf >400: 0.7 ± 0.2 μg/mL, in Sf 20-400: 1.5 ± 0.2 μg/mL and in Sf <20: 6.2 ± 0.9 μg/mL, respectively).

Approximately 40% of subjects exhibited exaggerated postprandial lipaemia following the PO meal (HR group)(recruitment flow diagram in the Supplementary section). No difference in fasting apo B-48 concentration was observed between the hyper-responders and normo-responders (manuscript Table 2).

Following the PO meal, approximately three-fold higher plasma apo B-48 IAUC was observed in the HR group compared to the NR group with half of the exaggerated apo B-48 response residing in the remnant Sf <20 fraction (manuscript Figure 1). A smaller increase in apo B-48 response within the Sf 20 – 400 and Sf >400 fraction was also observed in the HR group compared to that of the NR group. The IAUC apo B-48 response following either RBO or CO meal was comparable in both groups.

In the HR group, fat free mass was associated with IAUC apo B-48 in Sf >400 and Sf 20 – 400 fraction but not with Sf <20 across all meal challenges. In contrast, no significant

association between IAUC apo B-48 in any of the lipoprotein fraction with fat free mass was observed in the NR group. Fasting triglyceride concentration was not associated with any of the lipoprotein fractions across all dietary treatment arms in the HR group.

4.1.4 Discussion and conclusion in brief

Contrary to the conventional thought, the majority of chylomicron particles in the fasting state and their response in the postprandial state are found within the potentially pro-atherogenic Sf <20 fraction. This suggests that following dietary fat ingestion, a substantial proportion of circulating chylomicrons can directly penetrate the subendothelial space.

The results of this chapter showed that the magnitude of hyper-remnantemia is dependent both on the nature of dietary fatty acids ingested and genetic determinants. The greater postprandial chylomicron response following PO meal observed in a subset of normotriglyceridemic subjects but not in the NR group indicates differential expression of key apolipoproteins (i.e. apo C-II, apo C-III, apo E) or isoforms of lipolytic enzymes involved in lipolysis. However this may not be the case as the chylomicron and triglyceride postprandial responses were comparable following ingestion of either CO or RBO meal suggesting intact lipolysis/clearance machinery.

The association between apo B-48 and fat free mass index (inverse in fat mass index) suggests the possibility of modest hydrolytic defect in muscle tissue. It is possible that the defect is more pronounced when the subjects ingested PO meal. Ingestion of a meal rich in SFA content has been reported to decrease insulin sensitivity (Galgani, Uauy, Aguirre, & Díaz, 2008; Riccardi, Giacco, & Rivellese, 2004) via accumulation of diacylglycerol and ceramide in muscle, activation of protein kinase C, nuclear factor-κB and the subsequent inflammatory genes (Kennedy, Martinez, Chuang, Lapoint, & McIntosh, 2009).

Our findings reiterate the importance of assessing remnant lipoprotein concentration. The classical markers of plasma lipid homeostasis measured in this study provide no valuable insight into chylomicron metabolism and remnant homeostasis. Therefore classical markers may not represent an accurate estimation of atherogenic risk.

4.2 Manuscript

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Dietary fat and physiological determinants of plasma chylomicron remnant homoeostasis in normolipidaemic subjects: insight into atherogenic risk

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Abstract

TAG depleted remnants of postprandial chylomicrons are a risk factor for atherosclerosis. Recent studies have demonstrated that in the fasted state, the majority of chylomicrons are small enough for transcytosis to arterial subendothelial space and accelerate atherogenesis. However, the size distribution of chylomicrons in the absorptive state is unclear. This study explored in normolipidaemic subjects the postprandial distribution of the chylomicron marker, apoB-48, in a TAG-rich lipoprotein plasma fraction (Svedberg flotation rate (Sf) > 400), in partially hydrolysed remnants (Sf 20–400) and in a TAG-deplete fraction (Sf < 20), following ingestion of isoenergetic meals with either palm oil (PO), rice bran or coconut oil. Results from this study show that the majority of fasting chylomicrons are within the potentially pro-atherogenic Sf < 20 fraction (70–75 %). Following the ingestion of test meals, chylomicronaemia was also principally distributed within the Sf < 20 fraction. However, approximately 40 % of subjects demonstrated exaggerated postprandial lipaemia specifically in response to the SFA-rich PO meal, with a transient shift to more buoyant chylomicron fractions. The latter demonstrates that heterogeneity in the magnitude and duration of hyper-remnantaemia is dependent on both the nature of the meal fatty acids ingested and possible metabolic determinants that influence chylomicron metabolism. The study findings reiterate that fasting plasma TAG is a poor indicator of atherogenic chylomicron remnant homoeostasis and emphasises the merits of considering specifically, chylomicron remnant abundance and kinetics in the context of atherogenic risk. Few studies address the latter, despite the majority of life being spent in the postprandial and absorptive state.

Key words: **Dietary fatty acids: Chylomicron remnants: ApoB-48: Postprandial lipaemia**

The majority of humans spend most of their 24-h-d in the postprandial state. A typical 24 h TAG profile in individuals consuming three to four meals and snacks per d, showed a 70 % increase in TAG concentration that reached baseline for only 3 h in the early morning[1]. Therefore, humans are essentially in a constant state of absorptive lipaemia. Following ingestion of dietary fats there is an elevation of TAG-enriched chylomicrons, however this elevation is ordinarily transient. Insulin-stimulated expression of lipoprotein lipase (LPL) bound to glycosylphosphatidylinositol anchored HDL-binding protein 1 mediates the hydrolysis of TAG from chylomicrons, resulting in rapid release of NEFA that can be taken up by endothelial cells via the membrane protein cluster of differentiation 36[2]. The post-hydrolysed TAG depleted remnants of chylomicrons and VLDL, are then removed via high-affinity receptor pathways, principally in the liver[3].

Postprandial hypertriacylglycerolaemia is indicative of the net effect of dietary lipid absorption, biogenesis and secretion of *de novo* chylomicrons and lipolysis by endothelial lipases. Modest intakes of dietary TAG can be mostly accommodated in the constitutively secreted chylomicrons, and may result in only minor changes to particle number[4,5]. However, rather than the TAG-rich lipoproteins (TRL) secreted following fat ingestion, accumulation of the post-hydrolysed remnants in plasma poses a greater atherogenic risk[6]. Indeed, fasting hypertriacylglycerolaemia, or postprandial dyslipidaemia, is not a requisite surrogate marker of chylomicron remnant accumulation *per se*[7–9], and measurement of chylomicron particle number, rather than TAG concentration provides a more complete marker of pro-atherogenic remnant accumulation[10].

Regulating the plasma abundance of pro-atherogenic chylomicron remnants principally relies on lipoprotein clearance capacity through receptor mediated pathways[11]. Chylomicrons share the lipolytic cascade and high affinity clearance pathways with TRL of hepatic origin (VLDL). An accumulation of VLDL

Abbreviations: CO, coconut oil; HR, hyper-responder; IAUC, incremental AUC; LPL, lipoprotein lipase; NR, normo-responder; PO, palm oil; RBO, rice bran oil; Sf, Svedberg flotation rate.

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remnants, or indeed LDL as a consequence of receptor insufficiency, often serves as a surrogate marker of clearance deficits of chylomicron remnant lipoproteins, because chylomicron remnants are primarily removed from blood by the apo B/E receptor (LDL-receptor). Although apo B-48, the equivocal marker of chylomicron abundance is not always reported, it is reported to be substantially elevated in subjects with diabetes/the metabolic syndrome, familial hypertriacylglycerolaemia, familial hypercholesterolaemia and in normolipidaemic subjects with coronary artery disease[12–15]. Lipoproteins that are reduced to remnants with a diameter of <70 nm contribute to atherosclerosis through their capacity to deliver cholesterol to the subendothelial space and retention in extracellular matrices[16–18]. Indeed, fully hydrolysed chylomicron remnants with diameters of 55 nm have been reported and contain approximately forty times more cholesterol than LDL per particle[19,20].

In healthy Caucasians, the plasma distribution of fasting apo B-48 is principally realised in the small, dense and potentially more atherogenic Svedberg flotation (Sf) <20 lipoprotein fraction[21]. However, in this study, we report that this abundance was not normally distributed, suggesting inter-individual variability in basal rates of chylomicron synthesis and/or remnant clearance. Moreover, there is likely to be potential interactive effects with dietary fats and the genetic regulation of chylomicron homoeostasis[22]. However, the hypothesis of differential synergistic fatty acid/genetically mediated effects on chylomicron remnant abundance *per se* has never been explored directly.

Dietary fatty acid type has been reported to influence chylomicron metabolism by modulating chylomicron assembly (i.e. microsomal transfer protein activity, lipid droplet formation and stability), LPL mediated hydrolysis and high-affinity clearance pathways[23,24]. Among the different types dietary fatty acid there is an abundance of evidence that SFA are pro-atherogenic via pathways that include undefined aberrations in chylomicron metabolism; a broader hypercholesterolaemic induction effect and subsequently heightened vascular inflammation[25–28]. Differential effects of fatty acid type on postprandial lipaemia have been widely reported. Exaggerated postprandial lipaemia may be due to overproduction of intestinally derived and hepatically derived TRL, slower clearance by LPL, a rate-regulatory hydrolysis and removal of TAG or accumulation of partially hydrolysed remnant lipoproteins[1,23,24]. However, the comparative response to different dietary fats on chylomicron size and importantly remnant abundance is less clear. To address the latter, this study was designed to explore plasma apo B-48 distribution in normolipidaemic but otherwise healthy subjects given different meals rich in either SFA, long-chain *n*-6 PUFA or in medium chain fatty acids.

Methods

This crossover designed study was conducted at Curtin University, Australia, between August 2014 and December 2015 and approved by the Curtin University Human Research Ethics Committee (HR 151/2013). All participants provided signed informed consent at initial screening. The study was registered with Australian New Zealand Clinical Trials Registry (12614000352606).

Subjects

Male and female healthy participants aged between 20 and 70 years were recruited by means of standard media and web portals. Before acceptance in the study, potential interested participants were invited to meet the investigators and attended an initial screening. The exclusion criteria were fasting TAG 1·7 mmol/l or more, pregnant, lactating, smoking, excess alcohol intake (>20 g alcohol/d), the use of lipid lowering medication, reported history of cardiovascular, hepatic, renal disease, gastric disturbances or use of drug that affect gastrointestinal motility, diabetes mellitus, unwillingness to avoid consumption of fish oil supplements 3 d before and during the study days, > ±5 kg changes in body weight in the previous 6 months, on a dietary advice and allergy or intolerance to any ingredients of the test meals.

