University of Alberta

Characterization of a Novel Model of Intestinal Lipoprotein Overproduction and the Impact of n-3 PUFA Supplementation

by

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Abstract:

Overproduction of intestinal chylomicrons (CM) has been proposed to contribute to fasting and post-prandial (PP) dyslipidemia and may accelerate the development of cardiovascular disease (CVD) during obesity, insulin resistance (IR) and diabetes. However, the impact of morphological changes in intestinal mucosa structure have not been investigated during IR and intestinal dyslipidemia. The first objective of this thesis was to characterize intestinal villi morphology and to determine whether a morphological relationship exists with enterocytic apoB48 (a marker of CM), and intestinal lymph secretion of apoB48 in the obese and IR JCR:LA-cp rat. The second objective was to assess the impact of n-3 PUFA supplementation on PP dyslipidemia in the JCR:LA-cp rat. Intestinal hypertrophy was observed in IR rats, corresponding to an increase in intestinal and lymphatic apoB48 expression. Further, a dietary intervention of n-3 PUFA showed lower PP plasma concentrations of apoB48 and PP plasma inflammatory markers. We conclude that intestinal hypertrophy may contribute to intestinal CM overproduction during obesity and IR. Additionally, dietary n-3 PUFA improves PP lipemia and the associated PP inflammatory response in the JCR:LA-cp rat model.

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List of Abbreviations:

ACC	Acetyl CoA Carboxylase
Apo	Apolipoprotein
AUC	Area Under the Curve
BMI	Body Mass Index
CEPT	Cholesterol Ester Transfer Protein
CM	Chylomicron
CRP	C-reactive Protein
CVD	Cardiovascular Disease
ECL	Enhanced Chemiluminescence
ELISA	Enzyme Linked Immunosorbent Assays
ER	Endoplasmic Reticulum
FAS	Fatty Acid Synthase
HDL	High Density Lipoprotein
Hp	Haptoglobin
iAUC	Incremental area under the curve
LCAT	Lecithin Cholesterol Acyl Transferase
LBD	Lipid Balanced Diet
LBP	Lipopolysaccharide Binding Protein
LDL	Low Density Lipoprotein
LPL	Lipoprotein Lipase
PUFA	Polyunsaturated Fatty Acids
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SREBP	Sterol Regulatory Element Binding Protein
TG	Triglyceride
VLDL	Very Low Density Lipoprotein

CHAPTER 1: Literature Review

1.0 Atherosclerosis:

Atherosclerotic cardiovascular disease is the number one cause of death in Western societies and is rapidly becoming prominent in underdeveloped nations (Stipanuk, 2000). Atherosclerosis is a complex pathological process that is characterized by the deposition of cholesterol, fatty acids, cellular waste, fibrin and calcium within the arterial wall forming a hardened lesion or plaque. Eventually, this process results in the narrowing and hardening of the arteries (Libby et al., 2002). For more than 100 years it has been well recognized that atherosclerotic lesions are laden with lipids (Ignatowksi, 1908). Indeed, the development of arterial lesions involves the retention and oxidation of cholesterol-rich lipoprotein particles within the arterial endothelium (Libby et al., 2002) (Figure 1-0).

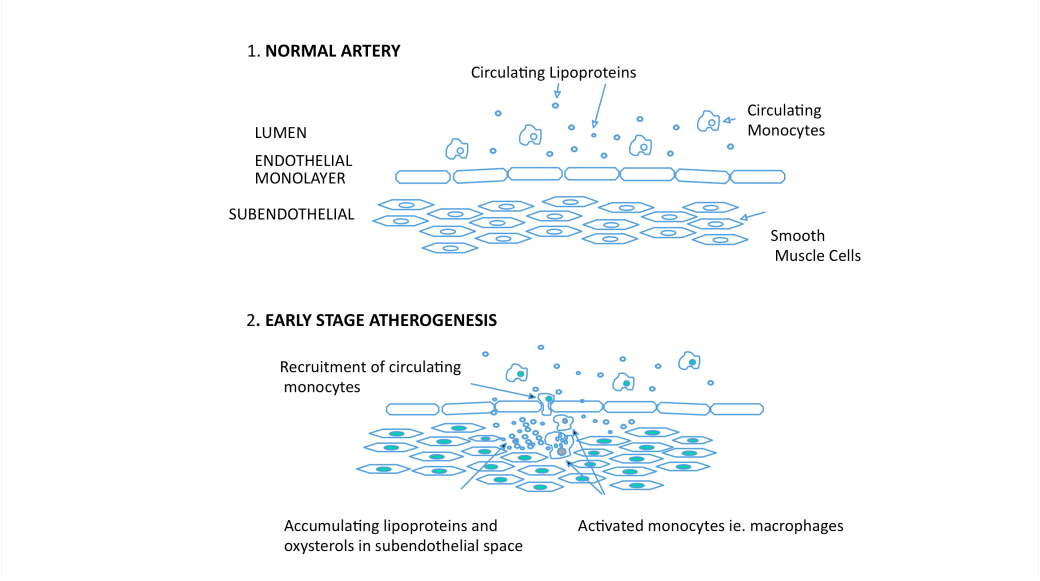

Figure 1-0. Initial Stages of Atherosclerosis. Early stages of atherosclerotic development involves the accumulation of lipoproteins into the subendothelial space followed by the recruitment of monocytes.

A rapidly growing base of knowledge in the cellular and molecular biology of the artery demonstrates that lipids and lipoproteins have a central role in understanding the atherogenic process (Nordestgaard and Tybjaerg-Hansen, 1992). It is thought that the retention and accumulation of oxidatively modified lipoproteins signal endothelial cells and induce the recruitment of cell adhesion molecules and monocytes to the arterial vessel. Circulating monocytes transmigrate into the subendothelial space and are activated and transformed into macrophages (Glass and Mitzhum, 2001). Macrophages further oxidize and can internalize lipoproteins through one or more receptor-mediated pathways in order to regulate and/ or remove the accumulation of lipid and cholesterol from blood vessels.

However, the over-accumulation of cholesterol-loaded lipoproteins induces uncontrolled macrophage differentiation, resulting in the development of foam cells (Glass and Mitzhum, 2001). The formation of foam cells is followed by the proliferation of smooth muscle cells, resulting in the thickening of the arterial wall. Over time, luminal growth of the arterial lesion occludes the supply of blood to the tissues leading to ischemia and infarction.

The current understanding of atherogenesis considers that the passage and retention of lipoproteins in the arterial subendothelial space is the first critical step in the atherosclerotic process. As a result, risk factors such as plasma concentration of low-density lipoprotein (LDL) have been used to assess the risk of developing cardiovascular disease (CVD) (Genest et al., 2003). In the fasted state, the LDL cholesterol (LDL-C) represents the major fraction in blood and a concentration greater than 130 mg/dL or 3.3 mmol/L corresponds with borderline to high risk of developing CVD (American Heart Association, 2008). More recently, there is evidence during the fed state (or post-prandial state) that chylomicron (CM)-remnants play a significant role in atherogenesis. Along with LDL, CM-remnants can permeate and are retained in the arterial wall, which is hypothesized to initiate and course progression of atherosclerosis (Proctor et al., 2002, Proctor et al., 2004). Impaired lipoprotein metabolism is suggested to contribute to increased arterial exposure and arterial retention of CM (Proctor and Mamo, 2004; Nordestgaard and Tybjaerg-

Hansen, 1992). However, the mechanisms involved in impaired CM metabolism, intestinal CM production/clearance and their contribution to atherosclerosis are still not well understood, these concepts form the focus of this MSc thesis. In order to appreciate the context of my hypotheses, Chapter 1 includes the outline of the fundamentals of lipid and lipoprotein metabolism.

1.1 Lipoprotein Metabolism

1.1.1 Lipid Metabolism

Lipids are often classified into triglyceride (TG), free fatty acids (FFA), phospholipids and cholesterol, each having a variety of biological functions (Nelson and Cox, 2000). Triglycerides are involved in energy metabolism as well as energy storage, while FFA can function as intracellular signals (eicosanoids) (Stipanuk, 2000). Similarly, phospholipids are important components in cell signaling, behaving as signal transduction molecules. In addition, phospholipids are essential to cell membrane synthesis and structure (Stipanuk, 2000). Cholesterol is also a structural component of cell membranes and is essential for the synthesis of bile acids and hormones (steroids) (Stipanuk, 2000).

Lipid metabolism involves the digestion, absorption, synthesis (intestinal and hepatic) as well as the transport of TG, fatty acids, phospholipids and cholesterol. To fully understand how aberrant lipid metabolism contributes to CVD it is important to have a fundamental appreciation of the normal physiological processes of lipoprotein metabolism (Figure 1-1).

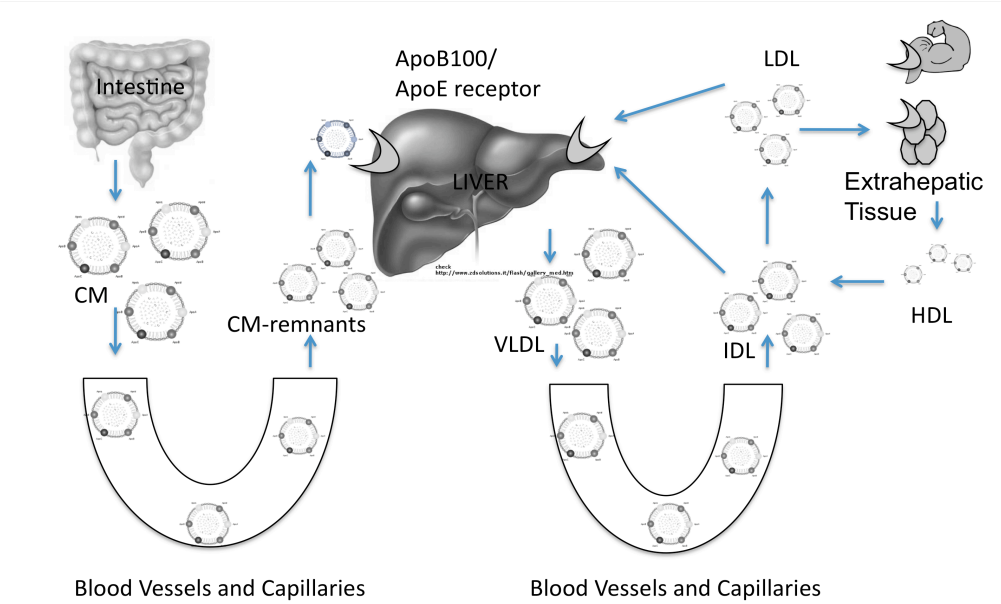

Figure 1-1. Lipoprotein Metabolism in Humans. Exogenous lipids move through the body in intestinally derived chylomicrons (CM) that distribute TG. The hydrolyzed CM, CM-remnant, is then recycled back to the liver where it is reassembled with endogenous lipids to form very-low density lipoprotein (VLDL). VLDL moves through the body as lipases delipidate the particle and form intermediate density lipoproteins (IDL) and eventually low-density lipoproteins (LDL). LDL is taken up by extra-hepatic tissue and cholesterol is distributing. Excess cholesterol deposited into extra-hepatic tissue is removed by high-density lipoprotein (HDL) (Voet, Voet and Pratt, 2002).

1.1.2 Lipoproteins

The hydrophobic nature of lipid requires that these molecules be transported throughout the body by lipoproteins (Nelson and Cox, 2000). Structurally, the outer membrane of lipoproteins are composed of phospholipid and apoprotein(s) while the internal core carries cholesterol, fatty acids and lipophylic vitamins (Voet, Voet and Pratt, 2002). Lipoproteins are classified based on their density but can also be identified

by their TG and cholesterol composition and/or associated apoproteins as seen in Table 1-1.

Lipoproteins	Particle Size (nm)	Core TG (%)	Core Cholesteryl Ester (%)	Apoproteins
CM	100-1000	84-89	3-5	A-I, A-II, B-48, C-I, C-II, C-III, E
VLDL	30-80	50-65	10-15	B-100, C-I, C-II, C-III, E
LDL	18-28	7-10	35-40	B-100
HDL	5-15	3-4	12	A-I, A-II, C-I, C-II, C-III, D, E

Table 1-1. Characteristics of the major classes of lipoproteins in human plasma. (Modified from Voet, Voet and Pratt, 2002).

1.1.2.1 Chylomicrons

Chylomicrons (CM) are continuously synthesized in the intestine and are responsible for transporting exogenous and endogenous lipid from the intestine to the circulation. CM are considered the least dense of all lipoproteins ($d < 1.006$ g/mL) and are heterogeneous in size (native CM having a diameter between 100-1000 nm and CM-remnants with a diameter of 50-80 nm) with an internal core consisting of 85-89% TG and 3-5% cholesterol ester (Hussain et al., 1996). Despite the various apoproteins associated with the intestinally derived lipoprotein, in humans apolipoprotein-B48 (apoB48) is unique to CM. ApoB48 is essential to the assembly of intestinal lipoproteins and is not transferred to other lipoproteins during lipolysis (Kane et al., 1980). We also know that there is

only one apoB48 molecule per particle (Kane et al., 1980). Consequently in humans, apoB48 is used as an exclusive marker of CM particle concentration.

For the purposes of hypotheses to be tested in this thesis a rodent model, (the JCR:LA-*cp* rat) is used, therefore it is also important to discriminate between the metabolism of apoB48 in humans *vs*. rodents. In rats and mice, apoB48 is synthesized in both the intestine and the liver (Liu et al., 1991) and is therefore associated with both intestinal and hepatic derived lipoproteins (CM and very-low density lipoproteins (VLDL)). Hepatic production of apoB48 by rodents is taken into consideration and accounted for throughout this thesis.

1.1.2.1.1 Chylomicron Production and Secretion:

Chylomicron assembly has been considered as a two-step process that involves the synthesis of primordial lipoproteins and core expansion (Cartwright and Higgins, 2001; Hussain, 2000). Cellular fractionation experiments demonstrate that TG and cholesterol are mainly associated with the trans-Golgi fraction whereas phospholipids and apoB48 are retained in the membrane fraction of the smooth endoplasmic reticulum (Cartwright and Higgins, 2001). The findings suggest that particle assembly begins in the membrane of the smooth endoplasmic reticulum while modification of the primordial lipoprotein takes place in the Golgi apparatus of the enterocyte (Figure 1-2).

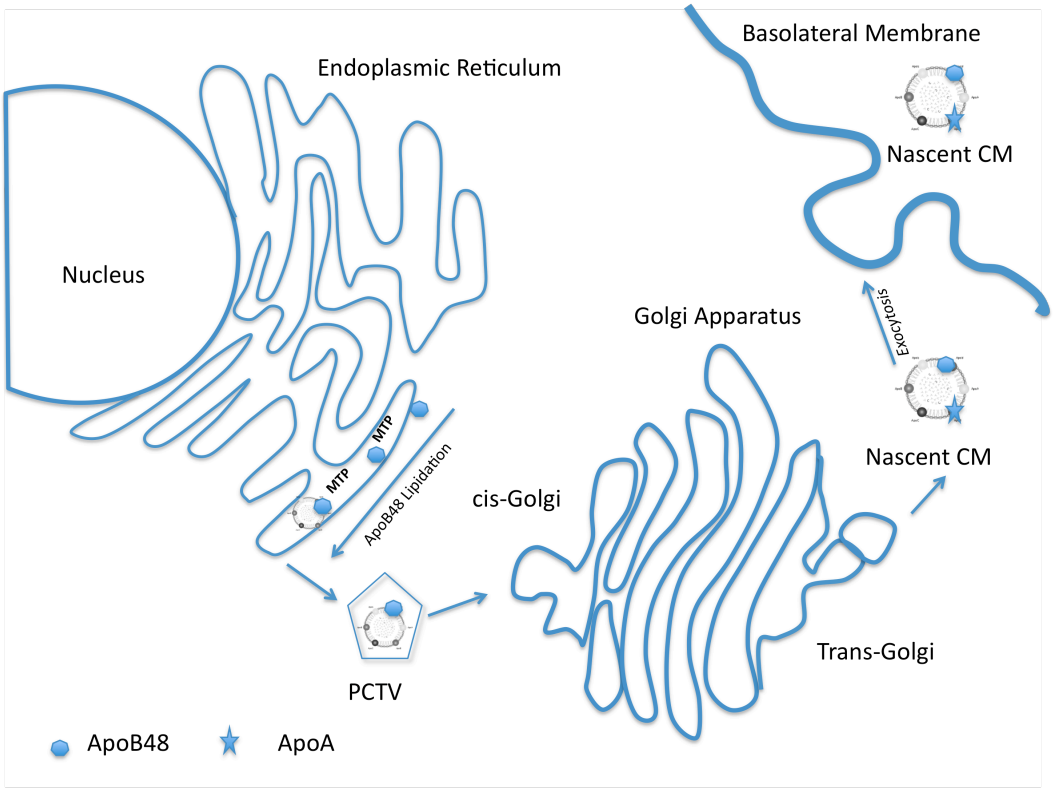

Figure 1-2. Chylomicron Production. CM assembly begins with the internalization and lipidation of apoB48 by MTP in the lumen of the smooth endoplasmic reticulum (ER). Pre-chylomicron transport vesicles (PCTV) are released from the ER and translocated to the Golgi apparatus where fatty acid and protein modification occurs. Modification of the lipoprotein is then followed by exocytosis of nascent CM from the basolateral membrane of the enterocyte.

ApoB mRNA editing occurs post-transcriptionally in the enterocyte and gives rise to a stop codon resulting in the synthesis of a protein that is 48% of the larger apoB100 molecule. It is generally believed that the apoB gene is constitutive and that apoB concentration is regulated by co- and post-translational mechanisms. *In vitro* studies indicate that regulation of apoB secretion can occur in the post-translational state (Dixon and

Ginsberg, 1993) while apoB mRNA concentration does not change (Hussain et al., 2005). Alternatively, Singh *et al.* (2002) has shown that modest changes, such as transforming growth factor-beta (TGF-β) in cells, affects the transcription of the endogenous apoB gene, which could potentially affect apoB48 production and secretion.

The co-translational lipidation of apoB48 (Hussain et al., 2005) is an essential process in CM production. The addition of lipid to the nascent CM occurs via fusion with pre-formed lipid droplets or via molecule-by-molecule addition of lipid by transfer protein, microsomal triglyceride transfer protein (MTP) (van Greevenbroek and Bruin, 1998; van Greevenbroek et al., 1998) (Figure 1-2). The incorporation of lipid during CM assembly results in the mutual solubilization and stabilization of the protein-lipid (apoB48 and TG) complex (Dixon and Ginsberg, 1993). An unstable protein-lipid complex impairs translocation of the hydrophobic portion of apoB48 resulting in degradation of the protein at the ER membrane (Davidson and Shelness, 2000).

Microsomal triglyceride transfer protein (MTP) is a heterodimeric protein consisting of a large MTP subunit (98 kDa) and protein disulfide isomerase (PDI) that exists in the lumen of the endoplasmic reticulum. This transfer protein is responsible for the transfer of lipids from one membrane to another. MTP and apoB48 physically interact early during the translocation of apoB48 across the endoplasmic reticulum membrane. The absence of MTP activity in cells has been shown to result in the

complete abolishment of the apoB48-containing lipoprotein secretion (Wetterau et al., 1992). In the small intestine, MTP may also facilitate further lipidation of nascent CM beyond the first apoB48 rate-limiting initiation in the endoplasmic reticulum.

Nascent CM are transferred from the endoplasmic reticulum to the Golgi-apparatus by a specialized, adenosine triphosphate (ATP)-mediated, complex vesicular compartment proposed as the pre-chylomicron transport vesicle (PCTV) (Figure 1-2) (Kumar and Mansbach, 1999; Siddiqi et al., 2006, Neeli et al., 2007). In the Golgi apparatus, apoB48 glycosylation is modified, lipid composition is altered and apoA attaches to the nascent CM before it is exocytosed from the basolateral surface of the enterocyte (Mansbach and Gorelick, 2007) into lymphatic vessels (lacteals) that run medially to the core of each villus. From the lymphatic circulation, CM drain into the plasma compartment via the thoracic duct. Circulating nascent CM interact with (high-density lipoprotein) HDL at which time HDL donates additional apoCII (a cofactor for lipoprotein lipase (LPL) activity) and apoE to the mature CM particle.

Recent research methods have used apoB48 protein expression as a determinant of CM production in the enterocyte. It is an important notion to appreciate that intestinal apoB48 content per se is only a measure of abundance at one point in time and is not indicative of the CM production response ‘functionally’ overtime (fasted and post-prandial state). Only by collecting CM is one able to investigate the net production of CM, TG and

corresponding cholesterol from this fraction, which are critical elements to understanding the total contribution of CM to lipid homeostasis during physiological and patho-physiological conditions. Therefore, in this thesis lymphatic cannulation (or fistula) has been employed to assess CM production. Lipid secretion into the lymph takes into account the "net" secretion of CM and lipid directly from the intestine. This methodological approach has not yet been used to explore the putative aberrations of CM metabolism during conditions of insulin resistance and/or the Metabolic Syndrome (MetS) and thus forms one of the primary objectives of this thesis.

1.1.2.1.2 Chylomicron Clearance:

CM are hydrolyzed and cleared from the circulation by endothelial lipoprotein lipase (LPL) and to a lesser extent hepatic lipase (Ginsberg, 1998). LPL is transported by ill-defined mechanisms to the surface of cells where it interacts with the triglyceride-rich lipoprotein (CM and VLDL) (Goldberg, 1996; Fisher et al., 1993). Similarly, hepatic lipase functions to hydrolyze TG from CM and augment the uptake of CM-remnants by the liver (Cooper et al., 1982) (see Figure 1-3).

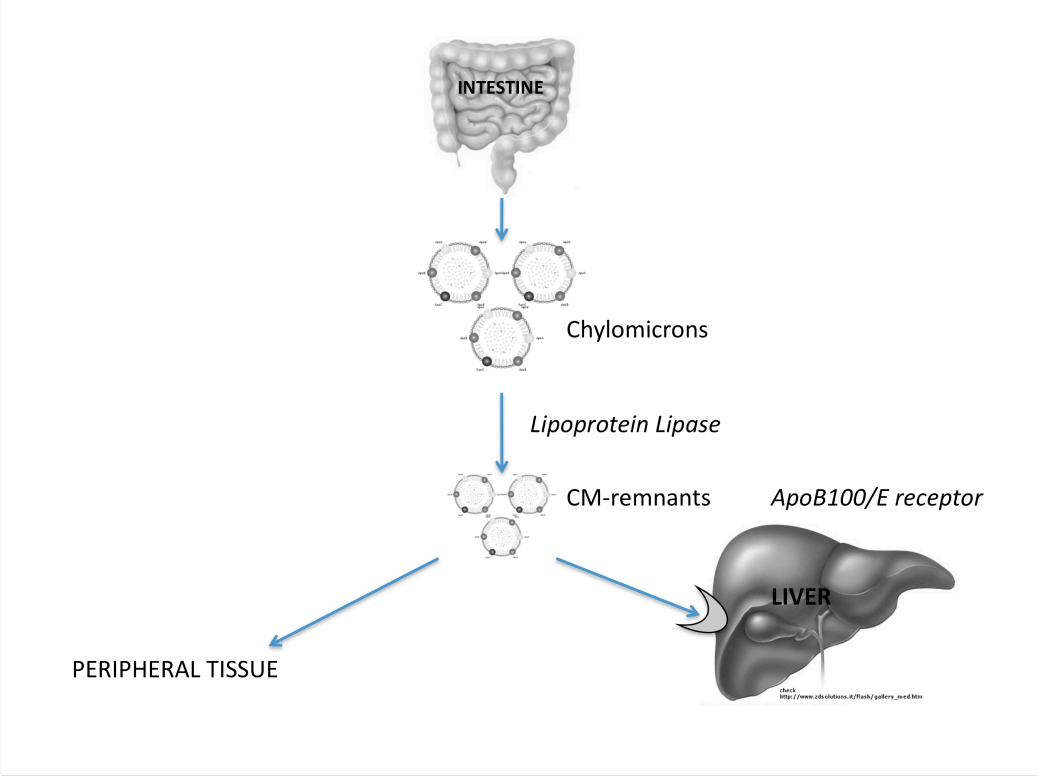

Figure 1-3. Chylomicron Clearance. The schematic depicts the hydrolysis and uptake of CM with lipoprotein lipase and apoB100/E receptor, respectively (Ginsberg, 1998).

