University of Alberta

The Role of Ezetimibe and Simvastatin in Modulating Intestinal Cholesterol Transport, Chylomicron Profile and Chylomicron-Remnant Uptake by the Arterial Wall in a Rodent Model of the Metabolic Syndrome

by

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Abstract

Intestinally derived chylomicron remnants (CM-r) may contribute to atherogenic dyslipidemia during the Metabolic Syndrome (Mets). However, at present the combined effects of ezetimibe (EZ) and simvastatin (SV) on post-prandial (PP) dyslipidemia during MetS remains unclear, nor is it known whether the combination has a synergistic anti-atherogenic effect on arterial retention of CM-r. The first objective was to delineate the effects of EZ+SV therapy on intestinal cholesterol flux and CM PP metabolism in the JCR:LA-*cp* rat, a model of MetS. The second objective was to quantify the impact of EZ+SV therapy on arterial retention of CM-r and subsequent myocardial lesion development in the JCR:LA-*cp* rat. EZ+SV therapy decreased net intestinal cholesterol absorption in MetS rats. Furthermore, EZ+SV therapy reduced arterial retention of CM-r and frequency of myocardial lesions in MetS rats. In conclusion, EZ+SV therapy reduces arterial retention of CM-r and myocardial lesion development possibly through its beneficial effects on cholesterol transport and PP-metabolism.

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List of Abbreviations

ABCG5	adenosine triphosphate binding cassette protein 5
ABCG8	adenosine triphosphate binding cassette protein 8
Apo	apolipoprotein
AUC	area under the curve
BMI	body mass index
CETP	cholesterol ester transferase protein
CHD	coronary heart disease
CM	chylomicron
CM-r	chylomicron remnant
cp	corpulent
CVD	cardiovascular disease
DGAT	diacylglycerol acyltransferase
ECL	enhanced chemiluminescence
ELISA	enzyme linked immunosorbent assays
ER	endoplasmic reticulum
EZ	ezetimibe
FAS	fatty acid synthase
FAT	fatty acid translocase
FFA	free fatty acid
H&E	hematoxylin and eosin stain
HDL	high density lipoprotein
HDL-C	high density lipoprotein-cholesterol
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
iAUC	incremental area under the curve
IDL	intermediate density lipoprotein

IR	insulin resistance
LBD	lipid Balance diet
LDL	low density lipoprotein
LDL-C	low density lipoprotein-cholesterol
LDL-r	low density lipoprotein-receptor
LRP	low density lipoprotein receptor related protein
LPL	lipoprotein lipase
MetS	metabolic syndrome
MGAT	monoacylglycerol acyltransferase
M-S	mucosal to serosal
MTP	microsomal transfer protein
MTT	meal tolerance test
NPC1L1	Niemann Pick C1-like-1
nm	nanometers
ObR	leptin receptor gene
OTC	oral fat challenge
Papp	apparent intestinal permeability
PG	proteoglycan
PP	post-prandial
RT	room temperature
SCAP	SREBP-activating protein
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.M	standard mean error
SREBP	sterol regulatory element binding protein
S-M	serosal to mucosal
SREs	activate sterol-regulatory enzymes

SR-B1	scavenger receptor-B1
TG	triglyceride
TICE	trans-intestinal cholesterol excretion
VLDL	very low density lipoprotein

Chapter 1: Literature Review

1.1 Introduction to Cardiovascular Disease

Continued Public Health Crisis:

Cardiovascular disease (CVD) is the primary cause of premature death and disability globally. The World Health Organisation estimated that there were 17.5 million CVD related deaths in 2007, which represents approximately 30% of all global mortality (WHO 2007d). Thus, CVD can no longer be considered a western phenomenon, and the continued rise in frequency suggests that we are far from understanding the underlying causes.

CVD contributes significantly to escalating costs of health care and as a result remains a huge economic burden. The most recent statistics published by the Health Agency of Canada (2002) stated that CVD accounts for $7.3 billion per annum in direct costs of illness (drugs, research, hospitalization, medical expenditure) and $12.3 billion per annum in indirect costs (loss of future earnings due to premature death, loss of productivity due to disability and illness) (Public Health Agency of Canada 2002). CVD is multi-factorial and the continued incidence suggests that we are yet to fully understand the disease. The premise of the disease is that lipid and sterols accumulate in the lining of blood vessels over time causing an inflammatory response, eventually leading to an occlusion (Ross 1999). The lipid and sterol that accumulates in arterial vessels is thought to be derived from circulating lipid sources such as those described as ‘bad cholesterol’

or low density lipoprotein [LDL] (Williams, Tabas 1998, Steinberg 2006). However, despite aggressive pharmaceutical targets to lower circulating LDL concentration, the frequency of CVD is still high, suggesting that other sources of cholesterol may contribute significantly to the development of the disease.

Cardiovascular Aetiology:

The term CVD is a definition that encompasses several vascular pathologies including coronary artery disease (CAD), rheumatic arterial disease, deep vein thrombosis, congenital heart disease, cerebrovascular disease, peripheral artery disease and pulmonary embolism (WHO 2007d). Atherosclerosis is thought to be the primary pathological process responsible for the development of most CVD. It is important to note that atherosclerosis can begin early in childhood but is not observed clinically until later in adult life (McGill et al. 2000). The current understanding of atherosclerosis is defined by the accumulation of cholesterol within the subendothelial space of the arterial wall resulting in a fatty lesion and advanced plaque formation (Mediene-Benchekor et al. 2001). The cholesterol deposited is thought to be derived from circulating lipoproteins, primarily apolioprotein-B (apoB) containing particles which permeate the vessel wall (Purcell-Huynh et al. 1995). The 'response-to-retention' hypothesis is considered a central paradigm in understanding the pathogenesis of atherosclerosis. The hypothesis describes the process whereby apoB-rich lipoproteins permeate and are retained by the arterial wall and undergo modification via oxidation and enzymatic processes (See figure 1.1) (Libby 2002). In turn, the accumulation of

lipids and cholesterol results in an inflammatory response that further exacerbates the complexity of lesion development in the arterial wall leading to a narrowing of the lumen and a hardening of the vessel wall (Libby 2002). Importantly, complications of the atherosclerotic processes are known to be chronic, slow and cumulative, also suggesting that repeated sub-threshold 'insults' to the vascular bed is a contributing factor in the development of atherosclerosis (Libby 2002).

The process of lesion maturation can be considered in 4 stages:

In a normal artery (stage 1) lipoproteins and monocytes circulate in the lumen of the vessel. Early atherogenesis (stage 2) is induced by retention of lipoproteins which may undergo oxidation and accumulate within the subendothelial space of the arterial wall (Libby 2002). Circulating monocytes are recruited to the endothelial wall and transmigrate into the subendothelial space (Muller, Randolph 1999). Here monocytes are activated and undergo transformation into macrophages via lipid accumulation. Macrophages undergo further oxidation and internalize lipoproteins to prevent lipid and cholesterol accumulation within the arterial wall (Kruth 2001). However, when there is over accumulation of cholesterol-rich lipoproteins, the macrophages are unable to metabolize the excess intracellular lipid and as a result necrotize to form 'foam cells' so that advanced fatty streaks (stage 3) are formed (Kruth 2001, Shibata, Glass 2009). The progression of atherosclerosis involves the development of fatty streaks into fibrous plaques which are concealed by caps composed of collagen, extracellular matrix and platelets (Libby 2002). Rupturing of the fibrous plaque forms a complicated lesion (stage 4), at which point a cardiovascular event can occur (Strong, McGill 1963).

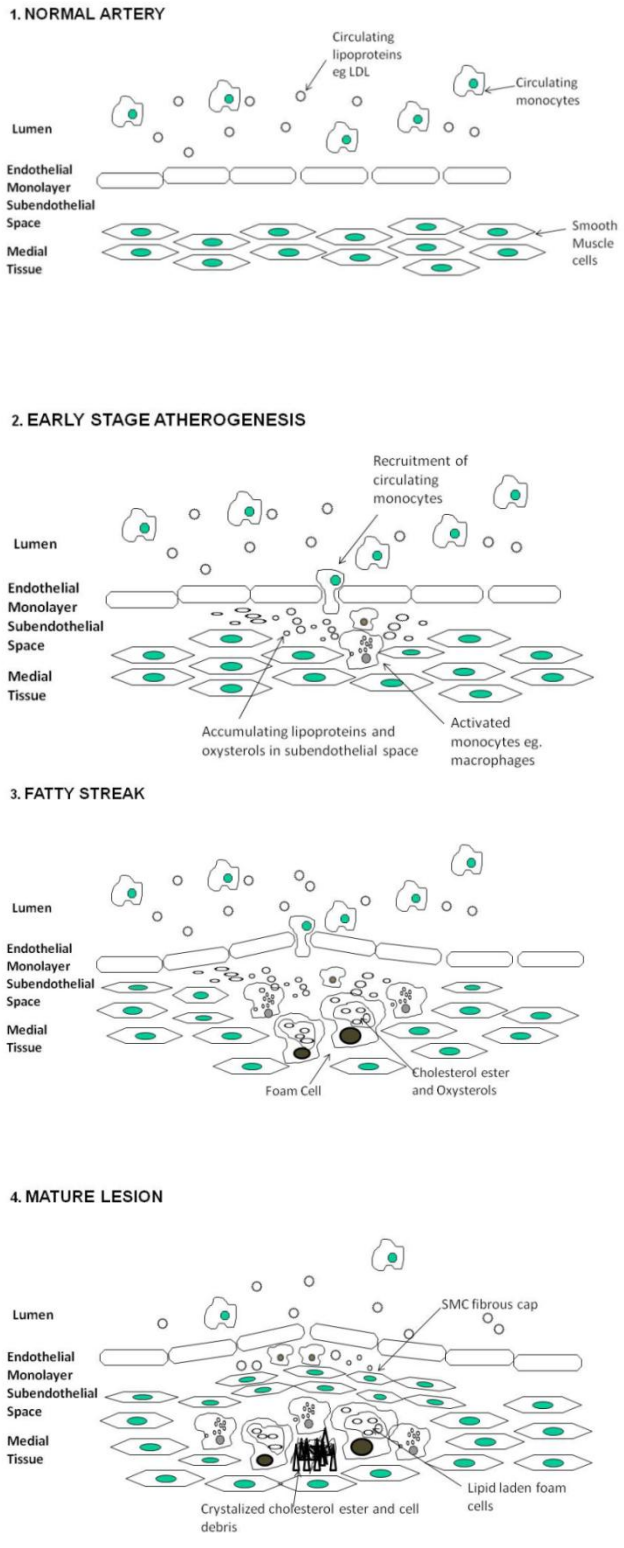

Figure 1-1. The progressive stages of atherosclerosis.

Relationship of Circulating Cholesterol and Formation of Atherosclerosis:

A large body of evidence has shown a direct positive correlation between elevated total plasma cholesterol levels and CVD risk. Both primary and secondary intervention trials to lower plasma cholesterol demonstrate reduced CVD related events and mortality (LIPID study group 2002, Sacks et al. 1996, Downs et al. 1998, Gylling 2004). Current understanding of the 'response to retention' hypothesis suggests that cholesterol deposition within the arterial wall is proportional to the level and duration of arterial exposure to circulating cholesterol-rich lipoproteins (Ross 1999, Williams, Tabas 1998, Libby 2002). Thus elevated plasma cholesterol levels are thought to increase arterial delivery (permeability) and accumulation of cholesterol-rich lipoproteins. Hepatically derived apoB-100 containing LDL plasma levels have been predominately used to assess CVD risk (WHO 2007c). LDL-cholesterol (LDL-C) levels greater than 130mg/dL or 3.3mmol/L correspond with borderline to high risk of developing CVD (WHO 2007c). However emerging evidence over the last decade has also implicated cholesterol dense chylomicron-remnants (CM-r) in the development of CVD (Zilversmit 1979, Mamo, Proctor 1999, Proctor, Vine & Mamo 2002).

1.2 Lipoprotein Metabolism

1.2.1 Lipoproteins

Lipoproteins are macromolecules containing varying amounts of triglyceride (TG), phospholipid (PH), cholesterol ester (CE), free cholesterol and proteins (Nelson, Cox 2000). Their basic biological role is to transport and deliver hydrophobic lipids in the circulation to tissues, by encapsulation of the lipid core with a surface of apoprotiens (Nelson, Cox 2000). Lipoproteins are named and separated according to their density but can also be identified by their cholesterol and TG composition and/or associated apoproteins (see table 1-1) (Kritchevsky 1986).

Lipoprotein	**Major Apolipoproteins**	**Density (g/ml)**	**Size (nm)**	**Triglyceride (% lipid)**	**Cholesterol (% lipid)**	**Phospholipid (% lipid)**
Chylomicron	B48, CI, CII, CIII, E	<0.95	200-1000	86	3	9
CM-remnants	B48, E	<1.006	45-60	70	13	11
VLDL	B100, CI, CII, CIII, E	<1.006	30-90	54	17	19
LDL	B100	1.019-1.063	15-30	7	46	22
HDL	AI, AII, CI, CIII,D	1.063-1.21	5-10	5	23	24

Table 1-1. Composition and physical properties of circulatory lipoproteins (Modified from: Kritchevsky D. Atherosclerosis and nutrition. Nutr Int. 1986;2:290-297).

1.2.2. Chylomicrons

Chylomicrons (CM) are continuously synthesized by the intestine and transport both exogenous (dietary) and endogenous (synthesized in the enterocyte) lipid. CM secreted following a meal can be up to 1000nm in size, have a density of d<1.006g/mL and hence are the least dense of all lipoproteins (Hussain et al. 1996). Chylomicron remnant (CM-r) particles (derived from their larger CM counterparts) are approximately 45-60nm in size (Kritchevsky 1986). CM are primarily composed of a TG core (88%) (Olson 1998) and some cholesterol and cholesterol ester which accounts for 2-5% of the total plasma cholesterol pool (Proctor, Vine & Mamo 2002, Mamo, Proctor 2002). The outer phospholipid bilayer of intestinally derived lipoproteins is embedded with unesterified cholesterol and apoproteins. In humans, apoprotein-B48 (apo-B48) has been shown to be the unique protein pertaining to CM and is formed exclusively in the intestine after tissue-specific post-editing of apoB100 mRNA (Kane, Hardman & Paulus 1980). ApoB48 is essential for CM assembly and has been shown to be non-transferable to other lipoproteins during lipolysis (Kane, Hardman & Paulus 1980). However, in rats and mice, apoB48 is synthesized by both the liver and intestine and thus is associated with lipoproteins of both hepatic (VLDL) and intestinal (CM) origin (Liu, Fan & Redinger 1991). Thus, discrepancies between metabolism of apoB48 in humans verses rodents will be taken into account throughout this thesis which utilizes the JCR:LA-*cp* rodent model.

1.2.2.1. Chylomicron Production and Secretion

Currently, CM assembly is thought be a three-step process which involves the assembly of primordial lipoproteins, the formation of lipid droplets and core expansion (Hussain 2000). Intracellular sites of CM assembly were identified by cell fractionation experiments conducted by Cartwright and Higgins (Cartwright, Higgins 2001, Cartwright, Plonne & Higgins 2000). TG and cholesterol were mainly associated with the trans-Golgi fractions whereas apoB48 and phospholipids were predominantly located in the membrane fraction of the smooth endoplasmic reticulum (Cartwright, Higgins 2001). These findings indicate that CM particle assembly begins in the membranes of the smooth endoplasmic reticulum and that further modification of the primordial particle takes place in the golgi apparatus of the enterocyte (Cartwright, Higgins 2001, Hussain et al. 2005).

The molecular processes underlying CM assembly and secretion have been well established over the past decade (Hussain et al. 2005). ApoB48 is the core apoprotein and is essential for the synthesis of CM. In man, apoB48 is formed via post-transcriptional editing of the apoB100 mRNA sequence in the enterocyte resulting in the synthesis of a protein that is 48% of the apoB100 molecule (apoB48) (Mamo, Proctor 1999). Microsomal transfer protein (MTP) is a heterodimeric protein complex consisting of a large subunit (97kDa), which is responsible for the lipidation of apoB48. CM assembly begins with the co-translational lipidation of apoB48 (Hussain et al. 2005) by MTP (van

Greevenbroek, de Bruin 1998, van Greevenbroek et al. 1998). If apoB48 is not-lipidated it is targeted for proteosome-mediated degradation (Davidson, Shelness 2000). However, binding of MTP to apoB48 encourages correct folding and lipid acquisition which prevents proteosome degradation of this apoprotein (Davidson, Shelness 2000). In the small intestine, MTP may facilitate further lipidation of CM beyond the first apoB48 rescue step, through the incorporation of TG and cholesterol esters in the endoplasmic reticulum (ER). ApoA-IV, a lipid binding protein expressed predominantly in the mammalian small intestine is added to the surface of the CM in the ER (Hussain et al. 2005, Karathanasis, Yunis & Zannis 1986). ApoA-IV facilitates the formation of a larger CM particle by surface stabilization and/or retention in the ER to allow additional core lipidation (Hussain et al. 2005). CM are then transported from the ER to the Golgi via a specialized vesicular compartment known as the pre-chylomicron transport vesicle (PCTV) (Kumar, Mansbach 1999, Siddiqi et al. 2006a, Siddiqi et al. 2006b, Neeli et al. 2007). Within the Golgi, apoA-I attaches to the CM particle to form a mature CM containing apoA-I, apoA-IV and apoB48 (Mansbach, Gorelick 2007). Following maturation of the CM in the golgi, the lipoprotein is exocytosed from the enterocyte at the basolateral membrane into the lymphatic vessels. From the lymphatic circulation, CM enter the plasma compartment at the subclavian thoracic duct.

ApoB48 quantitation is used as a means of determining CM intestinal production and clearance from the circulation

ApoB48 protein expression in the intestine or directly from mesenteric lymph can be used as methods of determining CM production in the enteroctye as CM particles can be identified by the structural apoprotein B48, in a ratio of one apoB48 molecule : one CM particle (Young 1990). However, these methods of measuring CM production have limitations in that they may only determine apoB48 abundance at one point in time or in one compartment – they are not a measure of the dynamic nature of CM metabolism. In particular, it does not represent the post-prandial profile of CM over a period of time following a meal. In this thesis, a combination of methods to measure CM production and clearance will be used including apoB48 concentration of intestinal lymph and plasma in the fasted and post-prandial state. Analysis of CM secretion into the lymph and plasma over time takes into consideration the "net" secretion and clearance of CM and lipid directly from the intestine.

1.2.2.2. Chylomicron Clearance

CM clearance involves lipolysis and uptake by tissues. The process of CM lipolysis is initiated by the actions of lipoproteins lipase (LPL), located on the surface of endothelial cells in all tissues, including skeletal muscle and adipose tissue (Ginsberg 1998, Goldberg, Merkel 2001, Goldberg 1996). LPL hydrolyses TG from CM thus liberating TG, free cholesterol and free fatty acids from the core of the particle (Goldberg, Merkel 2001, Goldberg 1996). Hydrolysis of CM (200-1000nm in size) leads to the formation of small dense CM-r with a diameter

of 45-60nm (Ginsberg 1998, Redgrave 2004). The resulting CM-r particles formed are composed of 70% less TG than CM, and have fewer phospholipids but a greater proportion of cholesterol ester (13%).

During CM lipolysis, CM apoprotein composition is modified in addition to changes in lipid content. Delipidation of CM liberates apoA and apoCII from the particle returning the apoproteins to HDL (Cooper et al. 1982). Of great significance is the retention of apoE by CM-r, as apoE is the primary protein to interact with the hepatic apoB100/apoE (LDL) receptor (Mahley, Innerarity 1983). CM-r interact with the apoB100/apoE receptor through apoE, which requires a cluster of four receptors to enable cellular internalization (Innerarity, Mahley 1978). ApoB100/apoE receptors are expressed at the surface of hepatic tissues and CM-r are removed via this way (Mahley, Innerarity 1983). The high affinity of apoE for the apoB100/apoE receptor compared to apoB100 may partially explain the enhanced clearance of CM-r as compared to LDL. In addition, CM-r may also be cleared via the LDL-like receptor protein (LRP) also expressed on the surface of hepatic tissues (Rubinsztein et al. 1990).

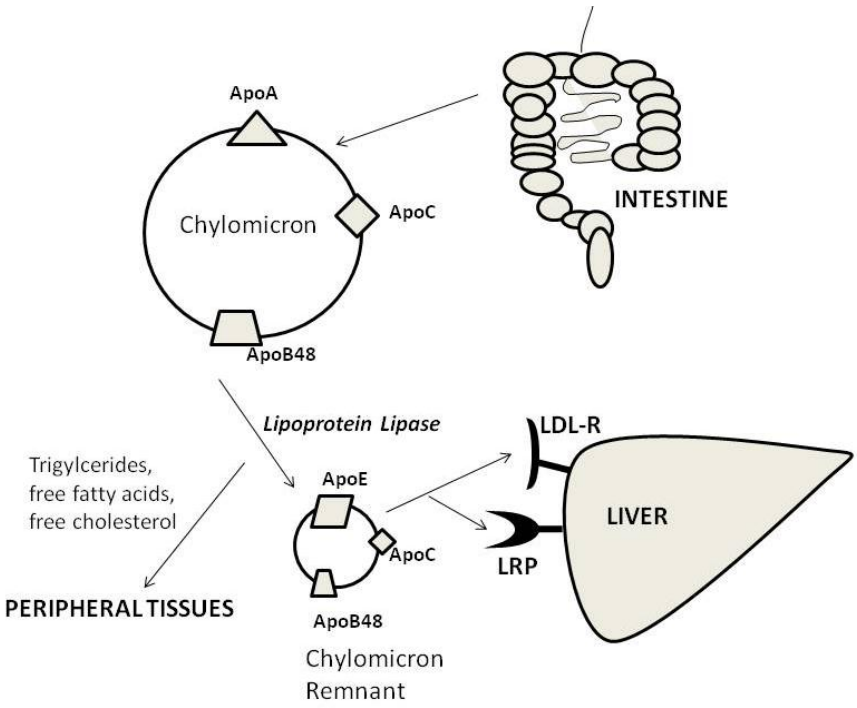

Figure 1-2 Schematic diagram of chylomicron hydrolysis and clearance by receptor mediated processes.

Chylomicrons and the apoB100/E receptor (LDL-receptor):

Under normal conditions, more than 90% of circulating lipoproteins (including LDL and CM-r) are cleared via the hepatic apoB100/apoE -receptor (Bowler, Redgrave & Mamo 1991, Choi, Cooper 1993, Ishibashi et al. 1996). The remainder of CM-r are taken up by other tissues through the vascular endothelium and flux through the tissue to the vasovasorum. The impairment of the apoB100/apoE (LDL) receptor (e.g. reduced LDL-receptor expression), delays CM-r clearance and in turn elevates the concentration and duration of CM-r remaining in plasma (Cabezas et al. 1998). Prolonged exposure of cholesterol dense CM-r to the vascular endothelium is proposed to result in increased cholesterol deposition within the arterial wall (Mamo, Proctor & Smith 1998).

In conditions of reduced apoB100/apoE -receptor expression, LDL clearance is observed to be normal whilst CM-r clearance is impaired (Bowler, Redgrave & Mamo 1991). As previously mentioned, delayed CM-r clearance is thought to be due to CM-r requiring a cluster of four receptors to be internalized by the apoB100/apoE-receptor (Innerarity, Mahley 1978). LDL particles on the other hand only require binding of one-ligand to one-receptor and thus during conditions of reduced apoB100/apoE expression, LDL particles are cleared more efficiently than CM-r.

Chylomicrons and the LDL-receptor related protein (LRP):

The LDL-receptor related protein (LRP) is a member of the LDL-receptor gene family that is highly expressed in the liver and mediates the removal of several ligands including LDL and CM-r. Unlike the LDL-receptor, interaction of the LRP with CM-r not only requires apoE but also requires heparin sulphate proteoglycans (Weisgraber et al. 1986). The importance of the LRP pathway in CM-r clearance was highlighted by several studies demonstrating that LDL-receptor deficient/KO models do not display impaired postprandial metabolism (Rubinsztein et al. 1990, Ishibashi et al. 1996, Kita et al. 1982). Thus, it was proposed that additional clearance pathways such as the LRP pathway also play a key role in CM-r clearance. Human studies have supported these findings as LDL-receptor deficient subjects have a normal post-prandial response suggesting that alternative pathways such as the LRP are involved in CM-r clearance (Rubinsztein et al. 1990).

1.2.3. Very Low Density Lipoproteins (VLDL)

Very low density lipoproteins (VLDL) are synthesized exclusively by the liver and play a key role in the transport of endogenous cholesterol and TG to peripheral tissues. VLDL particles are resemblant of CM in terms of their density (0.95-1.006g/ml), TG content and Svedberg flotation (S_f) rate (20-400) (Olson 1998). However, VLDL particles are currently distinguished from CM by the presence of apoB100 on their surface, whilst apoB48 is uniquely pertained to the CM particle. On average, VLDL particles consist of 5-15% cholesterol, 10-20% phospholipid, 90-95% lipid, 50-55% TG and 7-10% protein.

Lipolysis of VLDL by lipoprotein lipase (LPL) located on the surface of endothelial cells results in the formation of smaller, denser VLDL-remnant particles or intermediate-density lipoproteins (IDL) (Ginsberg 1998). IDL is considered to be a 'transient lipoprotein' as it has a short half-life and represents an intermediate stage during the conversion of VLDL to LDL.

1.2.4. Low Density Lipoproteins (LDL)

Low density lipoproteins (LDL) are generated via plasma catabolism of VLDL and IDL and/or metabolized by the liver. LDL particles are composed of 20-25% phospholipid, 78% lipid, 22% protein, 5-15% TG, 40-50% cholesterol and are indentified by the structural apolipoprotein B-100 in a ratio of one apoB-100 molecule : one LDL particle (Ginsberg 1998). The functional role of LDL is to transport cholesterol to the all tissues of the body. The clearance of LDL from plasma is predominantly determined by the availability of the LDL receptors

(LDL-R) (Goldstein, DeBose-Boyd & Brown 2006, Goldstein, Brown 1990). After LDL interacts with the LDL receptor at the liver, endogenous hepatic cholesterol synthesis is inhibited via down-regulation of sterol regulatory element binding protein (SREBP) and thus cholesterol homeostasis is, in part, maintained by this pathway (see section 1.3.2) (Brown, Goldstein 1997).

In normal individuals, 60-80% of LDL can be cleared via the high affinity LDL receptor mediated pathways, whilst the remainder are cleared via non-receptor pathways (e.g. fluid phase endocytosis) (Dietschy, Woollett & Spady 1993, Spady et al. 1986). Furthermore, other receptors involved in the sequestration of LDL-cholesterol include; LRP and scavenger receptors (inclusive of those receptors which recognize oxidatively modified LDL) (Ginsberg 1998). However, in situations where LDL-R expression is decreased, cellular cholesterol metabolism is unregulated resulting in markedly increased LDL plasma cholesterol levels (Choi, Cooper 1993, Ishibashi et al. 1996, Brown, Goldstein 1984).

Literature to date has found a significant epidemiological association between raised levels of LDL-C and CVD risk (Mediene-Benchekor et al. 2001, Colhoun et al. 2004). The association between LDL-C plasma concentrations and CVD risk is attributed to the LDL fraction carrying more than 70% of plasma cholesterol at any one time. Due to the small size of LDL particles (22.5-27.5nm), these lipoproteins can easily permeate the arterial wall, undergo oxidative modification and stimulate foam cell formation; a hallmark feature of

atherosclerosis (Morel, DiCorleto & Chisolm 1984, Henriksen, Mahoney & Steinberg 1983, Hiramatsu et al. 1987).

Although there is an accumulating body of evidence that implicates LDL-C in the pathogenesis of atherosclerosis, up to 40% of individuals diagnosed with CVD have normal levels of LDL (Vessby 2003, Mooradian 2003). However, there is now emerging evidence that CM-r may play a significant role in the pathogenesis of atherosclerosis in subjects who are normolipidemic (i.e. have normal range LDL-C) (Proctor, Mamo 2003) which will be discussed in further detail in section 1.5.

1.2.5. High Density Lipoproteins (HDL)

Nascent high density lipoproteins (HDL) are synthesized by the liver and small intestine. Typically, HDL is composed of 40-50% cholesterol ester & phospholipid and 10-18% unesterified cholesterol & TG (Ginsberg 1998). Nascent HDL particles secreted by the liver and intestine take up free cholesterol derived from the extracellular surface of cell membranes and other lipoproteins. Nascent HDL has the enzyme lecithin cholesterol acyltransferase (LCAT) located on its surface which converts cholesterol and the phosphatidylcholine of VLDL and LDL to cholesterol ester (Ginsberg 1998). Formation of mature spherical HDL occurs as a result of cholesterol ester accumulation in the core of the nascent HDL particle (Bruce, Tall 1995).

Reverse Cholesterol Transport:

Mature HDL particles may either transfer cholesterol ester to other lipoproteins and tissues or be directly metabolized and removed from the plasma via the liver. The transfer of cholesterol ester to other lipoproteins (e.g. CM and VLDL) and tissues is mediated by the actions of cholesterol ester transfer protein (CETP). Thus, when CM and VLDL are cleared by the liver, CETP-transferred cholesteryl ester can be taken up as well (Tall 1990).

Long standing epidemiological evidence has shown an inverse correlation between low plasma HDL concentrations and increased CVD risk (Colhoun et al. 2004). HDL removes excess cholesterol from blood, tissues and cholesterol loaded cells in atherosclerotic plaques. Therefore, mechanisms to elevate HDL levels are currently a target for future drug treatments (Ferns, Keti 2008). Epidemiological studies have provided consistent evidence that for every 0.03mmol/L (1mg/dL) decrease in HDL-C, there is a 2-3% increase in CVD risk (Wilson, Abbott & Castelli 1988, Gordon, Rifkind 1989). The Canadian Working Group on Hypercholesterolemia and Other Dyslipidemias has recommended cholesterol:HDL ratio as a second target therapy to lowering LDL-C plasma levels (Genest et al. 2003).

1.2.6 Hepatic and Intestinal Contributions to Cholesterol Homeostasis

Historically, hepatic regulation of cholesterol homeostasis and its contribution to CVD has been the primary focus of scientific research. However, pioneering breakthroughs in the understanding of how the intestine complements the liver during whole body cholesterol homeostasis are providing potential new therapeutic targets for anti-atherogenic treatments. Consequently, we are beginning to appreciate that both the liver and the intestine are major contributors to whole-body cholesterol homeostasis. In an effort to appreciate these two organs in the context of my thesis questions, mechanisms involved in cholesterol homeostasis including synthesis by the liver and absorption via the small intestine and their implications to hypercholesterolemia (elevated plasma cholesterol) will now be discussed.

1.3 Cholesterol Homeostasis

1.3.1 Introduction

Cholesterol ($C_{27}H_{46}O$) (see figure 1-3) is an essential component of mammalian cell membranes as it is required to maintain membrane integrity and fluidity (Maxfield, Tabas 2005). Cholesterol is also the precursor molecule of steroid hormones (Kruit et al. 2006). Maintenance of cholesterol homeostasis in the body requires accurate metabolic cross-talk between processes that regulate dietary cholesterol intake, *de novo* (endogenous) cholesterol synthesis and cholesterol absorption/excretion. Both dietary and endogenously synthesized cholesterol are transported within the circulation in lipoprotein particles.

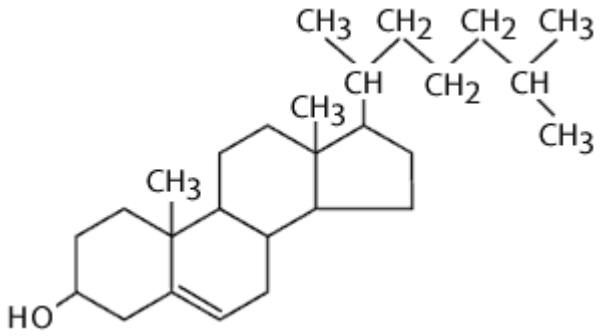

Figure 1-3. Molecular structure of cholesterol. ($C_{27}H_{46}O$).

Although cholesterol is essential for life, imbalances in processes mediating cholesterol homeostasis can lead to a dis-balance, in particular elevated plasma cholesterol levels which are associated with CVD (WHO 2007c). Keys et al were the first to demonstrate the link between dietary cholesterol and cholesterol metabolism in humans (Keys 1975). The study revealed that individuals who consumed diets rich in cholesterol and saturated fatty acids had raised blood cholesterol concentrations and were at greater risk of developing CVD (Keys 1975). Later studies confirmed high cholesterol diets decrease cholesterol uptake via the liver by suppressing production of the LDL-receptor and LRP which mediate clearance of LDL and CM-r (Spady, Woollett & Dietschy 1993). As a result of delayed clearance of cholesterol-rich lipoproteins from plasma, these atherogenic particles increase in concentration in the circulation.

Consequently, exacerbated delivery of cholesterol-rich lipoproteins, LDL and CM-r, and deposition of cholesterol may occur in arterial vessel walls. Accumulation of cholesterol within vessel walls leads to atherosclerosis, the primary pathological process responsible for the development of CVD. Treatments to lower serum cholesterol levels (including both lifestyle and drug interventions) have been shown to result in decreased CVD related morbidity and mortality (Sacks et al. 1996, Downs et al. 1998, Gylling 2004).

1.3.2 Hepatic Biosynthesis of cholesterol

It is commonly believed that the liver plays a crucial role in whole body cholesterol homeostasis and is often termed the 'control centre'. However, at present, controversy exists as to how much the liver contributes to *de novo* cholesterol synthesis. Studies by Dietschy et al which examined the independent contribution of the intestine, liver and extra-hepatic tissues to cholesterol synthesis revealed that the liver contributes as little as 15% to endogenous cholesterol synthesis and that extrahepatic organs contribute as much as 85% (Dietschy, Turley & Spady 1993, Dietschy 1997). However, most studies indicate that the liver is the primary site of cholesterol synthesis within the body (Kruit et al. 2006).