Subjects were randomised using the random-number generator function RANDBETWEEN in Microsoft Excel software for the order of the test meals. Sample size required was determined from the sample size of twenty-four in a comparable study in which difference in apo B-48 postprandial response between postprandial hyperlipidaemia and normolipidaemia subjects were detected with a power of 80 % and α of 0·05[29].

Test meal composition

Each participant consumed an isoenergetic breakfast meal (3100 kJ, 43·4 g fat, 69·4 g carbohydrate and 13·6 g protein) on three occasions in which 40 g of the fat was from one of the following three different cooking oils: palm oil (PO) (Bimoli; PT Indofood Sukses Makmur Tbk), coconut oil (CO) (Eco Food Organics) and rice bran oil (RBO) (Alfa One™ Rice Bran Oil; Hansell Food Group) (Table 1). The test meal was a mixed breakfast meal consisting of pasta (150 g) (Large spiral pasta; San Remo Pasta Ltd), tomato pasta sauce (50 g) (Dolmio Traditional Recipe Classic Tomato Pasta Sauce; Mars Food) added with 40 g test oils, white bread (40 g) (white toaster; Mias Bakery) and 160 ml orange juice (Just Juice, orange juice). Each meal provided 53 % of total energy as fat. Fatty acid content of the different oils is provided in Table 1. Fatty acid composition of the cooking oils were analysed in the form of fatty acid

Table 1. Fat content of the test oils measured by GC

Fatty acid content (%)	Total SFA	Total MUFA	Total PUFA	12:0 (lauric acid)	14:0 (myristic acid)	16:0 (palmitic acid)	18:0 (stearic acid)	18:1*n*-9 (oleic acid)	18:2*n*-6 (linoleic acid)
Palm oil	60	31	9	0·1	4·3	42·1	9·8	30·4	8·8
Coconut oil	92	8	0	47·2	27·2	13·3	4·4	7·8	0·0
Rice bran oil	28	40	32	0·1	0·1	24·7	3·2	39·9	32·2

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methyl esters by GC (Perkin Elmer AutoSystem XL) equipped with an autosampler, a split-splitless injector, SGE BPX70 GC column and a flame ionisation detector using a method developed in our laboratory.

Study protocol

Participants attended three postprandial study days separated by at least 4 weeks between each visit. Before each study day, participants were asked to restrain from vigorous physical activity, alcohol and consumption of high-fat meals. They were asked to consume the same amount and food items for breakfast and lunch on the day before each postprandial test day and were provided with a standard commercially available ready meal (SunRice pack meal, 15 g fat; SunRice) for their evening meal before study day. After an overnight fast for at least 12 h, participants were admitted to the research unit. Upon arrival, their body weight, height, waist circumference and blood pressure (Omron) were measured. An initial fasting blood samples were collected before consumption of the test meal. Following consumption of the test meal, participants were given access to plain water with no other food and drinks to be consumed. The test meal was consumed within 15 min and subsequently postprandial blood samples were collected periodically at 4 and 8-h after the test meal for measurement of apo B-48, TAG and cholesterol concentration. Body composition measured by bioelectrical impedance analysis (SECA mBCA 515; SECA GmbH & Co. KG), was determined on the first fat challenge. Fasting and postprandial venous blood samples were collected into BD Vacutainer^{R} EDTA tubes (Becton Dickinson) and underwent low-speed centrifugation. Plasma samples were isolated and stored in aliquots at −80°C.

Lipoprotein fractionation

Sequential floatation ultracentrifugation was used to collect lipoprotein fractions of Sf > 400 and Sf 20–400 of plasma samples using a modified method that has been used in our laboratory[30,31]. In brief, 1 ml plasma was overlayed with a density solution (1·006 g/ml) in 5 ml thin walled tubes (Beckman Coulter). The buoyant chylomicron fraction (Sf > 400) was collected following 30 min of ultracentrifugation at 40 000 rpm, 20°C (AH-650 swing-out rotor; Thermo Scientific) by aspirating approximately 1 ml of the top layer. To collect Sf 20–400, the tube was then topped with density solution (1·006 g/ml) and ultracentrifuged again under the same condition for 20·5 h. Approximately 1 ml of the top layer was aspirated to isolate this fraction. The infranatant remaining in the tube was regarded as the remnant fraction (Sf < 20). To calculate the concentrations of apo B-48, TAG and cholesterol of these fractions, the volume of all supernatants and infranatant collected were taken into account by measuring the collected supernatants or infranatant using a glass bore syringe (Hamilton).

Lipid, insulin and glucose assays

Fasting and postprandial TAG and total cholesterol in plasma and lipoprotein fractions were measured with an enzyme-based colorimetric reagent (Trace; Thermo Fisher Scientific Inc.; CV < 5 %). HDL was collected using a precipitation method by the addition of phosphotungstic acid in the presence of Mg ions (HDL-cholesterol precipitant Reagent; Randox Laboratories Limited). A modified version of Friedewald formula was used to estimate LDL-cholesterol[32]. Fasting insulin and glucose concentrations were analysed by an accredited clinical laboratory (PathWest Laboratory, Fiona Stanley Network) using their automated procedures. The level of insulin resistance was assessed by homoeostasis model of insulin resistance (HOMA-IR)[33]. All samples were assayed in duplicate and run within a single batch.

Apo B-48 determination

Apo B-48 concentration was measured by using a commercial sandwich ELISA method (Shibayagi Human apo B-48 ELISA Kit; Ishihara; CV < 3 %). This method has been validated[34]. The determination of apo B-48 concentration in plasma, Sf > 400 and Sf 20–400 fractions was completed based on the manufacturer's instruction with some modification on the dilution factors to ensure that the apo B-48 concentration in those fractions within the standard curve. Fasting and postprandial plasma, Sf > 400 and Sf 20–400 samples were 1 in 300, 1 in 50 and 1 in 100 diluted, respectively. Apo B-48 concentration in Sf < 20 was calculated from the difference between the concentration of apo B-48 in plasma and in the less dense fractions (Sf > 400 and Sf 20–400).

Statistical analysis

All data were analysed using SPSS version 21 (SPSS Inc.). Data were checked for normality and were natural log transformed where necessary. Postprandial response (incremental AUC (IAUC)) was calculated using the trapezoidal rule[35] with some modifications. The IAUC was estimated as the difference between the area under the plasma curve between 0 and 8 h and the area defined below the baseline concentration. However in responses where the 8 h concentration was lower than that at baseline, the baseline AUC represented the area below a line connecting the baseline and 8 h concentration levels. The IAUC represents the increase in area after the response of the fat load above fasting concentrations. The primary outcome of this study was IAUC apo B-48 in plasma, Sf > 400, Sf 20–400 and Sf < 20 fraction. The secondary outcome of this study was IAUC TAG in plasma and lipoprotein fractions and the association between IAUC apo B-48 and a number of metabolic determinants. Postprandial differences between groups following each meal challenge were assessed with Mann–Whitney *U* test. Differences in postprandial responses between meals (PO, CO and RBO) were analysed by linear mixed model with Bonferroni *post boc* tests to detect significant pairwise differences corrected for multiple comparisons. Spearman correlations were computed to assess the association between parameters. A P value <0·05 was considered as significant.

Results

Totally, twenty-six participants with normal fasting TAG concentration took part in the single blind, randomised crossover

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study, of which twenty-three completed all three meal challenges. In all, three subjects only attended two postprandial visits. None of the participants reported any adverse effects to the test meals. To explore putative interactive effects of the dietary fat challenge with a genetically determined chylomicron distributional response, subjects were considered based on the postprandial TAG response 4 h after consuming the positive lipaemic control SFA-enriched meal (PO) were examined. Subjects were classified as hyper-responders (HR) if plasma TAG exceeded 1·7 mmol/l at the peak absorptive phase of 4 h. Of the twenty-six subjects studied, ten had concentrations ≥1·7 mmol/l (HR). Totally, sixteen subjects were found to have a 4-h TAG concentration <1·7 mmol/l following the SFA-enriched meal (normo-responders (NR)).

Table 2 shows that the HR and NR groups had comparable fasting apo B-48. In addition, HR and NR groups were normolipidaemic and comparable for plasma TAG, total cholesterol, LDL-cholesterol and non-HDL-cholesterol. The HR group had a lower concentration of HDL-cholesterol than NR, however this was otherwise within the normal reference range. Both NR and HR groups were normoglycaemic and there was no evidence of insulin resistance based on HOMA or fasting plasma insulin. However, the HR group had significantly greater BMI, waist circumference and fat-free mass than the NR group.

The putative effect of dietary fatty acids on the distribution of chylomicrons in lipoprotein fractions in NR and HR subjects is depicted in Fig. 1. In NR, the plasma IAUC for apo B-48 was comparable for the PO-enriched fat challenge *v.* the RBO diet (23·9 (SE 4·9) μg × 8 h/ml in PO *v.* 27·2 (SE 3·9) μg × 8 h/ml in RBO) (meal effect $P = 0·068$). However, there was a modestly lower plasma apo B-48 IAUC response in NR subjects following consumption of the CO diet (18·4 (SE 2·6) μg × 8 h/ml). In contrast, HR subjects had approximately 2–3-fold greater IAUC apo B-48 plasma response following ingestion of PO (63·2 (SE 13·4) μg × 8 h/ml) relative to the meal challenge with RBO (35·3 (SE 6·2) μg × 8 h/ml) or CO (24·4 (SE 4·8) μg × 8 h/ml) (meal effect $P = 0·003$, PO–CO $P = 0·016$, PO–RBO $P = 0·267$).

Distributional analysis of the heightened apo B-48 IAUC in HR subjects following ingestion of the PO-enriched meal, indicated that approximately half of the exaggerated apo B-48 response was realised in the remnant Sf < 20 fraction (32·1 (SE 6·6) μg × 8 h/ml in HR *v.* 13·4 (SE 4·3) μg × 8 h/ml in NR; $P = 0·02$). Smaller increases in the apo B-48 IAUC were also observed in HR subjects compared with NR within the Sf 20–400 fraction (14·4 (SE 4·5) μg × 8 h/ml in HR *v.* 6·6 (SE 1·4) μg × 8 h/ml in NR; $P = 0·138$) and the larger more buoyant lipoprotein isolate Sf > 400 (17·3 (SE 4·8) μg × 8 h/ml in HR *v.* 4·2 (SE 1·4) μg × 8 h/ml in NR, respectively; $P = 0·024$). Distributional analysis of the apo B-48 IAUC in HR and NR subjects following ingestion of a meal rich in either RBO or CO, did not indicate significant differences between lipoprotein fractions. This finding is consistent with the notion that the disturbed response was specific to the PO fat challenge in HR subjects.