The hydrolysis of TG reduces the core volume and surface area of the CM. The mature CM is further hydrolyzed, becoming a smaller CM-remnant with a diameter of 50-80 nm (reduced from a diameter of 100-1000 nm) (Ginsberg, 1997; Redgrave, 2004). The composition of the CM-remnant contains 70% less TG than a nascent CM with fewer phospholipids but a greater proportion of cholesteryl esters (13%).

Along with changes in lipid content, apoprotein composition is also modified during CM hydrolysis. Delipidation of CM releases apoA and apoCII from the particle returning the apoproteins to HDL, while apoE is

retained within the CM-remnant (Cooper et al., 1982). ApoE plays a critical role in the high affinity uptake of the CM-remnant from plasma by interacting with low-density lipoprotein receptor (LDL-R) or the apoB100/E receptor (Mahley and Innerarity, 1983). CM-remnants interact with the apoB100/E receptor utilizing apoE, which requires a cluster of four receptors to enable internalization (Innerarity, 1978). The expression of apoB100/E receptors at the cell surface of hepatocytes removes remnants from circulation (Mahley and Innerarity, 1983). The greater affinity of apoE for the apoB100/E receptor compared to the apoB100 of low-density lipoprotein (LDL) may explain the enhanced clearance of CM compared to LDL. CM-remnant uptake can also occur by LDL-like receptor proteins (LRP) and surface proteoglycans (heparin sulfate proteoglycans) (Weisgraber, 1986), an area that is still debated in the literature.

1.1.2.2 Very Low Density Lipoproteins (VLDL)

Very low-density lipoprotein (VLDL) particles are synthesized in the liver and are responsible for transporting endogenous TG and cholesterol to peripheral tissues. Lipolysis of VLDL is facilitated by the binding of apoE to heparan sulfate proteoglycans that induce catabolism by endothelial bound lipoprotein lipase (Ginsberg, 1997). As lipolysis proceeds, VLDL becomes smaller and denser forming VLDL-remnants or intermediate-density lipoproteins (IDL). IDL distributes TG to tissues concurrently releasing apoproteins and ultimately forming low-density lipoprotein (LDL).

1.1.2.3. Low Density Lipoproteins (LDL)

Low-density lipoprotein (LDL) is formed from the hydrolysis of VLDL and IDL. Unlike CM and VLDL, which are mainly composed of TG, the composition of the internal core of LDL is abundantly cholesterol. Cholesteryl esters make up 35-40% of LDL (Olson, 1987). Furthermore, in humans, LDL is identified by apoB100, an apoprotein responsible for LDL clearance. As noted previously, in rodents the liver is able to produce both apoB100 and apoB48. In essence rats are able to secrete a VLDL apoB48 containing particle that ultimately is metabolized to become a LDL apoB48 containing particle. While the significance of this in rats is a potential confounder during the fasted state, we address the origin of apoB100 and apoB48 in the fed state in this thesis.

The function of LDL is to transport cholesterol to the liver and extrahepatic tissue via LDL receptor-mediated endocytosis. Receptor-mediated endocytosis is mainly determined by the availability (or activity) of LDL receptors (LDL-R) (Brown and Goldstein, 1984; Goldstein et al., 1983). In normal individuals, 60 to 80% of LDL is cleared from plasma through the binding of apoB100 to LDL-R, while the remaining 20 to 40% of LDL is cleared by both hepatic and extrahepatic non-receptor mediated pathways (Dietschy et al., 1993; Spady et al., 1986). Other receptors involved in sequestration of cholesterol include; LDL-receptor like protein (LRP) and scavenger proteins that recognize oxidatively modified LDL

(Ginsberg, 1998). Cholesterol can also be cleared from the circulation via non-receptor mediated pathways (i.e. cell surface proteoglycans).

Long standing evidence of lipid-laden arterial lesions has implicated elevated concentrations of LDL-cholesterol (LDL-C) as a primary contributor to the development of atherosclerosis (Genest et al., 2003). The small size of LDL (18-28 nm) as well as the cholesterol-rich composition is thought to premise that LDL can readily permeate the arterial wall where it can undergo oxidative modification stimulating the formation of foam cells. Although, LDL-C has become recognized as a well-established marker of CVD risk, it is also a critical clinical observation that 40% of coronary artery disease patients have normal plasma concentrations of LDL (Proctor and Mamo, 2003). Hence, this forms a formidable rationale that plaque-derived cholesterol may be sourced from other lipoprotein fractions than LDL alone.

1.1.2.4 High Density Lipoproteins (HDL)

The synthesis of nascent HDL begins in both the liver and small intestine as a small, cholesterol-poor, apolipoprotein-phospholipid complex (spherical or disc-like). The nascent HDL particle secreted by the liver and/or intestine accepts free cholesterol from the extracellular surface of cell membranes via interaction with ATP-binding cassette A1 (ABCA1). The plasma enzyme lecithin cholesterol acyltransferase (LCAT) allows for the sequestration of cholesterol into the lipoprotein core (Ginsberg, 1998).

As the HDL particle continues to circulate throughout the bloodstream accepting more cholesterol and phospholipids it is transformed into a mature HDL particle (Bruce and Tall, 1995; Glosmet, 1968).

The cholesterol associated with HDL is often referred to as “good” cholesterol. Essentially HDL is thought to have the opposite ‘transport’ function of LDL. Rather than depositing cholesterol into blood and tissues, HDL is thought to remove cholesterol from cells and peripheral tissues, a process referred to as reverse cholesterol transport (Berger, 1984).

Reverse cholesterol transport involves the continued uptake of free cholesterol, a process mediated by the actions of LCAT. LCAT esterifies free cholesterol on the surface of HDL and promotes the movement of hydrophobic cholesteryl ester into the lipoprotein core. As free cholesterol is internalized, more cholesterol is able to be absorbed onto the lipoprotein surface enlarging HDL (Kostner et al., 1987). The mature HDL particle has two fates; 1) transfer of cholesteryl esters to other lipoproteins and tissues; or 2) metabolism/ removal of HDL from the plasma (Tall, 1990). The former involves cholesterol ester transfer protein (CEPT) (Figure 1-4). CEPT mediates the transfer of cholesteryl ester from HDL to TG-rich lipoproteins such as CM and VLDL. Thus, when CM and VLDL are taken up by the liver CEPT-transferred cholesteryl ester can be cleared as well (Tall, 1990). The process of reverse cholesterol transport is the reason that HDL is deemed the anti-atherogenic lipoprotein. Also noteworthy, is the fact that rodents do not express CEPT, which renders them a ‘HDL

predominant' animal. In most rats and mice, cholesterol from HDL is not able to transfer back to other lipoprotein fractions in plasma. In rats and mice, cholesterol in the HDL fraction accumulates to a much larger extent than in humans and they rely on particle kinetics for cholesterol metabolism. The rat is often considered a poor animal model to study HDL particle metabolism per se, but curiously most rats/mice are also considered resistant to vascular disease due to their inherent lack of vascular dysfunction.

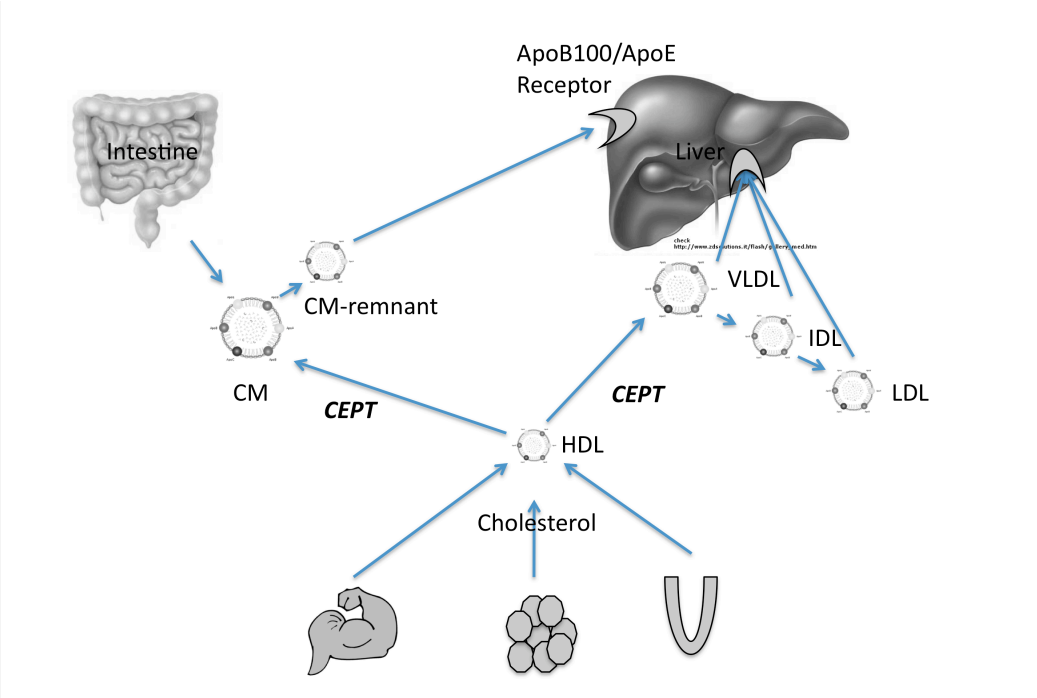

Figure 1-4. Reverse Cholesterol Transport. This process involves the uptake of excess cholesterol from the body by high-density lipoproteins (HDL). Cholesterol ester transfer protein (CEPT) transfers cholesterol from HDL to TG-rich lipoproteins (CM and VLDL). The uptake of CM and VLDL by the liver removes CEPT-transferred cholesterol from the body (Ginsberg, 1998).

1.2 Intestinal Structure and Function

1.2.1 Intestinal Anatomy and Physiology

The intestine plays a critical role in the metabolism of endogenous and exogenous fatty acids through lipoprotein assembly and secretion. To fully appreciate the intestine's role in lipid metabolism the structural and functional components of this organ must be understood. The small intestine can be divided into three anatomical regions; the duodenum, jejunum and ileum. The small intestine consists of four layers composed of muscle and epithelium; the mucosa, the submucosa and the muscularis externa, which is two layers of muscle tissue: circular muscle and longitudinal muscle (Figure 1-5) (Silverthorn, 2001).

The inner mucosa of the gastro-intestinal tract has several structural modifications to increase the absorptive surface area (Silverthorn, 2001). The intestinal mucosa projects into the lumen as small 'finger-like' extensions known as villi and increases the surface area for absorption (Figure 1-5). Tubular invaginations extending down into supporting connective tissue at the luminal surface of the intestine, referred to as crypts, also contribute to an increase the intestinal absorptive surface. Furthermore, intestinal cells (enterocytes) and their extending microvilli further add to the absorptive surface area (Figure 1-5).

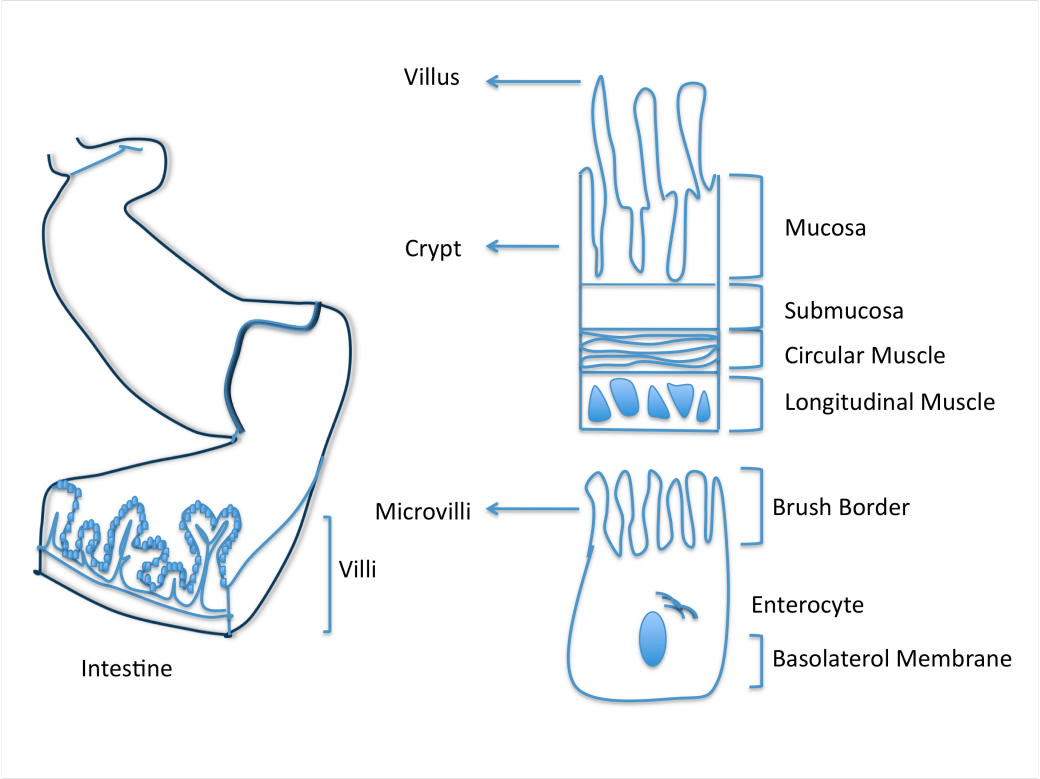

Figure 1-5. Intestinal Morphology. Intestinal surface area is enhanced by 'finger-like' extensions called villi that are covered by enterocytes (Silverthorn, 2001).

There are a variety of cells that populate the intestinal villus including; transporting epithelial cells (enterocytes), endocrine and exocrine secretory cells (goblet cells, enteroendocrine cells and paneth cells) and stem cells (Silverthorn, 2001). Stem cells are undifferentiated cells found in the crypt of the intestine that are responsible for continuously producing new epithelium. Stem cells in the crypt proliferate and the newly formed cells migrate toward the villus tip, eventually sloughing off at the tip (Marshman et al., 2002).

1.2.2 Intestinal Lipid Digestion and Absorption

The digestion of lipids involves the mechanical and chemical breakdown of food in the intestinal lumen. The absorption of lipids then requires further processing (Sections 1.2.2.1 and 1.2.2.2) before being taken up by enterocytes lining the villi in the intestinal lumen. The significance of the intestine in lipid metabolism was highlighted with the discovery of specific mucosal brush border transporters for cholesterol (NPC1L1, SR-B1, CD36) and long chain fatty acids (FATP4) (Kruit et al., 2006; Lally et al., 2007; Bonen et al., 2007). Lipid availability, from the diet and enterocyte lipid synthesis, is essential for proper CM assembly/secretion therefore enterocytic lipid transporters are thought to play a critical role in lipoprotein metabolism.

1.2.2.1 Triglyceride Digestion and Absorption

Digestion of TG begins in the stomach by gastric lipase. Gastric lipase cleaves fatty acids at the sn-3 position of the TG molecule. TG-containing medium chain fatty acids are hydrolyzed at a faster rate than TG-containing long chain fatty acids. In the stomach diacylglycerol and free fatty acids produced by the hydrolysis of dietary TG promotes the emulsification of dietary fats. Lipid emulsions then enter the small intestine as lipid droplets. In the proximal region of the intestine pancreatic lipase further digests TG by cleaving fatty acids at the sn-1 and sn-3 position forming 2-monoacylglycerol and free fatty acids (Stipanuk, 2000).

In the lumen of the intestine, hydrophobic lipid digestion products are solubilized by bile salts to form mixed micelles. Mixed micelles are soluble lipid vesicles that can readily cross the unstirred water layer that overlies the mucous layer at the brush border membrane of enterocytes lining the intestinal villi (Figure 1-6) (Stipanuk, 2000).

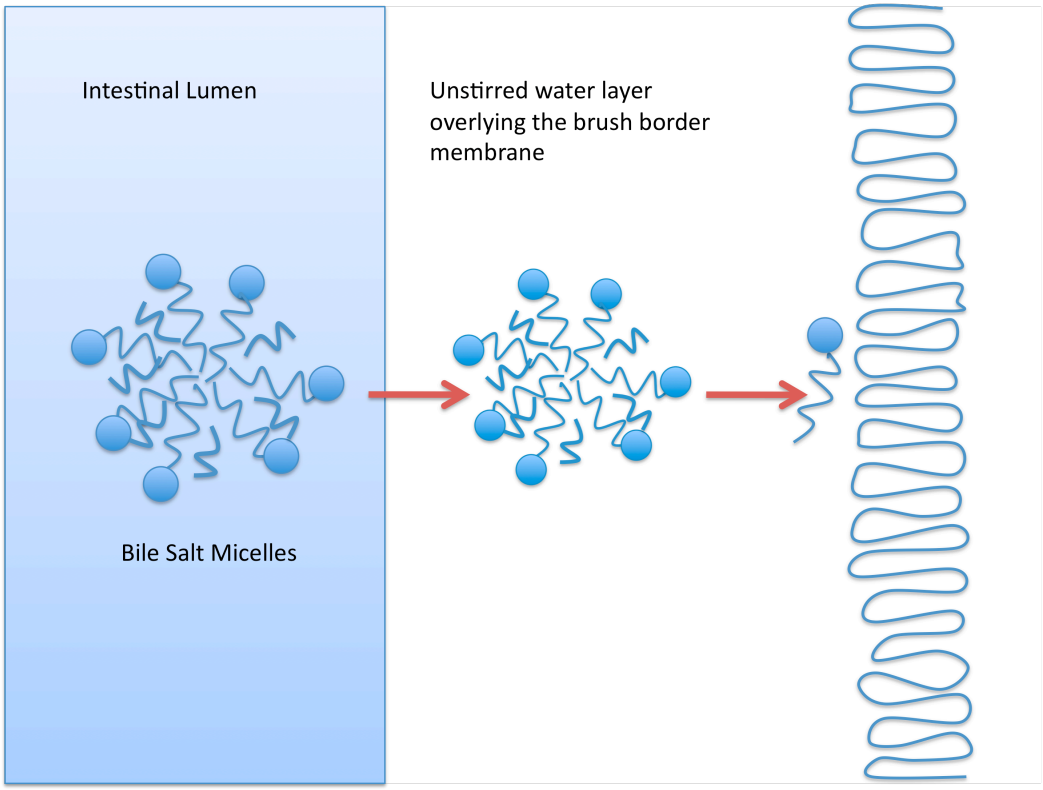

Figure 1-6. The role of bile acids in solubilizing hydrophobic lipids by forming micelles. Micelles are able to overcome the diffusion barrier associated with the unstirred water layer and deliver lipid molecules to the brush border membrane for absorption.

Absorption at the brush border occurs through a passive, fatty acid concentration gradient (Zakim, 1996) and/or by proposed carrier fatty acid transport proteins (Stremmel et al., 1992; Fitscher et al., 1996). Fatty acid

transporter protein 4 (FATP4) expressed in the apical membrane of enterocytes has been shown to be involved in intestinal fatty acid absorption (Shim et al., 2009). This is supported by the idea that the over-expression of FATP4 in HEK-293 cells, human embryonic kidney cells, enhances long chain fatty acid uptake (Lobo et al., 2007). Furthermore, a reduction in the expression of FATP4 by anti-sense oligonucleotides in primary enterocytes reduces fatty acid uptake (Stahl et al., 1999). However, the role of FATP4 *in vivo* still remains unclear.

1.2.2.2 Cholesterol Digestion and Absorption:

Most dietary cholesterol is present as free cholesterol, however, 10% to 15% is present as a cholesterol ester (Stipanuk, 2000). In the intestine, cholesterol esters are hydrolyzed to free cholesterol by cholesterol esterase, which is secreted by the pancreas as an active enzyme. Free cholesterol is then incorporated into mixed micelles, which provides an efficient vehicle for cholesterol to cross the unstirred water layer for eventual uptake by enterocytes. There are two major hypotheses by which intestinal epithelial cells absorb cholesterol. One long-standing hypothesis suggests that cholesterol is absorbed via an energy independent passive diffusion process regulated by a concentration gradient (Hui et al., 2008). The second hypothesis suggests cholesterol absorption is an energy dependent, protein-mediated process (Thurnhofer and Hauser, 1990). In support of the latter, scientific advancements have

identified proteins in the intestine that are directly involved in cholesterol absorption.

The importance of CD36 in cholesterol absorption was demonstrated in null-CD36 mice showing a significant reduction in cholesterol transport from the intestinal lumen to the lymph (Nauli et al., 2006). Another potential transporter involved in cholesterol uptake is scavenger receptor B1 (SR-B1). SR-B1 is highly expressed in the apical side of the enterocyte (Labonte et al., 2007) and when over-expressed is associated with increased cholesterol absorption (Bietrix et al., 2006).

Niemann Pick C1 like 1 protein (NPC1L1) is a polytopic transmembrane protein containing a sterol-sensing domain found on the apical membrane of intestinal epithelial cells (Yu, 2008). In 2004, the protein was identified to be essential for intestinal cholesterol absorption after being treated with the inhibitory drug ezetemibe (Altmann et al., 2004). Furthermore, NPC1L1 expression appears to be positively associated with plasma apoB48 and cholesterol content (Lally et al., 2006).

Finally, ATP binding cassette (ABC) transporters, ABCG5 and ABCG8 are present in the apical membrane of the enterocyte (Hui et al., 2008). These transporters limit the amount of cholesterol absorbed by effluxing cholesterol back into the lumen (Lee et al., 2001). Interestingly, studies in type-1 (insulin deficient) and type 2-diabetes (elevated insulin)

showed NPC1L1 mRNA is up regulated while ABCG5/8 is down regulated, a modification that is associated with an increase in plasma apoB48 and cholesterol content (Lally et al., 2007, Lally et al., 2006).

1.2.3 Intestinal Morphological Changes and Metabolic Disorders

The intestine is a very complex organ involved in a number of metabolic and physiological functions. For example, not only does the intestine play a pivotal role in lipid metabolism, it also functions as a protective barrier responding to and sensing harmful pathogens (Vaarala et al., 2008). However, it is important to note that the intestine is also a very dynamic and adaptable organ typified by the relatively short lifespan of enterocytes (48 hours) (Weiser et al., 1989). Therefore, disease states and/or metabolic disturbances can potentially impact the structure, function and integrity of the intestine.

Adaptations of the gut in type 1-diabetes have demonstrated aberrant intestinal microflora, increased intestinal permeability and an altered mucosal immune system (Vaarala et al., 2008). It is suggested that the aforementioned adaptations of the intestine surface barrier impacts the innate immune system. In addition, permeability of the intestine permits the unregulated passage of intestinal contents that can ultimately alter pro and anti-inflammatory cytokine patterns (Vaarala et al., 2008). Since CVD is considered a low-grade inflammatory disease, an altered immune state

due to aberrant adaptations of the intestine, in the diabetic condition, may potentially exacerbate the already increased risk of developing CVD.