Hepatic cholesterol synthesis is mediated by an extensive series of reactions. The major precursor for the synthesis of cholesterol is acetyl coenzyme A (acetyl-CoA) which combined with the actions of hydroxyl-methylglutaryl coenzyme A synthase (HMG-CoA synthase) gives rise to HMG-CoA. The rate-limiting step in the cholesterol biosynthetic pathway is the conversion of HMG-CoA to mevalonic acid which is catalyzed by HMG-CoA reductase (Bays et al. 2008). Once synthesized from mevalonic acid, hepatic cholesterol is esterified via the actions of acyl-CoA-cholesterol acyl transferase (ACAT).

Direct and indirect regulation of HMG-CoA reductase:

HMG-CoA reductase is the rate limiting step in cholesterol synthesis as it catalyses the conversion of HMG-CoA to mevalonate. The statin class of compounds (such as simvastatin) are currently the drug treatment of choice to help lower cholesterol levels and they function by inhibiting HMG-CoA reductase (Holdgate, Ward & McTaggart 2003, Wang, Liu & Liao 2008). The molecular mechanisms involved in direct and indirect regulation of HMG-CoA reductase will now be discussed.

Cholesterol Directly Regulates HMG:CoA Reductase activity:

Cholesterol itself is able to maintain negative feed-back inhibition of HMG-CoA reductase activity. *In-vitro* studies have revealed that following cholesterol accumulation within the endoplasmic reticulum membrane, HMG-CoA binds to Insig proteins resulting in ubiquitination and degradation of HMG-CoA reductase (Sever et al. 2003b, Sever et al. 2003a). Thus, further cholesterol synthesis is inhibited.

Cholesterol indirectly regulates HMG:CoA Reductase activity:

Cholesterol indirectly regulates HMG-CoA reductase expression via its actions on sterol regulatory element binding protein 2 (SREBP2). *In-vitro* studies have revealed that during periods of low circulating cholesterol levels, SREBP2 binds to SREBP cleavage activating protein (SCAP) which escorts SREBP2 to the golgi. SREBP2 is cleaved in the golgi, which results in its activation and

subsequently facilitates transcription of the HMG-CoA reductase encoding gene (Goldstein, DeBose-Boyd & Brown 2006). Contrastingly, when cholesterol accumulation occurs in the ER, the SCAP/SREBP complex binds to a resident ER protein, designated Insig, which blocks the transport of SREBP2 to the golgi (Goldstein, DeBose-Boyd & Brown 2006). As a result, transcription of genes declines and cholesterol synthesis and uptake is inhibited.

Hepatically derived cholesterol is packaged into VLDL particles:

Hepatically derived esterified cholesterol is incorporated into apoB-100 containing VLDL particles via MTP (White et al. 1998). VLDL particles are released into the circulation for delivery of lipids to tissues, where they undergo TG hydrolysis by endothelial LPL to release FFA. As a result, IDL particles are produced of which some are cleared via the liver and the remainder are lipolysed further to LDL by LPL and hepatic lipase. LDL are subsequently taken up by extrahepatic tissues and cleared by the liver.

1.3.3. Intestinal Cholesterol Absorption

Intestinal cholesterol absorption is an important factor towards determining circulating cholesterol levels (Bays, Stein 2003, Bays 2002, Turley, Dietschy 2003). The small intestine absorbs cholesterol from both endogenous (biliary) and exogenous (dietary) sources (Lu, Lee & Patel 2001). The former is believed to contribute ¾ to the cholesterol absorbed whilst the later ¼ (Dietschy 1997). The underlying mechanisms involved in intestinal cholesterol absorption are yet to be fully appreciated.

During digestion of food, bile acids are secreted from the liver and gall bladder into the intestine. Bile acids aid emulsification of lipids including dietary cholesterol, TG and phospholipid in food (Chiang 1998). Following emulsification, lipid micelles containing free cholesterol, phospholipids and fatty acids are formed. The micelles provide an efficient vehicle for cholesterol to be transported to the mucosal brush border membrane (BBM) of jejunal enterocytes. Cholesterol is transferred from lipid micelles to the enterocyte BBM and cholesterol is internalized by the enterocyte, however the mechanisms by which this occurs are unclear and yet to be clarified (Tso, Nauli & Lo 2004). Currently, there are two hypotheses by which intestinal epithelial cells are thought to uptake or absorb cholesterol. One hypothesis suggests that cholesterol is absorbed by an energy independent passive diffusion process regulated via a concentration gradient (Hui, Labonte & Howles 2008). The second hypothesis proposes that cholesterol is absorbed through an energy dependent, protein mediated process

(Thurnhofer, Hauser 1990). In agreement with the latter hypothesis, several intestinal transporters have been identified and will now be discussed.

1.3.3.1. Intestinal Cholesterol Absorption and Intracellular Transporters

Niemann Pick C1 Like 1 Protein:

Niemann Pick C1 like 1 protein (NPC1L1) is the main cholesterol transporter of interest as it is abundantly expressed in the intestine, particularly in the jejunum at the BBM (Altmann et al. 2004). NPC1L1 was first described by Davies et al and its name derives from it sharing 42% amino acid homology with Niemann-Pick type C1 protein (NPC1), a protein involved in intracellular cholesterol transport (Davies, Levy & Ioannou 2000). The NPC1L1 transporter is believed to facilitate luminal cholesterol absorption and transports cholesterol from the enterocyte mucosal membrane to the endoplasmic reticulum where cholesterol is re-esterified. However, the location of this transporter is currently under dispute. There is some evidence that post-translationally, NPC1L1 moves from internal membranes to the mucosal membrane during cellular cholesterol depletion facilitating absorption (Yu et al. 2006). Other studies suggest that NPC1L1 has a fixed location at the BBM of enterocytes and does not have the ability to move internally (Davis, Altmann 2009). Cholesterol absorbed into the enterocyte is esterified by the actions of ACAT, and the resulting cholesterol ester is packaged into nascent CM. Previous studies have shown that cholesterol rich diets suppress NPC1L1 in murine intestine, and cholesterol depletion enhances its expression in the porcine intestine (Altmann et al. 2004). Furthermore, NPC1L1 mRNA

expression appears to be positively correlated with plasma apoB48 and CM cholesterol content (Lally et al. 2006).

Impact of type I and type II diabetes on NPC1L1 mRNA expression:

Animal models of type I diabetes (streptozotosin-treated rats) and type II diabetes (Zucker diabetic fa fa rats) show increased NPC1L1 mRNA expression (Tomkin 2008). Genetic deficiency of NPC1L1 in apoE KO mice, an animal model that accumulates remnant like lipoproteins in plasma, lowers the cholesterol content of atherogenic lipoproteins by 80-90% and prevents development of vascular disease (Davis et al. 2007).

Human studies have supported animal data, as type II diabetic patients demonstrate increased NPC1L1 mRNA expression in the intestine. Even more intriguing is that type II diabetics with CAD have been shown to absorb cholesterol more efficiently compared to patients without CAD (Lally et al. 2006). At present there is little knowledge on NPC1L1 mRNA expression in the metabolic syndrome (MetS).

Scavenger receptor B1:

Scavenger receptor B1 (SRB1) has been shown to be highly expressed at the mucosal side of the proximal small intestine compared to the serosal side (Labonte et al. 2007). The role of this transporter in cholesterol absorption was first indicated by studies showing that SR-BI cDNA transfected cells display increased cholesterol uptake from micellular substrates compared with mock-control transfected cells (van Bennekum et al. 2005, Altmann et al. 2002).

Specific intestinal SR-BI over expression in transgenic mice has been associated with increased cholesterol absorption (Bietrix et al. 2006). Moreover, antibodies against SR-BI demonstrate abolishment of high affinity binding of cholesterol to brush border membrane vesicles that would normally be observed in NPC1l1-/- mice (Labonte et al. 2007). Despite these findings, there is evidence indicating KO SR-BI mice absorb cholesterol as efficiently as wild type mice (Altmann et al. 2004). Thus, it has been proposed by Hui et al (2008) that SR-BI may play a role in the initial step of cholesterol absorption by facilitating high affinity cholesterol binding to the mucosal brush border membrane but alternative cholesterol transporters may compensate for the absence of SR-BI in mediating cholesterol absorption in KO models (Labonte et al. 2007). To date there have been limited studies investigating the impact of MetS or type II diabetes on intestinal SR-BI expression. Ravid et al (2008) revealed that high glucose levels decrease SR-BI expression in CaCo-2 cell lines which is consistent with findings from hepatic cell studies (Murao et al. 2008).

FAT/CD36:

FAT/CD36 (translocase) a human analogue of SR-BI is believed to be expressed along the mucosal enterocyte membrane of the duodenum and jejunum. There is a large body of evidence showing that CD36 deficiency correlates with abnormal lipid processing in enterocytes (Hui, Labonte & Howles 2008). However, the potential role of CD36 in cholesterol absorption is far from clear. Studies have shown that cholesterol uptake is enhanced in CD36 transfected COS-7 cells as

compared to mock-control transfected cells (van Bennekum et al. 2005). Furthermore, Tso et al have shown reduced lymphatic transport of dietary cholesterol and reduced apoB-48 and CM assembly in CD36 KO mice (Nauli et al. 2006, Drover et al. 2005). Streptozotocin-induced type I diabetic male Sprague-Dawley rats have been observed to have increased FAT/CD36 mRNA expression in duodenum (2.2-fold), jejunum (1.8-fold) and ileum (1.5-fold) ($p<0.05$) (Chen et al. 2006). Insulin treatment in type I diabetic rats lowers FAT/CD36 mRNA expression suggesting that both insulin and glucose concentrations mediate the expression of intestinal transport FAT/CD36 (Chen et al. 2006).

1.3.3.2. Intestinal Cholesterol Efflux Transporters

ATP-Binding Cassette Protein 5/8:

ATP-Binding Cassette Protein 5 (ABCG5) and ATP-Binding Cassette Protein 8 (ABCG8) are located at the mucosal membrane of the enterocyte (Hui, Labonte & Howles 2008). Their expression is greatest in the duodenum and jejunum and they work in tandem to efflux cholesterol (mainly plant sterols) from the enterocyte back into the lumen for excretion (Graf et al. 2003). Blocks et al (2004) unveiled that the mRNA and protein expression of ABCG5 and ABCG8 was significantly decreased in the intestine of streptozotocin-treated rats (model of type I diabetes – no pancreatic insulin) and levels could be partially normalized by insulin supplementation (Blocks, Bakker-Van Waarde W.MM & Verkade 2004). Other

work showed that there was a negative correlation between ABCG5/8 and cholesterol content of CM in streptozotocin-induced diabetic male Sprague-Dawley rats (Lally, Owens & Tomkin 2007). Lally et al showed ABCG5/8 mRNA expression to be decreased by more than 50% in the diabetic animal compared to their lean counterparts which was correlated with an increase in cholesterol content of CM, but this data did not reach statistical significance (Lally, Owens & Tomkin 2007). Collectively, these results correspond with data from human studies indicating that type II diabetic patients have lower mRNA levels of these transporters as compared to controls, and a negative correlation exists between ABCG5/8 and CM cholesterol content (Lally et al. 2006). Insulin resistance in men has been linked to a q604E polymorphism in the ABCG5 gene, further suggesting that both ABCG5 and ABCG8 are associated with insulin and cholesterol metabolism (Gylling et al. 2004). Mutations of ABCG5 and ABCG8 in humans inhibits intestinal cholesterol efflux, and predisposes these individuals to atherosclerosis (Tomkin 2008, Gylling et al. 2004).

ATP-Binding Cassette Protein 1:

ATP-Binding Cassette Protein 1 (ABCA1) was first discovered as the defective gene in Tangier Disease (Levy et al. 2007). ABCA1 is located on the basolateral surface of intestinal cells and is crucial for intestinal secretion of HDL, wich accounts for 30% of HDL production in the body (Hui et al. 2008). In addition, ABCA1 also contributes to the efflux of cholesterol out of the enterocyte and back into the intestinal lumen (Vaisman et al. 2001).

Within the enterocyte cholesterol is packaged into chylomicron particles:

Following cholesterol absorption into the enterocyte, free cholesterol may either be returned to the intestinal lumen via the ABCA1 and ABCG5/8 transporters or alternatively it can be esterified by ACAT and packaged into CM particles via the actions of MTP within intestinal epithelial cells. CM are then secreted from the enterocyte into the lymphatic system.

1.3.4 Enterohepatic Circulation

Bile acids are synthesized from cholesterol in the liver where they are conjugated to glycine and taurine and subsequently stored in the gall bladder as the principal constituents of bile. After ingestion of a meal, bile acids are released from the gall bladder into the duodenum where they facilitate absorption of fat-soluble vitamins and cholesterol. In healthy individuals, approximately 95% of bile acids are efficiently reabsorbed from the intestine and returned to the liver via the enterohepatic circulation (Chiang. 1998). In the small intestine, bile acids are absorbed by both passive and carrier-mediated (active) mechanisms. Passive absorption of bile acids occurs down the length of the intestine, whilst carrier-mediated transport is restricted to the ileum. Carrier-mediated transport of bile acids consists of apical uptake from the intestinal lumen, intracellular trafficking to the basolateral membrane and subsequent basolateral efflux into the portal circulation. The intestinal bile acid transporter (IBAT) is proposed to be the primary transporter by which bile acids are absorbed by the enterocyte (Chiang

1998). Hepatic conversion of cholesterol to bile acids balances fecal bile acid excretion and this process represents a major route for cholesterol elimination from the body (see figure1-4).

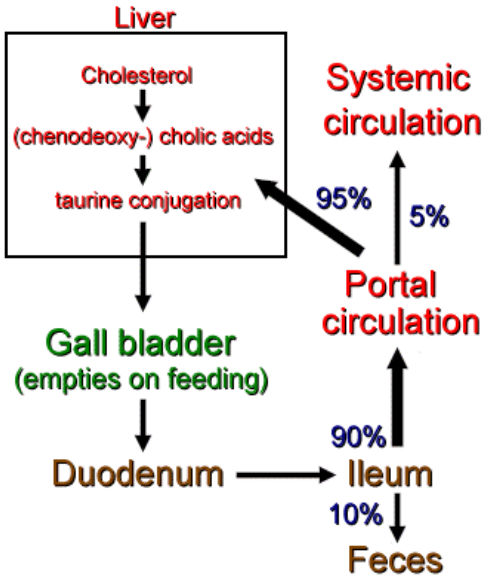

Figure 1-4. Schematic diagram of the enterohepatic circulation.

1.4 Chylomicron Metabolism and Association with Chronic Disease

1.4.1 Chylomicron Metabolism and Obesity

Obesity is an epidemic disease in which there is abnormal excessive body fat accumulation and this presents a risk to health. There is now significant epidemiological evidence showing that obesity is associated with an increased risk of developing CVD (Dagenais et al. 2003). The World Health Organization (WHO) estimated that in 2005, 400 million people worldwide were obese and the incidence would dramatically increase to 700 million people by 2015 (WHO 2007b). Obesity is the leading cause of preventable illness and death in North America and lifestyle interventions would help alleviate the progression of this epidemic. Currently, body mass index (BMI) is used as a crude measure of obesity (WHO 2007b). BMI is determined by weight and height of an individual (kg/m2) and is defined as having a BMI > 30kg/m2 (WHO 2007b). Alternatively, obesity can be measured by waist circumference and/or waist to hip ratio. A waist circumference of >102cm in men and >88cm in women, whilst waist to hip ratios >0.9 in men and >0.85 in women define central obesity (WHO 2007b).

Several studies have now indicated that plasma apoB48 concentrations are increased in obese individuals. Studies by Couillard et al (2002) examined the post-prandial response of CM in men following a test meal enriched in dietary fat (64% dietary fat). The study revealed that obesity was positively correlated with increased concentrations of post-prandial TG-rich, apoB48-containing lipoproteins (Couillard et al. 2002).

Moreover, there is emerging evidence that the distribution of body fat rather than weight plays a greater role in determining the association between obesity and post-prandial lipemia (Watts et al. 2001, Mamo et al. 2001). Previous studies have shown apoB48 plasma concentrations to be increased in viscerally obese individuals and CM catabolism was inversely related to waist to hip ratio (Watts et al. 2001). These findings are supported by those of Mekki et al (1999) who examined the effect of fat distribution on post-prandial lipemia in obese women. The results revealed that visceral adiposity correlated with elevated levels of apoB48-containing lipoproteins (Mekki et al. 1999). The prevalence of obesity in children is increasing and recent studies have shown an association between visceral obesity and elevated plasma apoB48 concentrations (Su et al. 2009, Nzekwu et al. 2007).

1.4.2 Chylomicron metabolism and type II diabetes

The WHO estimates that 180 million people worldwide have been diagnosed with diabetes, of which 90% are type II diabetic (WHO 2007a). The WHO projects that the incidence of type II diabetes will increase by 50% in the next ten years (WHO 2007a). Diabetes and its complications impose significant health and economic consequences to individuals, families and public health systems. Direct health care costs of diabetes range from 2.5% to 15% of annual health care budgets, depending on local diabetes prevalence and sophistication of treatment available (WHO 2007a). Furthermore, personal costs of diabetes are encountered,

such as reduced quality of life and the increased probability of developing further complications: kidney disease and CVD.

Non-Insulin Dependent Diabetes Mellitus (NIDDM) or type II diabetes is a disorder which results from the body's ineffective use of insulin i.e. the body's cells are unable to respond appropriately when insulin is present. As a result, blood glucose levels rise as the body's cells fail to respond to the normal insulin-mediated response to uptake glucose, insulin concentrations rise to try and compensate, and insulin resistance subsequently develops. Type II diabetes is currently defined as fasting plasma glucose concentrations >7.0mmol/L (126mg/dL), or a plasma glucose reading at 2-hr following a glucose tolerance test >11.1mmol/L (200mg/dL) (WHO 2007a).

Individuals with type II diabetes are 4 times more likely to develop CVD compared to the non-diabetes state. Moreover, CVD is responsible for 50-80% of deaths in individuals with type II diabetes (Kannel, McGee 1979). There is now an accumulating body of evidence showing that post-prandial lipemia is a prominent feature of individuals with diabetes (Hogue et al. 2007, Curtin et al. 1996). More specifically, subjects with type II diabetes demonstrate an increased production rate of intestinal apoB-48 containing lipoproteins (Hogue et al. 2007, Curtin et al. 1996). Hogue et al have confirmed and further extended these findings by demonstrating increased production rate of apoB-48 containing

lipoproteins and decreased catabolism in diabetic patients compared to non-diabetic controls (Hogue et al. 2007).

1.4.3 Chylomicron metabolism and insulin resistance/hyperinsulinemia

Insulin resistance (IR) is defined as the 'diminished' ability of cells in the body to respond to the normal actions of insulin (Reaven, Abbasi & McLaughlin 2004). As a result, pancreatic beta cells try to maintain homeostatic blood glucose concentrations in an IR state by over secreting insulin, a condition known as hyperinsulinemia (Reaven, Abbasi & McLaughlin 2004). IR is associated with increased fasting and post-prandial concentrations of CM attributed to an increased production rate and decreased clearance from the plasma (Duez et al. 2006, Harbis et al. 2001).

Insulin is proposed to play an important role in the regulation of CM metabolism. Human studies have revealed a positive correlation between plasma insulin concentrations and apoB-48 (Duez et al. 2006, Harbis et al. 2001). Correspondingly, IR subjects demonstrate CM overproduction (Duez et al. 2006, Duez, Pavlic & Lewis 2008). Furthermore, insulin infusion in subjects fed a carbohydrate-free meal during a 3-hr hyperinsulinemic/euglycemic clamp showed reduced plasma apoB48 concentrations (Duez et al. 2006). The molecular/cellular mechanisms of increased production rate of CM particles during IR are still unclear. There is some evidence that apoB-48 intracellular stability is increased as well as mass and activity of MTP leading to CM over-production (Duez, Pavlic

& Lewis 2008, Black 2007). Moreover, in IR-states, elevated free fatty acid influx into the intestine, and down-regulation of insulin signalling appear to stimulate CM production (Duez, Pavlic & Lewis 2008, Duez et al. 2008).

1.4.4 Chylomicron metabolism and the metabolic syndrome

The metabolic syndrome (MetS) is currently defined as central/visceral obesity and two of the following symptoms: hypertension, raised TG, reduced HDL cholesterol, increased plasma glucose and insulin concentrations (IDF 2006). Due to MetS being a pre-diabetic manifestation of the clinical parameters listed above, it has been difficult to obtain a homogenous population of subjects for the purpose of research. Moreover, there are few available animal models of MetS which has limited the ability of researchers to investigate the association between MetS and the early development of CVD.

The JCR:LA-*cp* rat is a well established animal model that mimics features of MetS in obesity, and spontaneously develops myocardial and systemic arterial lesions (Vine et al. 2007, Mangat et al. 2007, Russell, Graham & Richardson 1998, Vine, Glimm & Proctor 2008). The JCR:LA *cp/cp* strain has become the model of choice for the study of MetS and atherosclerosis. The JCR:LA-*cp* rat incorporates the autosomal recessive corpulent (*cp)* polygenic trait (or phenotype) first isolated by Koletsky (Koletsky 1975). JCR:LA-*cp* rats which are homozygous *(cp/cp)* are phenotypically obese, IR, and hypertriglyceridemic (Mangat et al. 2007, Russell, Proctor 2006, Tofovic, Jackson 2003). The *cp*

phenotype has been shown to encode a stop codon in the extracellular domain of the leptin receptor, leading to complete absence of the ObR (Ob-receptor) in the plasma membrane of *cp/cp* rats (Russell, Proctor 2006).

Our laboratory has recently shown that this model has impaired CM metabolism, as determined by plasma apopB48 concentration, with impaired CM clearance from the circulation compared to control animals (Vine et al. 2007, Mangat et al. 2007, Vine, Glimm & Proctor 2008). Moreover, the JCR:LA-*cp* rat has over-production of intestinal CM and this is associated with intestinal villus hypertrophy (Vine, Glimm & Proctor 2008). The initial study in this thesis will focus on the modulation of intestinal cholesterol flux and subsequent CM production in the JCR:LA-*cp* rat following ezetimibe and simvastatin treatment (two hypercholesterolemic drug treatments).

1.4.5 Chylomicron over-production and delayed clearance in Insulin Resistance

It was previously believed that the elevated concentration of circulating CM observed during IR was a result of impaired CM lipolysis attributed to defects in LPL, and delayed CM clearance associated with reduced activity of the hepatic LDL-receptor and LRP (Coppack 1997, Malmstrom et al. 1997). In addition, it has been suggested that apoE deficient CM are produced during IR states, resulting in lipoproteins having a lower affinity for clearance via hepatic receptors (Phillips et al. 2002). However, an accumulating body of evidence has shown that in addition to delayed CM clearance, CM production and secretion are up-regulated during IR (Duez, Pavlic & Lewis 2008, Duez et al. 2008, Vine, Glimm & Proctor 2008, Adeli, Lewis 2008).

Studies by Duez et al have investigated the relationship between IR and CM production (Duez, Pavlic & Lewis 2008). ApoB48 metabolism was measured in human IR subjects in the steady-state fed condition and blood samples were collected throughout the day (Duez et al. 2006). Findings revealed that there was a significant association between apoB48 production rates and fasting insulin levels (Duez et al. 2006).

Animal studies have also shown that the production of CM positively correlates with IR (Duez, Pavlic & Lewis 2008, Lewis et al. 2005, Zoltowska et al. 2003). It has been proposed that insulin may be involved in the direct and indirect regulation of apoB48 production and secretion (Mangat et al. 2007). The JCR:LA-*cp* rat has been shown to have a positive correlation between plasma

insulin levels and circulating apoB48 particles (Vine et al. 2007, Vine, Glimm & Proctor 2008).

More recently, CM over-production and secretion has been associated with IR. Studies utilizing stable isotope enrichment of apoB48 lipoproteins observed an increased intestinal production rate of these lipoproteins in hyperinsulinemic/insulin resistant subjects (Duez, Pavlic & Lewis 2008). The underlying mechanisms/cellular mechanisms of intestinal CM over-production are yet to be clearly established. Several studies have indicated that during IR, there are defects in insulin receptor signalling resulting in up-regulated *de novo* lipogenesis and modulations in CM assembly, including the PCTV (Duez, Pavlic & Lewis 2008, Adeli, Lewis 2008). Evidence from the Syrian Golden hamster, rendered IR by a high fructose diet, demonstrates increased CM secretion from isolated primary enterocytes, which is associated with increased apoB48 intracellular stability, *de novo* lipogenesis and increased activity and mass of MTP (Lewis et al. 2005, Haidari et al. 2002). Thus, the increased availability of lipids coupled with greater MTP expression stabilizes the apoB48 protein-lipid complex (Black 2007). As a result, there is increased intestinal production and secretion of apoB48-containing lipoproteins leading to elevated CM plasma concentrations in IR.

Furthermore, Federico et al demonstrated up-regulation of CM secretion in IR enterocytes, thought to be due to defective insulin receptor signalling (Federico et al. 2006). The insulin signalling pathway was observed to be impaired as there

was reduced insulin receptor substrate (IRS)-1 and Akt phosphorylation in IR enterocytes, whereas ERK signalling was amplified (Federico et al. 2006).

Alternatively, apoB48 overproduction has been attributed to enhanced *de novo* lipogenesis and increased mRNA SREBP-1c levels (Federico et al. 2006). As previously described in section 1.3.2, SREBP-1c is the key regulatory mechanism of lipogenesis and can regulate the activation of enzymes in the biosynthetic pathway; such as HMG-CoA reductase and fatty acid synthase. Thus, it is proposed that increased SREBP-1c mRNA during IR up-regulates *de novo* lipogenesis providing increased lipid substrate for apoB48 stability, which may ultimately contribute to increased CM secretion (Haidari et al. 2002). Further studies in the Psammomys obesus gerbil, a model of nutritionally induced IR, have supported these findings (Zoltowska et al. 2003). Cultured jejunal explants from IR Psammomys obesus gerbils demonstrated increased apoB48 biogenesis, *de novo* TG synthesis and monoacylglcerol acyl transferase (MGAT) (enzyme involved in TG synthesis) (Zoltowska et al. 2003). Therefore, these findings would also suggest that enhanced *de novo* TG synthesis enhances apoB48 stability, preventing its degradation and promoting CM over-production and secretion (Zoltowska et al. 2003).

In the present thesis, I have utilized the lymph cannulation procedure in the JCR:LA-*cp* rat to assess CM production and secretion from the intestine, directly into lymph overtime in both the fasted and fed state. The previous studies I have described in the Golden Syrian hamster and Psammomys obesus gerbil have been limited in their analysis, as they have assessed CM overproduction in an *ex vivo*

setting and at one point in time. However, the lymph cannulation procedure is dynamic and allows the direct measurement of intestinal CM production and secretion over time. In this thesis, we investigate the impact of ezetimibe and simvastatin on lymphatic CM secretion in the JCR:LA-*cp* rat, a model of obesity and MetS.

1.5 Role of Chylomicrons in Atherosclerotic CVD

1.5.1 Chylomicron Metabolism and CVD risk

Zilversmit was the first to suggest that CM-r are potentially atherogenic (Zilversmit 1979). There is now substantial evidence that elevated CM concentrations are a significant risk factor for CVD (Cohn, Marcoux & Davignon 1999, Tomkin, Owens 2001). Moreover, findings by Karpe et al have shown a positive relationship between post-prandial concentrations of CM-r and the rate of progression of coronary artery lesions in post-infarction male patients (Karpe et al. 1994). The underlying mechanisms in the relationship between CM, the development of atherosclerosis and CVD risk will now be discussed in more detail.

1.5.2 Arterial Delivery of Lipoproteins

Current understanding of the 'response to retention' hypothesis suggests that intimal deposition of lipoprotein derived cholesterol in the arterial wall is proportional to the duration of exposure to pro-atherogenic (cholesterol-rich) lipoproteins (Ross 1999, Williams, Tabas 1998, Libby 2002, Mamo et al. 1997, Steinberg, Gotto 1999). An increased plasma concentration and duration of cholesterol-rich lipoproteins is thought to explain increased risk of CVD through greater arterial delivery. Elevated plasma concentration of lipoproteins may be a result of overproduction/secretion or delayed clearance of lipoproteins from the plasma compartment. Consequently, pharmaceutical lipid lowering therapies aim to lower plasma concentrations of cholesterol-rich lipoproteins in order to reduce

arterial exposure (LIPID study group 2002, Gylling 2004, Bays et al. 2008, Grigore, Norata & Catapano 2008).

Delivery of lipoproteins to the arterial wall occurs via transcytosis and thus is partly mediated by particle size. Transcytosis describes the process by which vesicles are formed on the intimal surface of endothelial cells, migrate to the subluminal surface and release their contents by exocytosis (Nordestgaard, Tybjaerg-Hansen & Lewis 1992, Simionescu, Simionescu 1991). Siminonescu et al demonstrated that transcytosis can accommodate vesicles with a diameter of 70-80nm and hence only lipoproteins less than 70-80nm can be delivered to the arterial wall by this process (Simionescu, Simionescu 1991) (Proctor, Vine & Mamo 2002, Simionescu, Simionescu 1993, Nordestgaard, Tybjaerg-Hansen 1992, Mamo, Wheeler 1994, Nordestgaard, Wootton & Lewis 1995, Proctor, Mamo 1996). These findings aid our understanding of why there is greater of delivery of smaller lipoproteins such as CM-r (45-55nm), LDL (26nm), and HDL (10nm) as compared to larger lipoproteins: CM (>200nm) and VLDL (>80nm) (see figure 1-5). Approximately 85% of arterial lipoprotein delivery occurs via transcytosis, and to a lesser extent they can be delivered via gap junctions or other processes (Simionescu, Simionescu 1993).

Consistent with the transcytotic process are findings by Proctor et al (1998) who revealed that CM-r particles are delivered to the arterial wall uniformly (i.e. across all regions of the vessel wall). However, additional active transport mechanisms have also been proposed for the delivery of lipoproteins to the vessel wall. Heparin sulphate proteoglycans residing on the surface of the endothelial

monolayer are thought to interact with CM-r particles and facilitate binding and /or delivery to arterial tissue (Ji et al. 1993, Ji et al. 1994, Ji, Sanan & Mahley 1995). In addition, LPL can interact with CM and cell surface receptors and thus acts as a bridging molecule for lipoprotein uptake (Goldberg, Merkel 2001, Pentikainen et al. 2002).

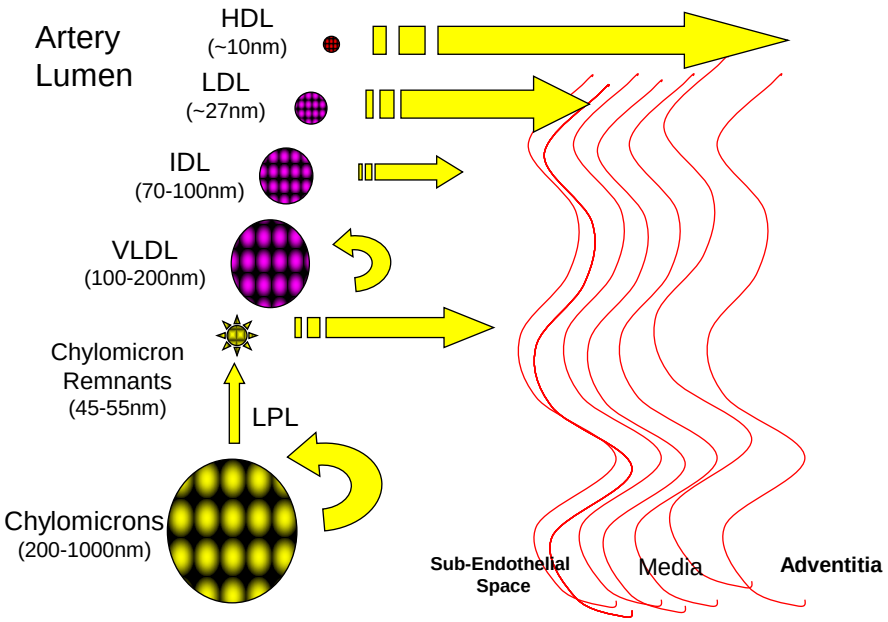

Figure 1-5. Proposed model of arterial uptake with respect to lipoprotein size adapted from Proctor, S.D. (2000). Lipoprotein delivery to the arterial wall is thought to occur via transcytosis. This process is dictated by vesicle size and hence only lipoproteins less than 70-80nm can be delivered to the arterial wall in this way. It is proposed that smaller lipoproteins such as HDL, LDL and CM-r are able to penetrate the arterial wall more readily than larger lipoproteins such as CM, VLDL and IDL.

1.5.3 Arterial Efflux of Lipoproteins

Lipoprotein efflux represents the number of lipoproteins that migrate out of arterial tissue. Regions which have greater lipoprotein delivery are observed to have greater lipoprotein efflux (Proctor, Vine & Mamo 2004). However, intriguingly, studies by Proctor et al (2004) have quantitatively shown that the rate of efflux is 3195 particles/min-1 and 163 particles/min-1 for LDL and CM-r, respectively. Thus, the efflux rate of LDL particle is 20 fold greater than the efflux of CM-r. These findings are consistent with the concept that efflux of lipoproteins may partly be dependent on particle size, as smaller particles (LDL) migrate out of vessels more efficiently than larger particles (CM-r), which efflux at comparatively a slower rate. In addition to particle size, other factors such as apoprotein complexing and arterial proteoglycan binding affinity to lipoproteins also play a vital role in influencing CM-r and LDL efflux rate.