The TAG response in lipoprotein fractions for NR and HR subjects in response to the dietary fat challenges is indicated in Fig. 2. In NR subjects, the plasma IAUC TAG response following the PO meal (1·7 (SE 0·3) mmol × 8 h/l) was comparable with the response following ingestion of RBO (2·1 (SE 0·6) mmol × 8 h/l)

Table 2. Subject characteristics
(Mean values with their standard errors; medians, 25th and 75th interquartile (IQR) ranges)

	Total (*n* 26)		NR (*n* 16)		HR (*n* 10)	
Parameters	Mean	SE	Mean	SE	Mean	SE
Men/women	11/15		4/12		7/3	
Age (years)	41	3	39	4	43	6
Apo B-48 (μg/ml)						
Median	6·3		7·1		5·1	
25th and 75th IQR	4·8–9·4		5·2–11·3		4·2–7·8	
TAG (mmol/l)	1	0·1	0·9	0·1	1·1	0·1
Total cholesterol (mmol/l)	5·5	0·2	5·4	0·3	5·4	0·4
LDL-cholesterol (mmol/l)	3·3	0·2	3·2	0·3	3·6	0·4
HDL-cholesterol (mmol/l)	1·7	0·1	1·8	0·1	1·4	0·1*
Non-HDL-cholesterol (mmol/l)	3·8	0·2	3·6	0·3	4·1	0·4
Glucose (mmol/l)	5·5	0·1	5·5	0·1	5·5	0·1
Insulin (mIU/l)						
Median	6·8		6·8		7	
25th and 75th IQR	4·2–11·1		4·1–9·8		5·1–12·2	
HOMA-IR						
Median	1·7		1·7		1·7	
25th and 75th IQR	1–3		1–2·3		1·3–3·2	
Waist circumference (cm)	83·7	2·7	77·9	3·2	93·1	2·8**
BMI (kg/m^2)	25·8	0·8	24·1	1	28·6	0·9**
Fat mass index (kg/m^2)	7·7	0·7	7·2	1	8·5	1·1
Fat-free mass index (kg/m^2)	18	0·5	16·8	0·5	19·9	0·7**
Systolic blood pressure (mmHg)	122	3	119	4	127	5
Diastolic blood pressure (mmHg)						
Median	72		71		73	
25th and 75th IQR	67–78		67–77		68–81	

NR, normo-responder; HR, hyper-responder; HOMA-IR, homeostasis model of insulin resistance.
* $P < 0·05$, ** $P < 0·01$; HR compared with NR group. *P* values were based on independent *t* test and Mann–Whitney test for mean and medians.

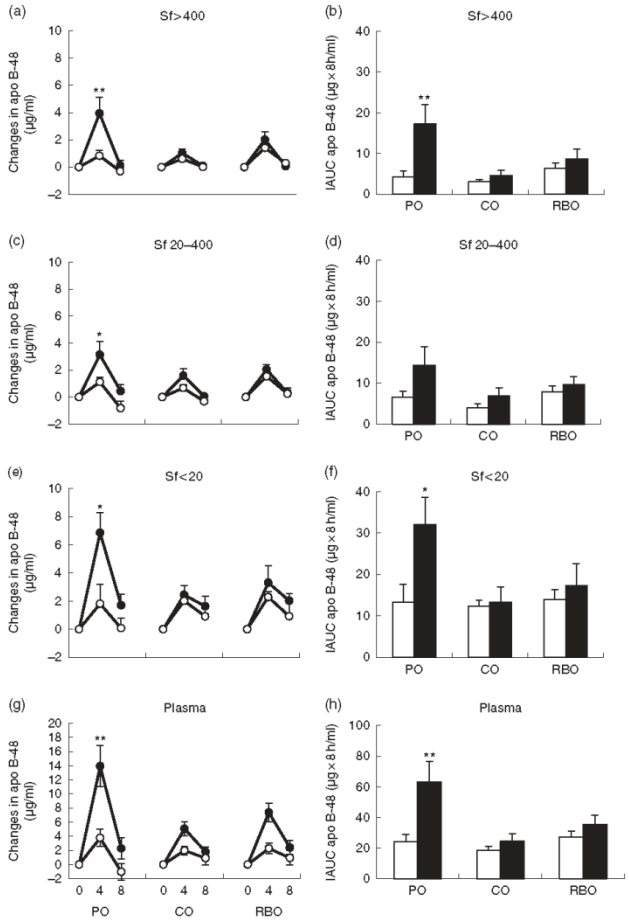

Fig. 1. Changes in apo B-48 concentration ((a), (c), (e), (g)) and incremental AUC (IAUC) of apo B-48 ((b), (d), (f), (h)) in Svedberg flotation rate (Sf) > 400, Sf 20–400, Sf < 20 fraction and plasma following mixed meal containing palm oil (PO), coconut oil (CO) and rice bran oil (RBO). ● and ■ represent hyper-responder (HR) group; ○ and □ represent normo-responder (NR) group. * *P* value <0·05, ** *P* value <0·01, against NR group.

or CO (1·4 (SE 0·3) mmol × 8 h/l) (meal effect $P = 0·414$). In contrast, in HR subjects the IAUC response for TAG following the PO-enriched meal was substantially greater than either the RBO (4·3 (SE 0·7) *v.* 2·8 (SE 0·5) mmol × 8 h/l, respectively) or the CO-enriched meal (1·7 (SE 0·5) mmol × 8 h/l) (meal effect $P = 0·001$). Distributional analysis of the heightened TAG IAUC response indicated in HR subjects was principally realised with a 3-fold increase in the largest and most buoyant lipoprotein fraction Sf > 400 compared with NR (3·1 (SE 0·6) *v.* 0·9 (SE 0·2) mmol × 8 h/l, respectively; $P < 0·0005$). Some increase in TAG IAUC was also observed within the Sf 20–400 fraction of HR subjects compared with NR (1·3 (SE 0·3) *v.* 0·5 (SE 0·1) mmol × 8 h/l, respectively; $P = 0·003$). Although relatively modest in the context of total TAG response, within the Sf < 20 fraction there was less of a TAG response in HR subjects compared with NR subjects (0·1 (SE 0·2) *v.* 0·4 (SE 0·2) mmol × 8 h/l, respectively; $P = 0·272$).

Suggested metabolic determinants of chylomicron homoeostasis in HR and NR subjects is depicted in Table 3. Fat-free mass determined by bioelectrical impedance analysis was

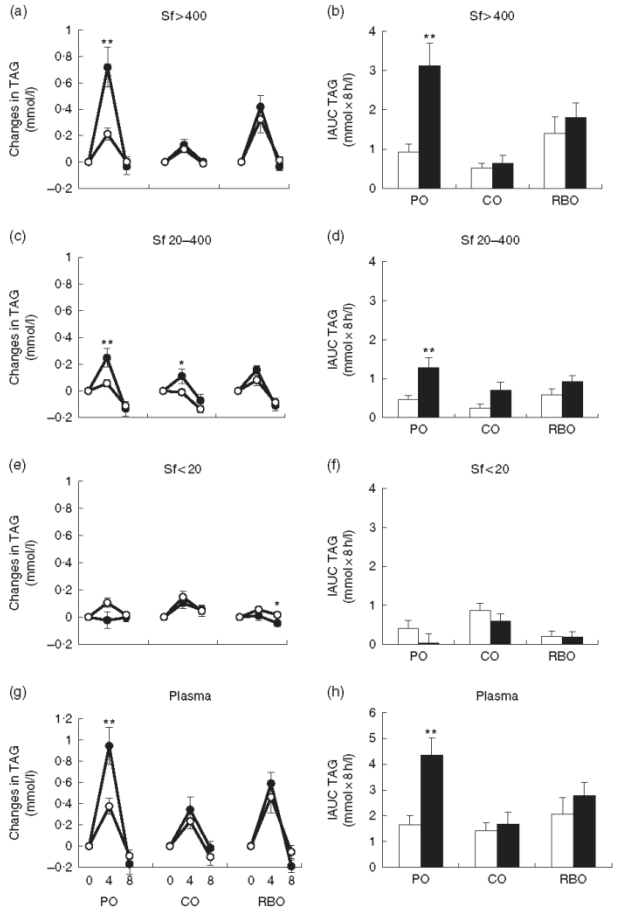

Fig. 2. Changes in TAG concentration ((a), (c), (e), (g)) and incremental AUC (IAUC) of TAG ((b), (d), (f), (h)) in Svedberg flotation rate (Sf) > 400, Sf 20–400, Sf < 20 fraction and plasma following mixed meal containing palm oil (PO), coconut oil (CO) and rice bran oil (RBO). ● represents hyper-responder (HR) group; ○ represents normo-responder (NR) group. * *P* value <0·05, ** *P* value <0·01, against NR group.

generally strongly associated with the apo B-48 IAUC for the Sf > 400 and the Sf 20–400 fractions in HR subjects across all dietary treatment arms. However, measures of fat-free tissue, or conversely adiposity, were not associated with the Sf < 20 remnant fraction in HR subjects. In the HR subjects, insulin sensitivity indicated by HOMA-IR score and fasting plasma insulin concentration were strongly associated with the apo B48 IAUC in the Sf > 400 and to a lesser extent the Sf 20–400 fraction in subjects given RBO or CO. However, the association was not evident in HR subjects when challenged with the PO-enriched meal. Fasting TAG, often considered a surrogate marker of chylomicronaemia, was not associated with chylomicron abundance in any of the lipoprotein fractions or dietary treatment arms considered in HR subjects. Instead, strong associations between fasting apo B-48 concentration and apo B-48 IAUC were observed across all dietary treatment arms in the HR group.