Furthermore, earlier studies have demonstrated morphological adaptations of the intestine during metabolic disorders such as insulin resistance (IR) and obesity (Estornell et al., 1995; Pillion et al., 1988; Mayhew et al., 1996). Intestinal hypertrophy, which is characterized by an increase in the mucosal protein/DNA ratio, is demonstrated in experimental animal models prone to obesity (Estornell et al., 1995; Sefcikova et al., 2008). In addition, studies in streptozotocin adult diabetic rats observed an increase in intestinal mass (Pillion et al., 1988). In contrast, Mayhew *et al.* (1996) studied the intestine of diabetic mammals and showed no changes in intestinal morphology but did show an increase in the number of enterocytes.

Few studies have investigated the structural and morphological changes of the intestine in the Metabolic Syndrome (MetS). The lack of evidence in this area limits our understanding of the influence the highly adaptable intestine may have on CM metabolism and the immune state. This thesis investigates the structural changes of the intestinal jejunal villi in the JCR:LA-*cp* rat, a model of MetS.

1.3. Chylomicron Metabolism, Metabolic Association with Chronic Disease:

1.3.1 Chylomicron Metabolism and Cardiovascular Disease Risk

In the 1970's, Zilversmit was the first to suggest that CM-remnants are potentially atherogenic (Zilversmit, 1979). Since then, elevated CM concentration has been established as a significant risk factor in CVD (Cohn et al., 1999; Tomkin and Owens, 2001). It is often under appreciated that most individuals in the Western world are in the post-prandial phase most of the day and impaired clearance of CM and their cholesterol-dense remnants may pose a continuous challenge to the arterial endothelium (Sniderman et al., 2002; Taskinen et al., 2003, Botham et al., 2005). A study by Karpe *et al.* (1994) demonstrated a positive relationship between the post-prandial concentration of CM-remnants and the rate of progression of coronary lesions in post-infarction male patients. The mechanisms characterizing the relationship between CM metabolism and CVD risk demonstrate both direct and indirect effects of CM on the development of atherosclerosis.

A direct relationship between CM and the development of CVD is exhibited in mechanistic studies by Proctor *et al*. (2002, 2004) showing that CM-remnants permeate and are retained in the arterial wall inducing the formation of foam cells and the development of atherosclerotic plaques (Proctor et al., 2002; Proctor et al., 2004). More recently, Nakano

et al. (2008) detected apoB48 (the only exclusive marker of CM particles in humans) in atherosclerotic lesions in patients of sudden cardiac death.

Other investigations have provided evidence that CM may increase the risk of CVD in an indirect manner. For instance, elevated concentrations of post-prandial TG enhance the exchange of TG between CM and HDL (Patsch et al., 1983; Patsch et al., 1984). Consequently, the compositional change from cholesterol-dense HDL to TG-rich HDL lowers the concentration of the cardioprotective HDL fraction, increasing CVD risk (Ginsberg, 1998).

Furthermore, recent evidence indicates that the post-prandial response influences the inflammatory state. Atherosclerosis has been often described as a low-grade chronic inflammatory disease (Libby et al., 2002). Emerging evidence suggests that the pro-inflammatory condition in atherosclerosis may be a post-prandial phenomenon (Alipour et al., 2007). van Oostrom *et al*. (2003; 2003) have shown that a post-prandial rise in TG increases neutrophil count with a concomitant production of pro-inflammatory cytokines. As we are in the fed state for the majority of the day, the impact of post-prandial lipemia and the associated post-prandial inflammatory state may have a direct and substantial effect in increasing the risk of developing CVD.

A well-established association between impaired post-prandial CM metabolism and the development of CVD has enticed researchers to

investigate the mechanisms and pathways related to the development of post-prandial lipemia. Consequently, it is critical to appreciate the metabolic parameters by which CM metabolism can become impaired, such as chronic conditions of obesity, diabetes, IR and the MetS.

1.3.2 Chylomicron Metabolism and Obesity

Obesity is an epidemic disease in which the accumulation of excess visceral body fat is associated with an increasing risk of developing CVD (Dagenais et al., 2003). As one of the leading preventable causes of death, it is becoming a serious public health issue as the rates of obesity in both adults and children continue to rise (The Obesity Society, 2009; Kosti et al., 2006). Obesity is defined by the body mass index (BMI) > 30 kg/m^2, which is determined by weight and height of an individual (kg/m^2). Obesity can also be measured by waist circumference and/or waist to hip ratio (The Obesity Society, 2009). A waist circumference of > 102 cm in men and > 88 cm in women, while waist to hip ratios >0.9 in men and >0.85 in women define central obesity (The Obesity Society, 2009).

Couillard *et al.* (2002) studied the post-prandial response of CM in obese men after feeding them a test meal containing 64% energy from dietary fat. The study showed that obesity was significantly associated with increased concentrations of post-prandial TG-rich, apoB48-containing lipoproteins ($r = 0.30\text{-}0.44$, $p < 0.05$).

Many investigators have found that the distribution of body fat, rather than weight, is far more important in determining the association between obesity and post-prandial lipemia. Watts *et al.* (2001) used a stable isotope breath test to determine the fractional catabolic rate (FCR) of CM particles and found that the rate of CM catabolism was inversely related to waist to hip ratio suggesting that elevated concentrations of CM is associated with visceral obesity. Further, Mekki *et al.* (1999) studied the effect of fat distribution on post-prandial lipemia in obese women, comparing android and gynoid phenotypes. The study found that visceral adiposity, as demonstrated by the android phenotype, showed elevated levels of small apoB48-containing lipoproteins (Mekki et al., 1999).

Emerging evidence in children have identified that childhood obesity is also associated with elevated plasma apoB48 concentrations. MRI imaging of visceral adiposity and western blot analysis of fasting plasma apoB48 (one apoB48 molecule per CM particle) showed increased abdominal obesity is associated with elevated apoB48 concentrations (Nzekwu et al., 2007, Su et al., 2009).

1.3.3 Chylomicron Metabolism and Diabetes:

Worldwide there are 246 million people diagnosed with type-1 or type-2 diabetes (IDF, 2009). In Canada, the financial strain of this disease on the healthcare system is estimated to become 15.6 billion dollars a year by the year 2010 (CDA, 2009). Furthermore, there are personal costs of diabetes such as a reduced quality of life and an increased likelihood of

suffering from further complications: coronary artery disease and stroke. While this thesis is not focused on frank diabetes per se, type-1 and type-2 diabetes will be introduced to help in the understanding of the pre-diabetic condition and how it relates to MetS.

1.3.3.1 Insulin Dependent Diabetes Mellitus (IDDM) or Type-1 Diabetes:

IDDM or type 1-diabetes is an autoimmune disease that results in the destruction of pancreatic β-cells that produce insulin. Currently, there is no known cure for type 1-diabetes. However, the disease can be treated with insulin replacement therapy along with dietary management. Pancreatic transplants have not been a practical means of treatment due to the availability of the organ and the technical difficulty involved in the surgical procedure (Meloche, 2007). Islet cell transplantation shows promise, however, chances of rejection and the long-term viability of the transplanted cells are still a concern (Meloche, 2007).

With regards to atherosclerosis, type-1 diabetics are at an increased risk of developing CVD (Kannel and McGee, 1979). However, despite the exacerbated CVD risk, the lipid profile of type-1 diabetics shows that classic fasting lipid parameters (HDL, LDL and TG) are often normal (Howard, 1987). Intriguingly, Su *et al.* (2009) investigated post-prandial CM concentrations in a population of type-1 diabetics and showed that in the absence of classic dyslipidemia (i.e. normal LDL-C), apoB48 concentrations were increased in the fasted and fed state (as calculated by post-prandial area under the curve).

1.3.3.2 Non-Insulin Dependent Diabetes Mellitus (NIDDM) or Type-2 Diabetes:

NIDDM or type 2-diabetes is a disorder in which the body's cells do not respond appropriately when insulin is present. Elevated blood glucose levels and increased concentrations of plasma insulin characterize type-2 diabetes. The World Health Organization (2007) defines type 2-diabetes as a raised blood glucose reading > 7.0 mmol/L in the fasted state and/ or a blood glucose reading > 11.1 mmol/L following a glucose tolerance test. The range of type 2-diabetes can exist from predominant insulin resistance with hyperinsulinemia to insulin resistance with an insulin deficiency due to an insulin secretory defect (Reaven, 2004; Wilcox, 2005).

Similar to type-1 diabetes, individuals with type-2 diabetes have a 3 to 4 fold increased risk of developing CVD compared to healthy individuals (Kannel and McGee, 1979). Additionally, individuals with type-2 diabetes have consistently demonstrated impaired lipid metabolism, to the extent that post-prandial lipemia is being recognized as an inherent feature of diabetes (Mero et al., 1998; Curtin et al., 1996, Hogue et al., 2007). Curtin *et al*. (1996) examined the post-prandial apoB48 response in patients with type 2-diabetes following a lipid-rich meal and showed that post-prandial apoB48-containing lipoproteins were significantly higher ($p<0.05$) in type 2-diabetic patients compared to control subjects. Further, Hogue *et al.* (2007) investigated the kinetics of apoB48 in type 2-diabetic (n=11) and non-diabetic subjects (n=13) during a 12h constant fed state. The results

showed that type 2-diabetic subjects had an increased production rate and decreased catabolic rate of intestinally derived apoB48-containing lipoproteins, as measured indirectly in plasma, compared to non-diabetic subjects.

1.3.4 Chylomicron Metabolism in Insulin Resistance/Hyperinsulinemia

Insulin resistance (IR) is defined as the 'reduced' ability of cells in the body to respond to the normal actions of insulin (Reaven, 2004). In an attempt to maintain homeostatic blood glucose levels in an insulin-resistant state, pancreatic β-cells over secrete insulin, a condition referred to as hyperinsulinemia (Reaven, 2004). Along with elevated plasma insulin concentrations, IR is associated with an increase in fasting concentrations of CM, as well as elevated post-prandial concentrations of CM (Kim et al., 2001; Abbasi et al., 1999; Ai et al., 2000; Harbis et al., 2001; Duez et al., 2006).

It has been previously proposed that insulin may be an important component in the regulation of CM metabolism. A two-part study in healthy volunteers demonstrated the potential regulatory effects of insulin (Harbis et al., 2001). The first phase of this two-part study investigated the effect of isolipidic meals that induce different degrees of hyperinsulinemia on post-prandial metabolism. The results showed a positive correlation between the concentration of apoB48 and plasma insulin (r_2 = 0.07; p=0.0001). The second phase of the study examined the post-prandial apoB48 response in subjects fed a carbohydrate-free meal during a 3-h

hyperinsulinemic/ euglycemic clamp. Findings showed reduced plasma apoB48 during insulin infusion was followed by late accumulation of plasma apoB48 (Harbis et al., 2001).

1.3.5 Chylomicron Metabolism and the Metabolic Syndrome:

The MetS is a cluster of pre-diabetic (hyperinsulinemic) pathologies that increase the risk of developing CVD (Gami et al., 2007). The clinical definition of the MetS, as defined by International Diabetes Federation, is central/visceral obesity and two of the following symptoms: raised TG, reduced HDL cholesterol, increased plasma glucose levels, and raised blood pressure (IDF, 2006). Earlier sections in this literature review have focused on post-prandial lipemia in obesity, diabetes and IR in order to contribute to the understanding of the effect of post-prandial CM metabolism during metabolic disorders associated with the MetS.

It is understandable that a broad definition of MetS adds heterogeneity to the characterization of subjects for clinical studies and has made it difficult to obtain a homogenous population of subjects. Additionally, the few available animal models of MetS has limited research to study the association between MetS and CVD. However, there is now a well-characterized animal model of the MetS, the JCR:LA-*cp* rat (Vine et al., 2007), which allows for the study of mechanisms associated with the physiological aberrations in the MetS.

Phenotypically, the JCR:LA-*cp* rat, homozygous (cp/cp) for the recessive *cp* (corpulent) trait is obese, IR, hypertriglyceridemic and, unlike other models of the MetS, spontaneously develops early atherosclerotic lesions associated with ischemic myocardial infarcts (Russell et al., 1989; Russell and Proctor, 2006). The plasma lipid profile of the JCR:LA-*cp* rat with the cp/cp phenotype has mildly higher total plasma cholesterol compared with their lean (+/?) counterparts (Russell and Proctor, 2006). The observed increase in cholesterol has been associated with elevated VLDL but not LDL, similar to the lipid profile observed in the pre-diabetic state in humans. More recently, work in JCR:LA-*cp* rat has characterized this model with severe chylomicronemia, as assessed by the post-prandial response of apoB48 following an oral fat challenge (Vine et al., 2007). Additionally, it has been shown that the exacerbated post-prandial apoB48 response in the JCR:LA-*cp* rat positively correlates with fasting plasma insulin concentrations (Vine et al., 2007). Collectively, the chylomicronemia associated with IR (pre-diabetes), along with the eventual development of atherosclerotic lesions in JCR:LA-*cp* rats allows for the unique opportunity to study dyslipidemia in MetS. The initial study in this thesis will focus on the contribution of intestine to the impaired post-prandial CM response in the JCR:LA-*cp* rat.

1.3.5.1 Chylomicron Overproduction in Insulin Resistance

The mechanistic factors contributing to impaired CM overproduction were studied in hyperinsulinemic and IR subjects (Duez et al., 2006). The

relationship between IR and CM production was investigated in subjects with a broad range of insulin sensitivity. ApoB48 metabolism was measured in the steady-state fed condition and blood samples were collected throughout the day. Small triglyceride-rich lipoprotein fractions were isolated via ultracentrifugation and apoB48 was quantified using SDS-PAGE. Intestinal lipoprotein production rates were determined by multiplying the slope of apoB48 and TG. The study found a significant correlation ($r = 0.558$; $p = 0.038$) between apoB48 production rates, and fasting insulin levels.

Emerging evidence in experimental animals showing that the production of CM positively correlates with insulin resistance (Lewis et al., 2005; Duez et al., 2008; Haidari et al., 2002; Zoltowska et al., 2003) suggests that insulin may be involved in the direct and indirect regulation of apoB48 production and secretion. Indeed data from our own group using the JCR:LA-*cp* rat model, has shown a positive correlation between plasma insulin values and circulating apoB48 particles (Vine et al., 2007).

Haidari *et al.* (2002) has also examined the production of intestinal lipoproteins (identified by apo-B48) in the fructose fed (diet induced IR) hamster and demonstrated overproduction of apoB48, from isolated primary enterocytes, associated with an increased intracellular stability of apoB48, enhanced de novo lipogeneis and an upregulation of microsomal triglyceride transfer protein (MTP). It is proposed that an increased abundance of MTP and an increased availability of core lipoprotein lipids

stabilize the apoB48 protein-lipid complex enhancing apoB48-containing lipoprotein production and ultimately contributing to elevated concentrations of plasma CM in IR.

Further studies using the fructose fed Syrian Golden hamster showed that the intestine was unresponsive to the inhibitory effects of insulin on apoB48 containing lipoproteins suggesting intestinal insulin resistance (Federico et al., 2006). In order to explain elevated apoB48 in the intestine Federico and colleagues (2006) examined the expression of sterol regulatory element binding protein 1-c (SREBP1-c) in intestinal enterocytes isolated from fructose fed hamsters and showed an increase in the mature form of SREBP-1c mRNA (Federico et al., 2006). SREBP-1c is the main regulatory mechanism of lipogenesis and can activate enzymes of the lipid biosynthetic pathway; such as HMG-CoA reductase and fatty acid synthase (FAS) (Brown and Goldstein, 1997; Streicher et al., 1996). Therefore, it can be speculated that SREBP is involved in enhanced de novo lipogenesis in the intestine (Haidari et al., 2002). Accordingly, studies in hepatic tissue have shown that the upregulation of SREBP in IR is associated with enhanced apoB production (Elam et al., 2001; Wang et al., 1997). Therefore, it can be suggested that the elevated expression of SREBP-1c in IR may enhance *de novo* lipogenesis, in turn increasing the intracellular stability of apoB48, which may ultimately contribute to the increased secretion of CM.

Further to this, Zoltowska *et al.* (2003) investigated the intracellular events that govern the production of apoB48-containing lipoproteins in the *Psammomys obesus* gerbil, a model of nutritionally induced IR and type 2-diabetes. Plasma biochemistry and findings in cultured jejunal explants from the IR *Psammomys obesus* demonstrated elevated apoB48 biogenesis accompanied by increased *de novo* TG synthesis and an increased abundance of monoacylglycerol acyl transferase (MGAT), an enzyme involved in TG synthesis. These findings are consistent with those reported in the fructose fed hamster, we speculate that enhanced *de novo* TG synthesis in the IR gerbil may enhance the stability of apoB48, protecting it from proteasomal degradation and encourage the assembly and secretion of CM (Zoltowska et al., 2003).

Current data in the fructose fed Golden Syrian Hamster and the *Psammomys obesus* gerbil is limited in their analysis of overproduction. In both these animal models, overproduction has been assessed in enterocytes or jejunal tissue in an ex vivo setting, which is only indicative of CM production at one point in time. However, in order to fully appreciate the physiological relevance of dynamic CM production: including lipid transport via the CM pathway, CM particle secretion overtime and/or in response to certain stimuli such as feeding; the process of CM production cannot be adequately measured in isolated enterocytes or jejunal explants. Therefore, in this thesis a surgical lymph cannulation approach has been used to assess CM production and secretion directly from the

intestine in both the fasted and fed states, strengthening the understanding of enterocytic CM production. In this thesis, CM production and secretion in the fasted and fed state are investigated in the JCR:LA-*cp* rat, a well-defined model of the MetS.

1.3.6 Chylomicron Metabolism and Inflammation

Atherosclerosis is considered a low-grade chronic inflammatory disease. It is well established that the penetration of lipoproteins into the arterial endothelial space induces foam cell formation, a process involving a complex inflammatory response. Within the past three to four years, emerging studies have suggested that the post-prandial lipid and lipoprotein response can induce a pro-inflammatory response (Alipour et al., 2007; Margioris, 2009). A post-prandial rise of TG as well as post-prandial apoB is associated with the activation of leukocytes, an upregulation of adhesion molecules, and the recruitment of neutrophils to the subendothelial space (Alipour et al., 2007; Alipour et al., 2008; van Oostrom et al., 2003, van Oostrom et al., 2004).

In normal conditions, inflammatory indices would demonstrate a steady increase following a meal with plasma inflammatory indices peaking at four hours followed by a steady decline of pro-inflammatory markers back to baseline (Figure 1-7). However, in conditions of impaired lipid metabolism elevated concentrations of plasma inflammatory indices

are maintained at peak levels during the post-prandial phase (Figure 1-7) (Margioris, 2009).

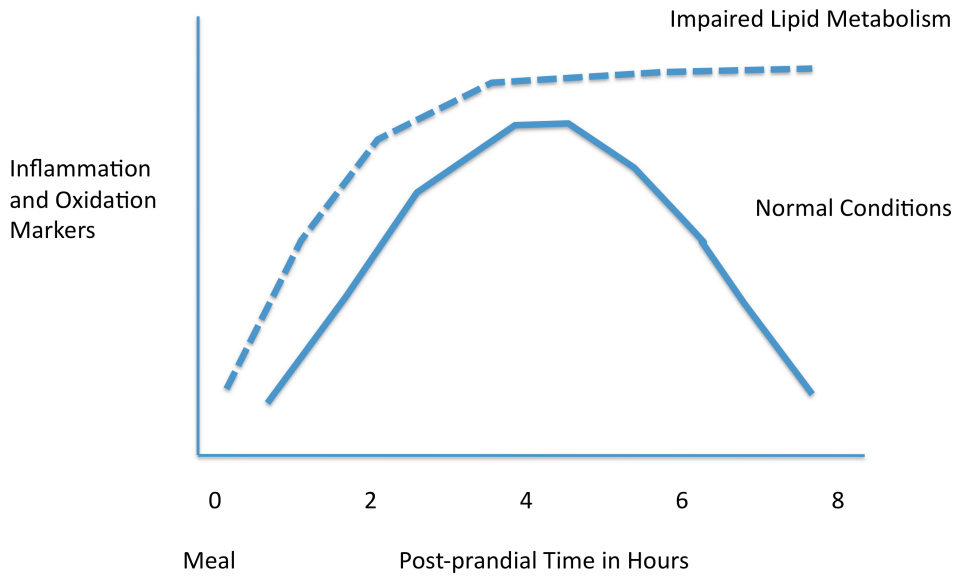

Figure 1-7. The post-prandial inflammatory response, as measured by inflammatory and oxidation markets, in normal and dyslipidemic conditions. Modified by Margioris, 2009.

Acute phase proteins secreted by hepatocyes have been used to assess inflammation in the post-prandial state. Plasma concentrations of acute phase proteins are modulated approximately 90 minutes after the onset of a systemic inflammatory reaction (Ramadori et al., 2001; Fey et al., 1990). Acute phase proteins of inflammation include C-reactive protein (CRP), haptoglobin (Hp) and lipopolysaccharide binding protein (LBP) (Ramadori et al., 2001; Fey et al., 1990). CRP and Hp are measured to assess the degree of inflammation in animals and have both diagnostic and prognostic value.

Clinically, CRP is a well-established marker of inflammation and can be useful in determining disease progress and the effectiveness of treatment (Di Napoli et al., 2005). Accumulating data for Hp has established a strong association of Hp, with diabetes, obesity and CVD (Suzuki et al., 2009; Brunetti et al., 2007; De Pergola et al., 2007). A cross-sectional study aimed at investigating the association between Hp and insulin in obese and overweight women (194 non-diabetic obese subjects) showed that Hp is positively associated with IR ($p < 0.001$) and obesity ($p < 0.001$) (DePergola et al., 2007). Further, Brunetti *et al.* (2007) assessed plasma Hp concentrations in 123 patients suffering from myocardial infarction. Within 12 h after the onset of chest pain Hp plasma concentration was measured. The findings showed that Hp concentration positively correlated with diminishing systolic function.

The up-regulation of LBP synthesis is associated with endotoxin infections and inflammation (Stoll et al., 2004). LBP has the ability to bind lipoproteins (Vreugdenhil et al., 2001; Schroder et al., 2004) since lipopolysaccharides are associated with the formation of CM (Ghoshal et al., 2009). Considering the association of LBP with lipoproteins, it might be expected that plasma concentrations of LBP are indicative of a stimulated pro-inflammatory state that is associated with aberrant post-prandial lipoprotein metabolism in the MetS.

1.4 Impact of n-3 Polyunsaturated Fatty Acids on Post-prandial Metabolism

1.4.1 Introduction

The cardiovascular benefits of fish oil has prompted the American Heart Association to recommend consumption of at least one to three servings a week of fish rich in n-3 polyunsaturated fatty acids (n-3 PUFA) (Kris-Etherton et al., 2002). Physiologically, eicosapentaenoic acid (EPA) (20:5n3) and docosahexaenoic acid (DHA) (22:6n3) cannot be synthesized by the body but can be obtained through dietary means or converted from α-linolenic acid, a nutritionally essential fatty acid. The best sources of EPA and DHA are salmon, mackerel, sardines and herring, all of which have high fat content. Other dietary sources include fish oils and some vegetable oils such as canola, flaxseed and walnut oil.