1.5.4 Arterial Retention of Lipoproteins

The 'response-to-retention' hypothesis is considered a central paradigm in understanding the pathogenesis of atherosclerosis. The hypothesis describes the process whereby apoB-rich lipoproteins are retained within the arterial wall and undergo modification via oxidation and enzymatic processes. In turn, lipid and cholesterol accumulation results in an inflammatory response that further exacerbates the complexity of the lipid build up in the arterial wall leading to narrowing and hardening of the arteries. Previously, increased CVD risk was associated predominantly with raised plasma lipoprotein concentrations, whereas

now it is appreciated that the arterial wall has differential affinity for lipoprotein classes, and thus lipoprotein delivery does not necessarily equate to retention (Proctor, Vine & Mamo 2002). It is now recognized that lipoprotein characteristics such a size, density, apolipoprotein complex and lipid composition play a vital role in determining arterial retention and the associated pro-inflammatory response (Proctor, Vine & Mamo 2002, Tomkin, Owens 2001, Krauss 2002, Krauss 2001, Yu, Cooper 2001).

The cholesterol found in atherosclerotic lesions was thought to be derived primarily from LDL and this led to the development of the 'response to LDL retention' hypothesis (Williams, Tabas 1998, Pentikainen et al. 2002). There is now both direct and indirect evidence that cholesterol deposited in atherosclerotic lesions can also be derived from CM-r (Zilversmit 1979, Mamo, Proctor 1999, Proctor, Vine & Mamo 2002, Yu, Cooper 2001, Proctor 2000). Studies by Proctor et al (2002) investigated the relative retention of both apoB48- and apoB100-containing lipoproteins and the associated cholesterol deposition. Findings revealed that fewer numbers of apoB48-containing particles (i.e.CM-r) are retained within the intima relative to apoB100-containing particles (Proctor, Vine & Mamo 2002). Contrastingly, the arterial cholesterol deposition associated with CM-r is four fold greater than that associated with LDL, despite fewer CM-r particles being retained within the intima (See figure 1-6) (Proctor, Vine & Mamo 2002). Human studies have revealed that apoB48-containing CM-r contain approximately 40 times more cholesterol per particle than apoB100-containing LDL particles which supports the findings of Proctor et al (Mamo 1995, Fielding

1992). Thus, CM-r pose a substantial atherogenic risk, particularly in conditions where plasma CM-r concentrations are raised.

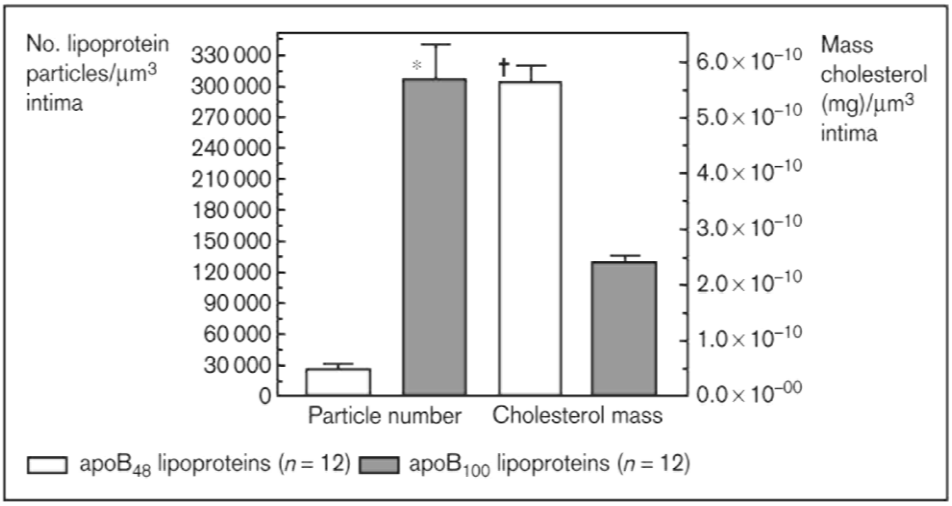

Figure 1-6. Quantification of fluorescent lipoproteins retained in arterial vessels adapted from Proctor, S.D. (2002). Number of lipoprotein particles retained within the intima of arterial vessels, with corresponding mass of lipoprotein-derived cholesterol. Normolipidemic concentrations of fluorescent lipoprotein preparations were perfused in situ through rabbit carotid arteries for 20 min under physiological conditions, followed by equivalent wash out period with buffer alone (i.e. retention). *$p<0.0001$, apoB100 particles versus apoB48 particles; †$p<0.0001$, mass of cholesterol derived from apoB48 particles versus mass of cholesterol derived from apoB100 particles.

Mechanisms for retention of apoB48- and apoB100-containing lipoprotein:

Earlier studies revealed that the sporadic retention and focal accumulation of lipoproteins corresponded with areas of lipid deposition and atherosclerotic lesion development. Skalen and colleagues (2002) were the first to provide evidence in *vivo* that the expression of matrix proteoglycans corresponded with the formation of atherosclerotic lesions. They revealed that apoB100 could directly bind with bigylcan and decorin in atherosclerotic mice models. Further studies by Flood et al revealed that apoB48 can also bind to arterial proteoglycans (including bigylcan) (Flood et al. 2002) . More recently, Boren and colleagues indentified putative binding sites within the apoB-molecule for select proteoglycans (Flood et al. 2002, Flood et al. 2004, Skalen et al. 2002). It would appear that the affinity of apoB48 for select proteoglycans is significantly greater than that of apoB100, due to potential masking of epiptope sites in the larger apoB100 molecule (Flood et al. 2004). These findings may help explain the differences observed in the retention of apoB48 versus apoB100 containing lipoproteins *in vivo* (Proctor, Vine & Mamo 2002).

In addition, earlier work from our laboratory using fluorescent *ex vivo* perfusion experiments have identified that the distribution of CM-r is parallel to the distribution of arterial proteoglycans (Proctor, Forbes & Mamo 2003).

1.5.5 Accelerated Atherosclerosis During Insulin Resistance, Type II Diabetes and The Metabolic Syndrome.

In healthy subjects, native CM are rapidly hydrolyzed within the circulation by LPL, liberating TG and free fatty acids to become smaller and denser CM-r. However, there is now accumulating evidence that both CM production and secretion may be up-regulated during disease states such as IR as discussed above (Redgrave 2004, Duez, Pavlic & Lewis 2008). Up-regulated *de novo* lipogenesis maybe a consequence of a number of factors including: intestinal insulin resistance, enhanced dietary lipid absorption, and/or increased expression and activity of proteins involved in CM assembly and secretion (Vine et al. 2007, Vine, Glimm & Proctor 2008, Hsieh et al. 2008). Moreover, IR subjects have demonstrated poor hydrolysis of CM and delayed clearance of these lipoproteins via hepatic receptor mediated pathways (Coppack 1997, Malmstrom et al. 1997).

The etiological significance of CM overproduction/secretion and delayed clearance is that there is increased exposure of the arterial wall to atherogenic cholesterol-dense CM-r (Proctor, Vine & Mamo 2002). Thus, there is an increased propensity for these particles to become entrapped and accumulate within the intima of the arterial wall (Proctor, Vine & Mamo 2002). As previously described in section 1.5.4, under experimentally controlled conditions, the net accumulation of cholesterol derived from CM-r is far greater than that deposited by LDL, despite these lipoproteins binding to extracellular proteoglycans with equal affinity (Proctor, Vine & Mamo 2002, Proctor, Vine & Mamo 2002, Proctor, Vine & Mamo 2004). Most intriguing is that over time

LDL particles appear to efflux more readily than CM-r from arterial tissue (Proctor, Vine & Mamo 2004).

Moreover, IR has been shown to induce vascular remodelling resulting in increased lipoprotein attachment in the subendothelial space (Camejo et al. 2002, Raines, Ferri 2005). Proliferation of vascular smooth muscle cells stimulates secretion of arterial proteoglycans, which can increase the capacity of lipoprotein binding (*in vitro*) (Tannock et al. 2002, Little et al. 2002). More specifically, transforming growth factor (TGF)-beta-1 has been identified in atherosclerotic lesions and stimulates proteoglycan synthesis by arterial smooth muscle cells (Little et al. 2002). Both animal models of IR (Bauer et al. 2004) and human IR subjects (Pandey, Loskutoff & Samad 2005) have been revealed to have elevated circulating levels of TGF-beta-1 which is thought to be responsible for vascular intimal medial thickening . Furthermore, TGF-beta-1 can increase proteoglycan-lipoprotein binding due to increased length of the glycosamionglycan chain (GAG) portion of the proteoglycan (Little et al. 2002).

1.5.5.1 Contributions of the JCR:LA-cp rat to understanding accelerated atherosclerosis during IR and Mets.

To explore these factors, our laboratory has recently established one of the few available models of intestinal CM over-production during MetS using the JCR:LA-*cp* rat (Vine, Glimm & Proctor 2008). In addition to greater mass of apoB48 secreted in lymph from MetS rats, we have observed an increase in CM-triglyceride, cholesterol and regulatory apolipoproteins (apoC-III) resulting in an increased binding to PG-biglycan *in vitro* (Mangat et al. 2007, Vine, Glimm & Proctor 2008). Complimentary data using the MetS JCR:LA-*cp* rat has shown elevated TGF-beta-1 concentrations and an increased mass of aortic biglycan relative to lean rats which increases linearly with age and serum insulin concentrations (Vine, Glimm & Proctor 2008). These results support the hypothesis that MetS can increase the net number of PG binding sites for atherogenic lipoproteins in arterial vessels. Collectively, it is apparent that the mechanisms and prediction of the degree of cholesterol entrapment in arterial vessels is complex and multi-factorial. It is now appreciated that many contributing factors are likely to be dys-regulated during MetS, and in turn significantly increase the potential for cholesterol-enriched remnant particles to accumulate in the arterial wall.

A conceptual model has been proposed for the deposition of cholesterol in arterial vessels; which is the sum of particle exposure (i.e. time), binding affinity of particle (i.e. permeability, attachment and removal), available PG binding sites and the particle cholesterol capacity (i.e. size and composition) (See figure.1-7).

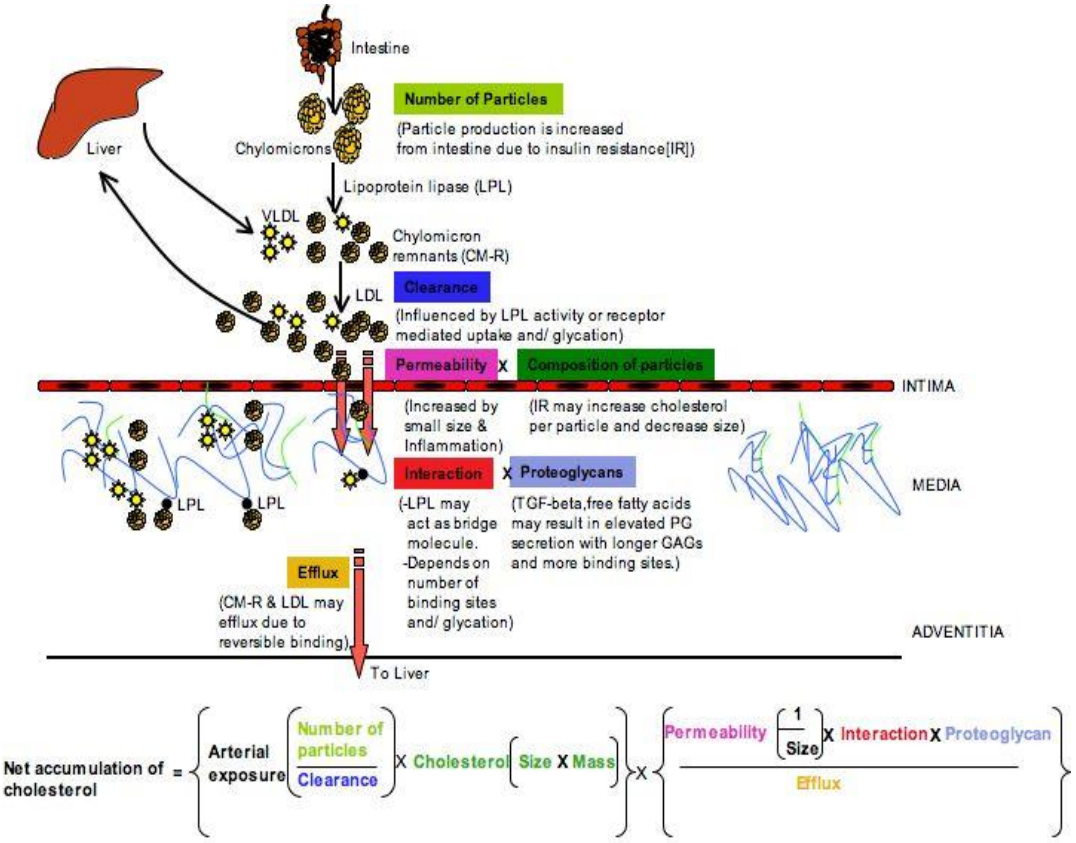

Figure 1-7. Proposed factors regulating net cholesterol accumulation in arteries. During conditions of IR and/or obesity intestinal CM production and secretion are exaggerated. This overproduction is thought to contribute to the accumulating presence of TAG-rich particles in plasma. Furthermore, delayed clearance of apoE-containing CM-r particles via the LDL-r (LDL-receptor) and the LRP-r (LDL related protein-receptor) pathways ensures continued exposure of atherogenic particles to the arterial wall. Small, dense, cholesterol-rich CM-r particles penetrate the vessel wall and become entrapped in the subendothelial space. Chronic IR and diabetes induce vascular remodelling of extracellular arterial PG resulting in an increase in the net number of PG binding sites for atherogenic lipoproteins. These factors facilitate increased binding of CM-r to the vessel wall. In addition, it has previously been shown that LDL-C effluxes more readily than CM-r suggesting that CM-r are preferentially retained within the arterial wall.

1.6 Drug Treatments for Hypercholesterolemia

1.6.1 Introduction

Hypercholesterolemia plays a significant role in the development and progression of atherosclerosis, and is a primary risk factor for cardiovascular disease (LIPID study group 2002). Both primary and secondary intervention strategies which aim to lower cholesterol levels have been shown to reduce the incidence of CVD (LIPID study group 2002, Sacks et al. 1996, Downs et al. 1998, Gylling 2004). Current available drug treatments for hypercholesterolemia fail to reach guideline LDL-C concentrations (See Table 1-2) (Schrott et al. 1997, Pearson et al. 2002). Furthermore, raised LDL-cholesterol does not account for increased atherosclerotic risk in other individuals predisposed to atherosclerosis and CVD, including those patients who are normolipidemic with CAD, type II diabetes and MetS (Vessby 2003, Mooradian 2003). Evidence has shown that impaired CM metabolism plays a causative role in atherosclerosis and could have significant clinical implications in conditions such as hypercholesterolemia, normolipidemia and MetS (Proctor, Vine & Mamo 2002, Karpe et al. 1994, Zilversmit 1995, Cabezas, Erkelens 2000, Huff 2003, Twickler et al. 2005). CM metabolism is currently not a part of routine clinical screening and to date there has been little focus on how drug treatments effect CM production and particle characteristics. This thesis will focus on elucidating how two drug treatments currently prescribed for individuals with hypercholesterolemia: ezetimibe and simvastatin, effect CM over-production and subsequent CM-r retention in the arterial wall of the JCR:LA-*cp* rat (a model of MetS).

1.6.2 Current Drug Treatments for Hypercholesterolemia

Drug Treatment	Mechanism of Action
Statins	Inhibit HMG-CoA reductase
Ezetimibe	Blocks NPC1L1 transporter
Bile Acid Sequestrants	Bind to bile acids
Fibrates	PPAR-alpha agonist
Plant Sterols and Stanols	Reduces cholesterol incorporation into mixed micelles
Niacin	Decreases fractional catabolic rate of apoA-I, inhibits DGAT2 and hormone sensitive triglyceride lipase

Table 1-2 Currently Available Drug Treatments For Hypercholesterolemia.

Bile Acid Sequestrants:

Bile acids are derived from cholesterol in the liver. Following delivery to the intestine, more than 95% of bile acids are re-absorbed by enterocytes and returned to the liver via enterohepatic recirculation. Bile acid sequestrants function by binding to bile acids in the intestine and facilitate increased bile acid excretion in the faeces. Thus, there is a decrease in bile acid returned to the liver and enzymes involved in bile acid synthesis from cholesterol are consequently up-regulated, leading to increased use of cholesterol substrate. Operative liver biopsies in patients treated with a bile acid sequestrant for three weeks have revealed that increased use of cholesterol for bile acid production induces a compensatory increase in hepatic LDL receptor expression, enhanced clearance of LDL-C, and lower LDL-C plasma levels (Einarsson et al. 1991). The Lipid Research Clinics Coronary Primary Prevention Trial revealed that both total cholesterol and LDL-C are decreased by 15-30% in men following treatment with bile acid sequestrants resulting in an overall decrease in clinical

cardiovascular events (Bays, Goldberg 2007). However, despite the ability of bile acid sequestrants to lower LDL-C, no additive beneficial cholesterol lowering effect is observed with increasing dosage. Thus, the 'statin' class of compounds are currently the first drug choice for treatment of CVD as they represent a more powerful means to lower LDL-C to a greater extent. Furthermore, although bile acid sequestrants have minimal side effects, they do reduce absorption of vitamins, A,D,E and K leading to deficiency of these essential vitamins.

Plant Sterols and Stanols:

Plant stanols are structurally similar to cholesterol and are incorporated into micelles in the intestinal tract. Plant stanols are more hydrophobic than cholesterol and hence displace dietary cholesterol from being incorporated into micelles. As a result, there is reduced absorption and enhanced excretion of cholesterol into faeces. However, the effectiveness of plant stanols further downstream in cholesterol metabolism is limited as they are have low incorporation into CM (Law 2000). For efficient incorporation into CM, sterols and stanols must be esterified. It is well established that sitosterol is not a poor substrate for ACAT (enzyme responsible for sterol esterification) and hence this explains why sterol absorption/incorporation into CM is low (Field, Mathur 1983). Thus, plant sterol and stanols have minimal effects on actual cholesterol incorporation into CM.

Fibrates:

Fibrates are PPAR alpha agonists and have been shown to increase fatty acid beta oxidation in the liver, LPL activity, circulating HDL levels and CM-r clearance whilst decreasing TG secretion in VLDL. However their efficacy to lower LDL-C levels has been shown to be minimal (Staels et al. 1998). Very recent evidence from "The Action Of Control Of Cardiovascular Risk in Diabetes (ACCORD) Lipid Trial" has revealed that fibrates fail to improve cardiovascular outcome in diabetic individuals (Chapman et al. 2010). Furthermore, in non-diabetic individuals, fibrates are associated with decreased non-fatal myocardial infarction but do not substantially affect all cause mortality (Chapman et al. 2010).

Niacin:

At present, niacin is the most potent drug treatment available for raising HDL levels. Niacin can increase HDL concentrations by 30-35%, even in subjects with isolated low HDL levels (Zema. 2000). The mechanism of action has only recently been elucidate and involves decreasing the fractional catabolic rate of apoA-I through reduced hepatocyte uptake, thus facilitating greater reverse cholesterol transport (Ganji, Kamanna & Kashyap 2003). Moreover, this B-vitamin has been shown to inhibit diacylglycerol acyltransferase 2 (DGAT2); a key enzyme in triglyceride synthesis, as well as an ability to inhibit adipose tissue lipolysis through inhibition of hormone-sensitive triglyceride lipase (Ganji, Kamanna & Kashyap 2003). Thus the net result of these combined effects includes increased HDL, and lowered plasma TG. Despite Niacin's lipid

lowering effects it has been shown to be poorly tolerated by patients (Ganji, Kamanna & Kashyap 2003). In addition, although niacin treatment has been associated with a significant decrease in cardiovascular events, its effect on coronary and cardiovascular mortality is minimal (Duggal et al. 2010).

Ezetimibe:

Mechanism of Action:

Ezetimibe (EZ) (1-(4-fluorophenyl)-(3R)-[3-(4-flurophenyl)-(3S)-hydroxypropyl]-(4S)-(4-hydroxyphenyl)-2-azetidinone) (SCH58235) and the analogue SCH48461 selectively inhibit intestinal cholesterol absorption through the NPC1L1 transporter (Davis et al. 2004). The NPC1L1 transporter regulates cholesterol luminal uptake into the enterocyte, where cholesterol may be esterified and incorporated into CM particles. Evidence from several studies suggests that NPC1L1 is the target of EZ (Bays et al 2002). NPC1L1 deficient mice demonstrate a 70% reduction in cholesterol absorption which cannot be lowered further with EZ treatment (Altmann et al. 2004). Moreover, in humans, NPC1L1 variants correlate with response to EZ treatment (i.e. EZ is observed to reduce cholesterol absorption to a greater extent in individuals with higher NPC1L1 mRNA expression than those with lower expression) (Simon et al. 2005, Hegele et al. 2005, Cohen et al. 2006). Studies have also indicated that EZ may increase cholesterol elimination into bile and thus increase the efficiency of reverse cholesterol transport via interaction with hepatic NPC1L1 proteins (Chan et al. 2008).

Although it is well documented that NPC1L1 is a major target of EZ treatment there is emerging evidence that this drug may have additional targets. NPC1L1 is believed to be primarily an intracellular protein that is translocated to the cell membrane mucosal surface under conditions of cholesterol depletion (see figure 1.6) (Yu et al. 2006). However, to date there have been no studies showing that NPC1L1 binds directly to cholesterol at the cell membrane surface. There are studies showing that BBM vesicles isolated from NPC1L1-/- mice have a similar high binding affinity to cholesterol and transport properties compared to BBM from wild type mice (Labonte et al. 2007, Knopfel et al. 2007). Thus, these findings would suggest that NPC1L1 does not function as a primary mucosal cell membrane transporter for cholesterol uptake. There is now emerging evidence that SR-BI and CD36 may also be potential targets for EZ. Both SR-BI antibodies and EZ treatment inhibit high-affinity cholesterol binding and transport to BBM vesicles, suggesting that EZ treatment interacts directly with SR-BI (Labonte et al. 2007). In addition, it has been shown that CD36 and SR-BI facilitated cholesterol uptake is similar in response to EZ treatment (van Bennekum et al. 2005).

Therefore, these findings would suggest that cholesterol absorption by enterocytes is protein-mediated at the mucosal membrane, and is followed by intracellular transport to the site of lipoprotein assembly. Moreover, there is increasing evidence that SR-BI may play a role in cholesterol uptake at the duodenal and jejunal mucosal membranes and CD36 at the jejunal and ileal mucosal membranes. NPC1L1 is also believed to facilitate intracellular cholesterol

trafficking. Collectively, it is proposed that EZ is able to target all three of these proteins which may help explain it's efficacy to reduce intestinal cholesterol absorption (Hui, Labonte & Howles 2008) (see figure 1-8).

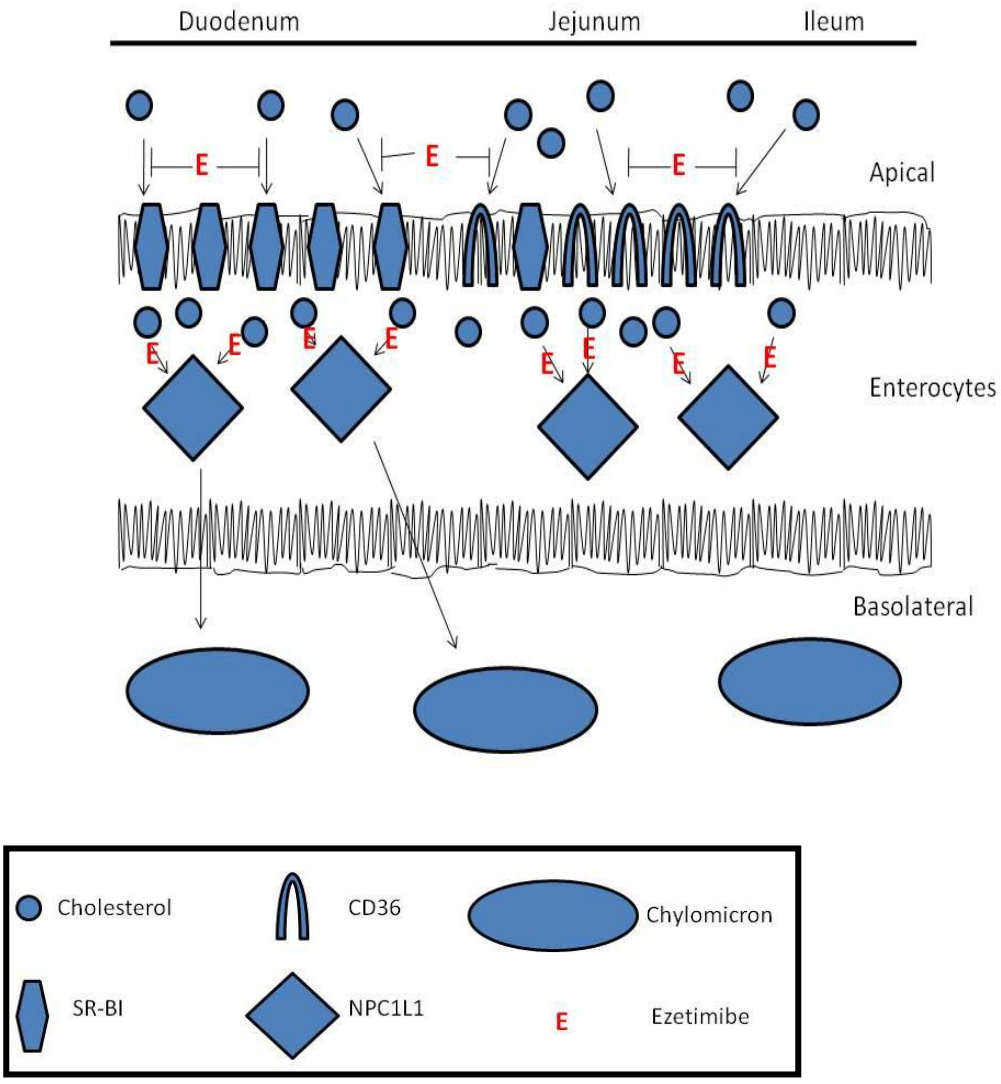

Figure 1-8. Potential targets of Ezetimibe treatment adapted from Hui et al (2007).

Ezetimibe has beneficial effects on chylomicron metabolism:

To date, there have been few studies examining the effects of EZ on CM production and CM composition. Previous animal studies have shown that EZ treatment decreases cholesterol absorption, resulting in cholesterol ester poor CM and less cholesterol being delivered to the liver via the CM-r pathway (van Heek, Compton & Davis 2001). As a result, a compensatory response occurs whereby hepatic LDL-receptor expression is up-regulated, enhancing LDL-C hepatic clearance and lowering plasma LDL-C levels (van Heek, Compton & Davis 2001)(Bays et al. 2008, Bays, Stein 2003, Davis et al. 2007, Davis et al. 2001). Acute dietary cholesterol feeding of EZ for a duration of seven days in Sprague-Dawley rats has been shown to lower lymphatic cholesterol content without any concomitant changes in hepatic or intestinal cholesterol synthesis (van Heek et al. 2003). Cholesterol synthesis was determined by administration of [14C]-cholesterol and [3H]-mevalonate intraduodenally into rats and analyzing mesenteric lymph for radiolabelled cholesterol and cholesterol ester content (van Heek et al. 2003). However, in this study, rodents were pre-gavaged with TG before lymphatic collection and basal particle size and composition of CM may have been influenced by the enterocyte being turned-on to lipid absorption and incorporation of lipids into the CM. Furthermore, this experiment only collected lymph for four hours which is the peak period of post-prandial CM secretion following a lipid rich meal/gavage. Thus, it is yet to be determined how EZ affects CM particle synthesis and composition during basal and post-prandial conditions. Further understanding of the effects on CM production and metabolism may help to elucidate the potential anti-atherogenic properties of EZ.

Hypercholesterolemic monkeys treated with EZ demonstrate a significant reduction (-69%) in cholesterol content of CM during the post-prandial phase (Heek. 2001). Briefly, blood samples were taken from monkeys at 5 hours during the post-prandial period, plasma was separated and CM isolated via density ultra-centrifugation. Cholesterol was determined by the method of Rudell and Morris (1973) which utilizes *o*-phthalaldehyde. Moreover, a recent study examined the effects of EZ on the *in vivo kinetics* of apoB48 in hyperlipidemic men. Subjects underwent a primed-constant infusion of a stable isotope [L-(5,5,5-D_3)-leucine] in the fed state which is incorporated into apo-B48 synthesis. Triglyceride rich lipoprotein apoB48 pool size was significantly decreased by 33%, but no difference in apoB48 production rate was observed (Tremblay et al. 2006). These results indicate that EZ is effective at reducing apoB48 pool size and that this effect is most likely mediated by enhanced CM clearance.

In this thesis the lymph cannulation method will be utilized to assess CM production and composition in the fasted and fed state following intervention with EZ.

Despite findings that EZ treatment reduces intestinal cholesterol absorption, there is some evidence that as a compensatory response, total cholesterol synthesis is up-regulated. In a randomized, double-blind, placebo-controlled cross-over study in 18 patients with mild to moderate hypercholesterolemia treated with 10mg/d of EZ, cholesterol absorption was reduced but a compensatory increase in total cholesterol synthesis was observed (Sudhop et al. 2002). Total cholesterol synthesis was determined by measuring fecal excretion of neutral sterols and the

ratio of lathosterol-to-cholesterol in plasma (the ratio of lathosterol-to-cholesterol is an indicator for hepatic HMG-CoA reductase activity and total cholesterol synthesis) (Sudhop et al. 2002). EZ treatment was observed to induce an 89% increase in cholesterol synthesis compared to the placebo group which is speculated to be mainly due to enhanced *de novo* hepatic cholesterol synthesis (Sudhop et al. 2002). The accumulating evidence that EZ monotherapy up-regulates total cholesterol synthesis may explain the favourable effects of co-administration of EZ with statins.

Statins:

Mechanism of Action:

Statins are currently the first drug treatment of choice for hypercholesterolemia due to their serum cholesterol lowering effects. They function by inhibiting HMG-CoA reductase; the rate limiting step in the cholesterol biosynthetic pathway. As 60-70% of plasma cholesterol is thought to be derived from hepatic cholesterol synthesis, inhibition of HMG-CoA reductase by statins induces a dramatic reduction in plasma LDL-C levels. Subsequently LDL-R expression is up-regulated and there is enhanced clearance of Apo-B containing atherogenic lipoproteins, LDL and CM-r. There have been many large clinical trials to observe the beneficial effects of statins, and there appear to be a number of off-target effects (Wang, Liu & Liao 2008). Statins have been shown to have pleiotropic effects as they inhibit the synthesis of isoprenoids, which are important lipid attachments for intracellular signalling molecules, such as Rho,

Rac and Cdc42 (see figure 1-9) (Goldstein, Brown 1990, Van Aelst, D'Souza-Schorey 1997). Hence, inhibition of isoprenoid synthesis has multiple effects on downstream signalling pathways. The beneficial effects of statins are thought to include: cholesterol lowering, improved endothelial function, decreased vascular inflammation, inhibition of smooth muscle proliferation and immunomodulation (Wang, Liu & Liao 2008).

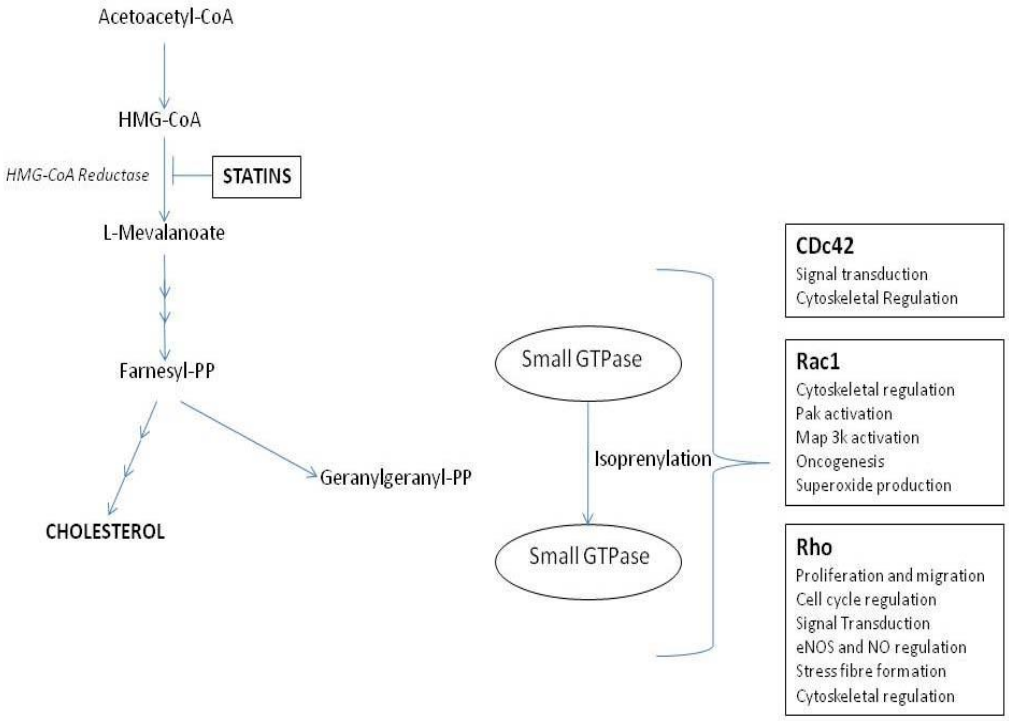

Fig.1-9 Pleiotropic effects of statins adapted from Wand, C.Y. (2007).