In NR subjects, fat-free mass did not correlate with apo B-48 IAUC for any of the lipoprotein fractions against any of the three meal challenges provided, contrasting with the findings in HR subjects. HOMA and fasting plasma insulin showed association

Table 3. Correlations between apo B-48 incremental AUC (IAUC) (0–8 h) in different fractions following palm oil (PO), coconut oil (CO) and rice bran oil (RBO) meal and baseline metabolic parameters in normo-responder (NR) (*n* 16) and hyper-responder (HR) (*n* 10) groups

R between apo B-48 IAUC and parameters	PO				CO				RBO			
	Plasma	Sf > 400	Sf 20–400	Sf < 20	Plasma	Sf > 400	Sf 20–400	Sf < 20	Plasma	Sf > 400	Sf 20–400	Sf < 20
NR												
Apo B-48	−0·211	−0·629	−0·132	−0·193	−0·246	−0·411	−0·343	−0·046	0·053	−0·071	−0·115	0·191
TAG	0·2	−0·211	0·154	−0·029	−0·075	0·139	0·171	−0·2	0·715**	0·456	0·429	0·635**
Total cholesterol	0·35	−0·046	0·221	0·086	−0·082	0·036	0·086	−0·161	0·471	0·3	0·476	0·321
LDL-cholesterol	0·611*	0·054	0·468	0·389	0·179	0·304	0·354	0·057	0·5*	0·279	0·479	0·406
HDL-cholesterol	−0·47	0·032	−0·484	−0·509	−0·377	−0·495	−0·399	−0·273	−0·205	−0·115	−0·13	−0·219
Non-HDL-cholesterol	0·525*	−0·054	0·386	0·329	0·122	0·227	0·273	0·013	0·527*	0·277	0·446	0·455
Glucose	−0·009	−0·307	−0·036	0·172	−0·057	0·084	0·025	0·001	0·001	0·087	0·041	−0·015
Insulin	−0·107	0·121	0·089	−0·439	−0·182	0·136	0·096	−0·425	0·432	0·553*	0·600*	0·059
HOMA-IR	−0·104	0·125	0·096	−0·429	−0·171	0·154	0·114	−0·429	0·421	0·559*	0·606*	0·026
BMI	0·3	−0·132	0·404	0·279	0·189	0·143	0·279	0·118	0·385	−0·091	0·079	0·612*
Waist circumference	0·418	0·068	0·575*	0·157	0·232	0·382	0·479	0·036	0·626**	0·226	0·432	0·662**
Fat-free mass index	−0·097	−0·311	0·184	0·154	0·157	0·197	0·175	0·055	−0·087	−0·093	−0·138	−0·105
Systolic	−0·011	−0·095	0·018	0·004	0·009	0·263	0·309	−0·075	0·187	0·175	0·108	0·196
Diastolic	0·141	0·032	0·404	−0·123	0·079	0·481	0·436	−0·166	0·394	0·202	0·252	0·284
HR												
Apo B-48	0·810*	0·643	0·952**	0·667	0·806**	0·588	0·721*	0·345	0·588	0·43	0·758*	0·261
TAG	0·357	0·452	0·5	−0·024	0·358	0·539	0·564	−0·224	0·37	0·418	0·479	−0·079
Total cholesterol	0·263	0·001	0·419	0·168	−0·085	−0·146	0·043	−0·122	−0·079	0·067	0·267	−0·328
LDL-cholesterol	0·214	−0·167	0·357	0·238	0·006	−0·152	0·042	0·079	−0·03	−0·018	0·248	−0·2
HDL-cholesterol	0·263	−0·228	0·12	0·311	−0·354	−0·591	−0·433	−0·03	−0·439	−0·396	−0·165	−0·299
Non-HDL-cholesterol	0·204	0·001	0·395	0·072	0·073	0·061	0·213	−0·073	0·122	0·182	0·359	−0·195
Glucose	0·216	0·335	0·539	−0·084	0·31	0·529	0·547	−0·146	0·322	0·675*	0·632*	−0·255
Insulin	−0·048	0·476	0·024	−0·357	0·321	0·770**	0·6	−0·2	0·176	0·721*	0·358	−0·261
HOMA-IR	−0·073	0·512	0·049	−0·439	0·264	0·742*	0·62	−0·313	0·117	0·706*	0·325	−0·325
BMI	−0·405	−0·619	−0·595	−0·333	−0·042	0·018	−0·164	0·333	0·079	−0·176	−0·273	0·188
Waist circumference	0·071	−0·405	−0·143	0·19	0·2	0·03	−0·115	0·612	0·285	−0·152	0·018	0·333
Fat-free mass index	0·635	0·731*	0·659	0·407	0·894**	0·833**	0·796**	0·353	0·766**	0·48	0·632*	0·498
Systolic	−0·095	−0·19	−0·095	−0·262	0·152	0·333	0·188	0·164	0·115	0·309	0·248	−0·224
Diastolic	−0·429	−0·452	−0·571	−0·357	−0·164	−0·055	−0·115	0·188	−0·382	−0·079	−0·212	−0·358

* $P < 0·05$, ** $P < 0·01$ (Spearman's correlation).

in the Sf > 400 and the Sf 20–400 for the RBO challenge, but this was not indicated for either the PO or CO meal challenges. In NR group, apo B-48 IAUC in Sf < 20 was also strongly associated with waist circumference and BMI following RBO meal.

For HR and NR subjects, there was no significant association between total cholesterol, LDL-cholesterol, HDL-cholesterol, fasting glucose or blood pressure and IAUC apo B-48 responses with the lipoprotein fractions indicated.

Discussion

This study explored the postprandial plasma distribution of apo B-48 in normolipidaemic subjects following ingestion of isoenergetic meals enriched in either PO, RBO or CO. Approximately 40 % of subjects studied demonstrated substantially exaggerated postprandial lipaemia in response to the SFA-rich PO diet (HR group), despite that these subjects did not exhibit an increased fasting concentration of chylomicron particles compared with the NR group. Moreover, significant heterogeneity was found within the responses of the otherwise healthy subjects with normal fasting plasma lipid concentrations, implying variation is due to the individual determinants of chylomicron homoeostasis.

The rationale to consider HR *v.* NR was driven based on the findings from Sato *et al.*[29]. Their results suggest that normolipidaemic subjects with TAG concentration postprandial ≥1·7 mmol/l may have a delayed clearance of TRL. The finding of the present study demonstrates that consideration of heterogeneity based on classical metabolic parameters such as the metabolic syndrome did not effectively segregate those with the exaggerated response to the SFA-rich meal. Hence analogous to classical dietary challenge assessment, we instead considered the postprandial response and set a cut-off value of 1·7 mmol/l TAG at peak postprandial phase based on recommendation below this concentration for fasting[36].

The study confirms that contrary to historic considerations, the majority of fasting chylomicrons are within the potentially pro-atherogenic Sf < 20 fraction (70–75 %)[37]. In both HR and NR subjects the most substantive apo B-48 IAUC response was realised in the Sf < 20 fraction, at least twice that compared with the Sf 20–400 or the Sf > 400 fractions. The findings indicate that following the ingestion of dietary fats, chylomicronaemia is principally realised in pro-atherogenic particles, which can be delivered through transcytotic processes directly to the subendothelial space. This study demonstrates that within normolipidaemic subjects significant heterogeneity in the magnitude and duration of hyper-remnantaemia is dependent both on the nature of the fatty acids ingested and the likely individuals' metabolic response. A significant subset of individuals studied had substantially elevated apo B-48 IAUC, specifically indicated when consuming a fat challenge rich in SFA. Paradoxically, the same HR subjects had comparable apo B-48 response and distribution between lipoprotein fractions to NR subjects when challenged with meals containing either RBO or CO. The metabolic aberration in these normolipidaemic HR subjects in response to a PO-enriched fat challenge was not detected in correlation analysis by indices of the metabolic syndrome.

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The study findings reiterate that fasting plasma TAG is a poor indicator of chylomicronaemia and potential postprandial atherogenicity[38]. It emphasises the merits of considering specifically, chylomicron remnant homoeostasis and response in the context of atherogenic risk. Few studies address the latter, despite the majority of life being spent in the postprandial and absorptive state.

Apo B-48, an equivocal marker of chylomicron homoeostasis and distribution, was principally indicated within the smallest and most dense plasma lipoprotein fraction measured (Sf < 20). These particles typically have a diameter 30–55 nm, sufficient to be captured within the non-specific transcytotic vesicles that deliver small constant amounts of plasma lipoprotein to the subendothelial space (<70 nm)[16,20]. It is a reasonable proposition therefore that a greater understanding of chylomicron atherogenicity would require better appreciation of distributional considerations in the absorptive state; a pathophysiological period that occupies the majority of our 24-h-d. In NR subjects, the plasma response and the distribution of apo B-48 was comparable for dietary challenges enriched in either PO, RBO or CO suggesting that vascular exposure was otherwise equivalent. In this context, atherogenic risk might be similar. However, other critical factors in the atherogenic cascade including entrapment, uptake by macrophages, mitochondrial respiratory activity or stimulation of inflammatory pathways may still render chylomicron remnants derived from PO a greater risk than remnants derived from RBO or CO in NR subjects.

In normotriglyceridaemic HR individuals, atherogenic risk may be exacerbated beyond the effects of the fatty acid *per se*, but as a consequence of heightened vascular exposure to chylomicrons. Despite the metabolic aberration in these individuals principally being indicated in the lipolytic cascade, nonetheless the substantive accumulation and response was for the large part realised within the dense Sf < 20 fraction. On the basis that chylomicronaemia was observed in HR subjects following the PO meal and not when challenged with either RBO or CO, we suggest that the metabolic defect is indicated specifically in the metabolism of the particles enriched in PO. Given that the response to PO was not exaggerated in all subjects (only in the HR group), one interpretation is that the defect may not be due to the fatty acid composition in *de novo* chylomicrons. Instead, the defects may be due to the complement of regulating apo that are associated with chylomicrons, which differs between individuals (between NR and HR groups). Differential expression of key apo involved in lipolysis (e.g. apo CII/CIII), or remnant clearance (apo E isoforms) may be a genetic point of synergistic regulation of PO-enriched chylomicrons[39,40]. Meal rich in SFA (PO-based meal) has been reported to modulate the amount of apoC-II, apoC-III and apo E than meal rich in MUFA and PUFA[40]. Since the exaggerated apo B-48 response was observed in the HR group following PO meal but not CO or RBO meal or in the NR group, this raises the possibility of interactions between type of dietary fatty acid (PO-based meal) and individual metabolic response. Alternatively, abundance or isoforms of lipolytic enzymes or receptor clearance proteins may explain the interactive effects with dietary lipids on chylomicron abundance. The latter seems less likely given that there was no difference between NR and HR in response to RBO- or CO-enriched meal challenges. Correlation analysis indicated for both NR and HR subjects, a consistently strong association between fat-free mass and apo B-48 homoeostasis, particularly within the Sf > 400 and the Sf 20–400 fractions. In the absence of a frank lipolytic defect in HR subjects (normal fasting TAG, normal response to RBO and CO), one explanation for the heightened apo B-48 response in HR subjects principally within Sf > 400 and Sf 20–400 (but not in Sf < 20) might be in a modest hydrolytic defect in muscle tissue-associated LPL, given the consistent association between fat-free mass (inverse association with fat mass) and apo B-48 abundance within larger more lipoprotein fractions. The latter is an interesting consideration given that HR subjects had greater fat-free mass and that muscle and adipose tissue LPL are differentially regulated[41]. Insulin stimulates adipose tissue LPL but inhibits expression of muscle tissue LPL. Meals high in SFA content have been reported to decrease insulin sensitivity[42,43] via accumulation of TAG and ceramide in muscle, activation of protein kinase C, NF-κB and the subsequent inflammatory genes[44]. By extension, muscle-mediated LPL activity may have been attenuated in HR subjects[45]. Nonetheless, the data from this study suggest that any such putative tissue-bed lipolytic defect in HR subjects would be a differential interaction between enzyme and substrate (chylomicron), because RBO and CO challenges were comparable between HR and NR subjects[11].