The benefits of dietary fish oil, long chain n-3 PUFA, were first seen in early epidemiological evidence from studies in Greenland Eskimos that reported a low incidence of CVD in a population consuming increased amounts of long chain n-3 PUFA (Bang and Dyberg 1972, Dyberg et al., 1975). Bang and Dyberg carried out a study in 50 subjects (25 males and 25 females) living in Greenland. The investigators collected samples of their daily food for 3 to 7 consecutive days. The composition of the food was analyzed for water, ash, protein, fat, individual fatty acids, cholesterol, and carbohydrate. Dietary composition of the Greenland Eskimos was then compared to the diets of the Danish population. The findings showed

that Eskimo diets were rich in long chain PUFA, predominantly n-3s. The researchers concluded that the reduced prevalence of CVD in the Greenland Eskimo population could be partly explained by the cardioprotective effects of n-3 PUFA, rich in their daily dietary consumption.

Since the early observations in Greenland Eskimos, the cardioprotective properties of n-3 PUFA have become of great interest to researchers. n-3 PUFA has been proposed to play an important role in protecting against the pathologies associated with CVD (Robinson et al., 2007). Preliminary work has shown that n-3 PUFA supplementation can downregulate TG levels (Harris et al., 1997; Mori et al., 2000) and improve immune function (Calder, 1998; Madsen et al., 2001. Mori and Beilin, 2004). However, the effect of n-3 PUFA on glucose tolerance in diabetes and insulin resistance is inconsistent (Grundt H, 1995; Sirtori et al., 1998; Kesavulu et al. 2002; Nettleton and Katz, 2005).

1.4.2 The Effect of n-3 PUFA supplementation on Insulin Resistance and Glucose Tolerance:

The effect of n-3 PUFA supplementation is controversial in conditions of IR and impaired glucose tolerance with studies showing inconsistent findings. A double-blind study in 935 type 2-diabetic patients showed that n-3 PUFA supplementation of 2 g/day had no effect on glycemic parameters (Sitori et al., 1998). Similarly, Kesavulu *et al.* (2002) observed no change in glycemic control in type 2-diabetic patients

following a two-month intervention of n-3 PUFA (1,080 mg of EPA and 720 mg of DHA per day). Alternatively, a study in sixty-three obese subjects being treated for hypertension observed a decrease in plasma glucose after having their diets supplemented with 3.65 g of n-3 PUFA daily for 16 weeks.

1.4.3 The Effect of n-3 PUFA Supplementation on Cholesterol:

The role of cholesterol in the development of atherosclerosis has been a major area of focus in CVD research for many years. Studies that have investigated the impact of increased dietary intake of n-3 PUFA on cholesterol levels in CVD risk have reported modest and inconsistent findings. Meta-analyses by Montori *et al*. and Freidburg *et al.* have reported that fish oil consumption is associated with a slight increase in serum LDL-cholesterol in patients with type 1 and type 2-diabetes (Montori et al., 2000; Freidburg et al., 1998). In contrast, Satoh *et al*. (2007) reported that LDL-cholesterol levels were significantly reduced in obese, type-2 diabetic subjects that consumed purified EPA.

Long standing evidence from epidemiological studies of individuals with genetic forms of hypercholesterolemia, clinical studies and studies in experimental animal models have suggested that LDL-cholesterol is the primary target in reducing the risk of CVD (Genest et al., 2003). However, supplementation of n-3 PUFA, a well-established nutritional factor shown

to decrease CVD risk, does not demonstrate a consistent benefit to reducing plasma cholesterol concentrations.

1.4.4 The Effect of n-3 PUFA on Fasting Triglyceride:

Hypertriglyceridemia is a hallmark feature of MetS and is a significant risk factor for the development of CVD. It is well established that n-3 PUFA supplementation can reduce fasting plasma concentration of TG in hypertriglyceridemic subjects (Calabresi et al., 2004; Harris and Bulchandani, 2006; Phillipson et al., 1985). Phillipson *et al.* (1985) supplemented the diets of 20 hypertriglyceridemic subjects with either a low-fat therapeutic diet, a diet rich in vegetable oil or a diet rich in fish oil (n-3 PUFA). Findings showed that subjects supplemented with n-3 PUFA had a 64% reduction in plasma TG compared to those consuming a low-fat therapeutic diet. Corresponding to the improvements of TG seen with n-3 PUFA supplementation, Calabresi *et al*. (2004) studied the hypolipidemic effects of n-3 PUFA in fourteen subjects with familial combined hyperlipidemia. Each individual consumed 1.88 g of EPA and 1.49 g of DHA per day. Findings of this study showed plasma TG was reduced by 44%.

1.4.5 The Effect of n-3 PUFA supplementation on Post-prandial TG:

The effect of n-3 PUFA supplementation on the post-prandial lipemic response has been shown to be very prominent. Weintraub *et al.* (1988) supplemented 8 normolipidemic subjects with 7.0 g/d of n-3 PUFA

while consuming an isocaloric diet. Findings demonstrated that n-3 PUFA supplementation reduced post-prandial TG, as measured by area under the curve (AUC), by 54%. Similarly, Williams *et al.* (1992) fed 2.7 g/d of n-3 PUFA to normal, healthy subjects and noted a 43.5% reduction in TG AUC. Longer-term studies with lower doses of n-3 PUFA also demonstrated an effective reduction in TG AUC. Agren *et al.* (1996) incorporated 2.3 g of n-3 PUFA into the diets of healthy volunteers for 15 weeks and observed a decline in TG AUC of 31.6%. Furthermore, Roche and Gibney (1996) supplemented the diets of healthy volunteers with 1.0 g/d of n-3 PUFA and recalled a 31.8 % decrease in TG AUC after 16 weeks.

1.4.5.1 The Effect of n-3 PUFA Supplementation on Post-prandial Chylomicron Clearance

A reduction in the post-prandial TG response with n-3 PUFA supplementation can be explained either by slowed CM production or enhanced CM clearance or both. Earlier work has suggested that the effect of n-3 PUFA in improving post-prandial TG is a result of expedited CM clearance (Harris et al., 1990; Nestel et al., 1984; Park and Harris, 2003). Park and Harris (2003) used healthy subjects (n=33) to study the effect of n-3 PUFA on CM clearance. Subjects were fed 4 g/day of EPA and DHA for 4 weeks. Results showed a reduction in post-prandial apoB48 by 16%, decreased CM size and enhanced lipoprotein lipase activity suggesting accelerated CM clearance.

The accelerated clearance of CM with n-3 PUFA supplementation may in part be a consequence of decreased hepatic VLDL production (Roche and Gibney, 1999). CM and VLDL compete for LPL-mediated removal from circulation (Karpe and Hutlin, 1995; Harris et al., 1990). Therefore, when VLDL production is diminished, circulating post-prandial CM has a greater possibility of interacting with LPL, thereby allowing for enhanced post-prandial clearance. Nestel *et al.* (1984) investigated the effect of n-3 PUFA supplementation on VLDL production in 7 subjects (5 normolipidemic and 2 hypertriglyceridemic). Over three weeks the subjects received 30% of their daily energy needs from fat, of which approximately 58% was EPA and DHA. Kinetic studies involved re-injected labeled apoB, after which VLDL production or flux was measured. The results showed that VLDL flux decreased anywhere from 22% in normolipidemic patients to 80% in hypertriglyceridemic subjects.

The depletion of plasma VLDL is supported in a study by Wong *et al.* (1987) who showed a marked decrease in the secretion of TG-VLDL and apoB in HepG2 cells (a liver cancer cell line) incubated with EPA. Additionally, experimental animals of MetS, such as the JCR:LA-*cp* rat, supplemented with 10% red fish oil have also demonstrated a reduction in the plasma concentration of VLDL by 44% (Dolphin, 1988).

1.4.5.2 The Effect of n-3 PUFA Supplementation on Chylomicron Production

Scientists have explored the effect of n-3 PUFA supplementation on the production and secretion of CM in experimental animal models and in cell lines and found that n-3 PUFA inhibits the production of CM (Levy et al., 2006; Murthy et al., 1992).

n-3 PUFA supplementation in the *Psammomys obesus* gerbil, an experimental animal of nutritionally-induced IR and type 2-daibetes, found diminished intestinal CM assembly and apoB48 biogenesis following n-3 PUFA supplementation with a concomitant reduction in TG *de novo* lipogenesis (Levy et al., 2006). The study suggested that the limited availability of TG impaired the assembly and secretion of CM (Levy et al., 2006). Furthermore, it has been suggested that limited TG availability can compromise the stability of the apoB48 protein leaving it susceptible to proteasomal degradation (Levy et al., 2006).

In vitro studies have demonstrated a similar reduction in CM secretion following incubation with n-3 PUFA. Ranheim *et al.* (1992, 1994) incubated Caco-2 cells, colonocytes of a cancer cell line, with EPA for 7 days. After 2 days cells exhibited a decrease in secreted TG. At the end of the study, TG and apoB secretion was reduced by approximately 50%.

Another study incubated Caco-2 cells for 48 hours with 1 mM of EPA or oleic acid. Similar to the findings by Ranheim *et al.* (1992, 1994), this study showed the cells incubated with EPA secreted less TG and

apoB. Furthermore, apoB mRNA abundance was decreased four-fold in cells exposed to EPA. Further analysis using pulse-chase experiments in Caco-2 cells showed that basolateral secretion of newly synthesized apoB was also reduced. The conclusion of these studies were that EPA impaired CM secretion in part by inhibiting the synthesis of apoB as indicated by a decrease in the abundance of apoB mRNA (Murthy et al., 1992).

One limitation of studies performed in Caco-2 cells is the difference in lipid metabolism between colonocytes and primary enterocytes isolated from the jejunum. The major TG synthesizing pathway in Caco-2 cells is the 3-glycerol phosphate pathway (Trotter et al., 1993), while the monoacylglycerol pathway is predominate in enterocytes (Tso and Fujimoto, 1991). Therefore, findings from Caco-2 cells may not reflect normal intestinal physiology. Furthermore, the expression of apoB quantified in Caco-2 cells is only indicative of cell production and secretion at one point in time and is not representative of apoB48 production of the cell during the dynamic phases of the fasting and fed state.

In addition, in this section thus far, we have reviewed studies demonstrating the ability of dietary n-3 PUFA to lower TG and plasma CM. Interestingly, despite reductions in CM, n-3 PUFA supplementation has not been shown to reduce plasma cholesterol associated with CM. Thus, this thesis will attempt to examine the role of IR in post-prandial

dyslipidemia and CM lipid composition with n-3 PUFA supplementation in the JCR:LA-*cp* rat.

1.4.6 The Effect of n-3 PUFA Supplementation on Fasting Parameters of Inflammation:

Cardiovascular disease is a chronic condition in which inflammation plays a critical role in disease development and progression. There is evidence to suggest that n-3 PUFA protect against CVD by modulating the immune response (Yaqoob and Calder, 2003; Mori and Beilin, 2004). Consumption of n-3 PUFA decreases the production of inflammatory cytokines, tumor neucrosis factor (TNF), interleukin-1 (IL-1), interleukin-6 (IL-6) and the activation of leukocytes (Calder, 2006; Massaro et al., 2008; Das, 2000; Kang and Weylandt, 2008). For the purposes of this thesis, the effect of n-3 PUFA supplementation on acute phase proteins have been reviewed as they are systemic markers most widely used to assess inflammation during the post-prandial phase (Devaraj et al., 2008).

Dietary treatments of n-3 PUFA have shown that the anti-inflammatory properties of EPA and DHA can reduce the plasma concentration of CRP in healthy volunteers, type 2-diabetic, hyperlipidemic and CVD patients (Micallef et al., 2009; De Luis et al; 2009). Micallef *et al.* (2009) used 124 healthy volunteers with varying plasma CRP concentrations (<1.0, 1.0-3.0 and >3.0 mg/l). Following n-3 PUFA supplementation results showed an inverse relationship between intake of n-3 PUFA and CRP. Similarly, in hypertriglyceridemic (> 200 mg/dL) and

type-2 diabetic subjects, De Luis *et al.* (2009) supplemented 16 males and 14 females with 2 capsules of 465 mg of EPA and 375 mg of DHA daily for 12 weeks. Results from this study showed a decrease in plasma CRP (5.98 +/- 3.9 vs. 3.9 +/- 1.6 mg/dl; $p < 0.05$) indicating that a dietary intervention of n-3 PUFA improves inflammatory parameters in patients that are hypertriglyceridemic and diabetic.

However, treatment of n-3 PUFA has also shown inconsistent results in CRP from patients with previous myocardial infarct and obesity (Madsen et al., 2007, Chan et al., 2002). In a double-blind study, forty-one patients were randomized to receive either a 5.2 g capsule of olive oil (control) or n-3 PUFA. The findings showed that patients receiving n-3 PUFA had a slight increase in CRP (2.46 vs. 2.70 mg/dL) but was not statistically significant. Furthermore, Chan and colleagues (2002) reported 4g/d of fish oil had no effect on CRP levels in subjects with dyslipidemia and obesity.

Few clinical studies have investigated the effects of n-3 PUFA supplementation on Hp. Most recently, EPA-treatment in diet-induced obese rats showed a decrease in IL-6 mRNA but showed no effect on Hp mRNA in adipose tissue (Perez-Echarri et al., 2008). There is also limited evidence exploring the potential effect of n-3 PUFA on the post-prandial LBP response.

1.4.7 The Effect of n-3 PUFA on Post-prandial Inflammation

Post-prandial inflammation is a new and emerging area of study in the field of CVD. A recently published review suggests that the adverse cardiovascular effects of some nutrients may result from modifications to the post-prandial inflammatory response following a meal (Margioris, 2009). The factors influencing post-prandial inflammation are both metabolic and nutrient-dependent (i.e. fatty acids). Obesity and type 2-diabetes are two conditions in which impaired metabolic conditions exacerbate post-prandial inflammation (Blackburn et al., 2006; Plat et al., 2007; Nappo et al., 2002; Esposito et al., 2007). Post-prandial inflammation in obesity is exponentially increased as the size of visceral adipocytes increase (Blackburn et al., 2006) (See Figure 1-8). Interestingly, the severity of post-prandial inflammation in obesity is reversible with weight loss (Jellema et al., 2004; Plat et al., 2007). Furthermore, patients with type 2-diabetes have shown that the magnitude of post-prandial inflammation positively correlates with the degree of IR, as determined by the homeostatic model assessment (HOMA) index (Napo et al., 2002, Esposito et al., 2007).

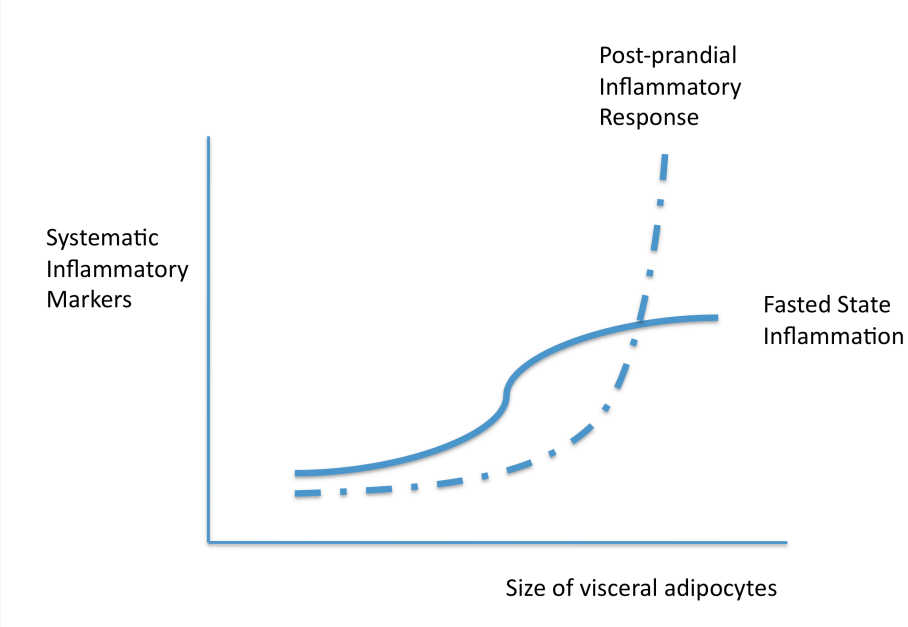

Figure 1-8. The inflammatory state and the post-prandial inflammatory response in conditions of increasing adiposity. Modified from Margioris, 2009.

One nutrient-dependent factor influencing the post-prandial response is the lipid profile of dietary fats (Napolitano and Bravo, 2005; Lui et al., 2007; Poppitt et al., 2008). Indeed, the quantity and quality of dietary fat may significantly impact the severity of the inflammatory response (Margioris, 2009). Since n-3 PUFA is a well-established hypolipidemic and anti-inflammatory agent, in this thesis the effect of n-3 PUFA supplementation on post-prandial lipemia and post-prandial inflammation using a model of obesity, IR and dyslipidemia is investigated.

CHAPTER 2: Rationale

2.1 Introduction

Every seven minutes a person dies of heart disease or stroke in Canada (Heart and Stroke Foundation, 2004). CVD affects more people than any other disease, accounting for 30% of all deaths in the country. Heart disease and stroke cost the Canadian economy 18 billion dollars in direct costs of treatment services (physical and hospital services) and indirect costs from lost wages and decreased productivity (Heart and Stroke Foundation, 2004). In order to relieve this medical and economic burden, focus on prevention and early diagnosis is needed to stop disease development and progression. A comprehensive understanding of the contributing factors to the dyslipidemia associated with atherosclerotic risk may lead to interventions in treatment and prevention of CVD.

2.2 Chylomicrons and Cardiovascular Disease:

Present concepts of atherogenesis consider the trans-endothelial migration of lipoproteins, which is the first critical step into the process of CVD development. LDL is a predominant lipoprotein observed during the fasted state and is thought to readily permeate through the endothelial layer. However, there is now evidence to suggest that CM-remnants can also permeate and be retained in the arterial wall. As described in Chapter 1, during the fed or post-prandial state CM are the predominant fractions and their metabolism is often impaired during chronic disease. Indeed,

Genest *et al*. (1991) has reported 40% of patients suffering from coronary artery disease have normal plasma LDL concentration but exhibit post-prandial CM lipemia. Similarly, numerous emerging clinical studies have demonstrated a relationship between post-prandial lipemia and the development of CVD (Patsch et al., 2000; Cohn, 1994; Weintraub et al., 1996).

Even though both LDL and CM are considered to accumulate in the subendothelial arterial space (Proctor et al., 2002; Proctor et al., 2004), impaired post-prandial CM metabolism may pose a greater risk to developing CVD. Compared to LDL, CM have been shown to efflux less readily from the artery and contain greater mass of cholesterol per particle (Proctor et al., 2003). It has been previously proposed that CM may exacerbate the amount of oxidatively modified cholesterol within the subendothelial space that would in turn accelerate the atherogenic cascade. To support this latter contention, the association between CVD risk and CM has been demonstrated in sudden death cardiac patients that report the accumulation of apoB48, the apoprotein unique to CM, in the coronary arteries of sudden cardiac death patients (Nakano et al., 2009).

2.3 Insulin Resistance and Cardiovascular Disease Risk:

It is now well recognized that chronic metabolic disorders such as IR and obesity increase the risk of developing CVD. However, the mechanism by which IR exacerbates the process of atherogenesis is not

completely understood. It has been suggested that IR may impact the homeostatic metabolism of intestinally derived lipoproteins. Karpe *et al.* (1994) showed that the progression of coronary lesions, in type 2 diabetic subjects, was related to post-prandial lipemia (Karpe et al., 1994). More specifically, fasting plasma concentrations of apoB48 in IR and obese men are elevated (24.3 $\pm$ 8.8 ug/mL) compared to apoB48 concentration in normal healthy individuals (6.8 $\pm$ 1.2 ug/ mL) (Mamo and Proctor, 2002).

Experimental animal models of IR, the fructose fed Syrian Golden Hamster and the *Psammomys Obesus* gerbil, have also demonstrated an increase in plasma concentrations of apoB48 compared to their healthy counterparts (Guo et al., 2005; Zoltowska et al., 2003). In our lab, we have shown that the JCR:LA-*cp* rat, a model of MetS (IR, obesity and dyslipidemia), has elevated fasting and post-prandial plasma concentrations of apoB48, compared to their lean littermates. Collectively, clinical and animal studies demonstrate that metabolic disturbances exacerbate the fasting concentration and the post-prandial response of CM, as measured by apoB48.

2.4 Chylomicron Overproduction in Insulin Resistance:

In the post-prandial state, lipoprotein metabolism is balanced by the production and clearance of CM. Earlier work studying impaired post-prandial lipoprotein metabolism focused on the inefficient clearance of lipoproteins (Mamo et al., 1998; Redgrave, 1977; Redgrave and Snibson,

1977). In conditions of post-prandial lipemia the competition between CM and VLDL for hydrolysis by lipoprotein lipase impairs the efficient clearance of CM particles, resulting in an increase in the number of potentially atherogenic circulating lipoproteins (Karpe, 1997; De Man et al., 1996). Furthermore, it has been demonstrated that conditions of IR and diabetes are associated with impaired lipoprotein hydrolysis (Maed et al., 2002), resulting in the inefficient clearance of CM from circulation (Figure 2-1).

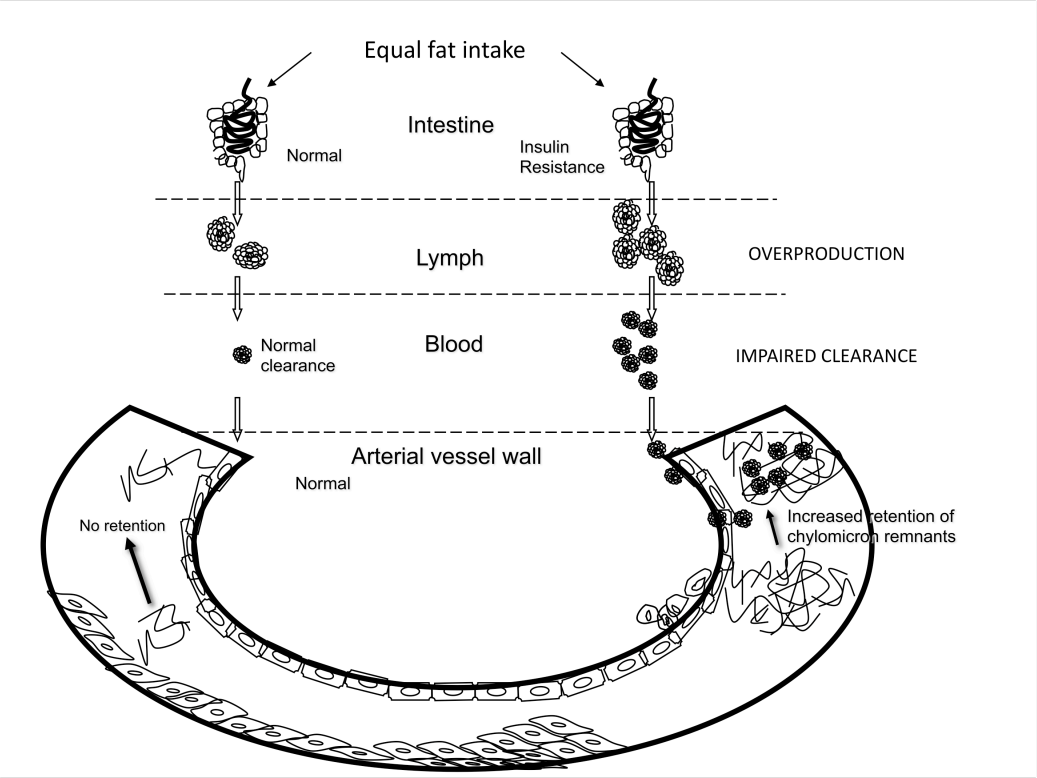

Figure 2-1. Chylomicron production and clearance in normal and insulin resistant conditions. The left side of this schematic represents a healthy individual with normal chylomicron production and clearance. The right side of the schematic represents the insulin resistant state, which is associated with chylomicron overproduction and impaired chylomicron clearance.