The anti-inflammatory actions of statins:

The vascular inflammatory response is a complex process that leads to atherosclerosis, thrombus formation and angiogenesis. C-reactive protein (CRP), TNF-alpha, and IL-6 are key markers of inflammation which have all been associated with prediciting CVD risk. Statins have been shown to decrease CRP levels following 6 weeks of treatment independently of LDL-C lowering and thus are proposed to have anti-inflammatory properties in humans (McFarlane et al. 2002). Furthermore, the "statin" class of compounds reduce the expression of endothelial and leukocyte adhesion molecules such as as P-selectin and intracellular adhesion molecule, ICAM-1 and thus attenuate the inflammatory process and consequent impact on CVD reduction (McFarlane et al. 2002).

Statins have beneficial effects on chylomicron metabolism:

Several studies have provided evidence that statins have beneficial effects on CM metabolism. Monotherapy of atorvastatin and rosuvastatin in hyperlipidemic men caused significant decreases in remnant lipoprotein cholesterol (-58.7%, -61.5%) and apoB48 (-37.5%, -32.1%), as compared to baseline respectively (van Himbergen et al. 2009). A recent study by Couture et al (2008) examined the effects of atorvastatin on the *in vivo* kinetics of apoB-containing lipoproteins (apoB100 & apoB48) labelled with a stable isotope (L-(5,5,5-D_3)-leucine] in subjects with type 2 diabetes mellitus with hypertriglyceridemia (Hogue et al. 2008). Atorvastatin was observed to decrease the triglyceride rich lipoprotein apoB48 pool size, possibly attributed to a decreased production rate. However, an

earlier study by Fu et al examined the effects of simvastatin (SV) on CM production and composition in apoE KO mice (Fu, Borensztajn 2006). CM were isolated by fractionation of plasma using fast protein liquid chromatography. These mice displayed enhanced hypercholesterolemia when treated with SV which was not due to increased CM-r particle number, but due to their cholesterol enrichment (Fu, Borensztajn 2006).

In addition, evidence has shown that statin treatment inhibits CM-r induced macrophage foam cell formation (Kawakami et al. 2005). The underlying mechanism for this is believed to be via inhibition of an apoB48 receptor located on macrophages (Kawakami et al. 2005).

Collectively, these results add to existing evidence showing that treatment of familial hypercholesterolemia with SV decreases plasma concentration of triglyceride-rich remnants as determined by decreased apoB-48 and remnant lipoprotein cholesterol plasma levels (Dane-Stewart et al. 2002).

Combined Ezetimibe and Statin Treatment:

The use of two lipid-lowering compounds; EZ and SV, with complementary mechanisms of action provides a powerful new approach to prevent and treat atherosclerosis. Several studies have shown that combined EZ+SV treatment has an additive improvement on lowering LDL-C, non-HDL-C, TG and ApoB, whilst raising HDL-C levels over statin monotherapy (Bays et al. 2008, Grigore, Norata & Catapano 2008). Combined treatment has been shown to up regulate LDL receptor expression which explains the observed reduction in LDL-C and total

Apo-B levels. Furthermore, in a miniature pig model of cholesterol metabolism, combination therapy caused a significant reduction in EZ- induced hepatic cholesterol synthesis and a marked increase in hepatic LDL receptor expression (Telford et al. 2007). ApoB-100 levels were also reduced which is attributed to a reduction in VLDL production and enhanced LDL-receptor mediated clearance of LDL-C (Telford et al. 2007).

To date there has been minimal literature documenting the effects of combined EZ+SV therapy on CM production and composition. Tremblay et al recently studied the effects of combined therapy on the *in vivo* kinetics of apoB48 and apoB100 in men with mixed hyperlipidemia (Tremblay et al. 2009). Plasma concentrations of total cholesterol, LDL and TG were significantly reduced by -43%, -53.6% and -44% respectively (Tremblay et al. 2009). ApoB48 pool size was shown to be decreased by -48.9% and production rate by -38% (Tremblay et al. 2009). Collectively, these results show that combined therapy decreases plasma TRL apoB48 levels which may be a result of reduced intestinal secretion of CM-apoB48.

Ezetimibe and Simvastatin Treatment on the Atherosclerotic Process:

Several clinical studies have demonstrated that statins can hinder the progression of atherosclerosis (Blankenhorn et al. 1993). However, it is unclear whether the clinical effects of statins can be solely attributed to their lipid lowering properties or whether direct effects such as vascular wall remodelling are involved. Previous studies have demonstrated that proliferation of smooth muscle cells stimulates the synthesis of large amounts of extracellular matrix *in vitro* (Tannock et al. 2002, Little et al. 2002). Proteoglycans are a major constituent of the arterial extracellular matrix and their increased synthesis facilitates a greater capacity for lipoprotein binding to the arterial wall (Little et al. 2002). In addition, smooth muscle cell growth and extra cellular matrix formation contribute to intimal thickening of the arterial wall; a key characteristic of the atherosclerotic process (Jawien et al. 1992). *In vitro* studies have revealed that SV treatment decreases proliferation of human arterial smooth muscle cells and proteoglycan synthesis and thus may potentially reduce arterial intimal thickening and lesion formation (Siegel-Axel et al. 2003, Riessen et al. 1999). More recently, SV treatment has been shown to have beneficial effects on the regression of atherosclerotic lesion development in men (Corti et al. 2001). The study revealed that SV induces vascular remodelling, as manifested by reduced atherosclerotic burden without lesion development (Corti et al. 2001).

Currently, there is no available literature examining the direct effects of EZ treatment on vascular wall remodelling and subsequent lesion development.

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Chapter 2: Rationale

2.1 Introduction

Obesity, insulin resistance (IR) and the metabolic syndrome (MetS) predispose individuals to the development of type 2 diabetes and increased cardiovascular disease (CVD) risk (the latter a primary cause of death and disability globally) (Desroches, Lamarche 2007). Cholesterol-associated dyslipidemia is a key feature of MetS, and human studies have revealed increased cholesterol absorption and elevated plasma cholesterol levels in individuals presenting with type I and type II diabetes (Gylling et al. 2004, Gylling, Miettinen 1996). We know that the intestine is a major site of cholesterol transport and metabolism. Specifically, there is new emerging evidence that the intestine has a predominant role in dyslipidemia associated with obesity and IR (Lally et al. 2006, Vine, Glimm & Proctor 2008, Adeli, Lewis 2008). However, there is currently limited knowledge on intestinal cholesterol transport per se under clinical settings or in animal models of MetS and/or how this may contribute to cholesterol-associated dyslipidemia. Recent discoveries support the concept that cholesterol is actively absorbed into the enterocyte through a number of transporters including; the Niemann Pick C1 Like 1 (NPC1L1), Scavenger Receptor-B1 (SR-B1) and fatty acid translocase (FAT/CD36) transporters (Hui, Labonte & Howles 2008). Indeed, previous studies have demonstrated net cholesterol absorption to be up-regulated in the diabetic state (Feingold et al. 1985), which is proposed to be a result of upregulated expression of intestinal cholesterol transporters (Lally et al. 2006, Lally, Owens & Tomkin 2007, Adeli, Lewis 2008). Once absorbed,

cholesterol is available for incorporation into apoB48-containing chylomicron (CM) particles that are secreted into the mesenteric lymph and delivered to the circulation at the site of the subclavian thoracic duct. Importantly, from a CVD perspective there is accumulating evidence that intestinally derived apoB48 remnant lipoproteins, CM-r, contribute to atherogenic dyslipidemia during conditions of IR and diabetes (Proctor, Vine & Mamo 2002, Karpe et al. 1994, Zilversmit 1995, Cabezas, Erkelens 2000, Huff 2003). Furthermore, very recent discoveries have revealed that IR can lead to the over-secretion of CM which may in turn contribute to both fasting and post-prandial dyslipidemia (Duez, Pavlic & Lewis 2008). As a result of elevated plasma CM concentrations, atherosclerotic risk has been proposed to be increased as there is an increased propensity for the cholesterol-dense CM-r to become entrapped within the intima of the arterial wall. Proctor et al previously demonstrated that under experimentally controlled conditions, arterial wall retention of cholesterol is greater from CM-r than from LDL, despite both lipoproteins having equal binding affinity to the vessel wall (Proctor, Vine & Mamo 2002). Therefore collectively, the synthesis and production of intestinal CM enriched with cholesterol and their catabolic product cholesterol-dense CM-r may play an important role in whole body cholesterol homeostasis, and moreover may also contribute substantially to CVD development and progression (Vine, Glimm & Proctor 2008).

Ezetimibe (EZ) is a novel pharmaceutical compound which selectively reduces intestinal cholesterol absorption by inhibiting the NPC1L1 transporter, without appearing to alter triglyceride and fat-soluble vitamin bioavailability (Bays et al.

2008, Davis et al. 2004, Garcia-Calvo et al. 2005). In contrast, the more well known 'statin' class of compounds such as simvastatin (SV) lower serum cholesterol by inhibiting the rate limiting step in the cholesterol biosynthesis pathway via HMG-CoA reductase (Bays et al. 2008, Holdgate, Ward & McTaggart 2003, Bays, Stein 2003, Shepherd et al. 1995).

It has previously been reported that acute dietary cholesterol feeding of EZ for a duration of 7 days in male Sprague-Dawley rats lowers lymphatic cholesterol content without any concomitant change in endogenous cholesterol synthesis (van Heek et al. 2003). However, in this study, rodents were pre-gavaged with triglyceride before lymphatic collection and basal particle size and composition of CM may have been influenced (van Heek et al. 2003). Furthermore, this experiment only collected lymph for 4 hours which is usually the peak period of CM secretion following an oral lipid load (van Heek et al. 2003).

Additional studies have shown that EZ treatment either alone, or in combination with SV can decrease both plasma LDL cholesterol and apoB48 remnant lipoproteins in hyperlipidemic men with type II diabetes (Tremblay et al. 2006, Tremblay et al. 2009, Simons et al. 2004, Davidson et al. 2004). However, studies conducted by Tremblay et al measured apoB48 concentration in plasma and thus have assumed that the production rate of apoB48 containing lipoproteins is a direct measure of intestinal production. At present, the combined effects of EZ and SV on postprandial dyslipidemia during IR remain unclear as both clinical and animal studies have not been able to consider intestinal secretion of apoB48 directly into lymph.

To date there is no study examining the effects of EZ and SV therapy on intestinal cholesterol transport, nor have effects on intestinal CM synthesis and cholesterol content been examined in the MetS. Alterations in cholesterol absorption and increased incorporation into CM may in part explain the apparent increased CVD risk observed during MetS. Thus, it is important to elucidate whether EZ and SV alter intestinal cholesterol transport with concomitant changes in CM synthesis and characteristics, and furthermore whether the atherogenicity of these particles is modulated. It is also yet to be determined how EZ and SV treatment affect CM synthesis and composition during basal and post-prandial periods, which would also determine how these pharmaceutical treatments modulate systemic CM metabolism from the intestine to the circulation during MetS.

The lack of suitable animal models has been one of the reasons limiting the knowledge of vascular complications in MetS. The JCR:LA-*cp* rat is one of the only available animal models that mimics MetS (Mangat et al. 2007, Brindley, Russell 2002) and spontaneously develops myocardial and systemic arterial atherosclerotic lesions, reflecting the human clinical condition (Russell, Graham & Richardson 1998, Russell, Proctor 2006). The JCR:LA-*cp* rat was recently established as a model of intestinal CM over-production during MetS (Vine, Glimm & Proctor 2008). The model has intestinal hypertrophy and altered intestinal transport of cholesterol (Vine, Glimm & Proctor 2008). More specifically, intestinal CM derived from the mesenteric lymph duct of MetS rats were greater in absolute number and cholesterol content compared to lean control rats in the fed-state (Mangat et al. 2007, Vine, Glimm & Proctor 2008).

At present, lowering LDL-cholesterol remains the target of large pharmaceutical trials. However raised plasma LDL-cholesterol does not account for increased atherosclerotic risk in individuals who are otherwise normolipidemic and /or predisposed to atherosclerosis in IR or MetS. Despite the evidence of CM-r and their causative role in the atherogenic process, there is currently limited knowledge on the potential effects of available hypercholesterolemic drug therapies on CM metabolism and CM-r atherogenicity in the MetS.

2.2 Thesis Aim

The overall aim of this study was to investigate the beneficial effects of ezetimibe and simvastatin in modulating CM metabolism, both at the site of intestinal synthesis and subsequent uptake of CM-r into the arterial wall in the JCR:LA-*cp* rodent model of MetS.

2.3 General Hypothesis:

Combined ezetimibe and simvastatin treatment will have beneficial synergistic effects on intestinal cholesterol transport and CM metabolism, and will reduce CM-r associated atherogenesis.

2.4 Specific Hypotheses:

Combined therapy of ezetimibe and simvastatin in the JCR:LA-*cp* rat model of MetS will:

I. Decrease intestinal mucosal to serosal cholesterol transport.
II. Decrease CM particle secretion and particle size.
III. Decrease lymph CM-associated cholesterol and total plasma cholesterol concentrations.
IV. Reduce cholesterol associated with CM-r retained within the arterial vessel ex-vivo.
V. Reduce the incidence of atherosclerotic myocardial lesions.

2.5 Specific Objectives:

To test the above hypotheses, the study design used was a single intervention study in JCR:LA-*cp* rats (6 weeks of age) randomized to either ezetimibe monotherapy (0.01%), or combined ezetimibe and simvastatin (0.01%) treatment for 8 weeks. Results of this study are presented in two separate chapters with specific objectives.

Chapter 3: Modulation of intestinal cholesterol transport and subsequent lymphatic chylomicron secretion following treatment with ezetimibe and simvastatin in the JCR:LA-cp rodent model of the metabolic syndrome.

Objectives:

I. To delineate the effects of combined ezetimibe and simvastatin therapy on intestinal cholesterol transport in the JCR:LA-*cp* rat by; determining mucosal-to-serosal and serosal-to-mucosal intestinal permeability of cholesterol following drug treatment. Permeability of radiolabelled marker compound [3H]-cholesterol in jejunal segments from JCR:LA-*cp* rats was determined using "modified Ussing chamber" techniques.
II. To determine the effect of ezetimibe and co-therapy with simvastatin on intestinal lymphatic CM production and particle characteristics following drug treatment by; evaluating intestinal lymphatic CM production (apoB48) and CM particle number, size and lipid composition.

Chapter 4: Effect of ezetimibe and simvastatin on chylomicron postprandial metabolism, and the arterial uptake of chylomicron-remnants and relationship to myocardial lesion development.

Objectives:

I. To determine the effect of ezetimibe and co-therapy with simvastatin on fasting and post-prandial lipid and CM metabolism following 8 weeks of treatment including;

   i) Fasting plasma apoB48 and blood lipid profile, including total cholesterol, LDL-C, HDL-C and triglyceride concentrations.

   ii) Fasting and postprandial glucose/insulin metabolism following a 'meal tolerance test (MTT)'.

   iii) Plasma lipid profile and apoB-48 response following an 'oral fat challenge (OFC)'.

II. To quantify the impact of ezetimibe and simvastatin following 8 weeks of treatment on arterial uptake of intestinal CM-r by:

   i) Determining the arterial retention of remnant lipoproteins via *in-situ* carotid perfusion of fluorescently labelled cy5 CM-r and semi-quantitative confocal microscopy.

III. To assess the effect of ezetimibe and simvastatin treatment on atherosclerotic lesion development in the JCR:LA-*cp* rat by:

i) Calculating the frequency and extent of atherosclerotic myocardial lesions using gross histological analysis and Mocha Image Analysis Software.

2.6 Chapter Format

Results of this study are presented in two separate chapters, which are formatted as manuscripts and include:

Chapter 3: Modulation of intestinal cholesterol transport and subsequent lymphatic chylomicron secretion following treatment with ezetimibe and simvastatin in the JCR:LA-*cp* rodent model of the metabolic syndrome.

Chapter 4: Effect of ezetimibe and simvastatin on chylomicron postprandial metabolism, and the arterial uptake of chylomicron remnants and relationship to myocardial lesion development.

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Chapter 3. Modulation of intestinal cholesterol transport and lymphatic chylomicron secretion following treatment with ezetimibe and simvastatin in the JCR:LA-*cp* rodent model of the metabolic syndrome

3.1 Introduction and Brief Rationale.

Subjects with the metabolic syndrome (MetS) have an increased risk of cardiovascular disease (CVD) (Desroches, Lamarche 2007). Cholesterol associated dyslipidemia is a prominent feature of MetS and is attributed to lower HDL-C concentrations rather than raised LDL-C (Desroches, Lamarche 2007, Alberti et al. 2005, Eckel, Grundy & Zimmet 2005). More recently, impaired postprandial lipid metabolism has been shown to be a powerful independent predictor of CVD risk (Proctor, Vine & Mamo 2002, Tomkin, Owens 2001, Higashi et al. 2001).

Plasma cholesterol concentration is mediated via metabolic cross-talk between endogenous (synthesis) and exogenous (dietary and biliary re-absorption) pathways. There is now an accumulating body of evidence that the intestine is a major site of cholesterol transport, synthesis and metabolism and has been proposed to play a significant role in the exacerbation of dyslipidemia in MetS (Lally, Owens & Tomkin 2007, Vine, Glimm & Proctor 2008, Adeli, Lewis 2008). Limited clinical studies have revealed that subjects with frank diabetes (either as insulin deficiency (type I)) or insulin resistance and hyperglycemia (type II diabetes) have increased intestinal cholesterol absorption that may contribute substantially to systemic dyslipidemia (Gylling et al. 2004, Gylling, Miettinen 1996). More recently, Gylling et al (2007) investigated cholesterol

metabolism in subjects with MetS by measuring serum non-cholesterol sterols, surrogate markers of synthesis and fractional absorption of cholesterol in plasma. Subjects with MetS were revealed to have greater cholesterol synthesis but lower cholesterol absorption efficiency compared to controls. Thus, these findings would suggest that cholesterol synthesis prevails over absorption in MetS (Gylling et al. 2007).

The absorption of cholesterol is the process whereby cholesterol is transported across the BBM into the enterocyte. The process is believed to be an active transport pathway involving several mucosal transporter proteins including; Niemann Pick C1 Like 1 (NPC1L1), Scavenger Receptor-BI (SR-BI) and fatty acid translocase (FAT/CD36) (Hui, Labonte & Howles 2008). In contrast, cholesterol is removed (effluxed) from the enterocyte into the intestinal lumen via the ATP-binding cassette (ABC) transporters ABCA1, ABCG5 and ABCG8 (Kruit et al. 2006, Hui, Labonte & Howles 2008, Lammert, Wang 2005). In addition, a newly described pathway suggests that cholesterol can also be transported directly from plasma, across the basolateral membrane of enterocytes and into the intestinal lumen for removal in faeces via the trans-intestinal cholesterol excretion pathway (TICE) (van der Velde et al. 2008). A recent study which administered stable isotopically labelled cholesterol combined with an isotopic approach to assess the fate of *de novo* synthesized cholesterol revealed that TICE is impaired in mice lacking ABCG5. Thus, these findings would suggest that the ABCG5/8 heterodimer may be involved in the TICE pathway. Following absorption into the enterocyte cholesterol is incorporated into

chylomicrons (CM) that are then secreted into the lymph. Recent evidence has suggested the coupling of NPC1L1 and FAT/CD36 transport of cholesterol and fatty acids with CM lipid composition (Drover et al. 2005, Lally, Owens & Tomkin 2007). For example, NPC1L1 mRNA levels appear to be positively associated with plasma CM apoB48 and cholesterol content (Lally et al. 2006). However, despite these findings, it remains unclear whether lipid transporter mRNA expression is reflective of function (substrate absorption) and whether this is further associated with direct lipid incorporation into the CM particle.

Intestinal cholesterol transport may be altered in subjects with diabetes and IR. A recent study by Lally et al examined NPC1L1, ABCG5 and ABCG8 mRNA expression in subjects with type II diabetes (Lally et al. 2006). The study revealed that ABCG5 and ABCG8 expression was significantly lower in diabetic subjects compared to control subjects, suggesting impairment in the efflux of cholesterol from the enterocyte into the lumen. In addition, NPC1L1 mRNA was significantly higher in the intestine of diabetic patients, suggesting that diabetic patients have increased cholesterol absorption. Furthermore, the alterations in transporter expression in type II diabetes were associated with increased plasma apoB-48 and cholesterol levels. However, the findings of this study are limited in that the use of mRNA as it is not a marker of transporter activity and may not equate to mucosal uptake of substrate.

More recently, our laboratory has developed and characterized an animal model (JCR:LA-*cp* rat) of IR and MetS to study the intestinal contribution to fasting and post-prandial dyslipidemia (Vine et al. 2007, Mangat et al. 2007, Vine, Glimm &

Proctor 2008). The JCR:LA-*cp/cp* rat demonstrates intestinal hypertrophy and CM over-production. Furthermore, CM isolated from the mesenteric lymph duct of JCR:LA-*cp/cp* rats are greater in number (based on apoB48 quantiation, one apoB48/particle) and cholesterol concentration compared to lean controls. Preliminary studies in the JCR:LA-*cp/cp* rat have attempted to elucidate whether alterations in cholesterol transport partly explain CM overproduction and cholesterol enrichment of these particles (Vine, Glimm & Proctor 2008). Using 'Ussing Chamber' techniques (isolated jejunum segments mounted in a diffusion chamber under physiological conditions, in order to determine substrate transport across tissue) it was revealed that mucosal to serosal flux of cholesterol is increased in the hyperinsulinemic state in the JCR:LA-*cp/cp* genotype. These findings are consistent with those of earlier studies showing altered expression of intestinal cholesterol absorption transporters (Lally et al. 2006, Vine, Glimm & Proctor 2008). The JCR:LA-*cp/cp* rat was also observed to have elevated serosal to mucosal flux of cholesterol, and further studies are required to delineate whether this is an adaptive response of the intestine and/or a different phenomenon of cholesterol transport in this animal model.

Ezetimibe (EZ) and its analogue SCH48461 selectively inhibit cholesterol absorption at the brush border membrane of enterocytes in the dudoenum, without altering triglyceride and fat-soluble vitamin bioavailability (Bays et al. 2008, Davis et al. 2004, Garcia-Calvo et al. 2005). There is now substantial evidence that EZ's mechanism of action is through binding to NPC1L1 (Davis et al. 2004, Garcia-Calvo et al. 2005, Lammert, Wang 2005). NPC1L1 is known to play a

key role in the absorption of cholesterol and phytosterols across the mucosal BBM to intracellular compartments for sterol esterification and incorporation into CM (Bays et al. 2008, Davis et al. 2004, Garcia-Calvo et al. 2005).

Previous studies which have delivered [3H]-cholesterol via intestinal cannula to rats following EZ treatment have shown that EZ inhibits cholesterol transport from the intestinal lumen and can dramatically reduce the appearance of intestinally derived cholesterol in plasma (Bays et al. 2008). Further studies in hypercholesterolemic rhesus monkeys which have isolated CM from plasma by sequential density ultra-centrifugation following EZ treatment have revealed that EZ reduces the cholesterol content of CM but has no impact on CM particle number (van Heek, Compton & Davis 2001). These studies have been limited in that intestinal CM production per say has not been measured directly. We know that CM production occurs constantly and CM incorporation of lipid is greater following feeding of a lipid-containing meal. Thus it is important to determine the effects of EZ on CM production and lipid composition in both the fasted and fed state.

Contrastingly, SV functions by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting step in the cholesterol biosynthesis pathway (Bays et al. 2008, Holdgate, Ward & McTaggart 2003, Bays, Stein 2003, Shepherd et al. 1995). As 60-70% of plasma cholesterol (at any one point in time) is thought to be derived from hepatic cholesterol synthesis, inhibition of HMG-CoA reductase offers a successful target to lower LDL-C concentration (Cuchel et al. 1997, Cortner et al. 1993). It has also been shown that SV can benefit non-

fasting lipids. For example, SV intervention in individuals with familial hypercholesterolemia decreased plasma concentration of apoB-48 and remnant lipoprotein cholesterol (Hogue et al. 2008, Tremblay et al. 2009). However, it is yet to be determined whether SV can directly affect intestinal cholesterol transport and CM production.

Cholesterol homeostasis is maintained by feedback mechanisms between contributions to the total cholesterol pool, including cholesterol synthesis and absorption. Monotherapy of SV has been shown to reduce cholesterol synthesis but in turn this may up-regulate intestinal cholesterol absorption (Hogue et al. 2008, Miettinen, Gylling 2003, Miettinen, Gylling 2002). Similarly, EZ treatment inhibits intestinal cholesterol absorption and results in up-regulation of intestinal and liver cholesterol synthesis (Altmann et al. 2004, Davis et al. 2004, Repa et al. 2005). Thus, the concept of using a combination of drug therapies with complementary targets (i.e. cholesterol synthesis and absorption) to regulate cholesterol homeostasis is attractive. Combined EZ and SV therapy has proven to be very effective at reducing total plasma cholesterol by 50% more than EZ treatment alone (Tomkin 2009).

To date there have been no studies directly examining the affects of EZ and SV therapy on intestinal cholesterol transport. Neither, have there been studies directly observing the effects of EZ and SV on CM particle composition, and production by assessing secretion of particles into lymph over time in the fasted and fed state in MetS. The assessment of CM particles secreted during fasted and post-prandial periods is critical in order to determine the impact of pharmaceutical

and dietary compounds in their metabolism. The aim of this study was assess to intestinal cholesterol transport and CM metabolism as potential mechanisms underlying the therapeutic application of combined EZ and SV therapy in the JCR:LA-*cp* rat, a model of MetS.

3.2 Methods

3.2.1 Animal Model

The JCR:LA-*cp* rat is a model that mimics clinical complications of the metabolic syndrome (MetS) including obesity, hyperinsulinemia, as well as myocardial and intimal atherogenesis (Mangat et al. 2007, Russell, Graham & Richardson 1998, Brindley, Russell 2002). The JCR:LA-*cp* strain of rat incorporates the autosomal recessive corpulent (*cp*) trait which has been shown to be a stop codon in the extra-cellular domain of the leptin receptor (ObR), thus rendering the homozygous (cp/cp) genotype to present with MetS. Heterozygous JCR:LA-*cp* rats are lean and observed to be metabolically normal. Vine et al recently reported that the JCR:LA-*cp* rodent is a useful model of impaired CM metabolism, with delayed clearance of CM from the circulation compared to their lean controls (Vine, Glimm & Proctor 2008).

3.2.2 Study Design

JCR:LA-*cp* rats at 6 weeks of age received a lipid balanced diet supplemented with 1% w/w cholesterol prepared using standard rodent feed formulation (Harlan Teklad). The diet was supplemented with lipid (30% w/w), consisting of flaxseed oil, tallow, sunflower oil and olive oil yielding a saturated-to-polyunsaturated fat ratio of 1:1. The composition of the diet (w/w) was carbohydrate 49%, crude protein 28%, moisture 10%, minerals 4% and fibre 6%. Animals were randomized to either ezetimibe (EZ, 0.01% w/w) (n=4) or combined EZ (0.01% w/w) and simvastatin (SV, 0.01% w/w) treatment (n=4) for 8 weeks. Rodents were on a 12

hour light/dark cycle, were caged individually and had ad libitum access to food and water. Food intake and body weight were measured daily. Animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the University of Alberta animal ethics committee.

3.2.3 Intestinal Cholesterol Transport Studies

Following 8 wks of treatment rats were sacrificed and the jejunum was removed distal to the ligament of the Treitz. The jejunum was immediately placed in Kreb's buffer (at 4^{o}C) and segments were mounted in modified Ussing chambers (Harvard Apparatus Inc, Holliston, MA), described previously in Vine *et al* (2002)(Vine et al. 2002a). Briefly, triplicate jejunal segments from each animal was used to determine, mucosal-to-serosal (M to S) and serosal-to-mucosal (S to M) transport of cholesterol and mannitol by measuring the apparent permeability (Papp). Tissues were exposed to oxygenated Krebs buffer at 37^{o}C and the exposed tissue surface area was 1.78cm^{2}. Tissues were allowed 30 mins to equilibrate. 100 µl of [14C]- mannitol (to determine intestinal integrity) and [3H]-cholesterol were added to the donor chamber at time 0. Subsequently, 200µl samples were removed from the receiver chamber to assess cholesterol and mannitol transport at times 0, 20, 40, 60 and 80 mins. Equal volumes of fresh supplemented Krebs buffer were replaced in the receiver chamber. Samples (200µl) were also taken from the donor chamber at the start and end of the experiment. Samples were added to a 1.5mL scintillation cocktail (Ultima, Perkin

Elmer, MA, USA) and radioactivity was measured using a liquid scintillation counter (LS 6500 Beckman-Coulter, Ont, Canada).

Apparent Permeability was calculated using the following equation:

P_{app}=dQ/dt X (1/A X C_0)

where Papp is the apparent intestinal permeability to the marker, dQ/dt equates to the appearance rate of radiolabelled marker in the receiver chamber, A is the exposed surface area of the tissue and C_0 is the initial concentration in the donor chamber.

Net cholesterol influx ratio was calculated using the following equation:

Net cholesterol influx= P_{app} M-S/ P_{app} S-M

where Papp M-S is the apparent intestinal permeability of cholesterol in the mucosal to serosal direction and, Papp S-M is the apparent intestinal permeability in the seroal to mucosal direction.

Net cholesterol efflux ratio was calculated using the following equation

Net cholesterol efflux= P_{app} S-M/ P_{app} M-S.

3.2.4 Biochemical and Metabolic Assessments

3.2.4.1 Lymphatic Chylomicron Collection and Analysis

Following 8 wks treatment, EZ (0.01 w/w) and EZ (0.01% w/w)+SV (0.01 w/w) animals were fasted for 16 hours to equilibrate gastro-intestinal contents and then re-fed equal quantities of standard rat chow prior to lymphatic surgery.

Chylomicron Isolation:

The superior mesenteric lymph duct was cannulated as previously described in Vine et al (2002) (Vine et al. 2002b). Animals were infused with saline (NaCl 154 mmol/l, 4% glucose) and then intralipid (20% intralipid, 4% glucose) into the duodenum. Lymph was collected from the mesenteric lymph duct following saline infusion (defined as the fasted state) for 4hr and then intralipid infusion (defined as the fed state) for 4hr. Lymphatic TG and cholesterol concentration was determined using commercially available colormetric assays (details below). Lymph CM particle size was measured using laser diffraction and standard particle size alogorithms (Beckman BI-90).

ApoB48 Quantification:

ApoB48 was quantified using an adapted western immune-blot method previously described by Vine et al (2002). In brief, lymph apolipoproteins from both fasted and fed states were separated by 3-8% tris-acetate SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis, Invitrogen, NuPage, CA, USA). Separated proteins were transferred onto a PVDF membrane (0.45um; 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 peroxidise (Santa Cruz Biotech, CA). ApoB48 bands were visualized using enhanced chemiluminescence techniques (ECL-Advance, Amersham Biosciences, UK) and band intensity was determined against a known mass of purified rodent apoB48 using Scion Imaging Technology.

3.2.4.2 Postpranidal Insulin-glucose response

At 12 weeks of age (after 6 weeks of treatment, n=4 for each group) rats were given an oral meal tolerance test following an overnight fast overnight. Animals were given 5.0g of standard rat chow as described previously (Russell, Graham & Dolphin 1999). Blood samples were taken using the standardized tail vein procedure at 0, 30 and 60 minutes. K_2EDTA (ethylene diamine tetraacetic acid, BD Franklin Lakes NJ USA, Cat#367835) tubes were used to collect blood and plasma was separated via centrifugation at 3000rpm at 4oC for 10 mins. These samples were then subjected to biochemical analysis for insulin and glucose using commercially available colormetric and enzymatic assays, respectively (details below).

3.2.4.3 Postprandial lipid and CM response

At 12 weeks of age (after 6 weeks of treatment, n=4 for each group) rats were given an oral fat load meal challenge. Rats were fasted overnight and the following morning fed a 5.0g pellet containing 30% (w/w) lipid. Following meal consumption, blood samples were taken via an established tail vein procedure (Vine et al. 2007) at times 0, 2, 4, 5, 6, 8 and 10h. K_2EDTA were used to collect

blood samples and plasma was separated by centrifugation at 3000 rpm at 4˚C for 10 min. Plasma collected was subjected to biochemical analysis, including lipids and apoB48.

3.2.4.4 Analysis of postprandial CM, lipid, insulin and glucose response

Graphpad Prizm (CA, USA) was used to determine total area under the curve (AUC) for plasma apoB48, TG, cholesterol, insulin and glucose. AUC corresponds to the postprandial total plasma concentration over the 10hr period for the oral lipid meal challenge and the meal tolerance test over the 60 min response period, respectively.

3.2.4.5 Plasma Biochemical Profile

Fasted samples from rats were assessed using enzymatic colorimetric assays: triglyceride (WAKO, Chemicals USA Inc., Richman, VA, USA, Cat#998-40391/994-40491), LDL (LDL-C, WAKO Cat#993-00404/999-00504), total cholesterol (TC, WAKO, Cat#439-17501), and HDL-cholesterol (HDL-C, Diagnostic Chemical Ltd., Charlottetown, Prince Edward Island, Cat#258-20). Insulin (ALPCO Diagnostics, USA, Cat#80-INSRT-E01) was measured using commercially available EIA kits and glucose determined via the glucose oxidase method (Diagnostic Chemicals Ltd., Cat#220-32).

3.2.5 Statistical Analysis

All results are expressed as the mean±S.E.M. Data were analyzed for statistical differences at a significance level of $p<0.05$ using one way ANOVA and post-hoc differences were analyzed using the Tukey method (GraphPad PRISM).