Measures of obesity suggested the HR subjects exhibited greater central obesity and were more overweight than the NR subjects, however there was no evidence of insulin resistance, or the metabolic syndrome based on current combined criteria *per se*. Insulin sensitivity is indicated in the context of glucose metabolism and plasma insulin homoeostasis, surrogate markers that may be inappropriate for identifying key regulatory determinants of chylomicron homoeostasis and metabolism.

In these otherwise healthy HR, the findings indicate that classical markers of plasma lipid homoeostasis provide no valuable insight into chylomicron metabolism, remnant homoeostasis and by extension atherogenic risk. Plasma TAG, cholesterol within the LDL, HDL and non-HDL-cholesterol were otherwise normal and not associated with apo B-48 IAUC measures.

The limitations of the present study are the relatively small number of participants and the unbalanced sex distribution between groups, nonetheless the study was sufficiently powered to demonstrate treatment effects. In the present study, the limited number of postprandial time points for blood collection in each postprandial course may miss an early or late apo B-48 or TAG peak resulting in underestimation or overestimation of apo B-48 postprandial response. However three time points blood collection for oral fat tolerance test have been shown to have high correlation with hourly blood collections following an oral fat tolerance test[46]. The current guidelines adopted by several countries have used non-fasting TAG concentration of 1·89 or 2 mmol/l or more as the cut-off value for increased risk of CVD events[47]. Further examination with a higher cut-off value (i.e. 2 mmol/l) is warranted. Analysis of apo C, apo E and LPL are necessary due to their role in lipolysis and remnant

uptake. In the present study, we did not examine LPL activity and those apo (apo C-II, apo C-III, apo E) therefore we could not determine which key apo or enzymes interacts synergistically with dietary fatty acid in the HR group.

In conclusion, we have demonstrated that some healthy normotriglyceridaemic individuals are susceptible to develop exaggerated chylomicron remnantaemia in response to meals enriched in SFA derived from PO. HR subjects were susceptible to a greater exposure of atherogenic small-sized remnants in response to PO-enriched meals with modest or no effects indicated for RBO and CO. Silent hyper-remnantaemia in response to PO-enriched meals in some normolipidemic subjects may contribute to atherogenic risk, but not in others.

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Conceived and designed the experiments: D. I., J. C. L. M., K. M. S.-C., M. J. S. and A. P. J. Performed the experiments: D. I. and A. P. J. Analysed the data: D. I. assisted by A. P. J. and J. C. L. M. Wrote the manuscript: D. I. and J. C. L. M. Reviewed and critically revised the manuscript for important intellectual content: J. C. L. M., K. M. S.-C, M. J. S. and A. P. J. All authors approved the final version of the manuscript.

The authors declare that there are no conflicts of interest.

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Supplementary result

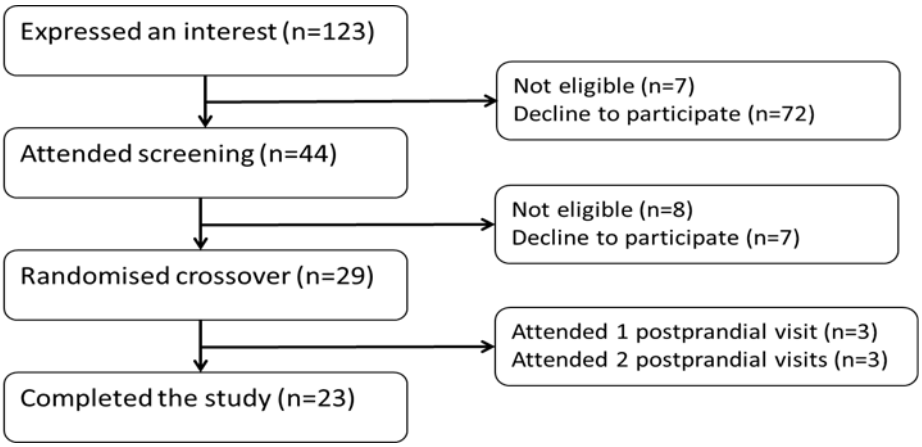

Figure 1. Participant recruitments flow diagram. Twenty-six participants were included in the data analysis. Of them, three subjects only attended 2 postprandial visits (2 subjects did not attend PO meal visit and a subject did not attend CO meal visit). Those who did not attend the PO meal were assigned to the HR group using intention to treat analysis.

CHAPTER 5

Chapter 5: Discussion, limitations and future studies

This chapter provides a general discussion of the findings presented in Chapter 2, Chapter 3 and Chapter 4. In the study described in Chapter 2 the association between apo B-48 concentration, a measure of chylomicron particle number, with a range of metabolic and anthropometric parameters was explored. Fasting chylomicron concentration was strongly associated with triglyceride concentration. Results outlined in Chapter 3 demonstrated the abundance and size distribution of chylomicrons in hypertriglyceridaemic and normotriglyceridaemic subjects. Furthermore in this study the contribution of an accumulation of chylomicron particles to the increased risk CVD in hypertriglyceridaemia was discussed. Whereas in Chapter 4 the effect of consumption of edible oil consumption (palm oil, coconut oil and rice bran oil) on postprandial chylomicron metabolism was investigated in a group of normotriglyceridaemic subjects. In this chapter the potential mechanism by which dietary lipids may regulate chylomicron remnant metabolism is discussed in relation to increased risk of atherosclerosis.

5.1 Chylomicron remnant metabolism and atherosclerosis

A central paradigm for the current atherogenesis model is the retention of lipoproteins in subendothelial tissue (K. J. Williams & Tabas, 1995; K. J. Williams & Tabas, 2005). Sustained concentration of remnant lipoproteins, particle size (Shaikh et al., 1991; M. Simionescu & Simionescu, 1991; Stender & Zilversmit, 1981; D. B. Zilversmit, 1995) and selectivity to the proteoglycans are crucial factors influencing chylomicron deposition in the subendothelial tissue (Flood et al., 2002). Indeed the preferential entrapment of apo B-48 containing lipoproteins in subendothelial tissue and detection of apo B-48 containing lipoproteins in animal and human atherosclerotic tissue have been reported (Nakano et al., 2008; Pal et al., 2003; Proctor & Mamo, 2003; Proctor & Mamo, 1996).

The presence of remnant lipoproteins in the subendothelial tissue will activate monocytes via several mechanisms: accumulation of cytosolic lipid droplets (Bentley et al., 2011), upregulation of apo B-48 receptor mRNA expression (Bermudez et al., 2012; Varela et al., 2011) and LRP1 (Gower et al., 2011), increase expression of leukocyte activation markers (CD11b) (Alipour et al., 2008), upregulation of a number of markers related to monocyte adhesion (Gower et al., 2011; Kawakami et al., 2002), increased production of reactive oxygen species (Bentley et al., 2011) and modulation of migratory and chemotaxis markers (IL-8, MCP1) (Bentley et al., 2011). These mechanisms result in migration and adherence of monocytes to the endothelium.

In subendothelial tissue, chylomicron remnants are taken up by macrophages via receptor-mediated LDLR and LRP1 apo E-dependent (Bejta et al., 2007), apo B-48R apo B-dependent (M. L. Brown et al., 2000), scavenger receptor CD36 (Bejta et al., 2007) and non-receptor mediated pathway via LPL (K. M. Botham & Wheeler-Jones, 2013; Palmer et al., 2005). The uptake of chylomicron remnants via phagocytosis has also been reported (Bejta et al., 2007; Elsegood & Mamo, 2006; Mamo et al., 1996). The lipid (mainly cholesteryl ester) originating from remnants will be esterified and stored in macrophages intracellularly forming foam cells. When these foam cells accumulate, fatty streaks, the hallmark of atherosclerosis, will be formed.

5.2 Metabolic determinants associated with chylomicron metabolism

Given accumulated evidence on chylomicron atherogenicity, remarkably studies that investigate the association between chylomicrons and other metabolic parameters are limited and are specific to a Japanese population (Kinoshita et al., 2009; Sakai et al., 2003; Sato et al., 2009).

As described in Chapter 2, our findings confirmed the result of previous studies that apo B-48 was strongly associated with triglyceride concentration (Kinoshita et al., 2009; Sakai et al., 2003; Sato et al., 2009). However despite a strong association between apo B-48 and triglyceride concentration, the contribution of triglyceride concentration to the variability of apo B-48 concentration adjusted for age and gender was low (30.4%). This suggests that triglyceride concentration is a poor surrogate marker of chylomicron remnant metabolism. The implication of this suggestion is that a subset of normotriglyceridaemic individuals may have an increased chylomicron concentration, and by extension, a higher atherogenic risk, however their susceptibility to develop atherosclerosis may not be detected by the conventional blood / health screening.