More recently, the overproduction of CM is being recognized as an equal, if not more significant contributor to the post-prandial lipemic state. There is limited evidence in humans showing CM overproduction due to the technical issues involved in human intestinal resection. However, indirect measures of CM production have shown that IR and type 2-diabetic patients exhibit increased production rates of apoB48-containing lipoproteins (Duez et al., 2006; Hogue et al., 2007) (Figure 2-1).

As detailed earlier in chapter 1, experimental animal models of IR have shown an increase in the biosynthesis of apoB48 at the level of the intestine and there is some evidence for the overproduction of CM particles (Haidari et al., 2002; Zoltowska et al., 2003). For instance, jejunal explants of *Psammomys obesus* gerbil demonstrated an increase in the synthesis of apoB48 and enhanced *de novo* lipogenesis (Zoltowska et al., 2003). Additionally, studies in the Golden Syrian fructose fed hamster showed that intracellular insulin signaling in enterocytes is altered in hyperinsulinemia and is associated with CM overproduction (Haidari et al., 2002; Federico et al., 2006; Duez et al., 2006).

The limitation of the aforementioned clinical and animal studies of intestinal overproduction is the use of techniques that either assess CM production indirectly through analysis of plasma concentrations of apoB48 or the use of ex vivo techniques that measure CM production at one point in time, respectively. This thesis will address more carefully the impact of

IR on the secretion of CM directly and overtime in both the fasting and post-prandial phases, using an adapted in vivo lymph cannulation method.

2.5 Insulin Resistance and the Intestine:

The intestine is a pivotal organ in lipid and lipoprotein metabolism and in conditions of metabolic disorders intestinal integrity can be modified. Briefly, studies in type 1-diabetes have demonstrated that the intestinal mucosal barrier, microflora and immune function are compromised (Vaarala et al., 2005). Furthermore, studies in conditions of obesity and hyperphagia have shown that the intestine adapts via hypertrophy (Estornell et al., 1995; Sefcikova et al., 2008; Williamson, 1982). However, to date, studies showing intestinal overproduction of CM in pathologies associated with the MetS have not considered the structural adaptations of the intestine. The intestine is a dynamic organ with cells turning over every 48 hours (Weiser et al., 1973). Therefore, it is reasonable to assume that chronic metabolic disorders could impact the structure and metabolism of the intestine.

In an effort to increase the understanding of the role of the intestine in aberrant lipoprotein metabolism, this thesis uses a rodent model of MetS, the JCR:LA-*cp* rat to investigate the structural adaptations of the intestine and the potential relationship between intestinal structural changes and CM overproduction.

2.6 The Effect of n-3 PUFA Supplementation on Post-prandial Lipid Metabolism:

The second phase of this thesis was to examine whether the post-prandial lipoprotein response in the JCR:LA-*cp* rat can be modified via nutritional intervention. It is well established that long chain n-3 PUFA supplementation can improve fasting TG concentrations (Harris and Bulchandani, 2006; Roche, 1999). Indeed, studies have shown that dietary n-3 PUFA supplementation can also improve the post-prandial TG response (Westphal et al., 2000; Williams et al., 1992; Williams, 1998; Roche, 1999), However, these studies have not delineated whether there is a direct effect of n-3 PUFA supplementation on the post-prandial CM response in the MetS.

Additionally, emerging evidence shows a substantial relationship between lipid metabolism and the inflammatory state (Marigoris, 2009). A post-prandial rise in TG and glucose stimulates and activates leukocytes and neutrophils, which elevate the plasma concentration of pro-inflammatory cytokines (Alipour et al., 2007). As a result, the stimulated pro-inflammatory state increases the plasma concentration of acute phase proteins: CRP, Hp, LBP (Brunetti et al., 2008). Since we spend the majority of our day in the post-prandial state constant exposure of inflammatory markers to arterial vessels plays a critical role in the initiation and progression of atherosclerosis. Therefore, in this thesis, we also propose to use the JCR:LA-*cp* to investigate the corresponding effect of n-3 PUFA supplementation on post-prandial inflammation.

2.7 Primary Thesis Aim:

The primary aim of this thesis was to investigate the morphological and lipidogenic adaptations of the intestine and the relationship of the intestinal changes to CM secretion and/or production in an established animal model of post-prandial lipemia and MetS. It is hypothesized that the intestine is hypertrophic under conditions of obesity and IR possibly augmenting the overproduction of CM. Additionally, it is proposed that dietary n-3 PUFA supplementation may improve the exacerbated post-prandial CM response and the associated pro-inflammatory response in conditions of the MetS. It is hypothesized that n-3 PUFA supplementation would improve the post-prandial lipid response as well as improve the corresponding post-prandial immune state.

2.8 Specific Hypotheses and Objectives:

1. Hypothesis: Increased CM secretion is associated with the MetS in the JCR:LA-*cp* rat.

Objective: A mesenteric lymphatic cannulation technique was used to determine the concentration of apoB48 (a marker of CM) and lipid (TG and cholesterol) secreted from the intestine in lean (non-IR) and IR JCR:LA-*cp* rats in both the fed and fasted state.

2. Hypothesis: The Metabolic Syndrome impacts the number and distribution of enterocytes along the villus-crypt axis. Additionally, apoB48

protein expression (production) per enterocyte is elevated in the IR JCR:LA-*cp* rat compared to their lean counterparts.

Objective: Immunohistochemistry and enterocyte fractionation coupled with an adapted western blotting method was used to determine the distribution of enterocytes and apoB48 protein expression along the intestinal villus-crypt axis in lean and IR JCR:LA-*cp* rats.

3. Hypothesis: Compared to lean control rats, the intestinal villi of IR JCR:LA-*cp* rats are greater in length, width and total surface area. Intestinal hypertrophy will correspond to an increase in the number of enterocytes lining the intestinal villus.

Objective: Gross intestinal length, width and mass, as well as jejunal villus length, width, surface area and enterocyte number were used to assess intestinal morphology of the jejunal region in the *JCR:LA-cp* rat aged to 6 months.

4. Hypothesis: Dietary n-3 PUFA supplementation reduces the post-prandial lipoprotein concentration of intestinally derived CM as well as improves the associated pro-inflammatory response of acute phase proteins in the MetS JCR:LA-cp rat.

Objective: JCR:LA-*cp* rats supplemented acutely (3 weeks) with n-3 PUFA were subjected to an oral fat challenge in order to assess the effect of dietary n-3 PUFA on the post-prandial CM response using apoB48

western blot analysis. Additionally, ELISA methods assessed the post-prandial inflammatory response of acute phase proteins; CRP, Hp and LBP.

CHAPTER 3: *Increased production of apolipoproteinB48 (apoB48) containing lipoproteins is associated with intestinal hypertrophy in the JCR:LA-cp rat model of the Metabolic Syndrome.*

3.1 Introduction

Impaired chylomicron (CM) metabolism is a contributing factor to the high prevalence of dyslipidemia (Avramoglu et al., 2003; Havel, 1994) associated with the Metabolic Syndrome (MetS). Elevated plasma CM concentration in the MetS exacerbates the risk of developing CVD. Since CM-remnants have the ability to permeate the arterial wall, these particles can contribute to the focal accumulation of cholesterol initiating and progressing the atherosclerotic process (Proctor et al., 2002; Proctor and Mamo, 1998). Atherogenic risk increases as a greater number of CM accumulate in circulation (Zilversmit, 1979; Karpe et al., 1994) increasing the exposure of CM-remnants to the arterial wall. Despite the mounting evidence implicating elevated fasting and post-prandial plasma CM concentrations to CVD risk, the mechanisms contributing to impaired CM metabolism are still not clearly understood.

In conditions of IR it has been suggested that impaired CM metabolism is a consequence of CM overproduction (Cabesaz et al., 1998, Redgrave, 2004; Duez et al., 2008). Duez *et al.* (2006) and Hogue *et al.* (2007) demonstrated an increase in the production rate of apoB48 in IR and diabetic patients, respectively.

Experimental animal models have attempted to examine the mechanistic adaptations of intestinal lipoprotein overproduction (Federico

et al., 2006; Zoltowska et al., 2003). Recently, Federico and colleagues (2006) showed that enterocytes of the IR Syrian Golden hamster have altered insulin-signaling pathways enhancing the process of CM assembly. Furthermore, Zoltowska *et al*. (2003) suggests that increased *de novo* lipogenesis and enhanced apoB48 biogenesis, in the intestine, are instrumental in oversecretion of CM.

These mechanistic studies are limited by the assumption that isolated enterocytes or intestinal explants are physiologically representative of CM metabolism, and do not necessarily reflect the net synthesis and transport of lipid. It is important to consider the secretion of CM particle number and lipid over time, particularly in response to stimuli such as feeding. In addition, it is especially important to appreciate that clinical studies and work in experimental animal models have not considered the potential morphological and adaptations of the intestine (i.e. an increase in the absorptive surface area of villi and/ or an increased number of enterocytes).

The aim of this study was to investigate the morphological modifications of the intestine as well as the enterocytic expression of apoB48, a marker used to identify CM. Lymphatic cannulation and enterocyte isolation techniques were used to directly assess the contribution of the intestine in CM production and secretion in the JCR:LA-*cp* rat, a model of MetS and impaired CM metabolism (Vine et al., 2007).

3.2 Methods

Animals and Diets

Male JCR:LA-*cp* MetS (*cp/cp*) (n=8) and lean (+/?) rats (n=8) were raised in our breeding colony at the University of Alberta. Rats were weaned to 3 weeks of age and housed with 12/12 h reversed light cycle to allow for study and testing during the dark phase of the rat's diurnal cycle. At that time, rats on protocol were transferred to state-of the-art individually ventilated caging environment (Techniplast^{TM}, Exton, PA, USA). Animals were allowed to age for approximately 6 months, by which time the established pathology for dyslipidemia is well developed (Russell et al., 1989). The animals had access ad libitum to standard laboratory rodent chow (Lab diet 5001, PMI Nutrition International, Brentwood, MO, USA) and water. The composition (w/w) of this diet consists of carbohydrate 49%, crude protein 24.0%, moisture 10%, minerals 6.5%, fibre 6.0% and fat 4.5%. All experimental animal work was conducted in accordance with the Canadian Council on Animal Care and the University of Alberta Animal Ethics Committee approved all experimental protocols.

Lymphatic Chylomicron Analysis

Animals of each phenotype (cp/cp and +/?) were initially fasted to equilibrate gastrointestinal contents and then re-fed an equal volume of chow before lymphatic surgery. The surgical cannulation procedure involved a gastric cannula used to infuse saline solution (NaCl 154 mmol/l,

4% glucose infusion) and intralipid (20% Intralipid, 4% glucose infusion) into the digestive tract. A collecting tube placed in the subclavian duct collected lymph before the intestinal secretions entered blood circulation. Lymph CM was collected from donor lean and MetS JCR:LA-*cp* rats during conditions of fasting (saline solution infused) and fed states (intralipid infused) with each infusion occurring over a period of four hours. Cholesterol and TG lymphatic concentrations were determined using commercially available kits. ApoB48 was quantified using an adapted western immuno-blot method (Smith et al., 1997; Vine et al., 2006) described in detail in method section *ApoB48 Quantitation and Localization*.

Intestinal Morphology

Following a 16 h fast, animals aged to 6 months, from each group, lean (n=8) and MetS (n=8) were sacrificed under halothane anesthetic. Intestinal jejunal sections of 30 cm in length were excised and flushed with PBS to remove residual digestive waste. A one-centimeter jejunal portion of the intestine was cut transversely and inverted. The sliced jejunal section was oriented in tissue embedding medium (VWR, Tissue-Tek OCT-Catalog #62550-01, USA) and preserved by snap freezing in liquid nitrogen and kept at -80°C for histological analysis. Histological characteristics of the intestinal villus were assessed in tissue cryosectioned (8 µm thick) and stained with hematoxylin (Sigma Aldrich Inc., Catalog # HHS16, USA) and eosin dyes (Sigma Alrich Inc., Catalog #

119830, USA). Morphological observations of the intestinal lumen (villus length, width and area) were quantified using Axiovision software 7.0 (Zeiss, Germany).

Enterocyte Isolation and Protein Extraction

Of the rats sacrificed under halothane anesthetic the excised 30 cm jejunal tissue is used for enterocyte isolation. Cells were isolated using the Weiser method that involves loosening intestinal attachments of enterocytes by incubations with dithiothreitol and citrate then freeing the enterocytes by shaking the intestinal tissue mechanically in EDTA-containing solutions (Weiser, 1973). More specifically, once the jejunum is flushed to remove digestive waste an EDTA, dithiothreitol (DTT) and citrate-containing solutions (0.438 g/L of EDTA and 0.077 g/L of DTT dissolved in PBS with a pH of 7.4) is poured into the jejunum and the jejunum is incubated in a shaking PBS bath set at 37°C for 4 min; weakening protein mediated, calcium dependent adhesion of cells to the monolayer and the basement membrane (Weiser, 1973). Following the DTT incubation, EDTA and citrate incubations of the intestine separate enterocytes from the luminal surface of the jejunum. Detached cells are collected in fractions with each progressive fraction being incubated for longer periods of time (fraction 1 = 2 min, fraction 2 = 2 min, fraction 3 = 4 min, fraction 4 = 5 min, fraction 5 = 6 min, fraction 7 = 7 min, fraction 8 = 10 min, fraction 9 = 15 min, fraction 10 = 20 min). Enterocytes are sequentially collected in 10 fractions with fraction 1 representing the

proximal (tip) portion and fraction 10 representing the distal (crypt) portion of the intestinal villus (Figure 3-1). Isolated cell fractions are centrifuged at 1000 rotations per minute for 2 min and the supernatant discarded. The pellets, containing enterocytes, are washed in PBS twice and re-suspended in 1 ml of PBS. Manual cell counts using a hemacytometer were performed on fresh cells from each fraction. Additionally, cell associated protein concentration was determined on lysed cells using a commercially available protein kit (ThermoFisher Scientific, USA, Catalog # 23250).

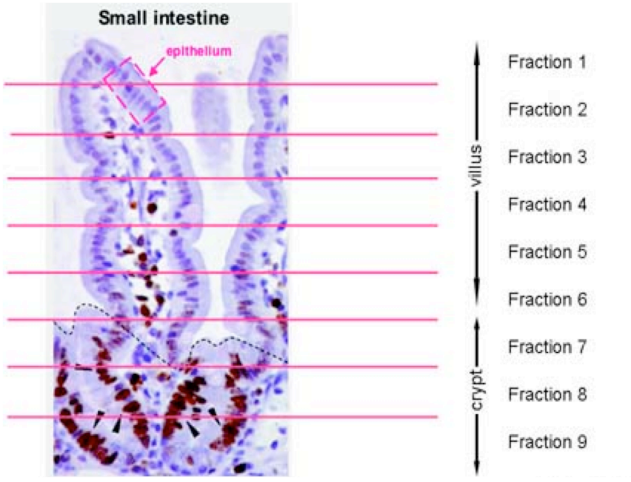

Figure 3-1. Cell Fractionation using the Weiser Method.

ApolipoproteinB-48 Quantification and Localization

Western immuno-blot. Cell-associated CM were quantified in the JCR:LA-cp rodent model by identification of apoB48 using a method previously described (Vine et al., 2007). Briefly, total plasma apolipoproteins were

separated by SDS-PAGE on a 3-8% tris-acetate polyacrylamide gel (Invitrogen, NuPage, CA, USA). The separated proteins were transferred onto a PVDF membrane (0.45 µm; ImmobilonP^{TM}, Millipore. MA, USA). Membranes were incubated with a goat polyclonal antibody specific for apoB (Santa Cruz Biotech, CA) and a secondary antibody tagged with hydrogen peroxidase (Santa Cruz Biotech, CA) was used to visualize apoB by chemiluminescence (ECL-Advance, Amersham Biosciences, UK). The intensity of the probed band was quantified using linear densitometric comparison with a known mass of the purified rodent apoB48 protein using Scion Imaging Technology (Vine et al., 2007).

Immunohistochemistry. To complement the observations seen with immuno-blotting, a newly developed immunohistochemical technique adapted for use with apoB antibody (Santa Cruz Biotech, CA) was used to demonstrate the distribution of apoB48 along the intestinal villus. Cryosections were rehydrated in xylene (2 x 10 min) and sequential ethanol washes (100% ethanol for 2 x 10 minutes followed by 95% ethanol, 70% ethanol, 50% ethanol and 30% ethanol for 2 min each). Intestinal sections were incubated with a commercially prepared primary antibody specific for apoB48 (Santa Cruz Biotech, CA) diluted to a 1:20 concentration with 1% donkey serum and kept in a hydrophobic chamber overnight at 4°C. Immunofluorescent secondary antibody, tagged with AlexaFluor 647 (Invitrogen, Catalog # A21447, USA) was incubated on sections for 30 min at room temperature at a concentration of 1:100 in 1%

donkey serum. Slides were mounted with an aqueous medium and the distribution of apoB48 along intestinal villus was visualized using a fluorescent microscope.

Statistical Analysis

Data was tested for normal distribution and differences between lean and obese animals were analyzed using unpaired t-test with significance set at $p<0.05$. (GraphPad PRISM). All results are expressed as the mean $\pm$ SEM.

3.3 Results

Intestinal Morphology

The intestine of MetS, JCR:LA-*cp* rats of 6 months of age were significantly greater in length (94 ± 9.8 cm vs. 111 ± 6.8 cm, $p < 0.05$) and jejunal weight (4.7 ± 0.3 g vs. 3.0 ± 0.1 g, p=0.001), as compared to lean rats. Accordingly, the mucosal villi in MetS rats were also significantly longer (631.9 ± 26.8 µm vs. 499.8 ± 17.8 µm, p=0.0007) with a larger villus area (106.50 ± 8.67 mm^2 vs. 79.53 ± 8.53 mm^2 p=0.04) compared to their lean counterparts, as shown in Table 3-1.

	Lean Rats (+/?)	**MetS Rats (cp/cp)**
Weight (kg)	432 ± 17.7	750 ± 10.4 **
Insulin (mU/L)	12.4 ± 1.6	423.5 ± 163.4 ***
Length of Intestine (cm)	94 ± 9.8	111 ± 6.8 *
Weight of jejunum (g)	3.02 ± 0.23	4.68 ± 0.72 *
Villus length (µm)	499.8 ± 17.75	631.9 ± 28.84 **
Villus width (µm)	193.72 ± 16.66	190.3 ± 9.36
Villus area (μm^2)	79530 ± 8532	106500 ± 8668 *

Table 3-1. Characterization of the Intestinal Morphology of obese and lean JCR:LA-cp rats. This table represents the phenotypic characteristics of both lean and obese rats by showing significant differences in body weight and plasma insulin concentrations. Measurements of the intestinal features represent significant differences between lean and obese rats as represented by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Enterocyte Number and Cell-associated Protein

Total protein from isolated enterocytes along the length of the villus was significantly higher in MetS rats (53.84 ± 5.65 mg/ml) compared with lean

rats (39.17 ± 3.77 mg/ml, p = 0.04). We observed a progressive increase in enterocyte number and cell-associated total protein concentration from the tip of the villus towards the villus crypt, with the greatest cell count and protein concentration measured in fractions 7, 8 and 9 in both groups (Figure 3-2 and 3-3). Moreover, enterocyte number in fraction 9 of the intestinal villus was greater in MetS rats compared to lean rats (2.83×10^7 ± 4.04×10^6 cells vs. 1.29×10^7 ± 2.33×10^6 cells, p=0.028, respectively) (Figure 3-1). Additionally, cell-associated protein concentration in fraction 8 and 9 of MetS rats is significantly greater compared to lean controls (8.13 ± 1.02 mg/ml vs. 5.01 ± 0.63 mg/ml, p =0.021 and 9.16 ± 1.46 mg/ml vs. 3.33 ± 0.45 mg/ml, respectively) (see Figure 3-3).

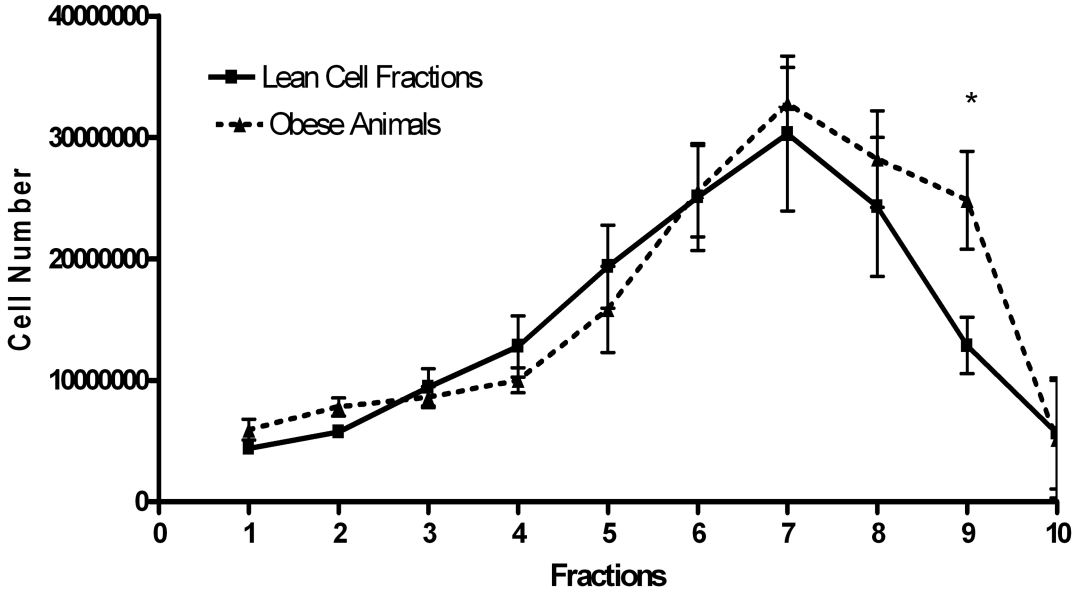

Figure 3-2. Number of enterocytes lining the intestinal villus. Fractions 1-10 represent the progression of cells collected from proximal tip of the villus towards the distal crypt, respectively. * p <0.05 represents the significant difference in the number of cells within the isolated fraction.