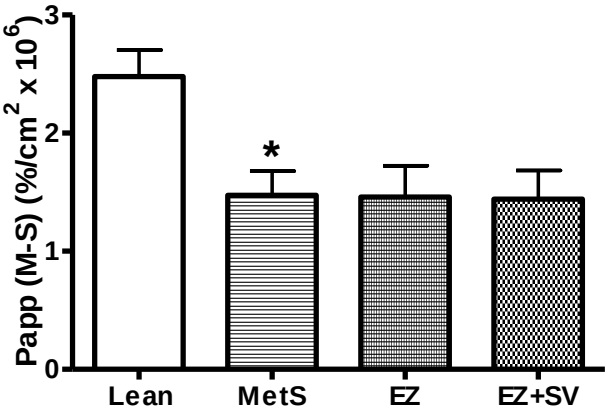
3.3 Results

3.3.1 Intestinal Transport of Mannitol and Cholesterol

Intestinal Transport of Mannitol:

Papp (apparent permeability) of mannitol across intestinal segments was used as a marker of intestinal tissue integrity and passive paracellular transport. The transport of mannitol in the mucosal to serosal (M-S; absorptive) direction was decreased by 41% ($p<0.05$) in jejunum isolated from MetS JCR:LA-*cp* rats as compared to their lean counterparts (Figure 3-1, graph A). Papp of mannitol in the serosal to mucosal direction (S-M; efflux) was decreased by 54% ($p<0.01$) in MetS rats compared with lean rats (Figure 3-1, graph B). No statistical difference was observed for Papp of mannitol in either the M-S or S-M direction in EZ and EZ+SV treated JCR:LA-*cp* rats relative to control MetS rats (see figure 3-1, graphs A&B).

(A)



(B)

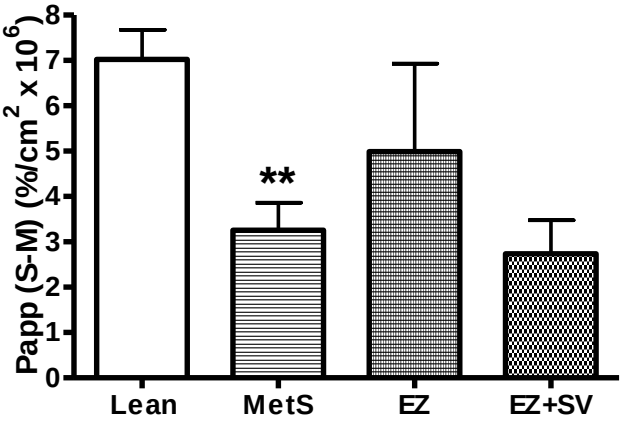
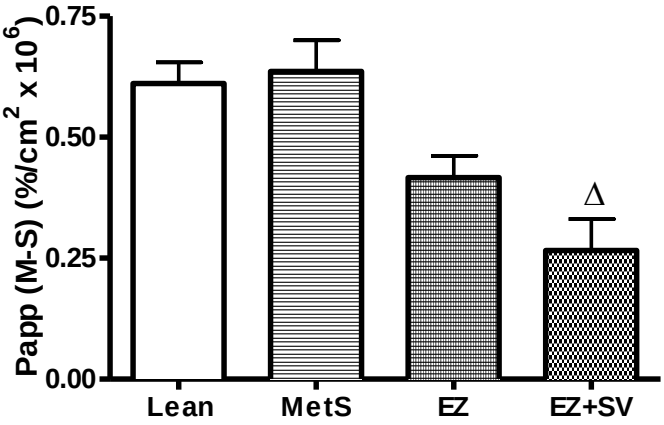


Figure 3-1. Intestinal transport of mannitol in treated and control JCR:LA-*cp* rats. JCR:LA-*cp* rats were randomized to a lipid balanced diet with or without EZ (0.01% w/w) or combined EZ (0.01%w/w) and SV (0.01% w/w) groups for 8 weeks. At sacrifice the jejunum was removed and segments mounted in modified *Ussing* chambers. Papp (apparent permeability) of [14C]-mannitol was determined for both mucosal to serosal (M-S) and serosal to mucosal (S-M) transport by rate of appearance of radiolabelled marker in receiver chamber. Mannitol transport was significantly decreased in MetS rats compared to lean rats for both M-S (graph A) and S-M flux (graph B). EZ and SV treatment either alone or in combination had no effect on M-S (graph A) and S-M (graph B) transport of mannitol. (*) $p<0.05$ and (**) $p<0.01$ denotes statistical significance between control MetS Vs. lean rats.

Intestinal Transport of Cholesterol:

No difference was observed in the M-S cholesterol transport (representing influx of cholesterol) between MetS rats and lean rats (Figure 3-2, graph A). There was a trend for EZ to reduce cholesterol M-S transport but this did not reach significance. However, combined treatment of EZ+SV significantly decreased M-S cholesterol transport (absorptive influx) by 58% compared to control MetS rats ($p<0.05$, Figure 3-2, graph A) The S-M cholesterol transport (representing efflux of cholesterol) was observed to be decreased in MetS rats by greater than 40% ($p<0.05$) compared to their lean counterparts, which is consistent with earlier findings from our laboratory (Vine, Glimm & Proctor 2008)(Figure 3-2, graph B). EZ monotherapy normalized S-M cholesterol transport in MetS rats. Addition of SV to EZ treatment had no effect on S-M cholesterol transport (efflux) compared to MetS rats (Figure 3-2, graph B).

(A)



(B)

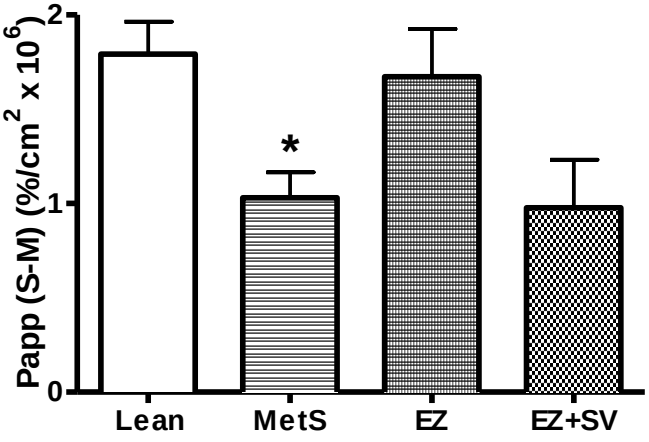


Figure 3-2. Intestinal transport of cholesterol in treated and control JCR:LA-*cp* rats. JCR:LA-*cp* rats were randomized to a lipid balanced diet with or without EZ (0.01% w/w) or combined EZ (0.01%w/w) and SV (0.01% w/w) groups for 8 weeks. At sacrifice the jejunum was removed and segments mounted in modified Ussing chambers. Papp (apparent permeability) of [3H]-cholesterol was determined for both mucosal to serosal (M-S) (graph A) and serosal to mucosal (S-M) (graph B) transport by rate of appearance of radiolabelled marker in receiver chamber. S-M cholesterol transport was significantly decreased in control MetS rats compared to lean rats (graph B). Combined EZ and SV therapy decreased cholesterol M-S transport compared to control MetS rats (graph A). (*) $p<0.05$ denotes statistical difference between control MetS Vs. lean rats. (Δ) $p<0.05$ denotes statistical difference between EZ+SV treated rats Vs. control MetS rats.

Intestinal Net Cholesterol Influx and Efflux:

Intestinal net cholesterol influx was greater in MetS rats compared to lean rats (0.74±0.2 vs. 0.47±0.2 respectively, $p<0.05$) (Figure 3-3). Net cholesterol influx was decreased following EZ treatment (60%, $p<0.01$) or in combination with SV (43%, $p<0.05$) compared to control MetS rats (Figure 3-3). Correspondingly, net cholesterol efflux was observed to be lower in MetS rats compared to lean rats (2.79±1.47 vs. 1.26±0.68 respectively, $p<0.05$) (Figure 3-4). EZ or SV+EZ treatment tended to normalize cholesterol efflux in MetS rats (Figure 3-4).

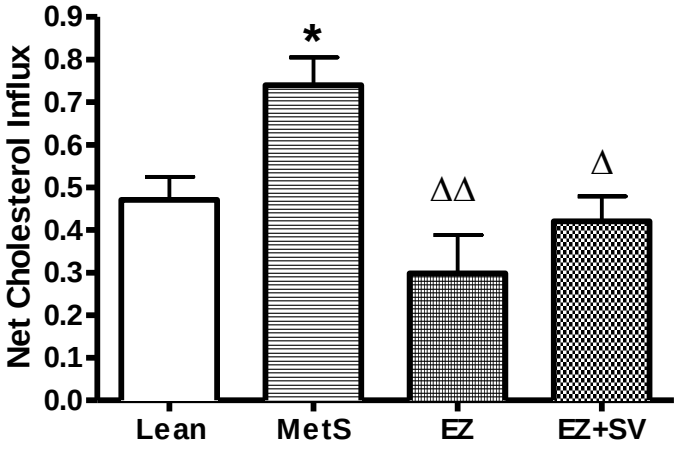


Figure 3-3. Intestinal net cholesterol influx in treated and control JCR:LA-*cp* rats. JCR:LA-*cp* rats were randomized to a lipid balanced diet with or without EZ (0.01% w/w) or combined EZ (0.01% w/w) and SV (0.01% w/w) treatment for 8 weeks. At sacrifice the jejunum was removed and segments mounted in modified Ussing chambers. Papp (apparent permeability) of [3H]-cholesterol was determined for both mucosal to serosal (M-S) and serosal to mucosal (S-M) transport by rate of appearance of radiolabelled marker in receiver chamber. Net cholesterol influx was calculated by the following equation: net cholesterol influx= Papp M-S/Papp S-M. Net cholesterol influx was significantly greater in control MetS rats compared to lean rats. EZ treatment either alone or in combination with SV therapy decreased net cholesterol influx. (*) *$p<0.05$* denotes statistical difference between control MetS Vs. lean rats. (Δ) *$p<0.05$* and (ΔΔ) *$p<0.01$* denotes statistical difference between treated rats (EZ,EZ+SV) Vs. control MetS rats.

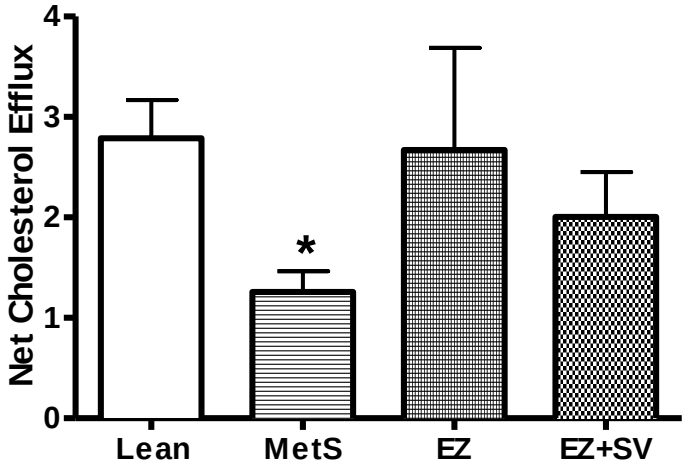


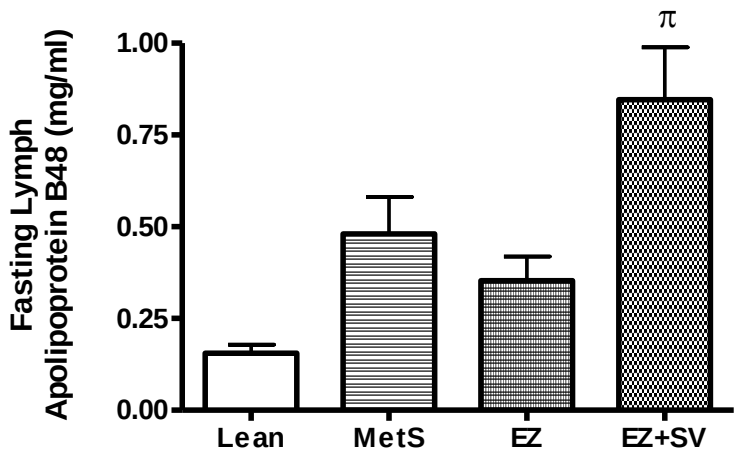
Figure 3-4. Intestinal net cholesterol efflux in treated and control JCR:LA-*cp* rats. JCR:LA-*cp* rats were randomized to a lipid balanced diet with or without EZ (0.01% w/w) or combined EZ (0.01% w/w) and SV (0.01% w/w) groups for 8 weeks. At sacrifice the jejunum was removed and segments mounted in modified Ussing chambers. Papp (apparent permeability) of [3H]-cholesterol was determined for both mucosal to serosal (M-S) and serosal to mucosal (S-M) transport by rate of appearance of radiolabelled marker in receiver chamber. Net cholesterol efflux was calculated by the following equation: net cholesterol efflux= Papp S-M/Papp M-S. Net cholesterol efflux was significantly lower in control MetS rats compared to lean rats. (*) $p<0.05$ denotes statistical difference between control MetS rats Vs. lean rats.

3.3.2 Intestinal lymph chylomicron production and lipid composition

Lymph ApoB48 concentration:

ApoB48 concentration in lymph following saline infusion (equivalent to fasting) was not statistically different between MetS controls Vs. lean controls or EZ treated rats (Figure 3-5, graph A). EZ+SV treated MetS rats showed a trend towards increased fasting apoB48 concentration compared to control MetS rats, but this did not reach statistical significance. Fasting lymphatic apoB48 concentration was significantly greater in the EZ+SV group compared to EZ group. However, following the intralipid infusion (equivalent to postprandial response following a lipid meal) apoB48 concentration in MetS rats was found to be significantly greater (120%, $p<0.05\%$) compared to their lean counterparts, which is consistent with previous studies (Vine, Glimm & Proctor 2008). Treatment of JCR:LA-*cp* rats with EZ monotherapy did not influence apoB48 concentration in lymph relative to control MetS rats. Intriguingly, combined treatment of EZ+SV increased apoB48 secretion in lymph during intralipid infusion compared to control MetS rats (54%, $p<0.05$) and EZ treated rats (108%, $p<0.001$) (as shown in figure 3-5, graph B).

(A)



(B)

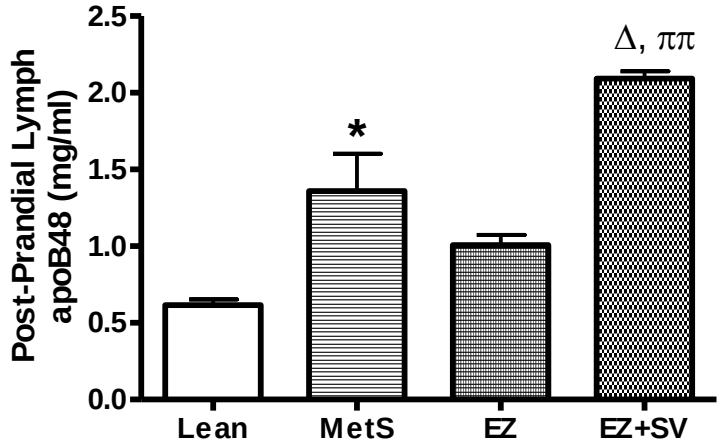
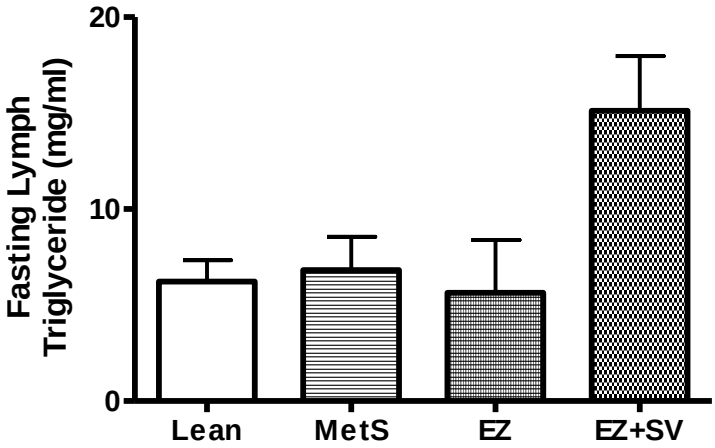


Figure 3-5. Apolipoprotein B48 in intestinal lymph from treated and control JCR:LA-*cp* rats following saline and intralipid infusion. JCR:LA-*cp* rats were randomized to a lipid balance diet with or without EZ (0.01% w/w) or combined EZ (0.01% w/w) and SV (0.01% w/w) groups for 8 wks. Lymph chylomicrons were collected for 4hrs following gastric infusion with saline (first 2 hours) and intralipid between (3^{rd} and 4^{th} hour). The graphs depict apoB48 concentration (mg/ml) in the lymph collected after saline (graph A) and intralipid (graph B) infusion, respectively for all groups. (*) *p<0.05* denotes statistical difference between control MetS rats Vs. lean rats. (Δ) *p<0.05* denotes statistical difference between EZ+SV treated Vs. control MetS rats. (∏) p<0.05 and (∏∏∏) *p<0.001* denotes stastical difference between EZ+SV Vs EZ treated rats.

Lymph Cholesterol and Triglyceride:

There was no statistical difference in the concentration of either cholesterol or TG in lymph isolated from treated, lean and control MetS rats following the infusion of saline (equivalent to fasting) (Figure 3-6 and 3-7, graphs A). Combined EZ+SV therapy showed a trend for increased TG and cholesterol concentration in lymph in the fasted stated compared to control MetS rats. However, following intralipid infusion (equivalent to postprandial response following a lipid meal), there was an increase in the concentration of TG (77%, $p<0.05$) in MetS rats compared to their lean counterparts (Figure 3-6, graph B). Lymphatic cholesterol in MetS rats was increased compared to lean rats (63%, $p<0.05$) following intralipid infusion (Figure 3-7, graph B). EZ treatment either alone or in combination with SV had no significant effect on lymphatic cholesterol and TG concentration compared to control MetS rats following saline or intralipid infusion (Figure 3-6 and 3-7).

(A)



(B)

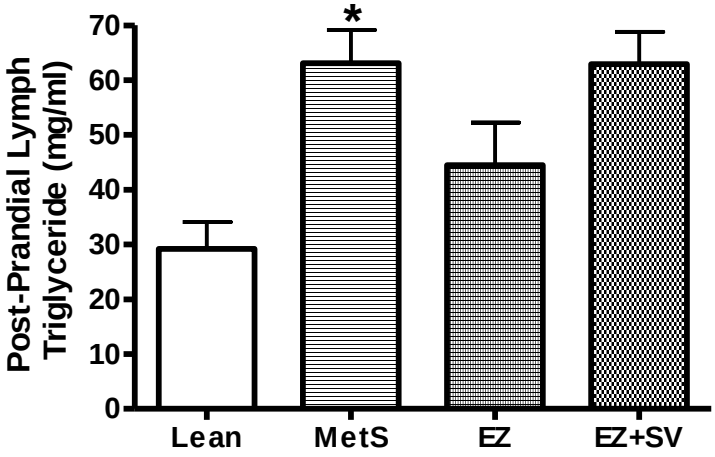
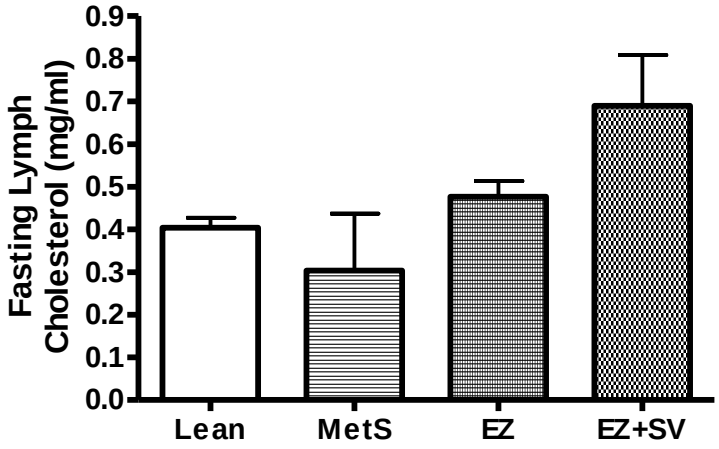


Figure 3-6. Concentration of total triglyceride in intestinal lymph from treated and control JCR:LA-*cp* rats. JCR:LA-*cp* rats were randomized to a lipid balance diet with or without EZ (0.01% w/w) or combined EZ (0.01%w/w) and SV (0.01% w/w) groups for 8 weeks. Lymph was collected from the mesenteric lymph duct cannulation procedure. Lymph was collected for four hours following gastric infusion with saline (first 2 hours) and intralipid (between 3rd and 4th hour) .The graphs depict total triglyceride concentration (mg/ml) in lymph collected after saline (graph A) and intralipid (graph B) infusion respectively for all groups. (*) $p<0.05$ denotes statistical difference between control MetS rats Vs. lean rats.

(A)



(B)

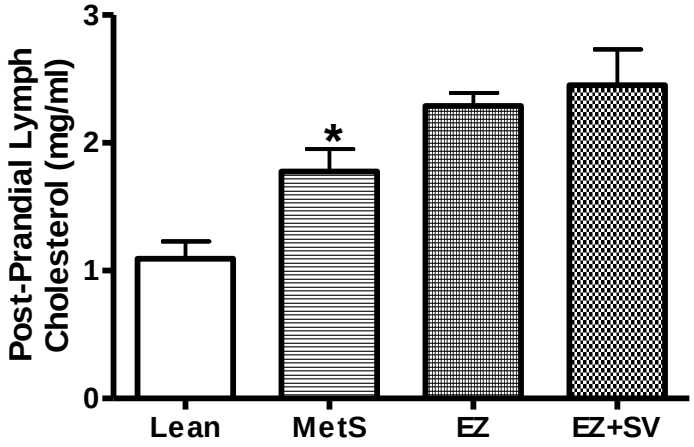


Figure 3-7. Concentration of total cholesterol in intestinal lymph from treated and control JCR:LA-*cp* rats. JCR:LA-*cp* rats were randomized to a lipid balance diet with or without EZ (0.01% w/w) or combined EZ (0.01% w/w) and SV (0.01% w/w) groups for 8 weeks. Lymph was collected from the mesenteric lymph duct cannulation procedure. Lymph was collected for four hours following gastric infusion with saline (first 2 hours) and intralipid (between 3rd and 4th hour). The graphs depict total cholesterol concentration (mg/ml) in lymph collected after saline (graph A) and intralipid (graph B) infusion respectively for all groups. (*)$p<0.05$ denotes statistical difference between control MetS Vs. lean rats.

Particle size and ratio of triglyceride and cholesterol to apoB48 in lymph:

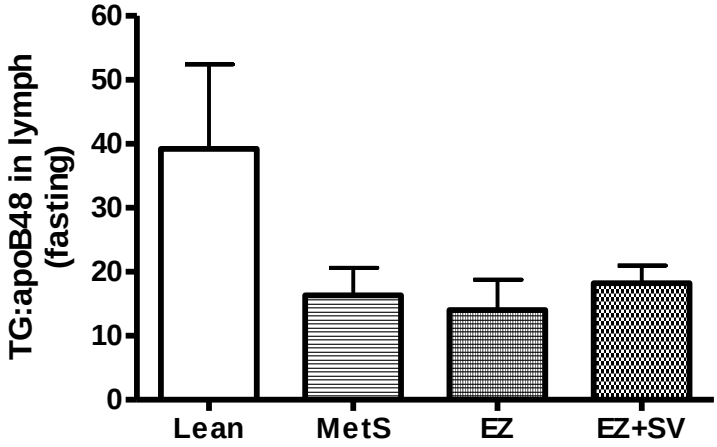
No statistical differences were observed in fasting particle size between control and treated rats. Following intralipid infusion, lymphatic CM particles from control MetS rats were larger in size relative to lean controls (185.2±9.4 vs. 97.96±12.37 respectively, $P<0.05$) (see table 3-1). EZ monotherapy, but not EZ+SV, caused a significant decrease in particle size compared to control MetS rats (Table 3-1) (88.6±16.14 vs.185.2±9.4 and 84.33±19.38 vs. 185.2±9.4 respectively, $P<0.05$). Control MetS rats showed a trend towards decreased TG:apoB48 ratio (TG per particle) compared to lean rats following saline infusion, but this did not reach statistical significance (Figure 3-8). No difference was observed in TG:apoB48 ratio between control MetS and lean rats following intralipid infusion (Figure 3-8). EZ treatment either alone or in combination with SV showed no statistical difference in TG:apoB48 compared to control MetS rats following either saline or intralipid infusion. However, following intralipid infusion, EZ+SV treatment showed a trend towards decreased TG:apoB48 compared to control MetS rats. Cholesterol:apoB48 ratio showed that lymph CM particles in lean rats contained a higher concentration of cholesterol per particle than MetS rats following saline infusion (2.385±0.1626 vs. 0.556±0.4538 respectively, $p<0.01$), consistent with earlier studies (Vine, Glimm & Proctor 2008) (Figure 3-9, graph A). However, following intralipid infusion, cholesterol:apoB48 ratio was similar between lean and MetS rats (Figure 3-9, graph B). Monotherapy of EZ increased cholesterol:apoB48 ratio ($p<0.05$) compared to control MetS rats following both saline and intralipid infusion (Figure 3-9, graphs A&B respectively). Contrastingly, combined EZ+SV therapy

decreased cholesterol:apoB48 ratio (-38%, $p<0.05$) compared to control MetS rats following intralipid infusion (Figure 3-9, graph B).

Lymph collected after saline infusion	**Particle Size (nm)**	**Lymph collected after intralipid infusion**	**Particle Size (nm)**
Lean	78±19.09	**Lean**	97.96±12.37
MetS	140.7±20.33	**MetS**	185.2±9.4*
EZ	75.86±10.40	**EZ**	88.6±16.14Δ
EZ+SV	111.0±23.27	**EZ+SV**	130.6±21.41

Table 3-1. Lymph chylomicron particle size following saline (fasted) versus intralipid (post-prandial) infusion. JCR:LA-*cp* rats were randomized to a lipid balance diet with or without EZ (0.01% w/w) or combined EZ (0.01% w/w) and SV (0.01% w/w) groups for 8 weeks. Lymph was collected from the mesenteric lymph duct cannulation procedure. Lymph was collected for four hours following gastric infusion with saline (first 2 hours) and intralipid (between 3rd and 4th hour). Size determined by BI-90,90-laser diffraction. (*) *$p<0.05$* denotes statistical difference between control MetS Vs. lean rats. (Δ) *$p<0.05$* denotes statistical difference between EZ treated Vs. control MetS rats.

(A)



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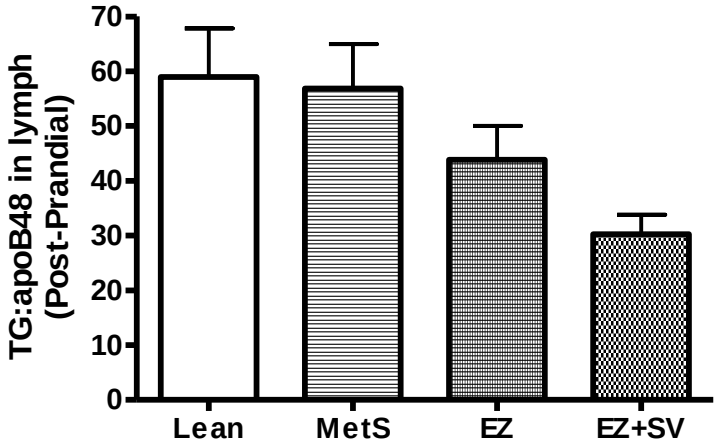
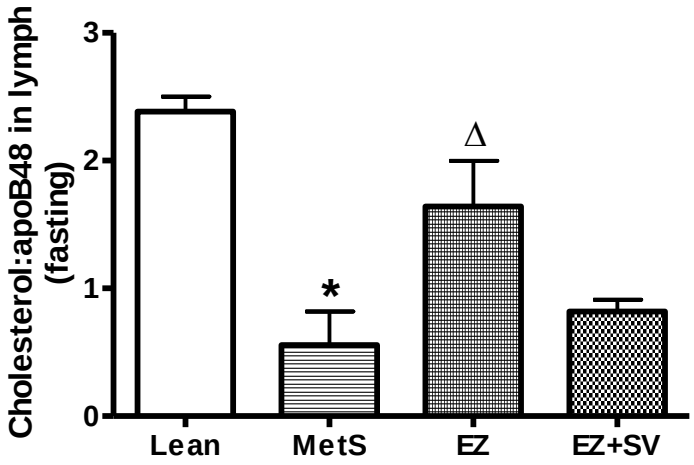


Figure 3-8. Ratio of trigylceride:apoB48 in lymph isolated from treated and control JCR:LA-*cp* rats. JCR:LA-*cp* rats were randomized to a lipid balance diet with or without EZ (0.01% w/w) or combined EZ (0.01% w/w) and SV (0.01% w/w) groups for 8 weeks. Lymph was collected from the mesenteric lymph duct cannulation procedure. Lymph was collected for four hours following gastric infusion with saline (first 2 hours) and intralipid (between 3rd and 4th hour).The graphs depict triglyceride to apoB48 concentration (mg/ml) in the lymph collected after saline (graph A) and intralipid (graph B) infusion respectively for all groups.

(A)



(B)

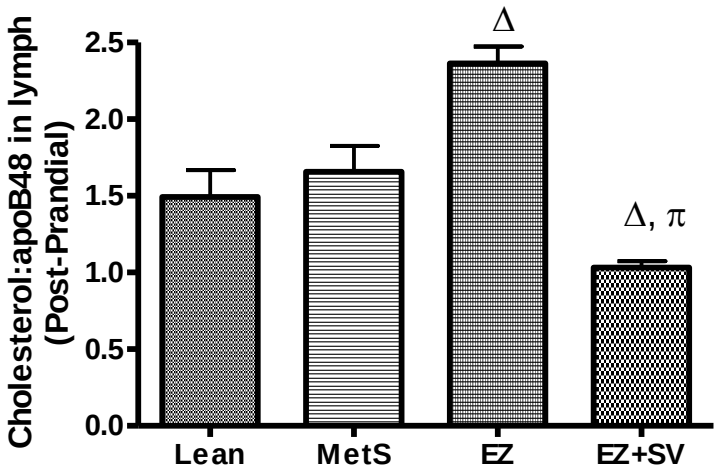


Figure 3-9. Ratio of cholesterol:apoB48 in lymph isolated from treated and control JCR:LA-*cp* rats. JCR:LA-*cp* rats were randomized to a lipid balance diet with or without EZ (0.01% w/w) or combined EZ (0.01% w/w) and SV (0.01% w/w) groups for 8 weeks. The graphs depict cholesterol:apoB48 concentration (mg/ml) in the lymph collected after saline (graph A) and intralipid (graph B) infusion respectively for all groups. (*) $p<0.05$ denotes statistical difference between control MetS Vs. lean rats. (Δ) $p<0.05$ denotes stastical difference between treated (EZ,EZ+SV) Vs. control MetS rats. (∏) $p<0.001$ denotes stastical difference between EZ+SV Vs. EZ treated rats.

3.4 Discussion

The primary objective of this study was to determine the effect of ezetimibe and co-therapy with simvastatin, on intestinal cholesterol transport, chylomicron particle composition and post-prandial lipid and chylomicron metabolism in the JCR-LA-*cp* model of MetS.

3.4.1 Intestinal Transport of Mannitol and Cholesterol

Intestinal Permeability of Mannitol:

Papp (apparent permeability) of mannitol across intestinal segments is currently used as a marker to assess both intestinal tissue integrity and passive paracellular transport . Increased mannitol permeability reflects a reduced integrity of the paracellular contribution, in particular, intercellular tight junctions, to the intestinal epithelial barrier (Tomkin 2008). Previous studies in animal models of diabetes and pre-diabetes have demonstrated increased uptake of mannitol indicating a reduced integrity and increase in permeability of the intestinal epithelial barrier (Bosi et al. 2006). In the current study, mannitol permeability was observed to be decreased in MetS rats compared to lean rats. It is speculated that these findings are a result of dietary cholesterol supplementation, as preliminary studies in our laboratory have shown mannitol permeability to be similar between MetS rats and their lean counterparts fed a lipid balanced low cholesterol diet. What is interesting, is the disparity between the lean and MetS animals in the change in permeability of mannitol during cholesterol supplementation.

Tight junctions (TJ) between epithelial cells are formed by adhesion proteins occludin, claudins and junctional adhesion molecules linked to cytosolic zonula occludens (Miyoshi, Takai 2005). Previous studies have shown that cholesterol depletion in the epithelial membrane of Caco-2 cells alters the distribution of TJ proteins claudin 3 and 4, as well as occludin, decreasing their association with one another, suggesting a reduction in TJ function (Lambert, O'Neill & Padfield 2005). Thus, cholesterol is thought to be involved in the distribution of proteins which form the TJ between epithelial cells. Cholesterol supplementation in the diet may alter the organization of TJ proteins differently depending on the metabolic phenotype. The measurement of tight junctional proteins and the permeability of other markers of paracellular transport, such as PEG, could be used to further elucidate the relevance and mechanisms associated with the increase in mannitol permeability in lean compared to MetS animals. No significant differences in mannitol permeability were observed between EZ and SV treatments and control MetS, demonstrating these interventions do not appear to influence paracellular transport pathways, at least using the marker mannitol.