A greater fasting apo B-48 concentration has been reported in individuals with MetS (Hanada et al., 2012; Kinoshita et al., 2009; Masuda et al., 2014). Alterations in chylomicron metabolism that lead to an accumulation of chylomicron remnants are poorly defined. However evidence from animal models and insulin resistant subjects demonstrated that the aberration of chylomicron metabolism in MetS is due to an increased production rate of chylomicrons as a consequence of intestinal hypertrophy (Duez et al., 2006) and reduced lipolysis due to a decrease in LPL expression (Annuzzi et al., 2008). In Chapter 2, the extent of association between apo B-48 and triglyceride was higher in subjects with MetS; nonetheless triglyceride concentration was still not predictive of chylomicron homeostasis. The low coefficient of variation for slope of association between

Z scores of apo B-48 and triglyceride in the total population (both MetS and no-MetS group) indicates that triglyceride is a poor surrogate marker of chylomicron homeostasis.

In addition to triglyceride concentration, decreased HDL cholesterol concentration has been recognised as a marker for increased CVD risk. The findings in Chapter 2 demonstrated that after adjustment for gender and age, there was no association between apo B-48 and HDL cholesterol concentration irrespective of the presence of MetS. This suggests that independent factors may implicate HDL cholesterol concentration (i.e. insulin resistance) (Li et al., 2014; Rashid et al., 2003).

The possibility of LDL cholesterol concentration as a predictor for remnant accumulation was also evaluated. Elevated triglyceride concentration in MetS is an indication of impaired lipolysis. Altered lipolysis promotes a longer residency time for TRL in the circulation and thus may affect lipid exchange and remodelling of lipoproteins. The increased transfer rate of cholesteryl ester from HDL to TRL would result in excessive cholesterol influx into the liver and eventually a build-up of cholesterol-rich chylomicrons due to down-regulation of LDLR (Tall, 1993). The clearance of these cholesterol-rich chylomicrons or chylomicron remnants by LDLR require a cluster of apo B/E receptors whereas LDL particles are cleared via a singular receptor interaction (Goldstein & Brown, 2009; Mahley, 1988). Therefore LDL cholesterol would be a poor predictor for remnant accumulation.

A strong association was also observed between apo B-48 and non-HDL cholesterol, a representation of cholesterol content in lipoproteins currently considered to contribute to atheroma development: chylomicron remnant, VLDL remnant and LDL particles (Blaha, Blumenthal, Brinton, & Jacobson, 2008; Miller, Ginsberg, & Schaefer, 2008). The strong correlation between apo B-48 and non-HDL cholesterol concentration indicates that accumulation of apo B-48 leads to the cholesterol enrichment in chylomicron remnants. In MetS where the CETP activity is increased, a greater proportion of cholesteryl ester would be diverted to TRL resulting in low cholesteryl ester content in HDL particles and an elevated concentration in acceptor lipoproteins (chylomicron and VLDL remnants) (Dullaart et al., 2011). The implication of this is that as chylomicron particles become smaller in size, they will carry more cholesterol and thus be more atherogenic (D. B. Zilversmit, 1995). These cholesterol-rich remnants can be sequestered by the arterial walls and induce the formation of lipid-laden monocytes/macrophages.

The NCEP ATP III guidelines have included non-HDL cholesterol concentration as a secondary target therapy taking into account the atherogenic potential of remnants in hypertriglyceridaemic individuals with triglyceride concentration of more than 200 mg/dL

(5.17 mmol/L) (Grundy et al., 2004). The findings in Chapter 2 have demonstrated a strong association between apo B-48 and non-HDL cholesterol concentration in the MetS group in which the average triglyceride concentration was 1.7 mmol/L. Considering that a 1:1 association between the percent of non-HDL cholesterol reduction and the percent of CVD risk reduction (Robinson, Wang, Smith, & Jacobson, 2009) and greater risk of myocardial infarction, ischemic heart disease and ischemic heart stroke in hypertriglyceridemic individuals (B. G. Nordestgaard, 2016) have been reported, it is possible that patients may benefit more from a lower triglyceride target.

Elevated triglyceride, LDL cholesterol and low HDL cholesterol are generally utilised as markers for atherogenic dyslipidaemia and increased cardiovascular risk. However based on our findings, these parameters are not suitable surrogate markers to assess chylomicron remnant homeostasis. The importance of measuring apo B-48 concentration for atherogenic risk remains in particular in groups with higher CVD risk such as MetS.

5.3 Chylomicron size distribution in hypertriglyceridaemia

As described in Chapter 3, subjects with normocholesterolaemic hypertriglyceridaemia exhibited a greater apo B-48 concentration in all fractions with the majority of the increased apo B-48 being in the lipid-poor Sf <20 fraction. It is estimated that lipoproteins found in this fraction have a diameter of less than 55 nm (Mills et al., 1984), a pro-atherogenic lipoprotein size phenotype (N. Simionescu & Simionescu, 1985). Accumulation of small-sized chylomicrons may partly explain the association between elevated fasting triglyceride concentration and increased risk of CVD.

The mechanisms for this association could be via altered chylomicron lipolysis and/or removal from the circulation. The build-up of small-sized chylomicrons may be indicative of altered remnant uptake by the liver. This defect could be due to accelerated de-sulfation of liver HSPG induced by insulin resistance (Chen et al., 2009; K. J. Williams & Chen, 2010). Hepatic over-expression of SULF2 is responsible for the removal of 6-O-sulfates from HSPG structure leading to cell surface and HSPG matrix degradation.

We proposed the possibility that impaired chylomicron metabolism in hypertriglyceridaemia was due to delayed TRL lipolysis. This contention is based on the findings of a greater apo B-48 and triglyceride concentration in the lipid-rich fraction (Sf >400 and Sf 20 – 400) and larger triglyceride to apo B ratio as observed in Chapter 3 indicating a larger lipoprotein size in that fraction (Ruderman, Richards, & Valles de Bourges, 1968). A defect in lipolysis would provide more opportunity for cholesteryl ester triglyceride exchange (Eisenberg, Gavish, Oschry, Fainaru, & Deckelbaum, 1984; Sniderman,

Tremblay, De Graaf, & Couture, 2012) leading to cholesterol enrichment in the TRL fraction (Sniderman et al., 2012). Our observation of a greater amount of cholesterol in the lipid-rich fraction in the hypertriglyceridaemic group supports this contention. Impaired transcription of LPL gene may explain the delay in lipolysis mediated by insulin resistance (Maheux et al., 1997).

From our findings in hypertriglyceridaemic subjects, it is unlikely that the accumulation of apo B-48 in the lipid-rich (Sf >400 and Sf 20 – 400) and lipid-poor (Sf <20) fractions was due to increased production rate of chylomicrons mediated by insulin resistance. In the fasting state, chylomicron production rates depend on the intestinal lipid availability such as from cytoplasmic lipid droplets (Shojaee-Moradie et al., 2013). The source of lipid within this droplets could be from previously ingested dietary fats or endogenous lipid (either from de novo lipogenesis or NEFA absorbed from circulation). We found no difference in NEFA concentration between groups.

5.4 The effect of dietary fatty acid composition on chylomicron remnant accumulation

The findings in Chapters 2 and 3 demonstrated a potential mechanism for the atherogenicity associated with hypertriglyceridaemia. We found that in the fasting state the majority of apo B-48 containing lipoproteins were present in the Sf <20 fraction (Irawati, Mamo et al. 2015). However the skewed distribution of chylomicron concentration suggests the possibility of genetic variability in chylomicron metabolism either in the basal production rates and/or clearance of chylomicron.

The findings in Chapter 4 confirm the results of Chapter 3 that showed the majority of apo B-48 in normotriglyceridaemic subjects in fasting was in the pro-atherogenic Sf <20 fraction. In the postprandial state, the apo B-48 response was substantially contributed by the apo B-48 in the Sf <20 fraction. This finding highlights the substantial exposure of small-sized pro-atherogenic chylomicron remnants on the arterial walls in the postprandial state. The surge of postprandial apo B-48-containing particles in the pro-atherogenic fraction signifies lipoproteins that can directly enter the subendothelial space. With the majority of time spent by humans in the postprandial state, this finding underscores the importance of postprandial remnantaemia on the increased CVD risk.

The findings from the study described in Chapter 4 also demonstrate that the heterogeneity in the extent of hyper-remnantaemia is influenced by the nature of fatty acids and the genetic determinants of the response. We identified a subset of subjects who despite being normotriglyceridaemic in the fasting state exhibit elevated postprandial triglyceridaemia (HR, hyper-responder group). The HR group exhibited a substantial

accumulation of chylomicron particles in the pro-atherogenic Sf <20 fraction following PO-derived SFA meal despite no evidence of chylomicron remnant accumulation in the fasted state. This implies an involvement of genetic determinants in chylomicron homeostasis in response to a meal.

Since the exaggerated response was only observed following PO meal challenge, not when challenged with either RBO or CO meal, our interpretation is that the metabolic defect of the HR subjects is specifically associated with the metabolism of chylomicron rich in PO-derived fatty acids. The possible genetic point of synergistic regulation of PO-rich chylomicrons is either differential expression of key apolipoproteins involved in lipolysis (i.e. apo CII/CIII) or clearance (apo E isoforms) (Schwarzova, Hubacek, & Vrablik, 2016) or a modest defect in hydrolysis mediated LPL in the muscle tissue. Meals rich in SFA have been reported to decrease insulin sensitivity (Galgani et al., 2008; Riccardi et al., 2004) via accumulation of diacylglycerol and ceramide in muscle, activation of protein kinase C, nuclear factor-κB and the subsequent inflammatory genes (Kennedy et al., 2009). Therefore muscle mediated LPL activity may be have been attenuated in HR subjects (Annuzzi et al., 2008). In addition, SFA-rich meals also induce greater apo C-III expression compared to unsaturated fatty acid (K. G. Jackson et al., 2005). The possible involvement of increased/decreased lipolytic enzyme or the presence of isoforms of receptor clearance proteins is less likely given the comparable apo B-48 response between NR and HR groups in response to RBO or CO meal challenge.

The current guidelines adopted by several countries have used nonfasting triglyceride concentration of 1.89 mmol/L or 2 mmol/L or more as the cut off for increased risk of CVD events. From our postprandial study, we observed that using a cut-off of plasma triglyceride concentration of 1.7 mmol/L or more at 4-hour postprandially was predictive of an accumulation of small-sized chylomicrons following a PO-rich meal challenge.