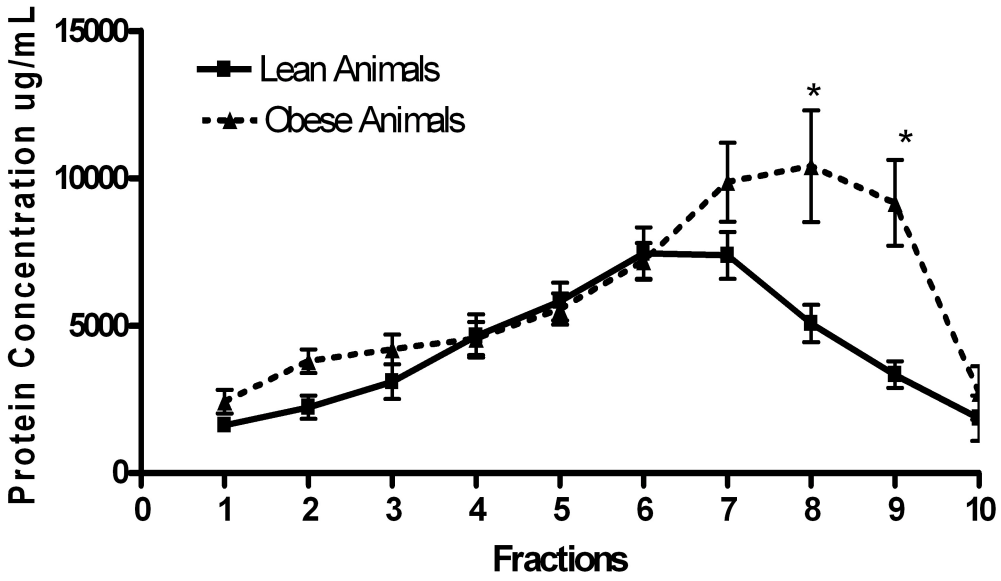

Figure 3-3. Cell-associated protein mass along the length of the intestinal villus. Total protein concentration in isolated enterocyte fractions from fraction 1- 10, the villus tip to the crypt. * $p<0.05$ represents a significant difference in total enterocytic protein with the fraction.

Apolipoprotein B48 Production and Distribution

Mass of apoB48 protein in enterocyte fractions showed a progressive increase in apoB48 concentration in enterocytes from the tip to the crypt of the villus. MetS rats had significantly more apoB48 at the crypt of the villus, specifically in fraction 9 (81.57 $\pm$ 21.22 µg/ml) as compared to their lean counterparts (26.86 $\pm$ 7.440 µg/ml, p=0.03).

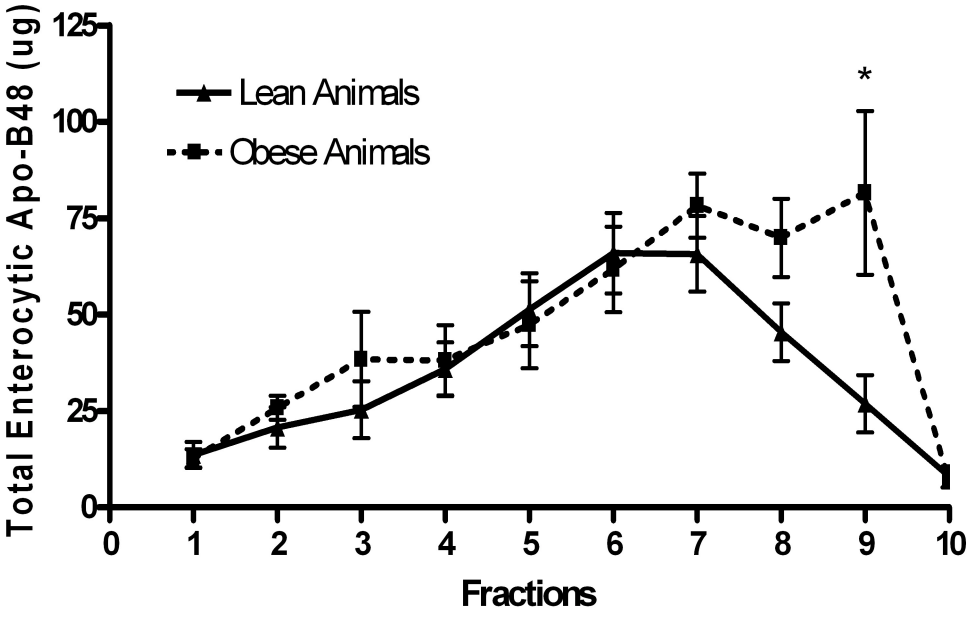

Figure 3-4. Cell-associated apoB48 mass along the length of the intestinal villus. ApoB48 mass was determined for each isolated cell fraction via immuno-blotting methods. * $p<0.05$ represents a significant difference in apoB48 from isolated cell fractions between phenotypically obese and lean rats.

To complement the western immuno-blotting approach, immunohistochemistry adapted for apoB48 was used to visually demonstrate the distribution of apoB48 along the intestinal villus. We observed that apoB48 accumulated in the crypt of the intestinal villus in both groups (lean and MetS). Further, the signal intensity observed visually along the villus of MetS rats was more intense than that observed in their lean counterparts.

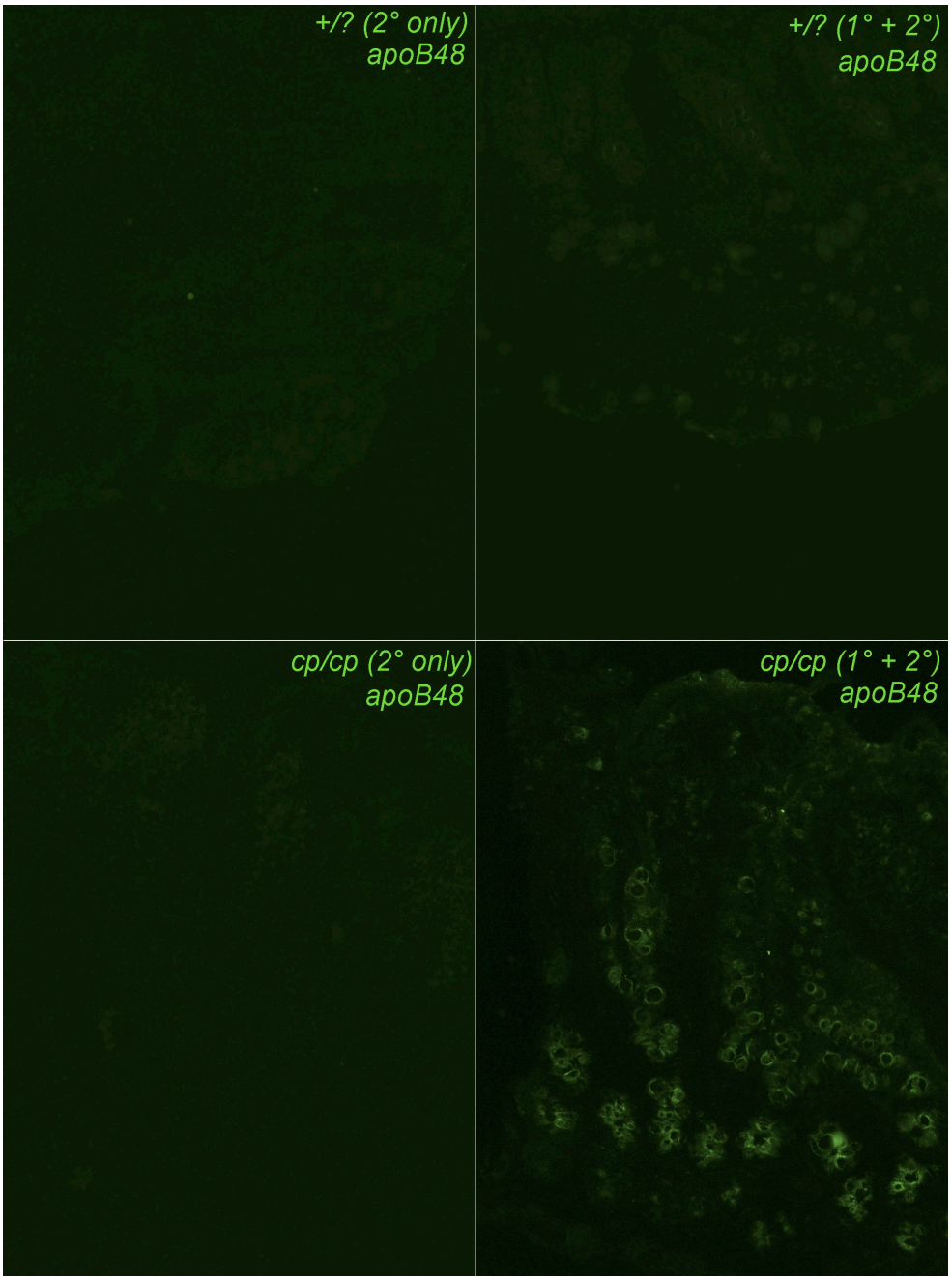

Figure 3-5. Immunohistochemical Analysis of Intestinal ApoB48 Distribution. Using a newly developed immunohistochemistry technique, fluorescent staining of the apoB48 in cryo-intestinal sections from obese JCR:LA-*cp* rats (cp/cp) (panel D) was more intense than that observed in lean rats (+/?) (panel B). Panels A and B are intestinal sections from lean animals (+/?) using secondary antibody alone or primary and secondary antibody, respectively. Panels C and D represent sections from an obese (cp/cp) rat treated with secondary antibody alone or primary and

secondary antibody, respectively. Images were captured at 20X magnification.

Lymphatic Chylomicron Production

Lymph isolated following a gastric saline (NaCl 154 mmol/L) infusion (fasted) demonstrated that lymph composition of the MetS JCR:LA-*cp* rat contained a greater concentration of apoB48, despite TG and cholesterol concentrations remaining similar compared to lean controls. In contrast, a gastric lipid infusion (fed state) resulted in an increase in lymphatic TG, cholesterol and apoB48 in both lean and MetS JCR:LA-*cp* rats. TG, cholesterol and apoB48 following the gastric lipid infusion were significantly elevated in MetS rats compared to their lean counterparts as shown in Table 3-2. The TG:apoB48 ratio (i.e., the size of apoB48 containing particles) was significantly lower in MetS rats compared with lean rats following both the saline and lipid gastric infusions. The cholesterol/apoB48 ratio showed that the particle composition of lean rats contained a higher concentration of cholesterol per particle than MetS rats in both the fasted and fed state. However, total particle number and total cholesterol secreted into the lymph was greater in MetS rats.

Fast 16 hr	Food equivalency 24hr (re-feeding)	Lymph cannulation (n=8/group)					
		Saline Infusion/3hr (4% Glucose)*		/B48 Ratio	20% Intralipid Infusion /3hr (4% Glucose)*	/B48Ratio (Δ vs Saline)	Δ Saline-Intralipid
Lean control →		TC mg/dL	61.7 ± 6.6	0.08	79.7 ± 10.2	0.075 (x0.9)	x1.25
		TG mg/dL	946 ± 106	1.25	2545 ± 282	2.4*1 (x1.9)	x2.5*1
Obese IR →		B48 ug/ml	750 ± 102	-	1074 ± 247	-	x1.4
		TCmg/dL (%Δ vs lean)	60.7 ± 3.6 (100%)	0.03ß1	161.9 ± 27.7*3 (200%ß2)	0.04ß1 (x1.3)	x2.8ß2
		TGmg/dL (%Δ vs lean)	1039 ± 113 (105%)	0.55ß2	3503 ± 419*3 (140%ß1)	1.0*1, ß2 (x1.8)	x3.5ß1
		B48ug/ml (%Δ vs lean)	1917 ± 346ß2 (250%)	-	3586 ± 554*1 (350%ß2)	-	x1.8ß1

*Normalized for lymphatic collection (mls lymph/hr)
*1=p<0.05, *2=p<0.01, *3=p<0.001 within phenotype
ß1=p<0.05, ß2=p<0.01, ß3=p<0.001 between phenotype

Table 3-2. Composition of lymph CM from IR and lean JCR:LA-*cp* rats. Lymphatic CM, TG and cholesterol were measured in both the lean and IR phenotype following saline (fasted state) and intralipid (fed state) infusions. The TG:apoB48 ratio is indicative of CM size.

3.4 Discussion

The key objective of this study was to investigate the relationship between intestinal structure and apoB48 and lipid production and secretion during the MetS and post-prandial lipemia. Our findings show that oversecretion of intestinal apoB48 corresponds with an increase in the protein expression of apoB48 along the intestinal villus which is associated with intestinal hypertrophy in the JCR:LA-*cp* rat.

The major limitation of existing clinical and animal studies assessing CM overproduction is that these studies have not been able to consider intestinal secretion of apoB48 directly into lymph. The intestine is a very dynamic organ and therefore to fully appreciate the role of the intestine in CM metabolism, apoB48 secretion needs to be assessed overtime during the fasted and/or fed states.

The strength of our study arises from the use of an adapted lymphatic cannulation procedure, which is a direct measure of intestinal lipid (TG and cholesterol) and apoB48 secretion during the fasting and post-prandial phase. Indeed, the results of lymphatic oversecretion of apoB48 in the present study are consistent with findings in clinical and IR animal studies (Duez et al., 2006; Haidari et al., 2002, Zoltowska et al., 2003).

Clinical work in hyperinsulinemic and diabetic patients has demonstrated that plasma insulin concentrations positively correlate with

an increase in the production rate of intestinally derived apoB48 (Duez et al., 2006; Hogue et al., 2007). Studies in the fructose fed Golden Syrian Hamster and the *Psammomys obesus* gerbil, both models of nutritionally induced IR, also show that IR is associated with enhanced intracellular stability of apoB48 and increased apoB48 biogenesis, as determined by isolated enterocytes and jejunal explants, respectively (Haidari et al., 2002; Zoltowska et al., 2003).

Furthermore, in the present study, increased apoB48 protein expression along the intestinal villus correlates with the intestinal oversecretion of apoB48 into the lymph. It has been suggested that enhanced apoB48 protein expression may be a consequence of intracellular apoB48 stability (Zoltowska et al., 2003). Generally, the efficient lipidation of apoB48 inhibits the proteasomal degradation of the apoprotein. The intracellular stability of apoB48 may be a consequence of upregulated microsomal triglyceride transfer protein (MTP). MTP is essential in the lipidation of apoB48. The absence or inhibition of MTP could severely hinder or abolish the assembly and secretion of CM (van Greevenbroek et al., 1998). Although, we have not investigated the expression of MTP in the present study work in other labs have reported that MTP is upregulated in IR (Phillips et al., 2006; Hseih et al., 2008). Duodenal biopsies, obtained during gastroscopy, in 27 diabetic and 24 non-diabetic subjects found that MTP mRNA abundance was significantly

higher in diabetic patients (25.0 ± 25.2 amol/μg) *vs.* non-diabetic subjects (13.1 ± 5.6 amol/μg) (Phillips et al., 2006).

Additionally it has been suggested that oversecretion of apoB48 in the fed state is associated with an increase in lipid availability in the enterocyte. Our findings support the concept of increased intestinal lipid availability due to the elevated lymphatic TG and cholesterol concentrations seen in MetS rats. A possible explanation of increased CM (apoB48) secretion and increased lymphatic lipid concentrations, in the fed state, is based on the influence of elevated plasma free fatty acids on lipoprotein production. In MetS, elevated plasma concentrations of lipids occurs as a result of saturated adipose tissue storage capacity and a decrease in the ability of insulin to suppress lipolysis. A study in humans that examined the effect of endogenous fat via an intralipid/heparin infusion showed that elevated plasma lipids is associated with increased plasma concentrations of apo-B48 containing lipoproteins (Duez et al., 2008).

Another possible explanation for elevated intestinal lipid secretion, specifically increased lymphatic cholesterol concentration, suggests that IR can impact the transport of cholesterol into the intestine. Recently, Lally *et al.* (2006, 2007) showed that intestinal cholesterol transporter NPC1L1 mRNA expression is increased, while ABCG5/8 (the transporter responsible for the efflux of cholesterol into the intestinal lumen) is decreased in both type 1 and type 2-diabetes. Additionally, CD36 is

upregulated in type 2-diabetes (Bonen, 2006; Hsieh et al., 2008), enhancing cholesterol uptake and absorption. Preliminary work in the JCR:LA-*cp* rat (Vine et al., 2008), using intestinal diffusion studies, showed that cholesterol transport is increased in the intestine of the MetS JCR:LA-*cp* rat. Therefore, the enhanced absorption and decreased efflux of cholesterol may explain the increased concentration of cholesterol and apoB48 in lymphatic circulation of MetS rat compared to lean controls.

To some extent, lipid availability may also be explained by enhanced *de novo* lipogenesis as demonstrated by an upregulation of intestinal lipogenic enzymes: SREBP-1c, monoacylglycerol transferase (MGAT) and diacylglycerol transferase (DGAT) that have been demonstrated in IR animal models (Duez et al., 2008; Zoltowska et al., 2003).

Lymphatic cannulation further revealed that CM produced in MetS rats were smaller in size, as determined by TG:apoB48 ratio. This finding is consistent with previous work from our laboratory reporting a decreased plasma TG:apoB48 ratio in the JCR:LA-cp rat (Vine et al., 2007). The small size of the CM and the elevated secretion of total cholesterol transported in these particles may heighten the atherogenic potential of these particles in the fed state, (i.e. cholesterol stays with the particle following lipolysis to form cholesterol dense CM-remnants). Cholesterol-dense CM-remnants then have the ability to penetrate the arterial wall (Proctor and Mamo, 2003). Although, the cholesterol:apoB48 ratio is lower in MetS rats compared to lean rats, the total cholesterol secreted by the

intestine is greater in MetS rats, which is likely to contribute to circulating levels of plasma cholesterol and increase the exposure of CM-derived cholesterol to the arterial endothelial space.

Furthermore, increased apoB48 production may be the effect of a pro-inflammatory state, a condition associated with the development of atherosclerosis and MetS (Holvoet, 2008). Qin *et al*. (2007) showed that TNFα increased microsomal-triglyceride transfer protein (MTP) mass and stimulated lipoprotein secretion. We speculate that IR and the elevated pro-inflammatory state, as seen in the obese JCR:LA-*cp* rat (Russell et al., unpublished data), may exacerbate intestinal CM production. In type 1-diabetes, modification in gut immunity and intestinal permeability exacerbates the pro-inflammatory state (Vaarala et al., 2008), therefore we speculate that inflammation can influence CM overproduction. Indeed, Su *et al.* (2009) showed that plasma fasting and post-prandial apoB48 concentrations is increased in patients with type 1-diabetes, while all other lipid parameters were unchanged between control and type 1-diabetic patients.

The impact of intestinal hypertrophy on CM overproduction may be of greater importance than we initially assumed, since our results indicate that apoB48 production per cell (data not shown) is not elevated in the MetS rat in the fasted state. Our findings of intestinal hypertrophy in the JCR:LA-*cp* rat are similar to the results seen in a study of streptozocin-diabetic rats that observed taller villi, an increase in the weight of the small

intestine and an increase in enterocyte number compared to normal rats (Zoubi et al., 1995).

Hyperphagia is also presumed to contribute to the hypertrophy of the intestine. Studies in streptozocin-induced diabetic rats with stimulated hyperphagia showed enhanced intestinal growth associated with an increase in cell number, while cell proliferation in the crypt of the villus doubled compared to non-diabetic rats (Miller et al., 1977). Consistently, the hyperphagic JCR:LA-*cp* rat also exhibits intestinal hypertrophy, increased intestinal absorptive surface area and an increase in enterocyte number at the crypt of the intestinal villus.

Alternatively, gut peptide glucagon-like peptide-2 (GLP-2), an important regulator in intestinal structure, may play a role in the adaptive morphology of the intestine during hyperphagia and MetS conditions. GLP-2 is released in response to food intake and plays a significant role in the regulation of intestinal mucosal integrity (Estall and Drucker, 2006). The major actions of GLP-2 stimulate enterocyte proliferation and prevent apoptosis (Estall and Drucker, 2006). It is reasonable to suggest that intestinal hypertrophy and increased enterocyte number in the MetS rat may be a consequence of hyperphagia, which stimulates the release and activity of GLP-2.

In conclusion, this study indicates that anatomical hypertrophy of the intestinal mucosa villi and an increase in the number of enterocytes, may

in part, contribute to the overproduction and oversecretion of CM during conditions of the MetS. Considering apoB48 production per cell was similar to lean controls in the fasted state, it is clear that the assembly and transport of CM is not solely dependent on apoB48. Other aspects of lipid transport, lipoprotein metabolism and CM assembly need to be further investigated in detail to fully understand the mechanistic adaptations (dysfunction) of the intestinal epithelium, in the hypertrophic gut in MetS.

CHAPTER 4: The Effect of n-3 PUFA Supplementation the Post-prandial Lipid and Inflammatory State in the JCR:LA-cp rat; a Model of Insulin Resistance and Obesity.

4.1 Introduction

Post-prandial lipemia and elevated plasma concentration of intestinally derived chylomicrons have been identified as significant and independent risk factors for the development of cardiovascular disease (CVD) (Karpe et al., 1994; Mamo et al., 1998; Cabesaz and Erkelens; 2000 Mamo and Elsegood, 1997). Increasing risk of CVD during conditions of postprandial lipemia has been attributed to the accumulation and inefficient clearance of apolipoprotein-B48 containing-chylomicrons (CM) and their remnants (CM-r) (Weintraub et al., 1996; Cabezas et al., 1998). Previous studies have demonstrated CM-r are associated with the formation of human atherosclerotic plaque, and have the ability to permeate the arterial endothelium and induce foam cell formation (Proctor et al., 2002; Proctor et al., 2004; Proctor and Mamo, 2003). Clinically, impaired post-prandial lipoprotein metabolism is thought to exacerbate the progression of CVD during conditions of obesity, insulin resistance and/or type-2 diabetes (Couillard et al., 1998; Blackburn et al., 2004; De Man et al., 1996; Taskinen, 2001).

Recent evidence has shown that an inflammatory response can ensue as a direct result of increased circulating CM and that this may be a normal physiological process (Alipour et al., 2007; van Oostrom et al., 2004). However under conditions where CM concentration is not abated,

this inflammatory response may become prolonged and exacerbated. A rise in plasma triglyceride (TG) concentration post-prandially, has been shown to stimulate the activation of neutrophils and monocytes (van Oostrom et al., 2003) resulting in a pro-inflammatory state. In addition, the post-prandial state facilitates the migration of immune cells and inflammatory cytokines that may contribute to endothelial dysfunction (Alipour et al., 2007; van Oostrom et al., 2004; van Oostrom et al., 2003).

It is well established that n-3 polyunsaturated fatty acids (n-3 PUFA) specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), found abundant in fatty fish, have the ability to improve an aberrant lipid profile (Lombardo and Chicco, 2006; Roche and Gibney, 1999; Nettleton and Katz, 2005). Supplementation of dietary n-3 PUFA has been shown consistently to reduce plasma TG concentrations (Roche and Gibney, 1999). The hypolipidemic effect of n-3 PUFA has been suggested to be a consequence of a decrease in the very-low density lipoprotein (VLDL) fraction (Harris et al., 1990; Nestel et al., 1984). Consistent with this hypothesis, studies have shown that EPA and DHA decrease VLDL production and secretion *in-vitro* (Wong et al., 1989). The current literature supports the rationale that EPA and DHA may have a similar effect on the post-prandial secretion of intestinal CM (Dolphin et al., 1988; Roche and Gibney, 1999; Harris, 1997), however, there have been limited studies addressing the effect of n-3 PUFA supplementation directly

on post-prandial lipemia during conditions of the metabolic syndrome (MetS).

We have recently identified the JCR:LA-*cp* rat as a useful model to study post-prandial lipemia associated with the complications of MetS (Vine et al., 2007; Mangat et al., 2007). Previous work using this model has shown that dietary supplementation of redfish oil can improve fasting hyperlipidemia, demonstrated by a reduction plasma TG and circulating concentrations of VLDL (Dolphin et al., 1988). Thus, the aim of this study was to investigate the effect of dietary supplementation of n-3 PUFA on the post-prandial metabolism of CM and the corresponding inflammatory response in the JCR:LA-*cp* rat. It was hypothesized that increased dietary n-3 PUFA would improve both fasting and post-prandial lipemia associated with CM following an oral fat challenge, and this would be associated with mitigation of the pro-inflammatory response during the post-prandial phase.