Intestinal Transport of Cholesterol:

The apparent permeability (Papp) of cholesterol was assessed to determine the bi-directional transport of cholesterol (i.e. Mucosal-Serosal, M-S and Serosal-Mucosal, S-M) in jejunal segments from JCR:LA-*cp* rats given either EZ monotherapy or combined EZ+SV treatment. The results of this series of experiments revealed that there was no significant difference in cholesterol M-S

Papp between MetS rats and lean rats. These findings are contradictory to previous studies in our laboratory which show M-S flux to be increased in MetS rats compared to lean rats (Vine, Glimm & Proctor 2008). However, in the present study, JCR:LA-*cp* rats were fed a lipid balance diet supplemented with 1% cholesterol (w/w). Thus, dietary cholesterol supplementation may alter M-S intestinal cholesterol transport, a finding that has not been previously reported.

The findings of this study also showed that EZ tended to decrease the absorptive transport (M-S) of cholesterol, although this did not reach significance. However combined therapy with EZ+SV decreased M-S transport in MetS rats significantly. EZ inhibits NPC1L1 mediated transport of cholesterol, by blocking the internalization of the NPC1L1/cholesterol complex induced by cholesterol loading (Chang, Chang 2008). Furthermore, studies have shown that EZ decreases intestinal cholesterol absorption via down-regulation of FAT/CD36 and/or SR-BI transporters (Hui, Labonte & Howles 2008). Although SV reduces cholesterol synthesis through hepatic HMG-CoA reductase inhibition, the rate-limiting step in cholesterol biosynthesis (Bays et al. 2008, Holdgate, Ward & McTaggart 2003, Bays, Stein 2003, Shepherd et al. 1995), the effects of SV on intestinal HMG-CoA reductase remain unknown. There is some evidence that HMG-CoA reductase inhibition by statins positively correlates with reduced intestinal NPC1L1 mRNA (Lally et al. 2006). A study by Tomkin et al revealed a 40% decrease in NPC1L1 mRNA in diabetic patients treated with statin but this did not reach statistical significance (Lally et al. 2006). Thus, the findings from this study showing reduced cholesterol M-S transport following EZ+SV treatment

may be associated with both decreased NPC1L1 mRNA expression, and inhibition of NPC1L1 activity. In addition, SV may inhibit intestinal HMG-CoA reductase activity which may contribute to a decrease in the availability of cholesterol for cellular transport. Further studies, examining the effects of EZ+SV on intestinal mRNA transporter and intestinal HMG-CoA reductase expression would clarify the mechanisms associated with these findings.

Cholesterol S-M permeability (efflux) was observed to be decreased in MetS rats compared to lean rats. These findings are inconsistent with preliminary studies in our laboratory which have shown S-M cholesterol transport to be elevated in MetS rats compared to their lean counterparts (Vine, Glimm & Proctor 2008). The contradictory findings within our own laboratory may be attributed to differences in dietary composition between studies. In the present study, JCR:LA-*cp* rats were fed a diet supplemented with 1% cholesterol (w/w) whereas earlier studies in our laboratory used a low cholesterol diet. Thus, dietary cholesterol supplementation may induce a compensatory response to remove cholesterol from the enterocyte by up-regulation of cholesterol efflux. However, findings in the present study are consistent with earlier studies in diabetic and hyperinsulinemic animal models which have shown reduced mRNA expression of the ABCG5 and ABCG8 cholesterol efflux transporters (Lally et al. 2006).

The results showed combined EZ+SV treatment had no significant effect on cholesterol S-M transport in MetS rats. These findings are contradictory to previous studies which have shown that SV increases the expression of intestinal efflux transporters (Lally et al. 2006). Statin therapy increases intestinal ABCG5

and ABCG8 mRNA expression in diabetic patients (Lally et al. 2006). However, to date there has been no study directly examining the effects of EZ on intestinal ABCG5 and ABCG8. Further studies measuring mRNA and protein expression of intestinal cholesterol absorption transporters (NPC1L1, SR-BI and FAT/CD36) and efflux transporters (ABCA1, ABCG5 and ABCG8) following EZ+SV therapy are needed to help understand these findings. Inhibition of cholesterol absorption transporters (NPC1L1, SR-BI and FAT/CD36) will help determine whether the observed effects of EZ+SV treatment on S-M cholesterol transport are solely attributed to the ability of these drugs to modulate efflux transporters. Similarly, inhibiton of efflux transporters (ABCA1, ABCG5 and ABCG8) will provide further understanding of whether the reduction in M-S cholesterol transport following EZ+SV treatment is solely attributed to the ability of these drugs to modulate activity of cholesterol absorption transporters or whether the findings of these findings are a result of the actions of EZ+SV treatment on bi-directional cholesterol flux.

Intestinal Net Cholesterol Influx & Efflux:

To help further explain M-S and S-M cholesterol transport results, net cholesterol influx and net cholesterol efflux were determined for treated and control JCR:LA-*cp* rats. MetS rats demonstrated an increased net cholesterol influx and a decreased net cholesterol efflux compared to lean rats. These findings are consistent with transporter expresssion studies which have shown NPC1L1 mRNA to be increased, and ABCG5 and ABCG8 mRNA to be decreased in type

II diabetic patients (Lally et al. 2006). EZ treatment either alone or in combination with SV decreased net cholesterol influx compared to control MetS rats. This is consistent with other studies showing that both EZ and SV have the potential to lower intestinal NPC1L1 mRNA expression, and SV can up-regulate ABCG5 and ABCG8 expression in diabetic subjects (Bays et al. 2008, Lally et al. 2006, Garcia-Calvo et al. 2005). In addition, EZ treatment alone appeared to normalize cholesterol efflux (S-M cholesterol flux) and net cholesterol efflux. These findings would suggest that EZ monotherapy may up-regulate the TICE pathway.

3.4.2 Effects of Ezetimibe & Simvastatin on Intestinal Lymphatic Chylomicron Production During MetS

To date there have been no studies examining the direct effects of EZ+SV on lymphatic CM production during MetS. Previous studies have shown that EZ lowers lymphatic cholesterol concentration without any concomitant change in endogenous intestinal cholesterol synthesis in male Sprague-Dawley rats (van Heek et al. 2003). These findings were limited in that rats were pre-gavaged with TG and thus only the effects of EZ at the peak period of post-prandial lymphatic CM secretion were reported, and there was no assessment of CM production during basal and postprandial conditions. The production of CM in the latter conditions are critical to understand in order to determine the direct impact of pharmaceutical and dietary compounds on CM metabolism. Clincial studies in hyperlipidemic men with type II diabetes have shown that EZ treatment either alone or in combination with SV decreases plasma apoB48 remnant lipoprotein

concentrations (Tremblay et al. 2006, Tremblay et al. 2009). However, clinical studies measuring apoB48 concentrations do not reflect a direct measure of intestinal CM production, as there are many other factors influencing plasma concentration of apoB48 lipoproteins including lipolysis and uptake by tissues.

Our study utilized an adapted lymphatic cannulation procedure that enabled us to directly measure intestinal lymphatic lipid (TG and cholesterol) and apoB48 secretion during the fasted basal and post-prandial state. In the present study, lymph isolated following saline infusion (fasted state), demonstrated a trend towards increased apoB48 concentration in MetS rats compared to lean rats. Previous studies in our laboratory have shown that Mets animals have significantly greater lymph apoB48 concentrations compared to their lean counterparts in the fasted state (Vine, Glimm & Proctor 2008). The lack of significance in the present study may be attributed to a small sample size. However, in the post-prandial state MetS rats demonstrate CM over-production (as determined by increased lymphatic apoB48 concentration) compared to lean animals which is consistent with earlier data from our laboratory (Vine, Glimm & Proctor 2008).Increased production of CM-apoB48 in the post-prandial state has been suggested to be a result of increased lipid availability in the enterocyte (Duez, Pavlic & Lewis 2008, Vine, Glimm & Proctor 2008). Correspondingly, increased lymphatic triglyceride and cholesterol concentrations are observed in the postprandial state in both MetS rats and lean rats, supporting the hypothesis that apoB48 overproduction is partly attributed to increased intestinal substrate availability in the enterocyte. However lymphtatic TG and cholesterol

concentrations were greater in the MetS animals compared to lean animals in the postprandial state. Net cholesterol absorptive influx was increased, and net cholesterol efflux was decreased in the MetS compared to lean animals, which may contribute to increased enterocyte intracellular substrate availability. An increase in intestinal and intracellular substrate availability in the MetS condition may also be explained by enhanced *de novo* lipogenesis and/or elevated storage capacity. Studies conducted by Duez et al and Zoltowska et al have shown intestinal lipogenic enzymes such as SREBP-1c, monoacylglycerol transferase (MGAT) and diacylglycerol transferase (DGAT) to be up-regulated in animal models of IR (Vine, Glimm & Proctor 2008). SREBP-1c, MGAT and DGAT mRNA determination in jejunal segments from this study would help to support the enhanced lipogenesis theory. Recent findings from our laboratory have shown that the JCR:LA-*cp* demonstrates anatomical hypertrophy of the intestinal mucosal villi and an increase in the number of enterocytes/cm^3 (Duez, Pavlic & Lewis 2008, Duez et al. 2008, Zoltowska et al. 2003). The intestinal hypertrophy in the JCR:LA-*cp* rat may in part contribute to the observed over production of CM and increased lipid secretion under of MetS conditions.

EZ monotherapy had no significant effect on lymphatic apoB48, TG or cholesterol concentration. However, EZ monotherapy increased the ratio of cholesterol:apoB48 following both saline and intralipid infusion. Thus, it may be speculated that the EZ induced reduction in net cholesterol influx in MetS may consequently up-regulate endogenous cholesterol synthesis or plasma cholesterol

uptake, resulting in increased cholesterol availability for incorporation into CM particles (Bays et al. 2008, Sudhop et al. 2002).

Combined EZ+SV treatment significantly increased lymphatic apoB48 concentration following intralipid infusion compared to control MetS rats. These results are contradictory to findings by Tremblay et al who have shown that co-administration of EZ+SV decreases triglyceride rich lipoprotein apoB48 pool size, mainly by lowering intestinal production rate of lipoproteins (Tremblay et al. 2009). We speculate that combined EZ+SV therapy may up-regulate microsomal triglyceride transfer protein (MTP) expression. It has been proposed that enhanced apoB48 production may be a result of intracellular apoB48 stability. The efficient lipidation of apoB48 by MTP inhibits proteosomal degradation of the apoprotein. Thus EZ+SV treatment may increase apoB48 production by up-regulating MTP which increases the intracellular stability of apoB48. Furthermore, addition of SV to EZ treatment ameliorated the EZ-induced increase in the ratio of cholesterol:apoB48, and significantly lowered cholesterol content of CM particles compared to control MetS rats. We speculate that these findings may also be a result of EZ+SV potential ability to increase MTP expression and consequently increase apoB48 production, thus lowering the cholesterol content associated with each particle. The ability of EZ+SV treatment to decrease CM particle cholesterol content may have implications on the atherosclerotic process as these particles are potentially less atherogenic due to lower cholesterol content

Thus, we propose that EZ+SV treatment may decrease cholesterol deposition within arterial walls due to reduced cholesterol content per CM particle.

In conclusion, the results of this study indicate that combined EZ+SV therapy decreases net cholesterol absorptive influx in the intestine. Furthermore, the study findings have revealed that combined EZ+SV therapy ameliorates an EZ induced increase in cholesterol:B48 of CM particles perhaps by up-regulation of apoB48 production. The results confirm that combined EZ+SV therapy which targets cholesterol absorption and biosynthesis is effective at lowering cholesterol content of CM particles and potentially lowers their atherogenic potential.

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Chapter 4. Effect of Ezetimibe and Simvastatin on the Arterial Uptake of Chylomicron Remnants and Myocardial Lesion Development.

4.1 Introduction and Rationale

Atherosclerosis is thought to begin with the focal accumulation of cholesterol rich lipoproteins within the arterial wall which initiates an inflammatory response (Ross 1999). Historically, plaque cholesterol was thought to be derived primarily from LDL (Mediene-Benchekor et al. 2001). However, pioneering work has provided further evidence that chylomicron-remnants (CM-r) (derived from intestinal chylomicron (CM) particles) contribute significantly to the atherosclerotic process (Proctor, Vine & Mamo 2002, Proctor 2000).

The uptake (otherwise known as delivery) of lipoproteins into the arterial wall is thought to be dependent primarily on particle size. Palade and Simionescu were the first to suggest that the primary mechanism by which lipoproteins can be 'delivered' to arterial tissue, occurs via transcytosis (Simionescu, Simionescu 1991, Palade, Simionescu & Simionescu 1979). Transcytosis describes the process whereby vesicles (70-80nm in size) are formed at a constant rate on the plasma membrane and migrate to the basolateral membrane where they expel their contents (Simionescu, Simionescu 1991). Lipoproteins smaller than 80nm such as CM-r (45-55nm) and LDL (26nm) are thought to traverse the endothelium via the transcytotic pathway (Proctor, Mamo 1996). However, additional associated transport mechanisms have also been implicated in the delivery of lipoproteins to the arterial wall, including heparin sulphate proteoglycans, lipoprotein lipase (LPL) and the LDL-receptor related protein (see figure 4-1) (Goldberg, Merkel 2001, Goldberg 1996, Ji et al. 1993, Ji et al. 1994, Ji, Sanan & Mahley 1995). It

has been speculated that smaller particles are delivered to the arterial tissue more efficiently, at a faster rate than larger particles, and thus are potentially more atherogenic (Proctor, Mamo 1996).

Arterial Retention of Lipoproteins:

The arterial retention of lipoproteins is thought to be the net result of the number of lipoproteins delivered and effluxed from the arterial wall. It has been proposed that particles that have a greater propensity to efflux from the arterial wall, are less likely to be retained. For example, LDL particles have been shown to have an efflux rate 20 fold greater than that of CM-r, and this is attributed to the smaller size of LDL particles (15-30nm) compared to CM-r (45-60nm) (Proctor, Vine & Mamo 2002, Proctor, Vine & Mamo 2004). Thus, the greater retention of CM-r within the vessel wall may partly be attributed to a slower efflux rate. Furthermore, it has been demonstrated that retention of CM-r within the vessel wall is not uniform and that there are focal points where remnant lipoproteins accumulate (Proctor, Mamo 2003). Previous studies have shown that the distribution of remnants within the vessel wall correlates spatially with the distribution of arterial proteoglycans (Proctor, Mamo 2003). Boren and colleagues revealed that apoB48 may have a greater affinity for select proteoglycans in comparison to apoB100, which may contribute to a greater retention of apoB48 containing CM-r (Flood et al. 2002, Flood et al. 2004, Skalen et al. 2002). Moreover, it has been shown that under experimentally controlled conditions, the net accumulation of cholesterol derived from CM-r within the arterial wall is four-fold greater than that deposited by LDL particles (Proctor,

Vine & Mamo 2002). These findings correspond with recent preliminary studies indicating that binding of intestinally derived CM-r to human biglycan is associated with an increased concentration of particle cholesterol compared to LDL (Mangat et al, 2008).

Despite these recent advancements in understanding remnant atherogenicity, there are still relatively few studies that have investigated the potential ability of current pharmaceutical interventions in hypercholesterolemia to reduce lipoprotein remnant derived cholesterol uptake by arterial vessels.

Increased deposition of lipoprotein-derived cholesterol during insulin resistance:
Obesity and insulin resistance (IR) in the MetS pre-dispose individuals to increased CVD risk and the development of type 2 diabetes which is also associated with enhanced cardiovascular complications (Tomkin, Owens 2001, Despres, Marette 1994). There is accumulating evidence that intestinally derived apoB48 remnant lipoproteins may contribute to atherogenic dyslipidemia during conditions of IR and diabetes (Proctor, Vine & Mamo 2002, Karpe et al. 1994, Zilversmit 1995, Cabezas, Erkelens 2000, Huff 2003). Recent animal and human studies have shown that IR can lead to the over-production of intestinal remnant lipoproteins which may contribute to both fasting and postprandial dyslipidemia (Duez et al. 2008). Duez et al. (2006) and Hogue et al. (2007) have demonstrated an increase in the production rate of apoB48 in IR and diabetic patients, respectively. In addition, impaired post-prandial clearance of CM and their remnants is associated with an increased risk of CVD due to the pro-longed circulatory time of CM-r and exposure to the arterial wall (Proctor, Vine & Mamo

2002). MetS and IR are further associated with an exacerbated risk of CVD associated with impaired post-prandial clearance.

The increased entrapment of particles within the vessel wall under clinical conditions of MetS and type 2 diabetes is attributed to the increased secretion of collagen and proteoglycans (PG) by local arterial smooth muscle cells (Tannock et al. 2002, Little et al. 2002). Studies have demonstrated that remodelling of the arterial matrix occurs in certain disease conditions such as diabetes, IR and hypercholesterolemia. Altered matrix PG content in diseased conditions can influence the rate at which lipoproteins accumulate in the vessel wall (Camejo et al. 2002, Raines, Ferri 2005). Select isoforms of proteoglycans are known to contain ligands for apolipoprotein E, C and B, and are a primary mechanism by which atherogenic lipoproteins bind to the arterial wall (Flood et al. 2002, Olin-Lewis et al. 2002). Similarly, certain lipid lowering treatments such as the 'statin' class of drug compounds have been shown to reduce the production of pro-atherogenic arterial proteoglycans. (Siegel-Axel et al. 2003, Riessen et al. 1999).

Collectively, the mechanisms associated with predicting the degree of cholesterol entrapment in arterial vessels is complex and multi-factorial. What we now appreciate is that these factors such as particle number, cholesterol content, arterial permeability and entrapment of particles are likely to be up-regulated during conditions of IR and diabetes, and in turn significantly increase the potential for cholesterol-enriched remnant particles to accumulate in the arterial wall.

A conceptual model has been proposed by Warnakula et al (2010) (*publication in progress*) and describes the deposition of cholesterol in arterial vessels as the sum of particle exposure (i.e. time), binding affinity of particle (i.e. permeability, attachment and removal), available PG binding sites and the particle cholesterol capacity (i.e. size and composition) (see Fig 4-1).

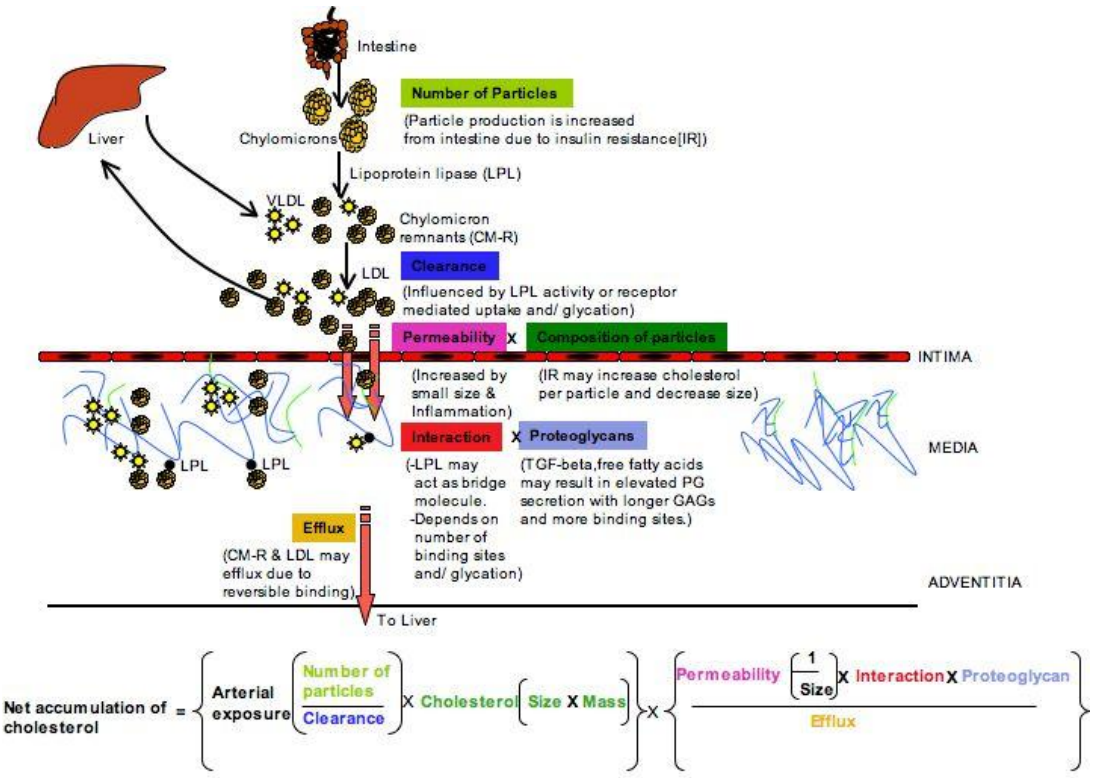

Figure 4-1. Proposed model of factors determining cholesterol accumulation. During conditions of IR and/or obesity intestinal CM production and secretion are exaggerated. CM overproduction is thought to contribute to the accumulating presence of TAG-rich particles in plasma. Furthermore, delayed clearance of apoE-containing CM-r particles via the LDL-r (LDL-receptor) and the LRP-r (LDL related protein-receptor) pathways ensures continued exposure of atherogenic particles to the arterial wall. Small, dense, cholesterol-rich CM-r particles penetrate the vessel wall and become entrapped in the subendothelial space. Chronic IR and diabetes induce vascular remodelling of extracellular arterial PG resulting in an increase in the net number of PG binding sites for atherogenic lipoproteins. These factors facilitate increased binding of CM-r to the vessel wall. In addition, it has previously been shown that LDL-C effluxes more readily than CM-r suggesting that CM-r are preferentially retained within the arterial wall.

Ezetimibe and simvastatin treatment:

Statin's such as simvastatin (SV) lower serum cholesterol by inhibiting the rate limiting step in the cholesterol biosynthesis pathway via HMG-CoA reductase (Bays et al. 2008, Holdgate, Ward & McTaggart 2003, Bays, Stein 2003, Shepherd et al. 1995). In contrast, ezetimibe (EZ) selectively reduces intestinal cholesterol absorption without altering TG or fat-soluble vitamin bioavailability (Catapano 2001). The key to EZ bio-functionality is the ability to reduce intestinal cholesterol absorption via the Niemann Pick C1 Like 1 transporter, which includes both biliary cholesterol and dietary cholesterol (Bays et al. 2008, Davis et al. 2004, Garcia-Calvo et al. 2005). In chapter three, it was revealed that combined EZ+SV treatment reduced net cholesterol absorption and cholesterol content of CM particles in lymph. In this chapter, the aim is to elucidate whether significant reductions in lymphatic CM cholesterol can influence the extent of CM-r binding to the arterial wall *in-vivo*.

Preliminary data from our group has shown that there is exacerbated arterial retention of CM-r lipoproteins in an animal model of IR (the JCR:LA-*cp* rat). At present the effects of EZ and combined EZ+SV therapy on the arterial retention of apoB48 remnant lipoproteins remains unknown.

Furthermore, the second aim of this chapter is to determine if combined EZ and SV treatment ameliorates atherosclerotic lesion development in the JCR:LA-*cp* rat. The JCR:LA-*cp* rat has previously been shown to spontaneously demonstrate early arterial and myocardial atherosclerotic lesion development (Mangat et al. 2007, Russell, Graham & Richardson 1998).

4.2 Methods

4.2.1 Animal Model and Overall Study Design

JCR:LA-*cp* rats were raised in our established breeding colony at the University of Alberta, as previously described (Vine et al 2007). At 6 weeks of age, male JCR:LA-cp/cp rats with MetS received a lipid balanced diet supplemented with 1%w/w cholesterol prepared from standard rodent formulation (Harlan Teklad). Dietary lipid was 30% w/w consisting of flaxseed oil, tallow, sunflower oil and olive oil yielding a saturated-to-polyunsaturated fat ratio of 1:1. The composition of the other nutrients (w/w) was carbohydrate 49%, crude protein 28%, moisture 10%, minerals 4% and fibre 6%. MetS rats were randomized to either one of the following treatments: EZ(n=4) (0.01% w/w) or combined EZ (0.01%w/w) and SV (0.01% w/w) (n=4) treatment for 8 wks at approximately 8.0mg/kg body weight. Rats were housed with a 12 hour light/dark cycle, caged individually and had ad libitum access to food and water. Food intake and body weight were measured daily. 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.

4.2.2 Study Design I and Methods

Study Design I*: To assess fasting and post-prandial plasma biochemistry following an oral fat challenge and meal tolerance test.*

JCR:LA-*cp* rats at 6 wks of age were randomized to either control, EZ (n=4, 0.01%) or EZ+SV (n=4, 0.01%) treatment for 8 wks. Following treatment, rats

were fasted overnight for 16 hrs and fasting plasma biochemical profile was determined. Postprandial insulin/glucose metabolism was assessed via a standardized 'meal tolerance test (MTT)' (Russell et al 1999). Postprandial apoB48, TG and cholesterol response were determined using a standardized 'oral fat challenge (OTC)' (Vine et al 2007) (See Figure 4-2).

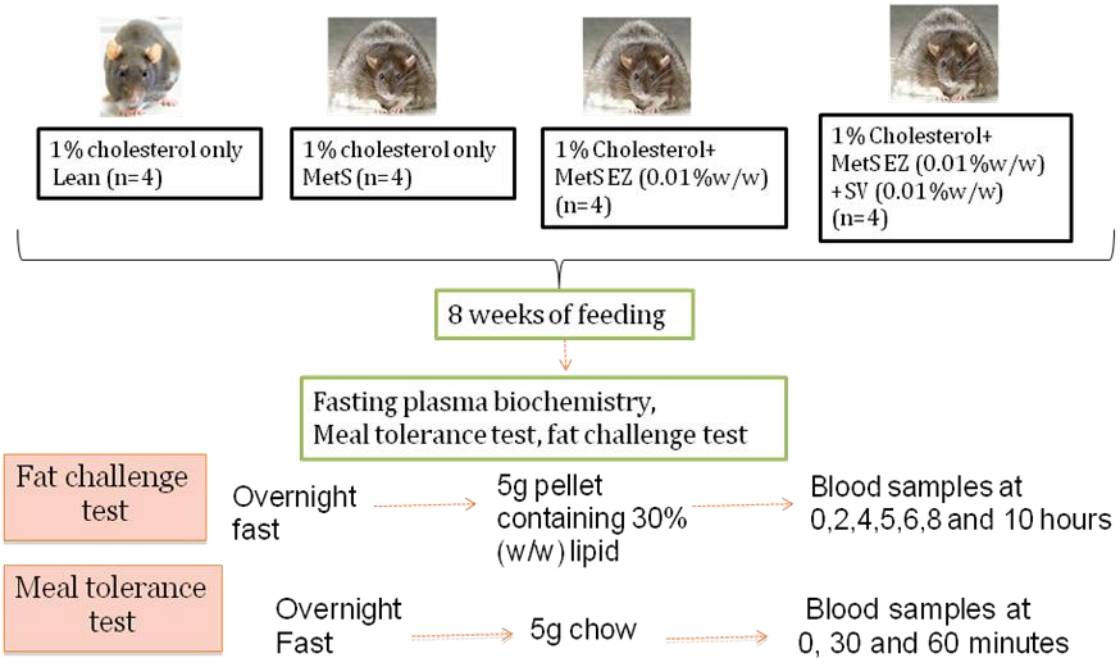

Figure 4-2. Study design I: To assess fasting and post-prandial plasma biochemistry following an oral fat challenge and meal tolerance test.

4.2.2.1 Metabolic Assessment

Fasting Plasma Biochemical Profile:

Plasma biochemical profile was assessed using commercially available enzymatic colorimetric assays. Direct enzymatic colorimetric assays were used to measure the following: triglyceride (WAKO, Chemicals USA Inc., Richman, VA, USA, Cat#998-40391/994-40491), LDL (LDL-C, WAKO Cat#993-00404/999-00504),

total cholesterol (TC, WAKO, Cat#439-17501), and HDL-cholesterol (HDL-C, Diagnostic Chemical Ltd., Charlottetown, Prince Edward Island, Cat#258-20). Insulin (ALPCO Diagnostics, USA, Cat#80-INSRT-E01) was measured using commercially available EIA kits and glucose determined via the glucose oxidase method (Diagnostic Chemicals Ltd., Cat#220-32).

Postprandial plasma insulin and Glucose Response to Meal Tolerance Test:

JCR:LA-*cp* rats were fasted overnight for 16 hours and re-fed 5.0g of standard rat chow as previously described by Russell et al (Russell, Graham & Dolphin 1999). Blood samples were taken using the standardized tail vein procedure at 0, 30 and 60 minutes. K_2EDTA (ethylene diamine tetraacetic acid, BD Franklin Lakes NJ USA, Cat#367835) tubes were used to collect blood and plasma was separated via centrifugation at 3000rpm at 4^{o}C for 10 mins. These samples were then aliquoted and frozen at -80^{o}C for for further analysis of insulin and glucose using commercially available colorimetric and enzymatic assays, as described above.

Postprandial Plasma Lipid and ApoB48 Response to Oral Fat Challenge:

JCR:LA-*cp* rats were fasted overnight and then were fed a 5.0g pellet containing 30% (w/w) lipid. Following the meal consumption, blood samples were taken via an established tail vein procedure (Proctor, Kelly & Russell 2005) at times 0, 2, 4, 5, 6, 8 and 10h. K_2EDTA tubes were used to collect blood samples and plasma was separated by centrifugation at 3000 rpm at 4˚C for 10 min. Plasma samples were then aliquoted and frozen at -80^{o}C for further lipid and apoB48 analysis using colorimeteric kits (as described above) and SDS-PAGE/ECL procedures respectively (as previously described in section 3.2.4.1, Vine et al 2007).

4.2.3 Study Design 2 and Methods

Study Design 2*: Ex-vivo retention of remnant lipoproteins and myocardial lesion development.*

JCR:LA-*cp* rats at 6 wks of age were randomized to either control, EZ (n=4, 0.01%) or EZ+SV (n=4, 0.01%) treatment for 8 wks. Cy5 labelled chylomicron remnants (Cy5-CM-r) were generated from donor lymph-cannulated rats from treated or control groups followed by rabbit hepatectomy procedure. *Ex-vivo* carotid perfusion of Cy5-CM-r preparations was carried out under physiological conditions of flow rate and pressure and assessed by semi-quantitative confocal microscopy (figure 4-3). Myocardial lesion development was assessed from hearts derived from treated and control JCR:LA-*cp* rats.

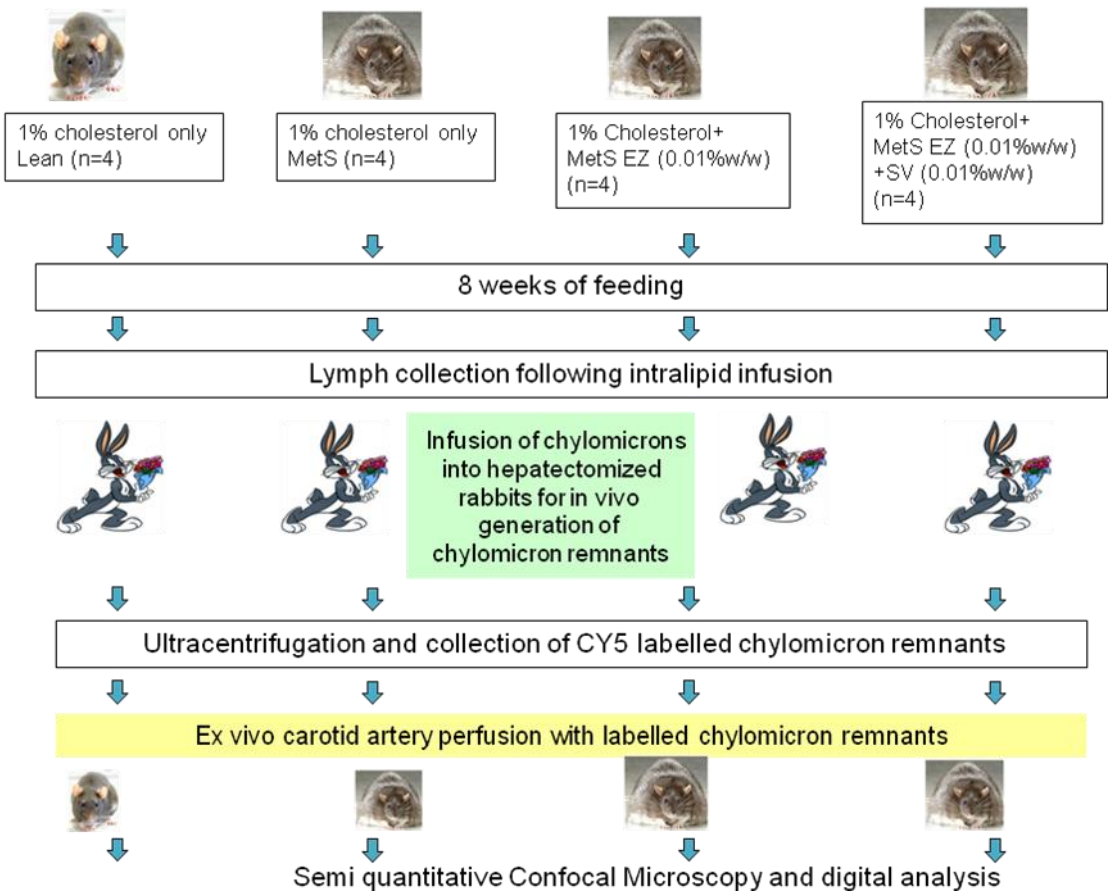

Figure 4-3. Study design II to assess *ex-vivo* retention of remnant lipoproteins.