These subjects would, according to the conventional CVD risk measures, have a very low risk of CVD. However our study provides evidence of a subset of these who exhibit impaired postprandial lipaemia following SFA-rich meals with an increased CVD risk expected due to an accumulation of postprandial chylomicron remnant particles. Furthermore, in this study, the classical markers of lipid homeostasis (triglyceride, HDL cholesterol, LDL cholesterol and non-HDL cholesterol) were within normal range in both NR and HR groups and those markers were not associated with apo B-48 response. This implies that those markers provide no valuable insight into chylomicron remnant metabolism and by extension the atherogenic risk.

5.5 Conclusion

An increased concentration of plasma triglyceride has been widely accepted as an independent risk factor for CVD. However it is unclear whether the higher risk of CVD is attributed to plasma triglyceride or the lipoprotein particles on which it resides. A large body of evidence has demonstrated the atherogenicity of chylomicrons/chylomicron remnants in animal models and human studies. To initiate atherogenesis, lipoproteins need to be retained in the subendothelial tissue. Obligatory properties for trapping remnants in the subendothelial matrix include a sustained concentration, appropriate particle size and selectivity of lipoproteins to subendothelial proteoglycan. Alterations in chylomicron assembly, lipolysis and clearance affect the particle size and the concentration in the circulation. The type of dietary fats is also known to affect lipid partitioning, lipolysis and clearance rate. Hence studies included in this thesis explored the interaction between metabolic determinants, dietary fats and chylomicron metabolism, specifically taking into consideration the distribution of apo B-48 in lipoprotein fractions.

Our observation confirmed that the fasting apo B-48 concentration, a measure of chylomicron particle number, was strongly associated with the fasting triglyceride concentration. We noted a stronger association in subjects with MetS. The well-reported relationship between elevated fasting triglyceride concentration and a higher risk of CVD may partly be explained by our observation of a chylomicron build-up in subjects with hypertriglyceridaemia including large and the small-sized pro-atherogenic remnants.

The association between apo B-48 the measured cholesterol profiles was variable. Apo B-48 was moderately or weakly associated with non-HDL cholesterol and HDL cholesterol concentration. In individuals with MetS, the association between apo B-48 concentration and non-HDL concentration was stronger whereas the association with HDL cholesterol was not significant suggesting independent factors implicate HDL cholesterol concentration. However those parameters (HDL cholesterol and non-HDL cholesterol) were poor surrogate markers for chylomicron homeostasis due to the low contribution of those markers on the variability of apo B-48 concentration. The need to measure apo B-48 concentration to determine atherogenic risk remains, especially in population with higher CVD risk such as in MetS.

In the postprandial state, the substantial chylomicron response is within the pro-atherogenic Sf <20 fraction indicating that following ingestion of fatty meal a considerable proportion of chylomicrons are able to directly enter the arterial walls. We also found that some healthy normotriglyceridaemic individuals (hyper-responders) exhibited exaggerated chylomicron remnantaemia in response to a meal rich in SFA derived PO substrate but not

when challenged with RBO or CO meal. This indicates the synergistic regulation between physiologic/genetic determinants and the nature of fatty acids.

The greater proportion of circulating apo B-48 in the dense fraction (Sf <20) observed in both fasting and postprandial states puts individuals at risk of exposure of small sized remnants on the vascular bed. In conditions where clearance of chylomicron remnants is altered, such as hypertriglyceridaemia, the exposure is heightened. However the concentration of circulating remnants cannot be predicted by the classic metabolic parameters or components of MetS. Furthermore we also found that some normotriglyceridaemic individuals exhibit silent hyper-remnantaemia in response to PO-enriched meals but not in others suggesting interactive effects of dietary fatty acid composition on remnant chylomicron response. Therefore our findings underscore the importance of examining chylomicron metabolism especially in people with heightened risk of CVD.

5.6 Limitation of the studies

There are limitations to the design and some of the methodologies in this thesis which need consideration. The cross-sectional study design in Chapter 2 provides strong associations but future intervention trials are needed for causative interpretation of our outcomes. In Chapter 4, we used PO meal challenge to assign subjects into hyper- and normo-responder group instead of utilising the commonly used liquid formula for fat tolerance test (Kolovou, Mikhailidis et al. 2011). This allowed us to test the response to a mixed meal (~40 g fat) that may typically be consumed by free living individuals (Silva, Wright et al. 2005). Consuming 75 g fat for assessing postprandial lipaemia as suggested may have overshadowed subtle differences due to an exaggerated postprandial response (Lairon 2008). In the postprandial study (Chapter 4), the postprandial response was assessed based on the changes in triglyceride or chylomicron concentration measured at three time points (0, 4 and 8 hours). The limited number of time points for the postprandial blood collection could have affected the calculation of the postprandial response if the postprandial peak was missed. However we chose three blood collection time points to minimise participant discomfort, given the evidence of a high correlation between three and the conventional hourly time points in oral fat tolerance tests (Maraki, Aggelopoulou et al. 2011).

The studies included in this thesis have used immunological based methods to quantify lipoprotein of interest, apo B-48. The limitation of this method is the potential of confounding effects originating from lipid interference with the binding of antibodies with protein epitopes. This would result in underestimation of protein concentration. In the

study in Chapter 3, there was a significant greater apo B-48 concentration between normotriglyceridaemic and hypertriglyceridaemic subjects. If there were lipid interference with the binding of antibodies and apo B-48 epitopes, the significant difference in apo B-48 concentration may not have been detected. One of the suggestions is performing delipidation however this requires complex procedure and may cause protein degradation (Mills, Lane et al. 1984). Apo B-48 concentration in Sf <20 fraction in the studies in Chapter 3 and 4 was determined by calculation rather than direct measurement. This may be the limitation of this study.

The participants recruited in this study are from the older age range population. Thus it may not represent the variation or the extent of association between apo B-48 and other metabolic and anthropometric parameters in general population. However Australian population is an ageing society and most metabolic issues that impinge on CVD are usually evident from middle age range. In this respect, the studies included in this thesis are very useful. The small number of participants in the MetS group (Chapter 2) may overestimate the association between apo B-48 concentration and metabolic determinants. The limited number of participants (in HR and NR group) was also happened when conducting the postprandial study (Chapter 4). The difficulty in recruiting participants for this study due to the nature of the study (cross-over design with long day visits) may limit the power of the study. There were a number of considerable differences in apo B-48 response observed following the test meals but these were not statistically significant. If we had more study participants, these differences might become significant. We are also unable to gender-match the subjects between groups in the postprandial study (Chapter 4). This would have enabled the identification of more subtle sex differences in lipid /apo B-48 metabolism that have been noted.

5.7 Future studies

This thesis for the first time provides evidence on the interactions between type of dietary fat, metabolic determinants and chylomicron size distribution in particular the dense fraction. This data is informative for research investigating postprandial lipaemia and CVD risk and may contribute to the method development in utilising fat tolerance tests in future research.

From our findings, with the same amount of fat ingested, the type of dietary fat affected the postprandial lipaemia suggesting the importance of the nature of ingested fatty acids on chylomicron response. The same amount of total fat in the SFA-rich meals but different chain lengths influenced the magnitude of postprandial lipaemia. Our findings also

showed that ingesting the same type and amount of dietary fat has differential effects on some normotriglyceridaemic subjects. Saturated fat is not a single entity and the merits in investigating the effect of SFA on health outcomes depend on the characteristics of the subjects consuming it, the dietary fatty acid composition within the meal, the food matrices and the comparators. This underscores the complex interactive effect of the nature of ingested fatty acids with genotype on chylomicron response and its risk for atherogenesis. We encourage further studies examining postprandial lipaemia following SFA challenge with different chain length and dose in a variety of metabolic conditions.

Studies are warranted to assess the association between small-sized chylomicron concentration with plasma inflammatory markers and lipid peroxidation and oxidative stress markers following SFA challenge.

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This thesis consists of 3 first author peer-reviewed articles (2 has been published and 1 under consideration) in quality international scientific journals. All articles included in this thesis are related directly to the objectives of this thesis. This appendix presents a statement of contribution of each article signed by author and co-authors. All authors have read and approved the final version of manuscripts before submission and publication.

Appendix A also presents copyrights for re-production of journal article used in and authorisation statements from copyright owners for figures used in this thesis.

Article 1:

Irawati D, Mamo JCL, Dhaliwal SS, Soares MJ, Slivkoff-Clark KM, James AP (2016). Plasma triglyceride and an inverse association with high-density lipoprotein cholesterol are poor surrogate markers of pro-atherogenic chylomicron remnant homeostasis in subjects with the metabolic syndrome. *Lipid and Health Disease*, In press.

Has been accepted, under revision, open access.

Deasy Irawati undertook the literature review, performed the laboratory analysis, wrote the first draft of the manuscript data analysis, presentation format and preparation of the tables. John C.L. Mamo contributed to the interpretation of the data, revised the manuscript for important intellectual content and contributed to the appraisal of the manuscript. Satvinder Dhaliwal assisted the data analysis and appraisal of the manuscript. Mario Soares contributed to the interpretation of the data and appraisal of the manuscript. Karin M. Slivkoff-Clark contributed to the appraisal of the manuscript. Anthony P. James conceived the idea of the study, contributed to the literature review, assisted the data analysis and appraised the manuscript.

Article 2:

Irawati D, Mamo JCL, Soares MJ, Slivkoff-Clark KM, James AP (2015). Hypertriglyceridemic subjects exhibit an accumulation of small dense chylomicron particles in the fasting state. *Atherosclerosis,* 243(1), 236-241.

Deasy Irawati undertook the literature review, the primary writing of the manuscript, performed the laboratory analysis, data analysis, presentation format and preparation of the figures. John C.L. Mamo developed the study design, consideration of the data and appraisal of the manuscript. Mario Soares assisted the data analysis and appraisal of the manuscript. Karin M. Slivkoff-Clark contributed to the appraisal of the manuscript. Anthony P. James conceived the idea of the study, contributed to the development of the study design, assisted the data analysis and appraised the manuscript.

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Irawati D, Mamo JCL, Soares MJ, Slivkoff-Clark KM, James AP. Dietary fat and genetic determinants of plasma chylomicron remnant homeostasis in normolipidemic subjects: Insight into atherogenic risk. *British Journal of Nutrition, 117*(3), 403-412.

Deasy Irawati undertook the literature review, designed the study, development of the test meals, recruited the participants, performed the clinical study and the laboratory analysis, wrote the first draft of the manuscript data analysis and interpretation of the data, presentation format and preparation of the tables and figures. John C.L. Mamo contributed to the design of the study, interpretation of the data, revised the manuscript for important intellectual content and contributed to the appraisal of the manuscript. Mario Soares contributed to the design of the study and data analysis and appraisal of the manuscript. Karin M. Slivkoff-Clark contributed to the development of the test meals and appraisal of the manuscript. Anthony P. James conceived the idea of the study, contributed to the development of study design, participant recruitment, interpretation of the data and appraised the manuscript.