4.2 Methods

Animal Model and Diets:

Obese (cp/cp), male rats of the JCR:LA–*cp* strain were raised in our established breeding colony at the University of Alberta, as previously described (Vine et al., 2007). Rats were weaned at 3 weeks of age and housed with a 12/12-hour reversed light cycle to allow for study and testing during the dark phase of the rats' diurnal cycle. Rats were transferred from the isolated breeding colony to a state-of-the-art fully individually ventilated caging environment (Techniplast^{TM}, Exton PA, USA). Animals were allowed to age for approximately 14 weeks, in order for the phenotype to fully develop (Russell et al., 1989). Animals were randomly allocated to one of three diets; an isocaloric, lipid balanced diet control diet (CD), (n=10) (15% w/w total fat, P/S = 0.4) or 5% n-3 PUFA (n=10) (15% w/w total fat, P/S = 0.4, 5% EPA+DHA incorporated into the diet while maintaining 15% w/w total fat) or 10% n-3 PUFA (n=10) (15% w/w total fat, P/S = 0.4, 10% EPA+DHA of total fatty acids) for three weeks with each consisting of 1% cholesterol in order to exacerbate hypercholesterolemia (Table 4-1). Animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care as approved by the University of Alberta Animal Ethics Committee.

	Control Diet (CD)	5% n-3 PUFA Diet	10% n-3 PUFA Diet
Nutrient Summary (per kg)			
Casein (g)	270	270	270
Starch (g)	214	214	214
Dextrose (g)	217	217	217
Non-nutritive Cellulose (g)	80	80	80
Vitamin Mixture (g)	9.5	9.5	9.5
Mineral Mix (g)	48	48	48
Choline (g)	2.75	2.75	2.75
Inositol (g)	6.25	6.25	6.25
L-methionine (g)	2.5	2.5	2.5
Linseed Oil (g)	3.0	3.0	3.0
Tallow (g)	91.71	91.88	94.73
Sunflower Oil (g)	55.29	40.13	24.27
Fish Oil (g)	0	15	28
Cholesterol (g)	10	10	10
Lipid Summary (% of Fat)			
Total Polyunsaturated Fat	24.7	24.3	27.7
Total Saturated Fat	64.5	66.0	65.0
P/S Ratio	0.4	0.4	0.4
Total n-6	23.4	17.4	17.1
Total n-3	1.2	6.9	10.6
Total EPA+DHA	0.0	5.3	9.4

Table 4-1. Nutrient and lipid summaries for each dietary group. Fatty acid composition of lipid balanced control diet (CD) and diets supplemented with fish oil containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); 5% n-3 PUFA diet and 10% n-3 PUFA diet, as determined by gas chromatography.

Assessment of Postprandial Lipemia

Following a 16 hr overnight fast, animals were subject to an adapted oral fat challenge as previously described (Vine et al., 2007). Briefly, experimental rats consumed a 5.0 g pellet made with 5001 laboratory chow. The pellet consists of carbohydrate 49%, crude protein

24.0%, moisture 10%, minerals 6.5%, fibre 6.0% and fat 4.5 %, but further supplemented with 25% w/w dairy fat from double cream (raising the total fat content of the 5.0 g meal to approximately 30% w/w).

Blood samples were collected in tubes containing Na_2EDTA from the tail at t= 0 and 2, 3, 4 and 6 hrs following consumption of the pellet meal. Plasma and serum were immediately separated from whole blood by centrifugation (3000 rpm, 4C, 10 min). Aliquots of plasma were stored at -80C for biochemical analyses.

Biochemical Determinations

Triglyceride (TG) concentration (WAKO Chemicals USA, Inc. Richman, VA, USA Cat #998-40391/994-40491), as well as total cholesterol (WAKO Cat#439-17501), LDL-cholesterol (WAKO, Cat#993-00404/999-00504) and HDL-cholesterol (Diagnostic Chemical Ltd, Canada Cat#258-20) concentrations were all measured using direct colorimetric chemical enzymatic reactions. Plasma glucose was assessed as per the glucose oxidase method (Diagnostic Chemical Ltd, Cat#220-32) and insulin concentration was measured at the University of Alberta Hospital using standard enzymatic colorimetric assay. Plasma leptin (Alpco Diagnostics, NH, USA Cat#22-LEP-E06) and adiponectin (Alpco Diagnostics NH, USA Cat#44-ADPR-0434) were analysed with commercially available enzymatic immunoassays for rodents. Acute phase proteins of inflammation were also measured using colorimetric

immunoassays for C-reactive protein (Alpco Diagnostics, Salem NH, USA Cat#41-CRPR-25ECRP), haptoglobin (Tridelta Development Limited, Wicklow, Ireland Cat#TP801) and lipopolysaccharide binding protein (HyCult Biotechnology b.v.Cat#HK503).

Chylomicron concentration was measured by quantifying apolipoprotein B48 (apoB48) using an adapted immuno-western blot method as previously described (Vine et al., 2007). Briefly, total plasma apolipoproteins were separated by SDS-PAGE on a 3-8% tris-acetate polyacrylamide gel (Invitrogen, NuPage, CA, USA). The separated proteins were transferred onto a PVDF membrane (0.45 µm; ImmobilonP^{TM}, Millipore. MA USA). Membranes were incubated with a goat polyclonal antibody specific for apoB (Santa Cruz Biotech, CA) and a secondary antibody tagged with hydrogen peroxidase (Santa Cruz Biotech, CA) was used to visualize apoB by chemiluminescence (ECL-Advance, Amersham Biosciences, UK). The intensity of the probed bands were quantified using linear densitometric comparison with a known mass of the purified rodent apoB48 protein.

Abundance of Lipogenic Proteins:

Proteins from whole liver homogenate were separated by SDS-PAGE electrophoresis on commercially prepared 3-8% tris-acetate polyacrylamide gradient gels (Invitrogen, NuPage, CA, USA) and immunoprecipitated for target proteins, fatty acid synthase (FAS) and

acetylCoA carboxylase (ACC). Separated proteins were transferred to a PVDF membrane (0.45 µm; ImmobilonP^{TM}, Millipore. MA USA) and incubated with primary antibodies specific to the protein of interest: FAS (1:5000) [Cell Signaling Technology Inc. Catalog # 3189] and ACC (1:25000) [Cell Signaling Technology Inc. Catalog # 3662]. Secondary antibodies tagged with hydrogen peroxidase were then used to visual the proteins of interest under chemi-illuminscent conditions.

Statistical Analysis

Data was tested for normal distribution and differences between control and n-3 PUFA treatment groups were analysed using unpaired t-test and one-way ANOVA with significance set at $p<0.05$. (GraphPad PRISM). Statistical correlation was also performed using pair-matched values for the postprandial curve. All results are expressed as the mean $\pm$ SEM.

4.3 Results

Food Intake, Body Weight and fasting lipid parameters: Body weight, food consumption and fasting lipid parameters for each treatment group are shown in Table 4-2. Rats supplemented with either 5% or 10% n-3 PUFA demonstrated a significant reduction in weight gain compared to controls ($p<0.01$ and $p<0.05$ respectively). Additionally, food consumption was reduced in rats receiving 10% n-3 PUFA treatment ($p<0.05$), but not 5% n-3 PUFA, compared to the food intake of control animals. Treatment with either 5% or 10 % n-3 PUFA was shown to reduce fasting plasma TG by 50% ($p<0.001$ and $p<0.001$, respectively) and cholesterol concentrations by 30% ($p<0.05$ and $p<0.01$, respectively) compared to controls. Additionally, plasma LDL-C was reduced by 30% in the 10% n-3 PUFA group ($p<0.01$) relative to controls, while HDL-C levels were not different between groups. Blood glucose concentrations in the 10% n-3 PUFA group were lower compared to the control group following the three-week dietary intervention. No difference in fasting plasma insulin concentration between treatment groups was observed. Notably, treatment with 10%, but not 5%, n-3 PUFA induced a lower fasting concentration of apolipoprotein B48 compared to the control diet ($p<0.01$).

Parameter (Fasting Plasma)	Control Diet (CD)	5% n-3 PUFA Diet	10% n-3 PUFA Diet
Food Consumption (grams)	34.0 ± 2.4	37.2 ± 1.6	27.9 ± 1.4 β
Body Weight (grams)	591.6 ± 5.2	556.4 ± 15.9 **	561.2 ± 5.7 *
Glucose (mg/dL)	149.58 ± 9.80	134.88 ± 3.50	119.82 ± 6.70 *
Insulin (mU/L)	97.36 ± 22.55	113.61 ± 24.80	114.36 ± 18.44
Cholesterol (mg/dL)	182.06 ± 10.80	144.95 ± 7.26 *	122.29 ± 5.76 **
Triglyceride (mg/dL)	300.33 ± 37.13	154.74 ± 10.40 ***	139.34 ± 15.56 ***
HDL (mg/dL)	46.16 ± 4.3	47.50 ± 3.9	43.31 ± 3.0
LDL (mg/mL)	42.21± 2.8	38.51 ± 6.2	25.28 ± 2.8 **
Apolipoprotein B48 (µg/mL)	65.83 ± 9.41	50.13 ± 7.91	30.13 ± 5.8 **

Table 4-2. Physical and fasting biochemical parameters of obese, male rats subject to a control diet (CD), a 5% n-3 PUFA or a 10% n-3 PUFA diet. Data are represented as mean ± SEM for each group.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ denotes statistical significance between obese rats on treatment diets (5% n-3 PUFA or 10% n-3 PUFA) vs. obese rats on a lipid balanced control diet (CD).

β<0.05 denotes a statistically significant difference in animals fed 10% n-3 PUFA compared to animals supplemented with 5% n-3 PUFA.

Fasting Inflammatory Markers: Fasting concentration of acute phase inflammatory proteins; C-reactive protein (CRP), haptoglobin (Hp) and lipopolysaccharide binding protein (LBP) are shown in Table 4-3. Fasting plasma CRP and Hp concentration were increased in the 10% n-3 PUFA diet, but not the 5% n-3 PUFA diet, compared to the control diet ($p<0.001$). In contrast, treatment with either 5% or 10% n-3 PUFA reduced LBP concentration compared to the control diet ($p<0.01$).

Fasting Adipokines (Table 4-3): Feeding with either the 5% or 10% n-3 PUFA reduced fasting plasma leptin concentration compared to the control group ($p<0.001$). Interestingly, despite the leptin receptor

deficiency of these obese animals, those treated with 10% n-3 PUFA showed a lower leptin concentration compared to the 5% n-3 PUFA group ($p<0.05$). Fasting concentrations of plasma adiponectin were increased (improved) in the 5% (but not 10%) n-3 PUFA diet relative to the control diet ($p<0.05$).

Postprandial Response: Following an oral fat challenge, animals fed the 5% or 10% n-3 PUFA diet showed a reduced (45% and 56%, respectively) post-prandial area under the curve (AUC) response for TG, compared to controls ($p<0.05$) (Figure 4-1). Incremental AUC (iAUC) for TG was also significantly lower in animals fed the 10% n-3 PUFA diet compared to controls ($p<0.05$).

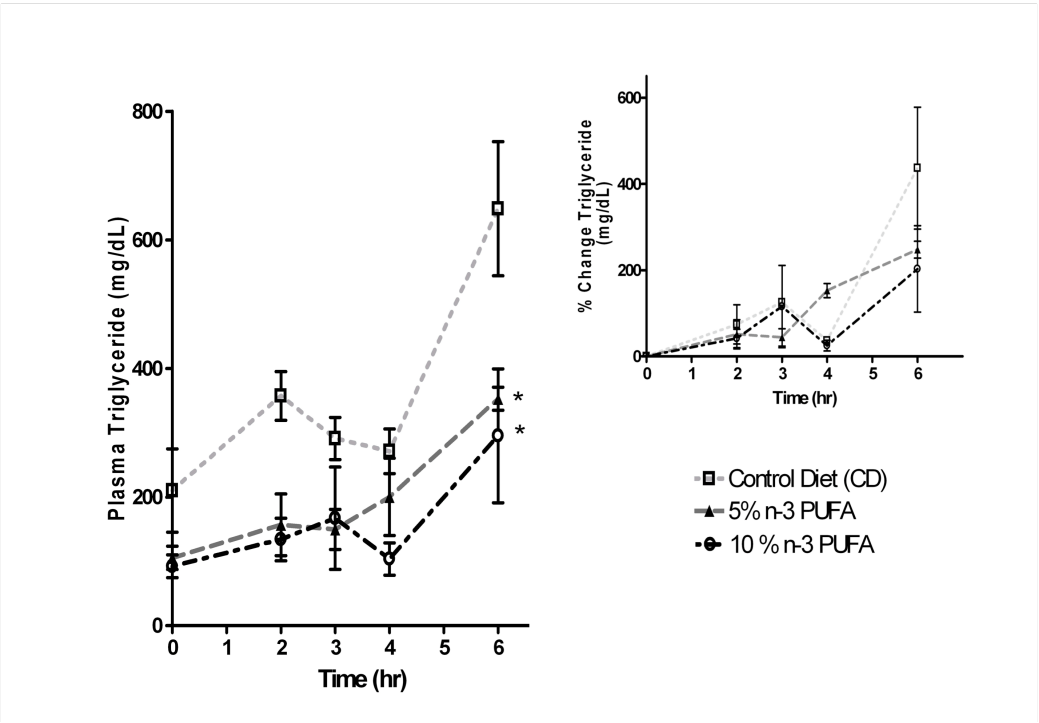

Figure 4-1. Postprandial response of plasma TG of obese, male rats in each of the treatment groups following an oral fat challenge. Inset. The incremental area under the curve for plasma TG concentrations.

*p<0.05 denotes a significant difference in area under the curve of fish oil fed groups (5% FO and 10% FO) to animals fed a lipid balanced control diet (CD).

The post-prandial AUC and iAUC response of apoB48 was lower (45% and 63% respectively), in animals fed 5% n-3 PUFA ($p<0.05$) and was reduced (by 57% and 76%, respectively) in the 10% n-3 PUFA diet ($p<0.05$ and $p<0.01$), in comparison to the control diet (Figure 4-2).

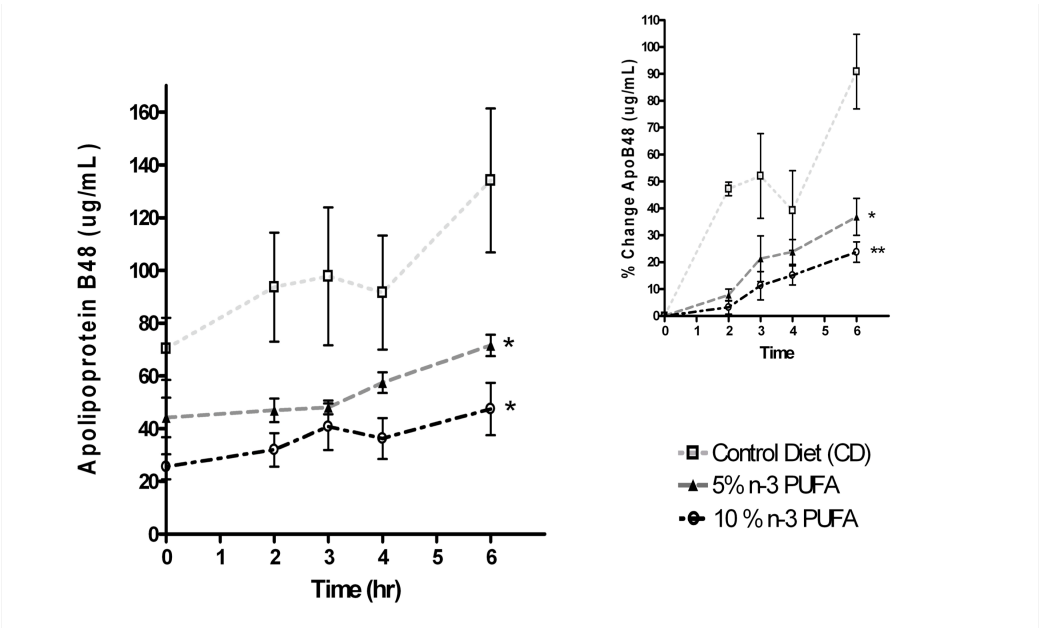

Figure 4-2. Postprandial apolipoprotein B48 response following an oral fat challenge in male, obese animals maintained on a control diet (CD), a 5% n-3 PUFA diet or 10% n-3 PUFA diet. Inset. The incremental area under the curve of plasma apolipoproteinB48 in all three groups.

*p<0.05, **p<0.01 denotes a significant difference in area under the curve of fish oil fed groups (5% n-3 PUFA and 10% n-3 PUFA) to animals fed a lipid balanced control diet (CD).

Notably, the post-prandial AUC of CRP was two fold higher for animals fed the 5% n-3 PUFA in contrast to the control diet (Table 4-3). Whereas, treatment with 10% n-3 PUFA diet resulted in a higher post-prandial AUC for Hp than both the control ($p<0.001$) and 5% n-3 PUFA groups ($p<0.01$) (Table 4-3). The post-prandial AUC for LBP was lower in animals fed either the 5% or 10% n-3 PUFA diet compared to controls ($p<0.001$) (Table 4-3).

Fasting Plasma	Control Diet (CD)	5% n-3 PUFA Diet	10% n-3 PUFA diet
Leptin (pg/mL)	15109 ± 1532.0	8710 ± 769.8 ***	4298 ± 225.4 ***, β
Adiponectin (ng/mL)	15.01 ± 2.426	20.15 ± 6.036 *	15.02 ± 2.572 β
CRP (µg/mL)	57.22 ± 6.69	63.04 ± 13.13 *	204.30 ± 2.07 ***, βββ
Haptoglobin (µg/mL)	1386 ± 92.33	1594 ± 31.74	2309 ± 134.20 ***, ββ
LBP (ng/mL)	27744 ± 3002	23699 ± 1369	18584 ± 870 **
Area Under the Curve (following lipid load)	**Control Diet (CD)**	**5% n-3 PUFA Diet**	**10% n-3 PUFA diet**
CRP (µg/mL/hr)	57.22 ± 6.69	63.04 ± 13.13 *	204.30 ± 2.07 ***, βββ
Haptoglobin (µg/mL/hr)	1386 ± 92.33	1594 ± 31.74	2309 ± 134.20 ***, ββ
LBP (ng/mL/hr)	27744 ± 3002	23699 ± 1369	18584 ± 870 **

Table 4-3. Fasting plasma concentrations as well as area under the (6-hour) clearance curve following a fat challenge of adipokines (leptin, adiponectin), and acute phase inflammatory proteins (C-reactive protein [CRP], haptoglobin and lipopolysaccharide binding protein [LBP]) from obese JCR:LA-*cp* male rats fed a lipid balanced control diet (CD), a fish oil supplemented diet (5% n-3 PUFA or 10% n-3 PUFA). Data is represented as average ±SEM.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ denotes statistical significance between obese rats on treatment diets (5% n-3 PUFA or 10% n-3 PUFA) vs. obese rats on a lipid balanced control diet (CD

β<0.05, β β <0.01, β β β <0.001, denotes a statistically significant difference in animals fed 10% n-3 PUFA compared to animals supplemented with 5% n-3 PUFA). CRP AUC was not significant between 5% and 10% fish oil groups.

Expression of Fatty Acid Metabolism Proteins: The abundance of hepatic fatty acid synthase (FAS) was increased in both the 5% or 10% n-3 PUFA diet groups relative to the control diet ($p<0.05$). In contrast, hepatic acetyl CoA carboxylase (ACC) was reduced in the 10% n-3 PUFA diet compared to the control and 5% n-3 PUFA diet ($p<0.01$ and $p<0.05$, respectively). Conversely, adipose tissue from animals fed the 5% n-3

PUFA diet showed a reduced (30%) protein mass of FAS (but not ACC) ($p<0.05$) relative to the control diet (Figure 4-3A and 4-3B).

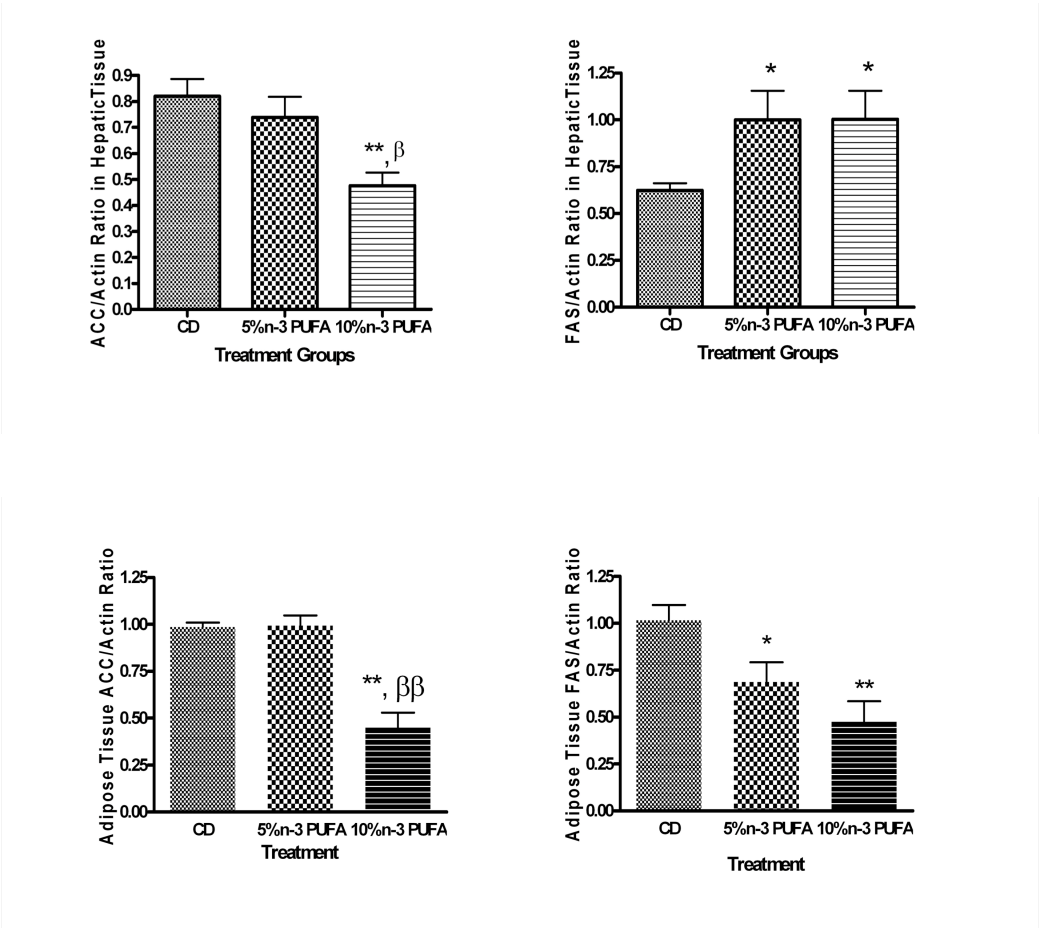

Figure 4-3. (A) Hepatic protein expression of lipogenic enzymes in obese JCR:LA cp rodents fed a control diet (CD), 5% n-3 PUFA diet and 10% n-3 PUFA diet. **(B)** Adipose tissue expression of lipogenic enzymes in 5% n-3 PUFA and 10% n-3 PUFA diet in obese, JCR:LA cp rats vs. obese rats fed a control diet (CD). Western blot probed for ACC and FAS are represented as a ratio to actin, which is used as a loading control. * $p<0.05$ and ** $p<0.01$ denotes a statistically significant difference of n-3 PUFA supplemented animals vs. CD controls. Whereas $\beta<0.05$ denotes a significant difference between animals fed 10% n-3 PUFA and animals maintained on 5% n-3 PUFA.