4.2.3.1 In-situ Perfusion of Fluorescent Chylomicron Remnants in Carotid Arteries of JCR:LA-cp Rats.

Lymph Cannulation and Nascent Chylomicron Isolation:

MetS JCR:LA-*cp* rats 6 wks of age were randomized to either one of the following treatments for 8 weeks: leans, MetS controls, MetS with EZ (n=4,0.01% w/w) or MetS with EZ (0.01%w/w) and SV (0.01% w/w) (n=4). Following treatment rats were fasted for 16 hrs to equilibrate gastro-intestinal contents. The superior mesenteric lymph duct and gastric-duodenal axis were cannulated. Animals were infused with an intralipid solution (20% intralipid, 4% glucose) into the duodenum. Lymph was collected from the superior mesenteric duct into EDTA (conc) tubes following intralipid infusion over a 4 hr period. Short speed centrifugation (3000rpm at 4^{o}C for 10 mins) was used to remove contaminating leukocytes and CM were isolated from lymph via density gradient ultracentrifugation as previously described (Proctor et al 1998).

Amine Conjugation:

1.5g of CM triglyceride was labelled with Amersham Fluorolink Cy5 Reactive Dye-5 pack (670nm Far Red Emission, Amersham catalogue number PA2500) as previously described (Proctor et al. 1998). Briefly, fluorescent cy5 dye was added drop wise to prepared CM (pH=7.0). Lymph CM were incubated with Cy5 dye at RT^{o}C for 1 hr with gentle agitation. Hydroxylamine pH=7.4 (Hydroxylamine: Cy5 dye 1:1 v/v) was added in small increments over a 45 min period at RT^{o}C to quench conjugation. Fluorescent CM were collected by elution through Econo-Pac 10PG columns (Biorad catalogue number 732-2010). Unbound fluorescence was removed by extensive dialysis against phosphate buffered saline (PBS)

pH=7.0. Proctor et al (1998) previously established that the Cy5 fluorophore does not exchange with other plasma macromolecules, is bound to the protein and does not alter the clearance kinetics of CM from plasma.

Preparation of Chylomicron Remnants:

Male New Zealand white rabbits were anaesthetized with ketamine/xylazine (30mg/kg and 5mg/kg i.m respectively). These rabbits were hepatectomized and administered 1.5g of Cy5 labelled CM triglyceride via a femoral vein catheter over a 5 minute period. The dose of CM triglyceride administered represents almost twenty times the normal endogenous pool of this lipid. The hepatectomy ensures the CM particles cannot be metabolized by the liver. Hypoglycaemia was prevented by 1.5ml/min glucose infusion through the femoral vein. CM were allowed to circulate in plasma for 3 hrs for lipolysis of CM by endothelial lipases, which prevents any further reduction in plasma triglyceride (Mamo 1996). Bowler et al previously validated that lipolysis is complete 2.5 hr after injection of CM into eviscerated rabbits and that no further reduction in plasma triglyceride can be observed. At 3 hours plasma was collected from the abdominal aorta. CM-r were isolated from plasma by density gradient ultracentrifugation at a density of 1.006g/ml ($2.256x10^6$g/hr) as previously described (Proctor et al 1998).

Analysis of Chylomicron Remnant Biochemical and Lipid Profile:

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine apoB48 concentration of remnants. Cholesterol (TC, WAKO, Cat#439-17501) and triglyceride (WAKO, Chemicals USA Inc., Richman, VA, USA, Cat#998-40391/994-40491) concentrations were determined for all CM-r preparations isolated from treatment and control groups. Particle size was determined using laser diffraction and standard particle size alogorithms (Beckman BI-90) and particles had a confirmed diameter of 45-55nm.

Ex-vivo perfusion of Cy-5 labelled remnant lipoproteins:

JCR:LA-*cp/cp* rats were anaesthetized with isoflourane and both the left and right carotid arteries were located and exposed by the clearing of connective tissue fascia. The carotid artery was chosen because it is a large artery with relatively few tributaries, minimising the duration of surgery and optimising in-situ perfusion conditions. Carotid artery segments were cannulated at the proximal and distal ends, and connected to a Harvard Perfusion apparatus (HSE UNIPER UP-100 Type 834). Flow rate and pressure were monitored at all times during perfusions through the Harvard Perfusion apparatus, as to reproduce the normal physiological conditions of flow rate (15mls/min) and pressure (90mmHg). The concentration of apoB48 in the perfusate was standardised to 100µg/ml for all experiments to ensure that the same number of particles were delivered to all vessels. Fluourescent CM-r were perfused within the carotid arteries for 25 mins and this was followed by a wash out period with phosphate buffered saline (PBS)

for 15 mins. The additional PBS perfusion enables the non-bound CM-r to efflux from the vessel wall, under normal physiological conditions allowing for the determination of CM-r arterial retention. Following perfusion, carotid vessels were rapidly removed and fixed in 2% Paraformaldehyde (Sigma®catalogue # P-6148) for 20 mins. Carotid segments were frozen in liquid nitrogen and sectioned by cryostat (approximately 3-4 microns thick) for analysis by 3D-confocal microscopy.

4.2.3.2 Quantification of Lipoprotein Arterial Uptake Using Fluorescent Digital Analysis

Confocal Laser Scanning Microscopy and Data Analysis:

Confocal laser (Kr/Ar) scanning microscopy (Zeiss 510) was used to visualize fluorescent lipoproteins and images were viewed by Zeiss LSM Image Browser Version 4.2.0.121. Confocal microscopy allows data to be collected in three dimensions (X,Y and Z) and through several Z-planes. Thus, intensity of fluorescence per unit volume of arterial tissue can be calculated (Figure 4-4)

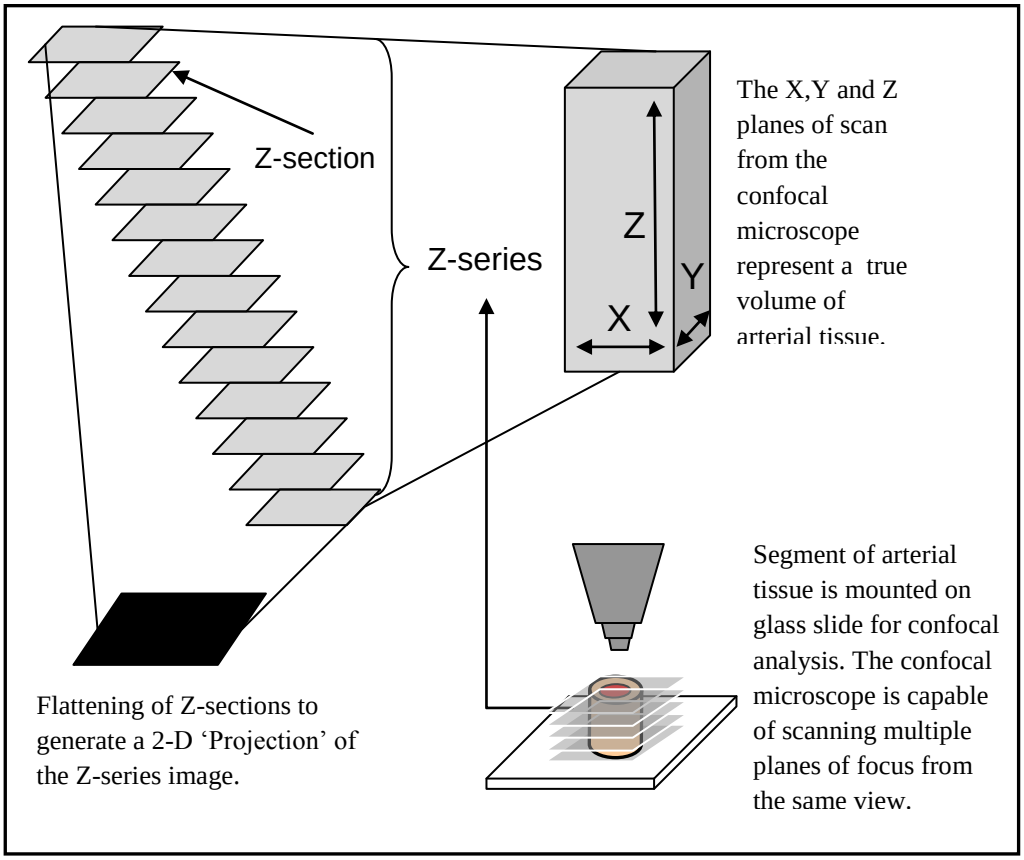

Figure 4-4. Laser Scanning of arterial segments using confocal microscopy adapted from S.D. Proctor (2001). Arterial segments perfused with Cy5 labelled chylomicron remnants are removed from JCR:LA-cp rats, frozen and sectioned. Arterial segments are mounted onto slides for analysis. The confocal microscope acquires fluorescence associated with both lipoproteins and the auto-fluorescence of the arterial section. The confocal laser scans through a designated volume of the arterial segment to generate a three dimensional Z-series image.

Fluorescence associated with the Cy5 fluorophore (670nm emission) was collected from arterial sections using the Far Red emission filter (i.e. 696-664nm). Arterial morphology images were collected using 540/32nm (green) emission filter which visualized 'auto-fluorescence' of carotid sections.

The final output from the confocal microscope was in the format of a 'LSM' image file that contained the complete Z-series of images.

Quantification of Fluorescence:

The fluorescence intensity associated with CM-r particles was determined by digital analysis using Image J software (version 1.36b) and the plug-in LSM reader. An area of interest (doughnut shaped) was traced on the carotid cross section image using the tools in image J which enabled exclusion of non-specific fluorescence from the image. The pixel intensity was determined for the traced section of each Z-section in the Z-series image and was calculated by the Image J 1.36b LSM Reader software. The final intensity was calculated as the sum of intensities on all the images of each Z-section. The interval between the z-sections was kept constant. The volume of each section was calculated as the product of: area, number of slices, and the interval. The total intensity and the volume were used to calculate the intensity per unit volume of each carotid section.

Retention of CMr-apoB48 and cholesterol ex-vivo:

The biochemical profile for CM-r was determined prior to each experiment. Direct enzymatic colorimetric assays were used to measure triglyceride (WAKO, Chemicals USA Inc., Richman, VA, USA, Cat#993-00404/999-00504) and total cholesterol (TC,WAKO,Cat #439-17501). ApoB48 was quantified using an adapted western immune-blot technique previously described in section 3.2.4.1.

The fluorescence intensity of the perfusate per unit volume was also collected for every experiment, under identical conditions in Z-series. The following formula was used to calculate the retention of apoB48 and cholesterol in the arterial tissue: Concentration of apoB48 or cholesterol in the perfusate / intensity per unit volume of the perfusate * intensity per unit volume of the tissue.

4.2.4 Statistical Analysis

ApoB48 and cholesterol retention within carotid arteries was compared between control and treatment groups using one-way ANOVA (Tukeys multiple comaparison, GraphPad PRISM). All results are expressed as the mean ±S.E.M.

4.2.5 Heart Histology and Atherosclerotic Lesion Analysis

Histological slides of cardiac tissue were subjected to analysis by an independent, external expert in the field of atherosclerotic myocardial lesions. Both the stage of the lesion and frequency were determined. Lesions were categorized by the following criteria previously determined by Russell et al (1998):

Stage 1: areas of necrosis without significant chronic inflammatory cell infiltration. Lesions are less than 1 day old. (Figure 4-5 panel A).

Stage 2: areas of cell lysis with active chronic inflammatory cell infiltration. Lesions are days to a week old. (Figure 4-5, panel B).

Stage 3: nodules of chronic inflammatory cells, without any visible dead monocytes. (Figure 4-5, panel C).

Stage 4: old, scarred lesions with no significant remaining inflammatory cell activity. Lesions are mature, contracted with prominent collagen bands and represent the end-stage of the reparative process at ages from 4 weeks and greater. (Figure 4-5, panel D).

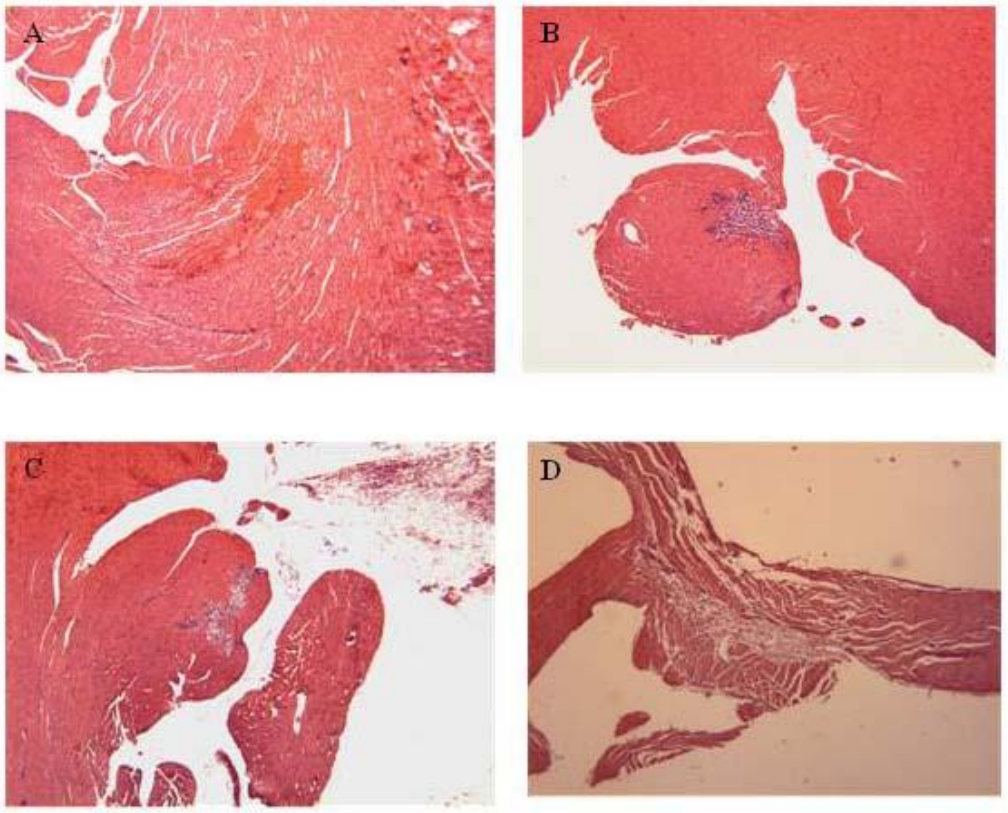

Figure 4-5.Representative micrographs of myocardial lesions of the heart from JCR:LA-*cp* rats (14 weeks of age). **A:** Stage 1 lesion; area of necrosis without long-term inflammatory cell infiltration. **B:** Stage 2 lesion; area of long-term inflammatory cell infiltration. **C:** Stage 3 lesion; area of active inflammatory cell activity and cell lysis. **D:** Stage 4 lesion: early scarred lesion with a small number of inflammatory cells or fibroblasts. All images at x2, H&E stained sections.

4.3 Results

4.3.1 Food intake, organ and body weights

Food intake and Body Weight:
At 6 wks of age, JCR:LA-*cp* rats were randomized to a 1% cholesterol supplemented lipid balanced diet (LBD) with either EZ (0.01% w/w), or both EZ (0.01% w/w) and SV (0.01% w/w) for 8 wks (n=4 for each group). No statistical difference was observed in food intake (figure 4-5) or body weight (figure 4-6) between treatments and the control MetS groups.

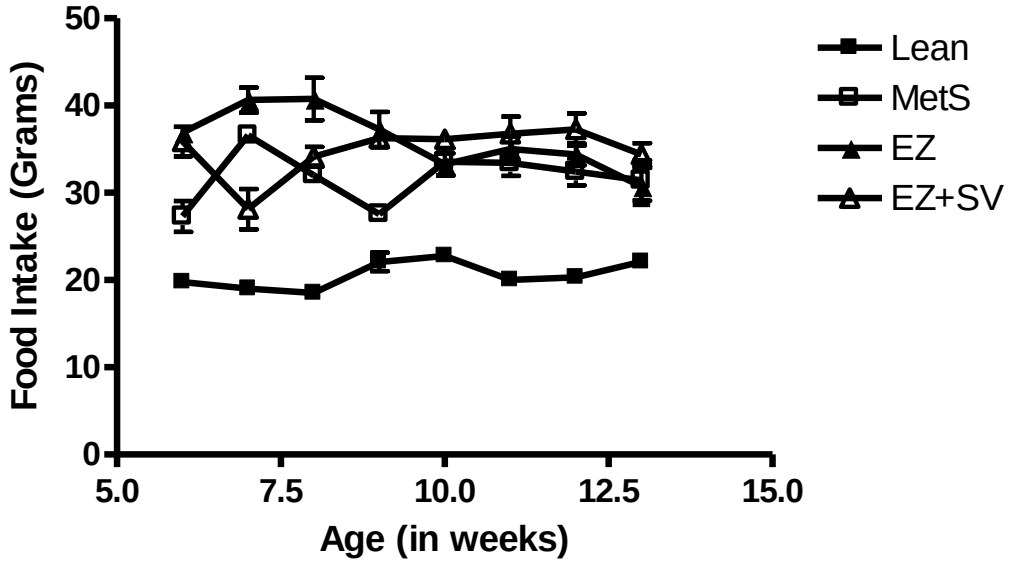

Figure 4-6. Food consumption of JCR:LA-*cp* rats over the 8 week period during EZ and SV treatment.

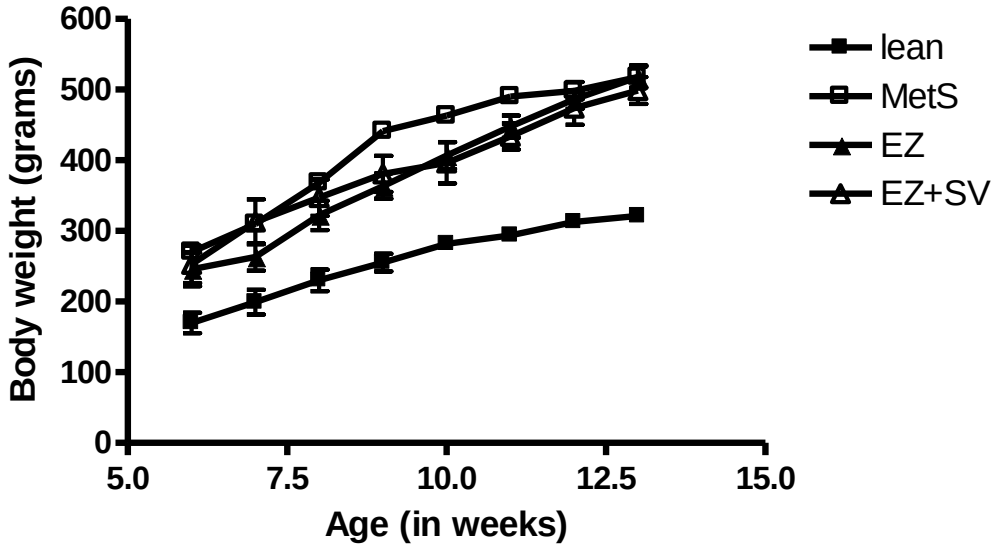

Figure 4-7. Body weight gain of JCR:LA-*cp* rats over the 8 week period during EZ and SV treatment.

Organ weights:

The weights of heart (1.15±0.02g vs. 0.9±0.02g), liver (15.63±0.77g vs. 8.26±0.48g) and kidney (1.5±0.01g vs. 1.1±0.01g) of control MetS rats were significantly greater compared to their lean counterparts. EZ treatment either alone or in combination with SV significantly reduced liver weight (see table 4-1). No statistical differences were observed in kidney weight between treatment groups and control MetS rats.

	Lean	**MetS**	**EZ**	**EZ+SV**
Heart (g)	0.9±0.02	1.15±0.02*	1.14±0.03	1.08±0.08
Liver (g)	8.26±0.48	15.63±0.77***	13.27±0.85$^{\Delta}$	12.34±1.06$^{\Delta}$
Kidney (g)	1.1±0.01	1.5±0.01**	1.44±0.14	1.21±0.08

Table 4-1. Organ weight of JCR:LA-*cp* rats following 8 week intervention with EZ and SV simvastatin. (*) *p<0.05*, (**) *p<0.01* and (***) *p<0.001* denotes statistical significance between control MetS rats Vs. lean rats. (Δ) *p<0.001* denotes stastical difference between treated (EZ, EZ+SV) rats Vs. control MetS rats.

4.3.2. Fasting plasma biochemical profile

MetS rats were observed to have significantly greater concentrations of TG, cholesterol, LDL and HDL compared to their lean counterparts (table 4-2). Both treatment groups reduced plasma total cholesterol compared to control MetS rats (EZ; -20% $p<0.05$) and (EZ+SV; -25% $p<0.01$). EZ treatment lowered plasma LDL-C either alone (-43%, $p<0.01$) or in combination with SV (-46% $p<0.05$), compared to the control MetS group (table 4-2).

Parameters	**Lean**	**MetS**	**EZ**	**EZ+SV**
TG (mg/ml)	0.35±0.09	1.7±0.21*	2.3±0.25	1.47±0.04
Cholesterol (mg/ml)	0.83±0.05	1.82±0.05**	$1.45\pm0.005^{\Delta}$	$1.36\pm0.08^{\Delta\Delta}$
LDL (mg/dl)	16.18±1.9	34.4±3.6*	$14.9\pm2.87^{\Delta\Delta}$	$15.68\pm3^{\Delta}$
HDL (mg/dl)	18.85±1.47	49.38±4.98**	39.41±2.95	35.21±2.73

Table 4-2. Fasting plasma biochemistry of JCR:LA-*cp* rats following 8 weeks intervention with EZ and SV. (*) $p<0.01$ and (**) $p<0.001$ denotes statistical significance between control MetS rats Vs. lean rats. (Δ) $p<0.05$ and ($\Delta\Delta$) $p<0.01$ denotes statistical difference between treated (EZ, EZ+SV) rats Vs. control MetS rats.

4.3.3. Post-prandial plasma response of apoB48, TG and cholesterol

The postprandial response of apoB48, as measured by total area under the curve (AUC) and incremental AUC (iAUC), was significantly increased in MetS rats compared to their lean counterparts. Total AUC indicated that circulating apoB48 mass over the post-prandial period was approximately 2-fold higher in control MetS rats compared to lean rats. Corresponding iAUC showed that the change in apoB48 particles during the post-prandial phase was 6-fold higher in control MetS rats compared to lean rats. EZ treatment either alone or in combination with SV decreased ApoB48 AUC (EZ; -60% $p<0.05$), (EZ+SV; -68% $p<0.05$) and iAUC (EZ; -81% $p<0.01$), (EZ+SV; -91% $p<0.001$) compared to the control MetS group (figure 4-8).

Furthermore, the post-prandial response of TG, as measured by AUC and iAUC was increased in MetS rats (10-fold higher and 11 fold higher, respectively) compared to lean rats. Both treatment groups decreased TG AUC (EZ; -58% $p<0.001$), (EZ+SV; -66%$p<0.001$) and TG iAUC (EZ; -71% $p<0.001$), (EZ+SV; -89% $p<0.001$) compared to control MetS rats (figure 4-9). The post-prandial response of cholesterol, as measured by AUC and iAUC was significantly increased in MetS rats (2.5-fold higher and 6 fold-higher, respectively) compared to their lean counterparts. Treated MetS rats demonstrated a decreased post-prandial cholesterol response as measured by AUC (EZ; -26% $p<0.05$) (EZ+SV; -31% $p<0.01$) and iAUC (EZ; -62% $p<0.001$) (EZ+SV; -73% $p<0.001$) compared to control MetS rats (figure 4-10).

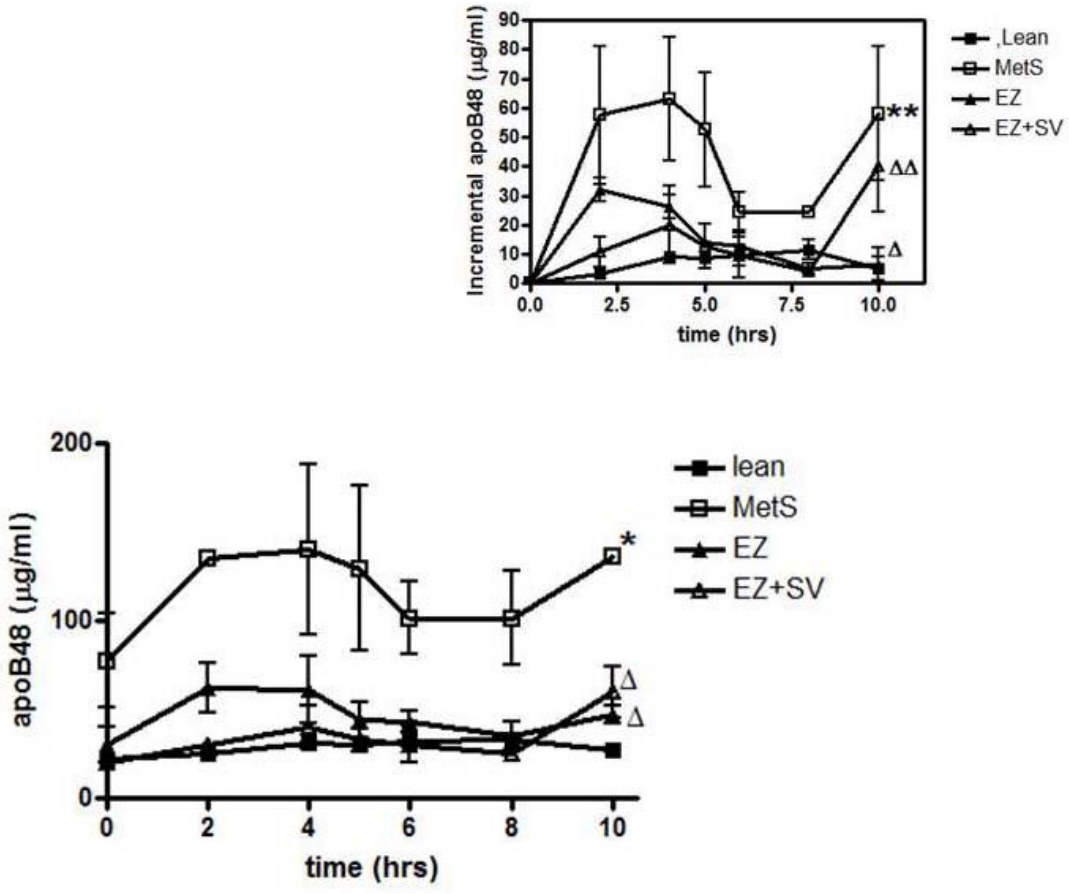

Figure 4-8. The post-prandial plasma apoB48 response (AUC) following an oral fat challenge in JCR:LA-*cp* rats. JCR:LA-*cp* rats were randomized to a lipid balanced diet with or without EZ (0.01% w/w) or EZ(0.01% w/w) + SV(0.01% w/w) groups for 8 weeks. Following an oral fat challenge, plasma was collected at two hour intervals over a ten hour period and plasma apoB48 concentration was measured. The total AUC and the change in apoB48 from fasted concentrations are shown (inset) and represents the incremental area under the curve (iAUC) . (*) *p<0.01* and (**) p<0.001 denotes statistical difference between control MetS rats and lean rats. (Δ) *p<0.01* and (ΔΔ) *p<0.001* denotes statistical difference between treated (EZ, EZ+SV) rats and control MetS rats.

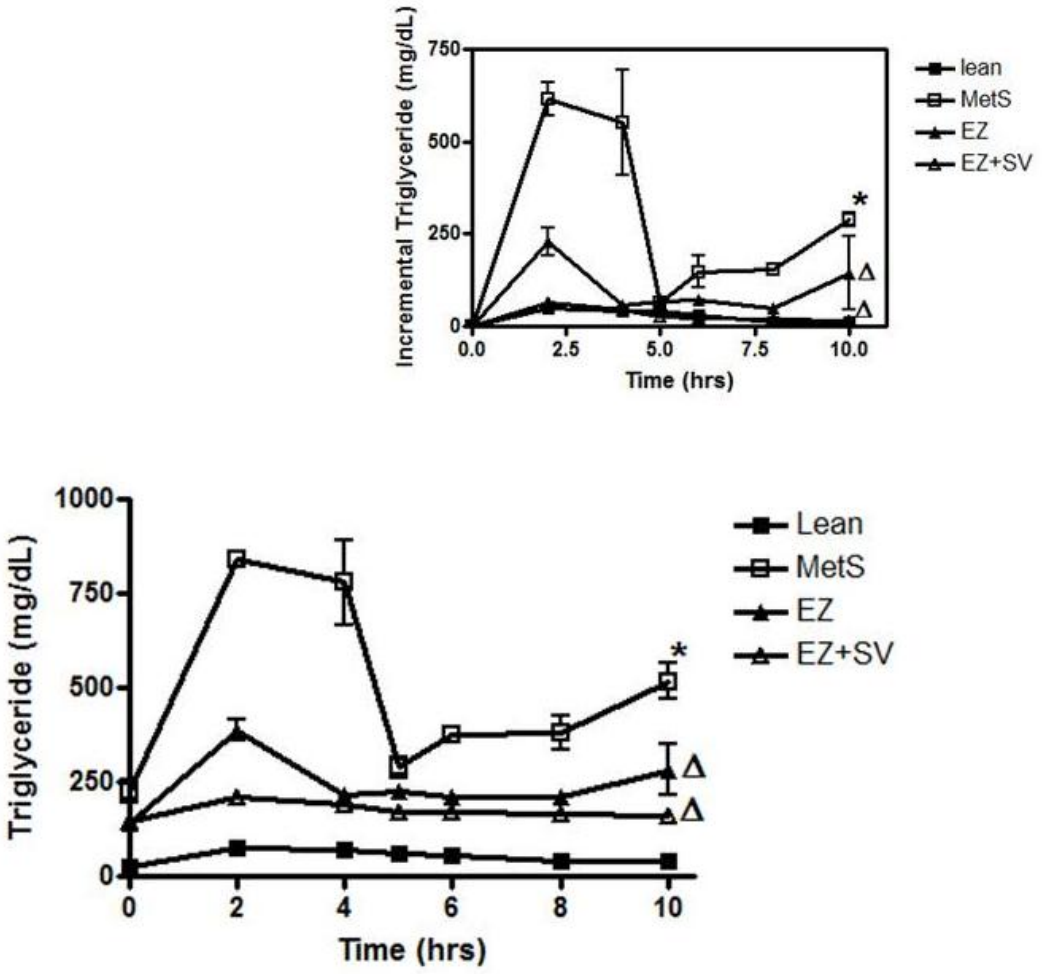

Figure 4-9. The post-prandial plasma triglyceride response (AUC) following an oral fat challenge in JCR:LA-*cp* rats. JCR:LA-*cp* rats were randomized to a lipid balanced diet with or without EZ (0.01% w/w) or EZ(0.01% w/w) + SV(0.01% w/w) groups for 8 weeks. Following an oral fat challenge, plasma was collected at two hour intervals over a ten hour period and plasma triglyceride concentration was measured. The total AUC and the change in triglycerides from fasted concentrations are shown (inset) and represents the incremental area under the curve (iAUC). (*) $p<0.001$ denotes stastical difference between control MetS rats Vs. lean rats. (Δ) $p<0.001$ denotes statistical difference between treated (EZ,EZ+SV) rats Vs. control MetS rats.

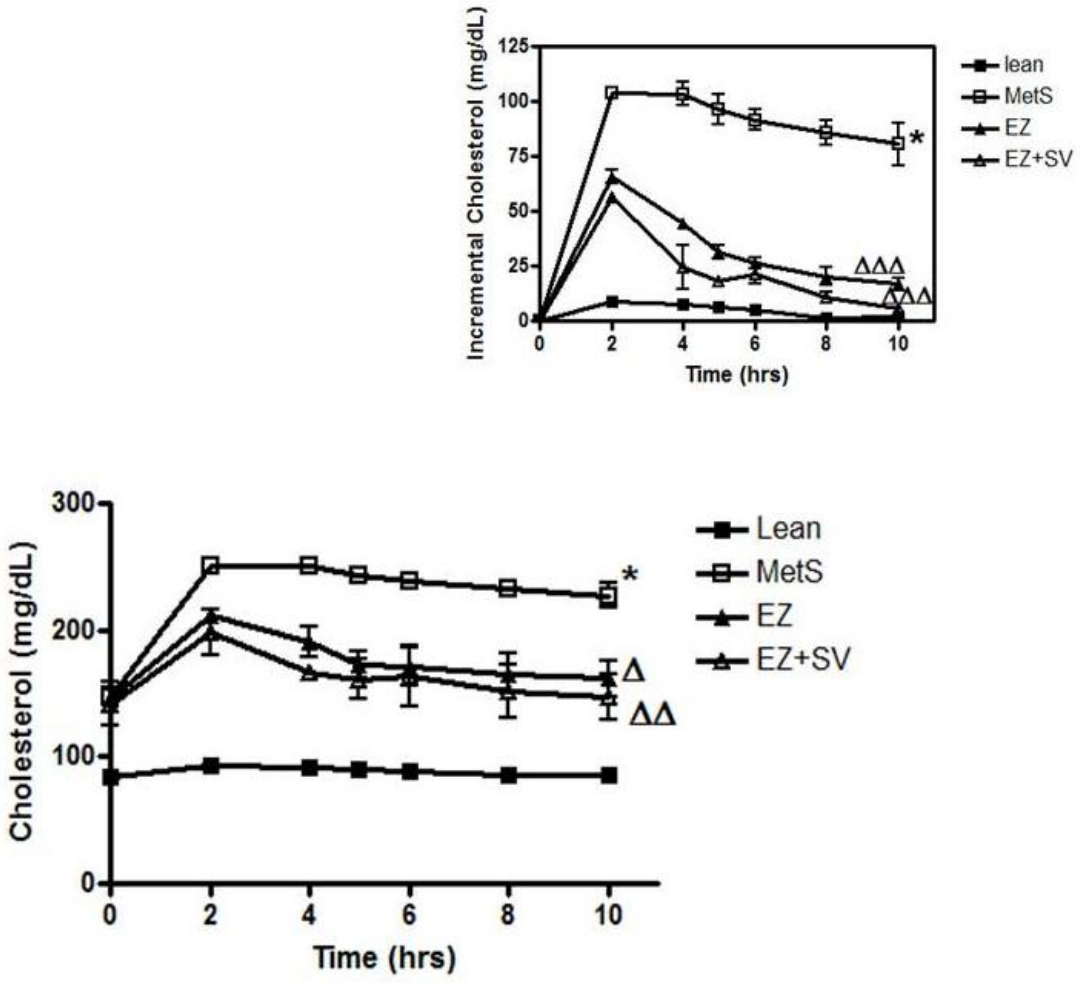

Figure 4-10. The post-prandial plasma cholesterol response (AUC) following an oral fat challenge in JCR:LA-*cp* rats. JCR:LA-*cp* rats were randomized to a lipid balanced diet with or without EZ (0.01% w/w) or EZ(0.01% w/w) + SV(0.01% w/w) groups for 8 weeks. Following an oral fat challenge, plasma was collected at two hour intervals over a ten hour period and plasma cholesterol concentration was measured. The total AUC and the change in cholesterol from fasted concentrations are shown (inset) and represents the incremental area under the curve (iAUC). (*) $p<0.001$ denotes statistical difference between control MetS rats Vs. lean rats. (Δ) $p<0.05$, (ΔΔ) $p<0.01$ and (ΔΔΔ) $p<0.001$ denotes statistical difference between treated (EZ,EZ+SV) rats Vs. control MetS rats.