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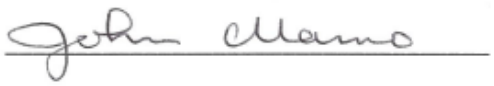

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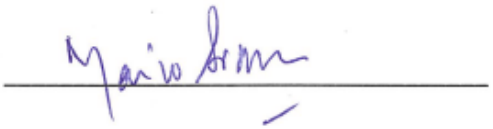

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Author:	Dennis D. Black
Publication:	Am J Physiol-Gastrointestinal and Liver Physiology
Publisher:	The American Physiological Society
Date:	Sep 1, 2007

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Appendix B: Study design and information for participants

INFORMATION TO VOLUNTEERS

Project title: Characterisation of chylomicron size distribution in subjects with metabolic syndrome: The effect of dietary fatty acid composition

"*This study has been approved by the Curtin University Human Research Ethics Committee (Approval Number* ***HR 151/2013****). The Committee is comprised of members of the public, academics, lawyers, doctors and pastoral carers. If needed, verification of approval can be obtained either by writing to the Curtin University Human Research Ethics Committee, c/- Office of Research and Development, Curtin University, GPO Box U1987, Perth, 6845 or by telephoning 9266 2784 or by emailing hrec@curtin.edu.au*"

Thank you for agreeing to participate in the above-mentioned study. This document is to assist you to understand what is involved in this project. There is a consent form attached, which you will be asked to sign at the time of your visit. Please be sure that you fully understand the nature of the study and any potential risks before signing.

Background information

Metabolic syndrome (MetS) is a cluster of metabolic abnormalities characterised by slightly elevated blood sugar levels, raised blood pressure, elevated triglyceride (blood fats) concentration, low high density lipoprotein (HDL) cholesterol level and obesity (particularly increased waist circumference). People with MetS have higher risk to develop diabetes and cardiovascular disease. In Australia, up to approximately one third of the population is diagnosed with metabolic syndrome. These numbers are only expected to increase in the coming years. The focus of this study is the size distribution of particles called chylomicrons which are responsible for transporting fat from our diet throughout the body. We are interested in monitoring this metabolism in people with and without metabolic syndrome, and also how the type of fats you consume affect this metabolism.

Study aim

The aim of this study is to determine whether different fats are metabolised differently in people with and without metabolic syndrome and to determine whether there is a greater risk of cardiovascular disease associated with consumption of certain dietary fats.

What does the study involve?

- You will need to attend a screening visit and 3 study days each lasting approximately 8 hours, separated by a period of 2 – 4 weeks.
- You will need to consume low fat diet on the day before each study day and consume the same amount and food items on those days.
- You will need to come on fasting on the study days.
- We will collect your blood before the meal and 2 times after the meal (4 and 8 hour after the meal), 12 mL (1 table spoon) of blood in each time point.
- You will need to complete a food frequency questionnaire at the beginning of the study to capture your long term dietary intake.
- You will need to fill in physical activity questionnaire during the study.

- You will receive a voucher as an appreciation for your time commitment at the completion of the study.

Study plan / requirements

Visit 1 (30 minutes) will give you an opportunity to meet the investigators and have the study procedures and protocol fully explained to you. We will ask you a series of questions about your medical history and current diet and also assess your veins in terms of their suitability for collection of multiple blood samples. If you agree to participate in the study, we will need you to sign the consent form and fill in a screening survey.

Visit 2, 3 and 4 will be 8-hour visits to the clinical rooms of the School of Public Health (room 209 building 400), Curtin University. The visits will be separated by at least 2 – 4 weeks between each visit. On each of these visits, you will be given the same type of breakfast meal but with different fatty acid composition.

Before each of these visits, you will be required to fast overnight for at least 12 hours (you can still drink plain water) and have a sufficient sleep (approximately 8 hours) on the night before the visit. On the day prior to each test day, you will need to consume a diet that is low in fat. We will also ask you to consume the same amount and food items for breakfast and lunch on the day before each postprandial test day. You will be provided with a suitable evening meal. This is to ensure that you consume the same amount and food items on that day and when you arrive on the test day, you have completely metabolised any fat that you consume previously. In addition, the evening before the test day, you will need to consume your dinner before 9 pm and fast from that time.

During the 8-hour visit, we will be taking fasting and postprandial blood samples of 12 mL (1 tablespoon) at 4 and 8 hours following the meal by venepuncture for chemical tests of changes in blood fat particles, cholesterol, triglyceride, non-esterified fatty acids, glucose and insulin. In total, we will only be taking around 40 mL (approximately 3 tablespoons) of blood in each visit. The blood collection will be conducted by a certified person (phlebotomist). Fat body composition measurement will be measured on one of the study days. In the rare event of a medical emergency, fully qualified physician and nursing staff from Curtin Health Service will be available.

Possible side effects

The blood collection on three separate time points in visit 2, 3 and 4 may cause discomfort as we will use a needle to collect the blood. Although we will try to collect the blood from different sites, it is possible that you may find the number of blood samples collected an uncomfortable experience. Therefore, it is important to inform us if this is the case and to know that you are free to withdraw from the study at any stage. For us, your comfort during the procedure is of more concern than our ability to collect all the blood samples. In some people, slight bruising and tenderness may appear afterwards at the site of venepuncture. These side effects are only minor and will return to normal in a matter of days.

The volume of blood collected should not cause you any risk of becoming anaemic. Moreover the visits have been spaced with minimal 2 week period between each visit so new red blood cells will be ready to enter the circulation. However we advise you not to donate blood during the period of study. If you need to do any routine clinical test with blood collection, this is not a problem.

Ability to withdraw from study

You should be aware that your participation in this study is entirely voluntary. You are free to withdraw from this study at any stage whether that is before, during or after any blood collection. You must not feel any pressure to complete the study.

Benefits to the participants

You will gain useful information about your current lipid and cholesterol profiles, anthropometric details (height, weight, body mass index) and fat body composition.

Your participation in this study will provide us with important data enabling us to investigate further the role the type of fatty acid composition consumed in one meal plays on cardiovascular disease risk factors. In Australia, cardiovascular diseases are a major cause of death and disability. It is expected that the findings of this study could provide practical outcomes in terms of nutritional advice based on the underlying mechanism involved in chylomicron metabolism and metabolic syndrome.

Confidentiality

Any information that you provide us regarding your personal information will be de-identified before being stored securely, to protect your privacy. The security of standards of all personal information follows Curtin data management guidelines. Although the design of this project is complete, it is possible that the results from this study being also used for collation purposes in future studies on a similar topic the results will not be identifiable.

FURTHER INFORMATION

All information from the participants is confidential. Participation is purely voluntary and you may withdraw from this study at any point in time.

If you have any further queries, please do not hesitate to contact:

Ms Deasy Irawati, MD. MSc

Telephone: 0452 606 488 OR email: deasy.irawait@postgrad.curtin.edu.au

REMINDERS FOR PARTICIPANTS

Project title: Characterisation of chylomicron size distribution in subjects with metabolic syndrome: The effect of dietary fatty acid composition

*"This study has been approved by the Curtin University Human Research Ethics Committee (Approval Number **HR 151/2013**). The Committee is comprised of members of the public, academics, lawyers, doctors and pastoral carers. If needed, verification of approval can be obtained either by writing to the Curtin University Human Research Ethics Committee, c/- Office of Research and Development, Curtin University, GPO Box U1987, Perth, 6845 or by telephoning 9266 2784 or by emailing hrec@curtin.edu.au"*

Thank you for agreeing to participate in the above-mentioned study.

Expected date for:

PP1: | **PP2**: | **PP3**:

To ensure the same study conditions occur before each visit, please follow the protocol outlined below.

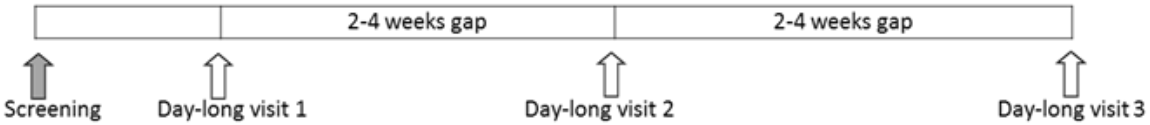
1. Do not undertake any vigorous physical activity 24-hours before the test day.
2. Do not undertake any exercise (not even a light walk) the evening before the visits.
3. On the day before visit 2, 3 and 4, please follow similar low levels of physical activity and consume the same amount and food items for breakfast and lunch. Dinner meal will be provided by the investigator.
4. Please finish eating dinner before 9:00 pm and only drink water (plain water).
5. Please ensure you have a good night sleep (≈ 8 hours).
6. The morning of the visits:
    a. Do not consume any food and only drink plain water.
    b. If possible have a friend or family member drive you to the centre at Curtin.
    c. If you drive yourself, you will be provided with a parking permit for the day.
    d. If you are running late, please do not panic (I need you to be calm on the morning of each visit).
    e. Wear light comfortable clothing.
    f. Please bring a book, magazine or iPod to make yourself comfortable during the visits.

If you have any questions about this study protocol, please do not hesitate to contact Deasy Irawati 0452 606 488 or leave a message on that number.

Procedure for CSIMS Study:

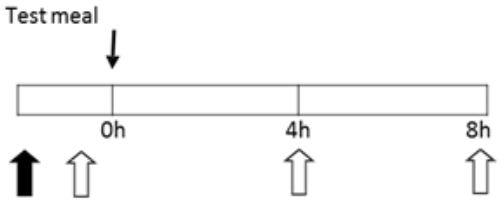
Venue: Building 400 room 209, School of Public Health, Curtin University

Time between visits (depend on your schedule):



During screening (30-45 minutes): you'll be measured for your body height, weight, waist circumference, fat body percentage, blood pressure and fasting blood sample will be collected.

During each day-long visit (8 am – 4 pm or 9 am – 5 pm):



⇧ Venous blood sample;

⬆ Measure your weight, waist circumference, and blood pressure.

Important notes:

- The blood will be collected by a certified phlebotomy
- Participants are free to withdraw from this study at any stage whether that is before, during or after any blood collection.
- Participant's comfort during the procedure is of more concern for us.