4.4 Discussion

The aim of this study was to investigate the metabolic effect of increased n-3 polyunsaturated fatty acid (n-3 PUFA) intake on post-prandial lipemia and the associated pro-inflammatory response in the obese JCR:LA-*cp* rodent model of the metabolic syndrome (MetS). Collectively, our findings support the hypothesis that the moderate (5%) dose of dietary n-3 PUFA used in our study can improve post-prandial lipid metabolism, while simultaneously improving circulating acute phase inflammatory proteins. Note that the equivalent amount of n-3 PUFA from 5% fish oil treatment in rats used in our study equates to approximately 5 g/fish oil/day for a 2500 kcal/day human diet. It is also interesting that some blood parameters (i.e. lipids) measured in this study offer support for a dose-dependent effect of increasing n-3 PUFA, while other parameters (i.e. related to inflammation), did not follow this trend. Unexpectedly, we also observed a reduction in body-weight gain over the three-weeks in rats treated with 5% n-3 PUFA. Whilst there has been limited data to support the notion that increased dietary n-3 PUFA intake may reduce body weight directly, a recent review by Li et al (Li et al., 2008) suggests that dietary n-3 PUFA may indeed exert anti-obesity properties. The lack of clarity in the literature with respect to the potential mechanistic action of n-3 PUFA on body weight control would suggest that this process is complex and involves multiple homeostatic pathways. For example there is some evidence to suggest that n-3 PUFA may stimulate CNS satiety signal(s) in

rats and that higher dose may ultimately result in appetite suppression (Dziedzic et al., 2007). One other possible confounder is the complexity that n-3 PUFA may modulate body weight during the underlying condition of obesity, hypercholesterolemia or both. It may well be that the combination of these metabolic conditions, such as those found in MetS, may have a greater tendency for reduced weight gain during increased dietary n-3 PUFA consumption.

Previous studies in the JCR:LA-*cp* rat have reported that intake of 10% (w/w) redfish oil (sebastes marinus) diet consisting of 4.9 $\pm$ 0.1% eicosapentaenoic acid (EPA) and 2.3 $\pm$ 0.5% docosahexaenoic acid (DHA), reduced circulating plasma TG concentration (Dolphin et al., 1988). Clinical studies have also shown that n-3 PUFA at a dose of 2.7 g/day for 2 months, followed by 1.7 g/day for an addition 4 months, lowers plasma triglyceride concentration by up to 25 % in subjects with type 2 diabetes and hypertriglyceridemia (Kesavulu et al., 2002), and by 50% in diabetic patients treated with anti-diabetic drugs (Clarke and Jump et al., 1994). The lipid lowering properties of dietary n-PUFA under these conditions have been ascribed to decreased hepatic lipidogenesis. It has also been reported that dietary n-3 PUFA supplementation may suppress the transcription of lipidogenic enzymes such as, fatty acid synthase (FAS) and sterol regulatory expression binding protein (SREBP) (Jump et al., 1999; Xu et al., 2001; Halvorsen et al., 2001) and increase β-oxidation in adipose tissue and liver (Halvorsen et al., 2001).

With respect to the cholesterol lowering properties of n-3 PUFA, the current study showed that intake of 10% n-3 PUFA to obese JCR:LA-*cp* rats reduced plasma LDL-C by 30%. This is in contrast to numerous clinical studies that have investigated the impact of increased dietary n-3 PUFA intake reporting either a modest and/or inconsistent effect on LDL-C (Nettleton and Katz, 2005). A compelling meta-analysis by Montori *et al* and Friedburg *et al* reported that increased dietary fish oil intake is associated with a slight increase in serum LDL cholesterol levels in those individuals with pre-existing type 1 or type 2-diabetes (Montori et al., 2000; Friedburg et al., 1998). However, there are other published reports that suggest n-3 PUFA intervention does not alter LDL-C in diabetic subjects (Woodman et al., 2002, MacLean et al., 2004). It is noteworthy that the JCR:LA-*cp* rat is both hyper-cholesterolemic and overtly obese which may be in contrast to studies in diabetic subjects that are often non-obese and/or normolipidemic. Recently, Satoh and colleagues (Satoh et al., 2007) showed that intake of purified EPA in 44 obese, type 2 diabetic subjects reduced serum LDL-C. We propose that dietary n-3 PUFA may have greater efficacy to reduce LDL-C under conditions inclusive of both obesity and hyper-cholesterolemia. Further, given the impact of n-3 PUFA directly on intestinal-derived lipids, it may be that a complex compensatory effect between the liver and intestine may exist in humans (as has been previous shown for intestinal cholesterol transport inhibitors).

Given the JCR:LA-*cp* rat has increased expression of hepatic SREBP-1 mRNA , resulting in increase production and secretion of VLDL (Elam et al., 2001), we propose that the reduced plasma LDL-C observed may be a consequence of dietary n-3 PUFA intake reducing VLDL production. Indeed studies by Deng *et al* (Deng et al., 2004) have shown that EPA/DHA (40% of calories from menhaden oil) intake in the JCR:LA-*cp* rat down-regulate hepatic mRNA expression of both FAS and ACC. In our current study intake of n-3 PUFA resulted in a reduced protein expression of hepatic ACC, but an increase in FAS. The discrepancy between our findings and that of Deng *et al* (2004) may be reconciled by potential differences in the fatty acid composition of diets used in each of the studies. Considering that Deng *et al* adjusted macronutrient content from predominantly carbohydrate to lipid is highly relevant, as it is known that FAS and ACC can be up-regulated by increased availability of glucose (Foufelle et al., 1996; Girard et al., 1994). It is thus plausible that hepatic lipidogenic enzymes such as FAS and ACC may be regulated by macronutrient substitution rather than directly by n-3 PUFA intake *per se*. Alternatively, n-3 PUFA intake may have differential effects on the post-translational regulation (mRNA verses protein expression) of hepatic FAS and ACC, however this remains to be determined.

It is also noteworthy that protein expression of FAS in adipose tissue from obese animals treated with n-3 PUFA was contrary to that quantitated in the liver. Reduced protein expression of FAS in adipose

tissue may decrease intracellular synthesis of TG and concomitantly reduce the storage of free fatty acid in adipose tissue. It is also reasonable to suggest that either a reduced size or abundance of adipocytes during n-3 PUFA intake may modulate the secretion of adiponectin (Lombardo et al., 2007; Drevon, 2005), which reduced plasma triglycerides by increasing VLDL catabolism (Qiao et al., 2008).

Emerging evidence suggests that intestinal apolipoprotein B48 (apoB48) containing-chylomicrons (CM) are pivotal contributors to dyslipidemic conditions. Levy *et al* (2006) reported that the jejunal secretion of apoB48 from diabetic and insulin resistant rats following dietary intake with n-3 PUFA was significantly reduced, and this was likely a result of post-translational degradation. Furthermore, intestinal/colonic-derived caco-2 cultured cells incubated with EPA have demonstrated a reduced expression of apoB mRNA and corresponding apoB secretion (Levy et al., 2006). Collectively, these results are consistent with our findings of reduced fasting and improved post-prandial clearance of apoB48 in the n-3 PUFA fed obese JCR:LA-*cp* animals. Furthermore, improved post-prandial clearance of CM observed following n-3 PUFA treatment may also be attributed indirectly to decreased total plasma TG concentration (Roche and Gibney, 1999; Harris, 2006; Ikeda et al., 2001), which may alleviate competition of lipolytic enzyme activity and subsequent high affinity receptor mediated clearance mechanisms by the

liver (Roche and Gibney, 1999; Park and Harris, 2003; Ranheim et al., 1992).

The physiological relevance of the post-prandial pro-inflammatory response has gained focus from studies that reveal a direct relationship with post-prandial lipid excursions and vascular dysfunction (Alipour et al., 2007; Burge and Calder, 2005). Clinical studies have shown consistently that there is an inverse relationship between C-reactive protein (CRP) and n-3 PUFA consumption (Madsen et al., 2007). In the present study, both fasting and post-prandial response of CRP to n-3 PUFA intake in the JCR:LA-*cp* rat was elevated and therefore not consistent with these clinical observations (Madsen et al., 2007). However, it has been suggested that in the rodent, CRP may not be an adequate measure of inflammatory homeostasis (Giffen et al., 2003). Various experimental conditions have shown that haptoglobin (Hp) is elevated in a more reproducible manner, implying that Hp is a more sensitive marker of acute inflammation in the rodent (also reviewed in Giffen et al., 2003). The rationale for increased Hp with higher dose of n-3 PUFA in our study, may suggest a pro-inflammatory response by the acute phase protein pathway. Consistent with our findings of reduced LBP and apo-B48 in the n-3 treated animals, LBP has been shown to bind with lipoproteins (Vreugdenhil et al., 2001; Schroden et al., 2004) and lipopolysaccharides have been associated with the formation of CM (Ghoshal et al., 2009). Based on the collective findings of acute phase proteins in this study,

there is preliminary evidence to suggest mitigation of the acute pro-inflammatory state by dietary n-3 PUFA may be limited to moderate consumption. Immunomodulatory effects of high doses of n-3 PUFA have raised important considerations of the potential adverse inflammatory effects. We also wish to raise the potential cofounding issue that it is not known whether the heightened inflammatory response observed in 10% n-3 PUFA treated group may influence metabolic parameters. For example, the greater (beneficial) adiponectin concentration observed in 5% n-3 PUFA treated, but not 10% treated rats, may be the result of an inflammatory suppression of adiponectin expression in adipose tissue. Therefore these findings are of particular interest to those subjects under conditions of increased CVD risk that often receive higher doses of n-3 PUFA; potentially exacerbating a pro-inflammatory response (Berstad et al., 2003; Eschen et al., 2004).

In conclusion, we report that a physiological dose of n-3 PUFA improves both fasting and post-prandial lipemia, and the associated pro-inflammatory response under conditions of hyper-cholesterolemia and obesity. We also provide evidence that dietary n-3 PUFA treatment may diminish weight gain in the obese hypercholesterolemic JCR:LA-*cp* rodent model. It would be valuable to reconcile optimal dietary consumption of n-3 PUFA and mechanisms by which n-3 PUFA improves parameters of CVD risk during human conditions of obesity, dyslipidemia and the Metabolic Syndrome.

CHAPTER 5

5.1 Collective Discussion:

The aim of this thesis was to investigate the morphological and lipogenic adaptations of the intestine in a model of post-prandial lipemia and MetS. In addition, this thesis examines the putative benefits of n-3 PUFA supplementation on post-prandial lipid metabolism and the associated pro-inflammatory response. The first phase of my thesis investigated the morphological characteristics of the intestine and the enterocytic production of apoB48, an essential structural protein in CM assembly.

5.1.1 Intestinal Morphology and Enterocytic Lipid and ApoB48 Production and Secretion in Insulin Resistance:

Emerging evidence has shown that IR and obesity impact intestinal lipoprotein metabolism (Haidari et al., 2002; Duez et al., 2006; Federico et al., 2006; Levy et al., 2002). In our lab we have shown the JCR:LA-*cp* rat has elevated post-prandial plasma concentrations of CM (apoB48) positively correlating with plasma insulin levels (Vine et al., 2007). The present work has shown that impaired post-prandial CM metabolism in IR may be a consequence of increased CM secretion from the intestine during the fasted and fed states. Intestinal hypertrophy and an increase in enterocyte number may, to some extent, explain the elevated concentration of lymphatic CM seen in the MetS, JCR:LA-*cp* rat.

In accordance with my specific hypotheses, we revealed that the JCR:LA-*cp* rat has hypertrophy of the intestinal villus in both length and

total surface area. This was accompanied by an increase in enterocyte number lining the intestinal villus at the crypt. Interestingly, enterocyte associated apoB48 mass/cell did not change in comparison to lean rats. Therefore, the increased concentration of lymphatic apoB48-containing lipoproteins might be explained by intestinal hypertrophy and an associated increase in enterocyte number.

Furthermore, our results showed that apoB48/ cell was similar in MetS and lean rats. Therefore, it is reasonable to speculate that enhanced CM secretion may be a consequence of the dysregulation of other components involved in CM production such as, MTP and/or excess enterocytic lipid availability. In a recent review, Hsieh *et al*. (2008) reported an upregulation of MTP in conditions of IR. Therefore, the upregulation of this enzyme may enhance the assembly of CM.

Also animal models of IR have demonstrated an increase in the expression of enzymes involved in lipid biogenesis in the intestine (Haidari et al., 2002; Zoltowska et al., 2003). An upregulation of lipogenic enzymes SREBP, MGAT and DGAT potentially increase *de novo* lipogenesis within the cell. An abundance of lipids in the enterocyte is associated with the intracellular stability of apoB48, an essential component of CM production, inhibiting the proteasomal degradation of the apoprotein (Zoltowska et al., 2003). Intracellular stability of apoB48 and the accumulation of enterocytic lipid enhances lipid assimilation into the primordial CM enhancing CM production.

More recently, it has been suggested that increased intestinal lipid availability may be a consequence of increased expression of lipid transporters. Lally *et al.* (2008) and Tomkin *et al.* (2008) have reported an upregulation of cholesterol transporter NPC1L1 in IR. Consequently, preliminary evidence in our lab shows that the process of intestinal lipid transport and cholesterol absorption is also exacerbated in the MetS JCR:LA-cp rat (Vine et al., 2008).

The mechanisms elucidating intestinal hypertrophy may primarily be explained by the causes of obesity in this animal model. Hyperphagia is a contributing factor in the development of obesity and may play a critical role in intestinal modification by encouraging villus growth and enhancing enterocyte proliferation. In a review discussing the factors influencing the morphological adaptations of the intestine, Williamson (1982) showed that hyperphagia leads to a prompt and persistent increase in intestinal mucosa mass. Furthermore, the impact of IR is theorized to be involved in intestinal adaptations. Studies in streptozotocin-diabetic rats had intestines of greater weight, taller villi and deeper crypts than normal rats. Indeed, an increase in the surface area of the intestinal lumen due to hypertrophy may allow for the incorporation of a greater number of transport sites (Karasov et al., 1983; Fedorak et al., 1987). To support this contention, hypertrophied intestines showed enhanced water and nutrient transport (Zoubi et al., 1995).

5.1.2 The Effect of n-3 PUFA on Post-prandial Lipemia and the Associated Inflammatory Response:

The second phase of this thesis examined the effects of n-3 PUFA supplementation on the post-prandial response of apoB48-containing lipoproteins and the associated inflammatory response. These findings are presented in Chapter 4.

Briefly, acute, moderate n-3 PUFA supplementation improved post-prandial CM metabolism in the dyslipidemic JCR:LA-*cp* rat with a concomitant improvement in the post-prandial inflammatory response of acute phase proteins.

Improvement of post-prandial lipemia following n-3 PUFA supplementation may be attributed to a down-regulation in apoB48 production. As mentioned earlier, lipids have been shown to act as a protective component for apoB48 proteasomal degradation (Zoltowska et al., 2003). Therefore, reduced fasting and post-prandial TG, as seen in the JCR:LA-*cp* rat supplemented with n-3 PUFA, may be indicative of decreased lipogenesis and/or absorption. Levy *et al*. (2006) have demonstrated that reduced lipogenesis and limited lipid availability in the enterocyte stimulates the degradation of apoB48, ultimately decreasing the production and secretion of CM. Comparable to lipid regulation in the intestine, a decrease in hepatic lipoprotein production following n-3 PUFA supplementation has been associated with a downregulation of SREBP, FAS and ACC (Jump et al., 1999; Jump and Clark et al., 1994).

Furthermore, recognizing that a post-prandial rise in TG is associated with the activation and recruitment of pro-inflammatory markers (Alipour et al., 2007), this thesis aimed to address the effect of n-3 PUFA supplementation on the post-prandial inflammatory response. Results suggest that excessive consumption of n-3 PUFA can have adverse affects on the immune response, while moderate n-3 PUFA consumption improves the response of post-prandial pro-inflammatory markers. Thus, improvement to the post-prandial lipoprotein response may impact the immune response.

Finally, it was interesting to see that, overall, moderate n-3 PUFA consumption showed the greatest physiological benefits compared to higher intake of n-3 PUFA. Nonetheless, increasing n-3 PUFA supplementation continued to show improvements in lipid parameters while the benefits to inflammatory parameters diminished with increasing consumption of n-3 PUFA. Therefore, we conclude that moderate n-3 PUFA consumption is most cardioprotective. Long term supplementation of n-3 PUFA has also suggested that 5% n-3 PUFA intake has the most benefits in the prevention of pathologies associated with CVD risk such as glomerulosclerosis and kidney dysfunction (Lu et al., 2009). Fortunately, recommended intakes of n-3 PUFA (EPA+DHA) are set at levels of 2-4 g/d to effectively lower TG (under a physician's care) (American Heart Association, 2009), the caloric equivalent consumed by rats fed moderate doses of n-3 PUFA in this study. However, the concern now arises in the

increased consumption of n-3 PUFA by critical care patients receiving clinical treatment, which may adversely effect immune status.

5.2 Future Directions:

The preliminary findings of this thesis provides the background necessary to further explore aspects involved in intestinal modification and CM production and secretion as well as the mechanistic pathways by which n-3 PUFA supplementation may be involved in regulating the expression of apoB48 in the intestine.

Based on the findings in this thesis it would be beneficial to investigate the magnitude of expression of enzymes (i.e. MTP) involved in CM assembly in the enterocyte as well as examine the theory of increased enterocytic lipid synthesis and/or absorption by investigating the expression of lipogenic enzymes: FAS, ACC, SREBP, MGAT and DGAT and lipid transporters: CD36 and NPC1L1. Furthermore, it would be compelling to examine the effect of moderate n-3 PUFA supplementation on intestinal morphology and apoB48 production and secretion. Preliminary evidence in our lab demonstrates that n-3 PUFA supplementation reduces intestinal apoB48 production per cell. Total intestinal cell-associated apoB48 was reduced in MetS rats fed 5% n-3 PUFA compared to obese control rats fed a control diet ($p = 0.039$). More specifically, rats supplemented with 5% n-3 PUFA had significantly lower apoB48 in enterocytes isolated at fraction 5, the middle portion of the

intestinal villus, compared to rats fed a LBD control diet (p=0.0054). ApoB48 protein mass along the intestinal villus, as represented by isolated enterocytes from the villus tip (fraction 1) to the villus crypt (fraction 10) is shown in Figure 5-1.

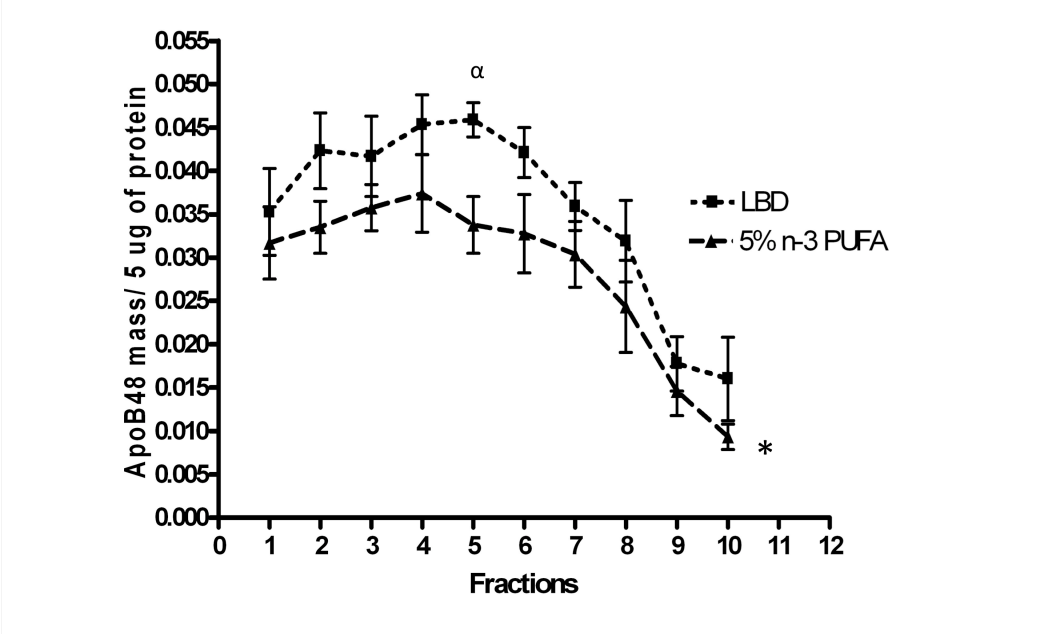

Figure 5-1. Intestinal expression of apoB48 in obese JCR:LA-cp rats fed a lipid balanced diet (LBD) and 5% n-3 PUFA diet. Western blot analysis probed for apoB48 along the intestinal villus with fraction 1 representing isolated enterocytes at the tip of the villus and fraction 10 representing cells from the crypt of the villus.

* p =0.039 denotes a statistically significant difference of total intestinal apoB48 mass in rats fed 5% n-3 PUFA compared to control rats fed lipid balanced diet (LBD). α p = 0.0054 demonstrates a statistically significant difference in cell-associated apoB48 protein mass in fraction 5 in rats fed 5% n-3 PUFA vs. rats fed LBD.

Considering the improvement in post-prandial CM metabolism and preliminary work demonstrating a reduction in apoB48 protein expression per enterocyte, it would be interesting to investigate changes in lymphatic apo48 secretion following a three week n-3 PUFA supplementation. It would also be interesting to investigate the acute impact of n-3 PUFA

supplementation. Gastric infusion of intralipid supplemented with n-3 PUFA would allow for the examination of the impact of n-3 PUFA consumption on CM (apoB48) and lipid secretion from the intestine.

Despite preliminary findings supporting the beneficial effects of n-3 PUFA on apoB48 production, the mechanisms by which n-3 PUFA exerts post-prandial hypolipidemic effects still remains unclear. Future studies could explore the molecular mechanisms (i.e. lipogenic enzymes, enzymes of CM assembly) impacted by dietary n-3 PUFA within the enterocyte. Tagging n-3 PUFA and assessing the localization of EPA and DHA in the intestine and within the enterocyte would be the primary step is assessing the target sites of action of n-3 PUFA. Furthermore, the beneficial effects of n-3 PUFA supplementation are of potential clinical importance in the MetS population at risk of developing CVD. Although, the optimal dose of dietary n-3 PUFA intake still needs to be determined for the human population the findings in this thesis have set the foundation for the design of a clinical trial.

5.3 Conclusions

In conclusion, post-prandial lipemia and the MetS are associated with an increase in lipid and CM secreted into the lymphatic circulation. The intestine adapts structurally in the MetS resulting in an increase in enterocyte number. It has been suggested that intestinal hypertrophy and an increase in cell number contributes to the elevated concentration of

apoB48 in lymphatic circulation and in turn further increases the risk of atherogenicity and CVD progression.

Furthermore, dietary n-3 PUFA lowers the concentration of post-prandial lipid and CM, and attenuates the post-prandial concentration of pro-inflammatory markers in MetS. These findings suggest that n-3 PUFA supplementation improves the exacerbated post-prandial lipoprotein response with a concomitant improvement to the post-prandial immune state.

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