4.3.4 Post-prandial plasma response of insulin and glucose

The post-prandial response of glucose, as measured by AUC and iAUC was increased in MetS rats (1.5-fold higher and 3-fold higher, respectively) compared to control lean rats. Glucose AUC (EZ; -23% $p<0.01$), (EZ+SV; -28% $p<0.001$) and glucose iAUC (EZ; -69% $p<0.01$), (EZ+SV; -52% $p<0.05$) was decreased in both treatment groups compared to control MetS group. The post-prandial response of insulin, as measured by AUC and iAUC was increased in MetS rats (2.5-fold higher and 3-fold higher, respectively) compared to control lean rats. Insulin AUC (EZ; -21% $p<0.05$), (EZ+SV; -74% $p<0.001$) and insulin iAUC (EZ; -26% $p<0.01$), (EZ+SV; -37% $p<0.01$) was decreased in MetS rats following treatment with EZ either alone or in combination with SV.

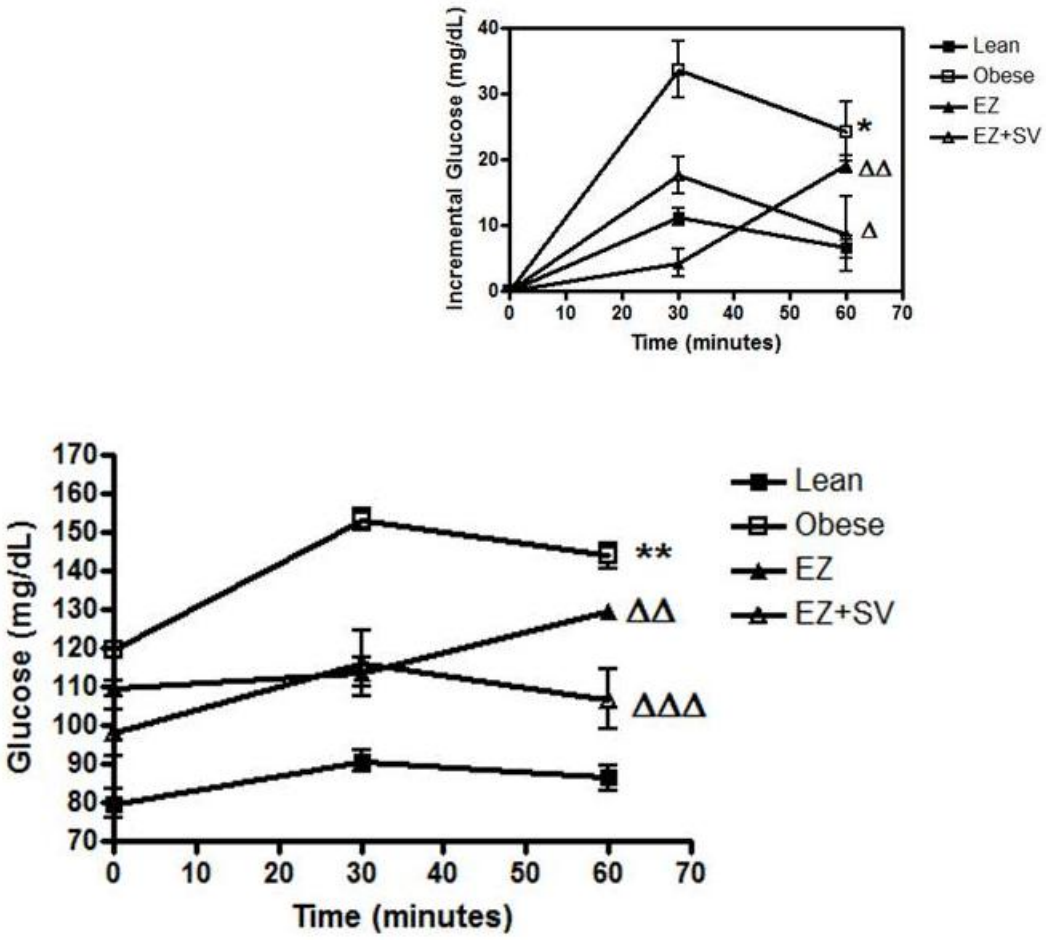

Figure 4-11. The post-prandial plasma glucose response (AUC) following a meal tolerance test in JCR:LA-*cp* rats. JCR:LA-*cp* rats were randomized to a lipid balanced diet with or without EZ(0.01% w/w) or EZ (0.01% w/w) + SV (0.01% w/w) groups for 8 weeks. Treated rats were subjected to a meal tolerance test and blood samples were taken at 0, 30 and 60 minutes. The total AUC and the change in glucose from fasted concentrations are shown (inset) and represents the incremental area under the curve (iAUC). (*) $p<0.01$ and (**) $p<0.001$ denotes statistical difference between control Mets rats Vs. lean rats. (Δ) $p<0.05$, (ΔΔ) $p<0.01$ and (ΔΔΔ) $p<0.001$ denotes statistical difference between treated (EZ, EZ+SV) rats and control MetS rats.

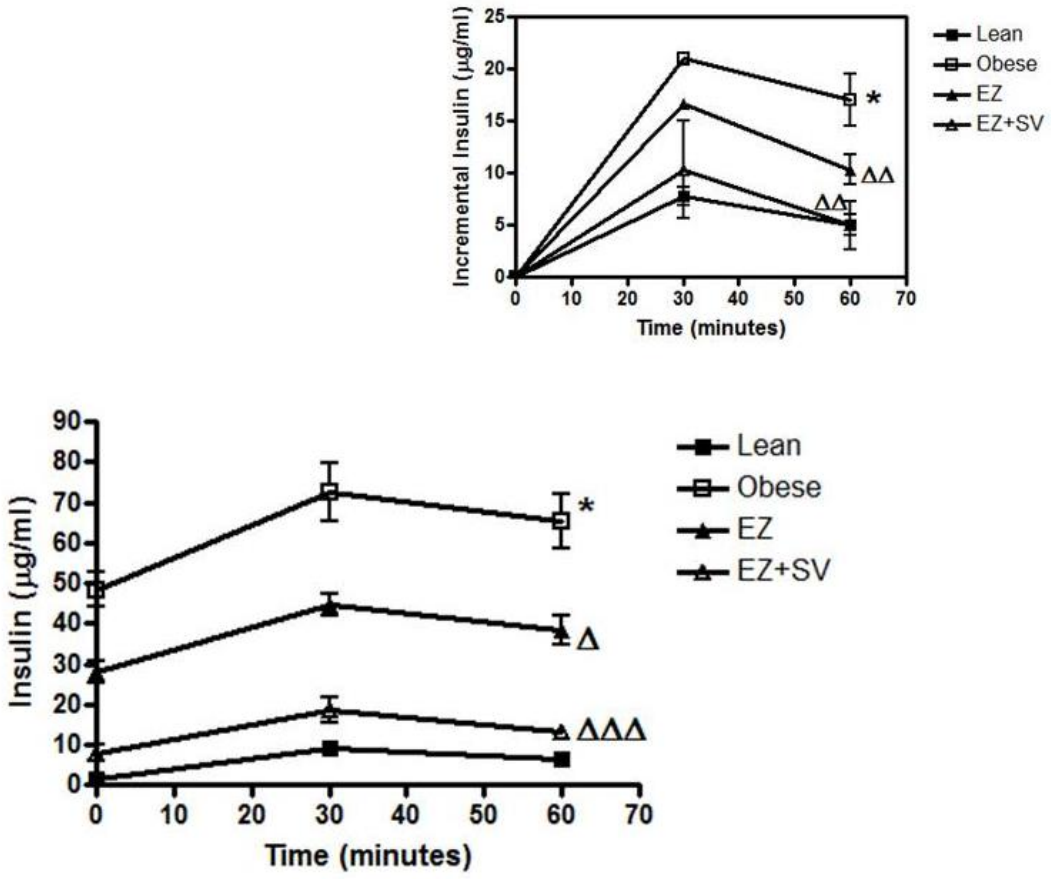

Figure 4-12. The post-prandial plasma insulin response (AUC) following a meal tolerance test in JCR:LA-*cp* rats. JCR:LA-*cp* rats were randomized to a lipid balanced diet with or without EZ(0.01% w/w) or EZ (0.01% w/w) + SV (0.01% w/w) groups for 8 weeks. Treated rats were subjected to a meal tolerance test and blood samples were taken at 0, 30 and 60 minutes. The total AUC and the change in insulin from fasted concentrations are shown (inset) and represents the incremental area under the curve (iAUC). (*) $p<0.001$ denotes statistical difference between control Mets rats Vs. lean rats. (Δ) $p<0.05$, (ΔΔ) $p<0.01$ and (ΔΔΔ) $p<0.001$ denotes statistical difference between treated (EZ, EZ+SV) rats and control MetS rats.

4.3.5 Chylomicron remnant lipid and apoB48

CM-r Cy5 labelled perfusate was n=1 for control and treated groups. Thus, there were no statistical differences in CM-r lipid and biochemical profile within control and treated groups.

	Cholesterol (mg/ml)	**Triglyceride (mg/ml)**	**apoB48 (mg/ml)**	**Particle Size (nm)**	**Cholesterol: apoB48**	**TG:apoB48**
Lean	0.62	0.89	0.40	48	1.55	2.22
Obese	0.75	1.56	0.21	55	3.57	7.43
EZ	2.19	15.10	0.43	49	5.10	35.11
EZ+SV	0.22	0.40	0.03	50	7.33	13.33

Table 4-3. Chylomicron remnant biochemical and lipid profile of JCR:LA-*cp* rats.

4.3.6. In-situ Arterial retention of chylomicron remnants.

The association of fluorescent CM-r in isolated segments from control and treated JCR:LA-cp rats is depicted in figures 4-13 and 4-14. The images suggest that arterial retention of CM-r is greater in control MetS rats as compared to control lean rats. Carotid segments from JCR:LA-*cp* rats treated with EZ either alone or in combination with SV are visually observed to have reduced fluorescence compared to control MetS rats. Statistical analysis of fluorescence intensity revealed that MetS rats have 77% greater retention of CM-r than lean rats ($p<0.01$). EZ treatment either alone or in combination with SV significantly reduced CM-r retention within carotid arteries (-64% and -90% respectively, $p<0.001$) (figure 4-15).

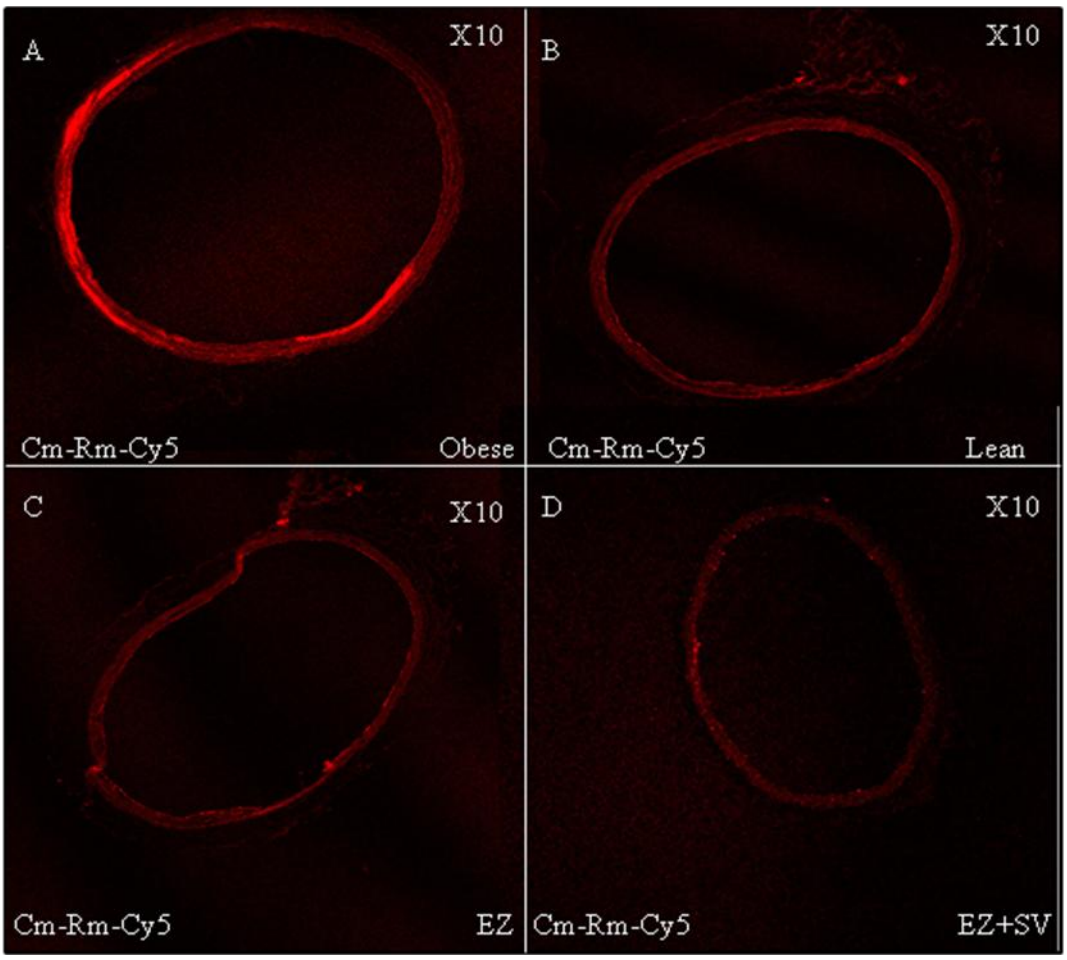

Figure 4-13. Image of arterial retention of chylomicron remnants in JCR:LA-cp rats (frames A-D). JCR:LA-cp rats were randomized to with or without EZ (0.01% w/w) or both EZ (0.01% w/w)+SV (0.01% w/w) groups for 8 weeks. Chylomicron remnants were labelled with Cy5 flourscence and subsequently normolipidemic concentrations of remnants were perfused through the cannulated carotid artery of treated and control group rats under physiological conditions of flow and pressure. Following perfusion, carotid vessels were removed and visualized by fluorescent confocal microscopy. Magnification at x10 as indicated. See Figure 4-15 and 4-16 for quantitative analysis.

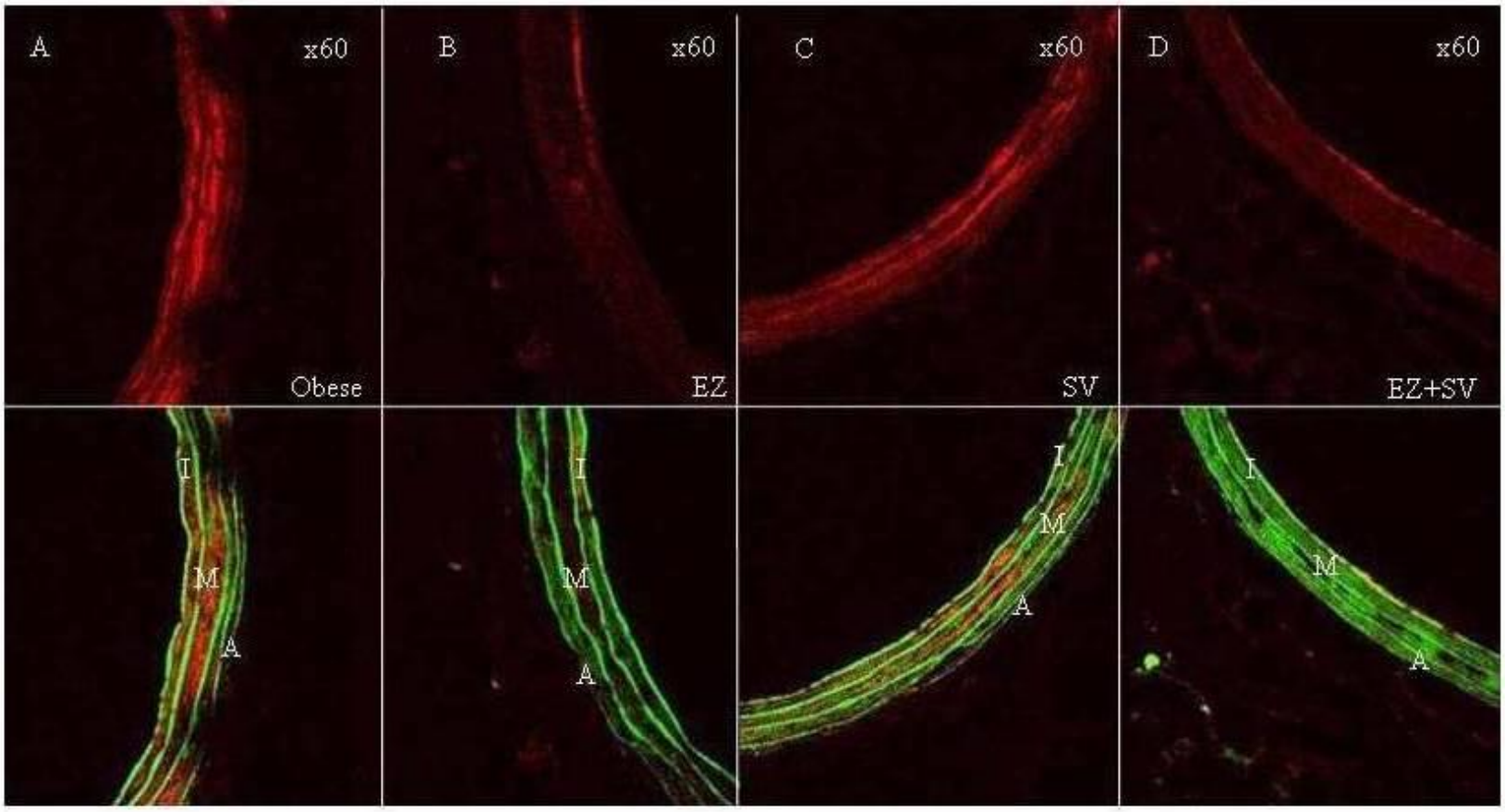

Figure 4-14. Image of arterial retention of chylomicron remnants in treated and non-treated JCR:LA-*cp* rats (frames A-D). The top panel (frames A-D) represent fluorescence associated with Cy5 labelled chylomicron remnants (red). The bottom panel (frames A-D) represent a composite overlay of fluorescence associated with chylomicron remnants (red) and morphology (green). Tissue were fixed and visualized by confocal laser scanning microscopy x60. I=Intima, M=Media and AV=Adventitia.

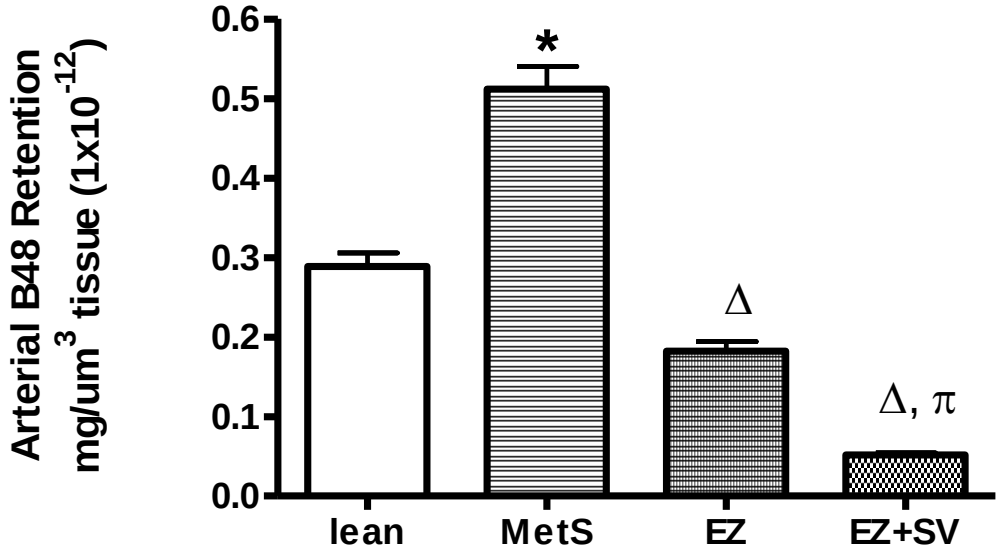

Figure 4-15.Arterial retention of apoB48 remnant lipoproteins in JCR:LA-*cp* rats following EZ therapy either alone or in combination with SV. Cy5 labelled chylomicron remnants were perfused through carotid arteries of treated and non-treated JCR:LA-*cp* rats under physiological conditions of flow rate and pressure. (*) *p<0.001* denotes statistical significance between control MetS rats and lean rats. (Δ) *p<0.001* denotes statistical significance between treated rats (EZ, EZ+SV) Vs. control MetS rats. (∏) *p<0.001* denotes stastical difference between EZ+SV Vs. EZ treated rats.

Arterial retention of cholesterol associated with apoB48 remnant lipoproteins in JCR:LA-cp rats:

MetS JCR:LA-*cp* rats demonstrated 4-fold greater arterial cholesterol retention associated with CM-r, compared to their lean counterparts ($p<0.001$). EZ therapy, either alone (-46%, $p<0.001$) or in combination with SV (-75%, $p<0.001$) significantly lowered arterial cholesterol retention compared to control MetS rats (figure 4-16).

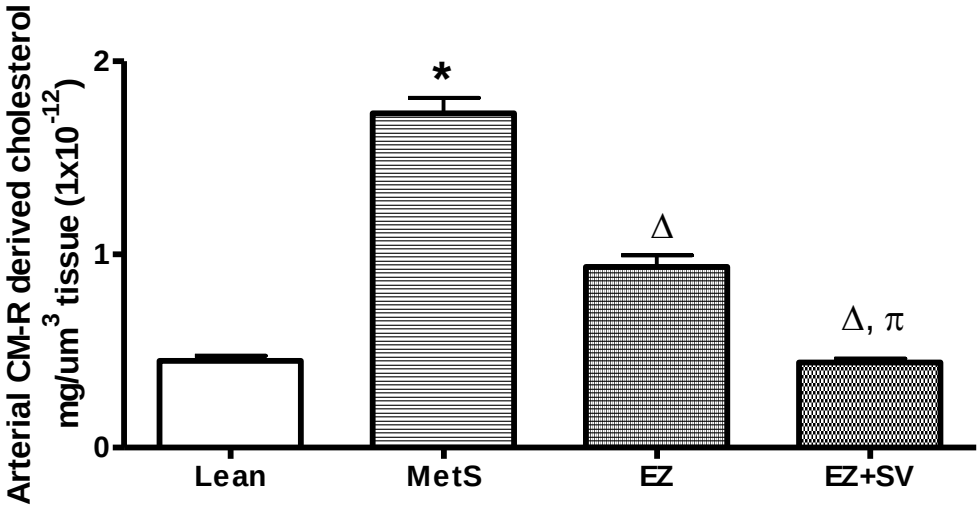

Figure 4-16. Arterial retention of cholesterol associated with apoB48 remnant lipoproteins in JCR:LA-*cp* rats following EZ therapy either alone or in combination with SV. Cy5 labelled chylomicron remnants were perfused through carotid arteries of treated and non-treated JCR:LA-*cp* rats under physiological condition of flow and pressure. (*) *$p<0.001$* denotes statistical significance between control MetS rats and lean rats. (Δ) *$p<0.001$* denotes statistical significance between treated rats (EZ, EZ+SV) Vs. control MetS rats. (∏) *$p<0.001$* denotes stastical difference between EZ+SV Vs. EZ treated rats.

4.3.7. Atherosclerotic lesion development:

The findings of this study revealed no statistical difference in frequency of stage 1, 3 and 4 lesions between treated and control MetS rats. However, control MetS rats showed a large number of early inflammatory lesions at stage 2 compared to lean rats, which showed no evidence of early lesions ($p<0.01$). Consistent with arterial retention data, EZ treatment either alone or in combination with SV demonstrated an 83% decrease in stage 2 lesion frequency compared to control MetS rats (See Fig 4-17).

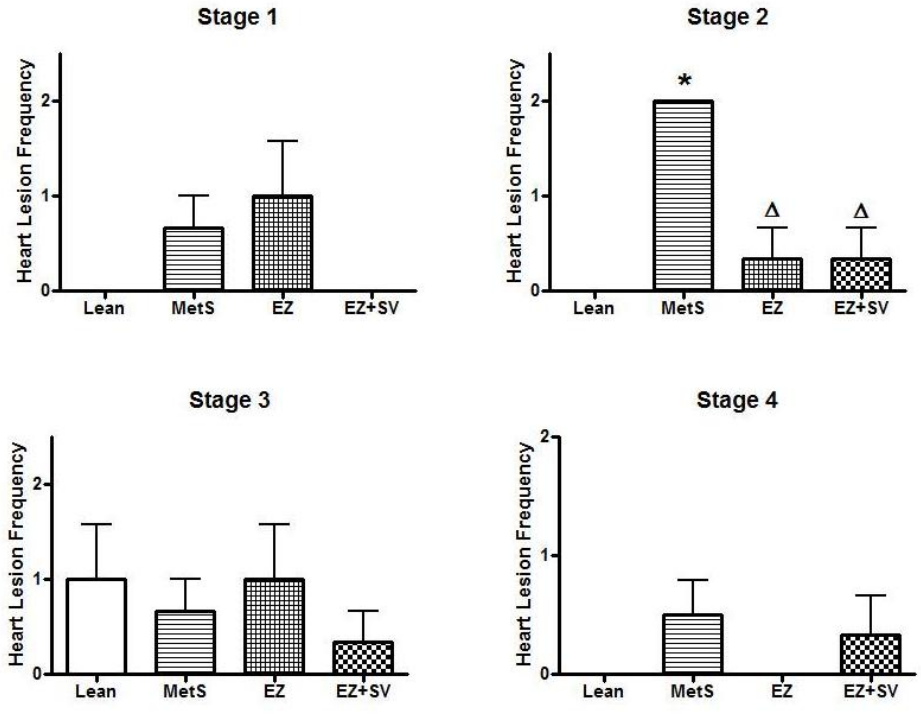

Figure 4-17. Frequency of myocardial lesions in 14 weeks old JCR:LA-*cp* rats. Homozygous (*cp/cp*) JCR:LA-*cp* rats 6 weeks of age were randomized to either EZ (0.01% w/w) or EZ+SV (0.01% w/w) treatment for 8 weeks. Following 8 weeks of treatment, the heart was removed from JCR:LA-*cp* rats and myocardial lesion frequency determined. (*) *p<0.01* denotes statistical significance between control MetS rats and lean rats. (Δ) *p<0.01* denotes statistical significance between treated rats (EZ, EZ+SV) Vs. control MetS rats.

4.4 Discussion

Atherosclerosis is thought to begin with the focal accumulation of remnant lipoproteins within the vessel wall, which consequently initiates an inflammatory response (Ross 1999). There is now direct evidence implicating CM-r in the atherosclerotic process (Proctor, Mamo 1996, Proctor 2000, Proctor, Mamo 1998). Impaired post-prandial clearance of CM and their remnants is associated with increased risk of CVD due to the pro-longed circulatory time of CM-r and exposure to the arterial wall (Proctor 2000, Vine 2007). MetS and IR are further associated with an exacerbated risk of CVD associated with CM over-producion and impaired post-prandial CM clearance (Vine 2007, Huff 2003, Zilversmit 1979). The key objective of this study was to determine the effect of ezetimibe (EZ) treatment, either alone, or in combination with simvastatin (SV) on post-prandial lipid, insulin and glucose metabolism, the arterial uptake of CM-r and myocardial lesion development.

Combined Ezetimibe and Simvastatin Therapy has Benefical Effects on Fasting and Post-prandial lipid and CM Metabolism in MetS.

Fasting Plasma Biochemistry:

MetS JCR:LA-*cp* rats were observed to have significantly greater concentrations of fasting TG, Cholesterol, LDL and HDL in plasma compared to their lean counterparts, which is consistent with previous findings from our laboratory (Mangat et al. 2007, Vine, Glimm & Proctor 2008). EZ treatment was observed to have no effect on TG concentration compared to MetS rats, which is

contradictory to earlier studies which have shown that EZ treatment can decrease TG concentrations by appoximately 5% (Mauro, Tuckerman 2003, Dujovne et al. 2002). EZ treatment either alone or in combination with SV decreased total plasma cholesterol and LDL-C. These results correspond with those of previous studies which have shown that EZ is capable of normalizing total plasma cholesterol concentrations and reducing LDL-C (Mauro, Tuckerman 2003, Dujovne et al. 2002, Gonzalez-Ortiz et al. 2006). Subjects with primary hypercholesterolemia who are treated with EZ monotherapy show reduced LDL-C, HDL-C and TG levels (Dujovne et al. 2002). Furthermore, recent clinical studies have revealed that co-administration of EZ with statins provides an efficacious treatment to lower LDL-C concentration in hypercholesterolemic patients with diabetes mellitus and/or MetS (Grigore, Norata & Catapano 2008). EZ's ability to reduce LDL-C is in part explained by its ability to inhibit cholesterol absorption through the NPC1L1 transporter at the level of the brush border of the intestine (Davis et al. 2004, Garcia-Calvo et al. 2005). Previous studies have suggested that inhibition of intestinal cholesterol absorption by EZ results in a reduction of the cholesterol content packaged into CM particles, which subsequently decreases the amount of cholesterol delivered in CM-r to the liver (van Heek, Compton & Davis 2001). As a result of decreased cholesterol delivery to the liver, the liver responds by up-regulating hepatic LDL-receptors, thereby enhancing LDL-C clearance and lowering plasma levels of LDL-C. However, in chapter 3 of this thesis, monotherapy of EZ was observed to increase cholesterol content of CM particles in lymph in both the fasting and post-prandial state.

Thus, collectively these findings would suggest that up-regulation of hepatic LDL-receptors by EZ may be induced by pathways independent of possible increased CM- associated delivery of cholesterol to the liver. In contrast, statins function by inhibiting HMG-CoA reductase, the rate-limiting step in cholesterol synthesis (Shepherd et al. 1995, Shepherd 2004). As a result, hepatocytes become depleted of cholesterol and respond by increasing LDL-C clearance from the blood via up-regulation of hepatic LDL-receptors. More recently, Gouni-Berthold et al (2008) revealed that combined EZ and SV treatment in healthy men for a duration of 14 days decreased LDL-receptor gene expression and LDL-C plasma levels (Gouni-Berthold et al. 2008). Thus, the combined actions of EZ and SV with complementary mechanisms of action provide a powerful approach to lowering LDL-C and total cholesterol concentrations.

Combined Ezetimibe and Simvastatin Therapy has Beneficial Effects on Post-prandial Lipid and CM Metabolism :

In this study, an oral fat challenge test was used to assess post-prandial response of TG, cholesterol and apoB48. The results showed that EZ therapy either alone or in combination with SV was effective at lowering post-prandial apoB48, cholesterol and TG. These findings are consistent with those of van Heek et al who showed that EZ treatment decreased post-prandial apoB48 and cholesterol concentrations in hypercholesterolemic monkeys (van Heek, Compton & Davis 2001). However, in chapter 3 of this thesis, we observed that combined EZ+SV therapy significantly increased apoB48, which was associated with increased cholesterol secretion into lymph compared to MetS rats. The apparent CM over-production in lymph (as observed in chapter 3), and the observation of reduced

CM-apoB48 plasma concentrations in the post-prandial state, following combined EZ+SV treatment, may indicate enhanced clearance of these lipoproteins via the liver. Enhanced hepatic clearance of CM would lead to a reduction in circulating plasma concentrations of CM-r particles. A recent study revealed that EZ+SV treatment in healthy men increased hepatic LDL-receptor gene expression which may partly explain findings of this study (Gouni-Berthold et al. 2008). Further studies assessing hepatic tissue uptake of radiolabelled CM would help determine whether EZ+SV treatment increases hepatic clearance of CM. In addition to the potential effects of EZ+SV on hepatic receptors, this combined therapy may mediate lipolysis and the expression of LPL. Previous studies *in vivo* and *in vitro* have shown LPL activity to be up-regulated following statin treatment (Schneider, Eynatten & Dugi 2005). The effects of EZ on LPL activity are yet to be determined.

Combined Ezetimibe and Simvastatin Therapy has Beneficial Effects on Fasting and Postprandial Insulin and Glucose Response:

Mets rats were observed to have elevated fasting plasma concentrations of insulin and glucose compared to their lean counterparts, which is consistent with previous studies in our laboratory (Vine, Glimm & Proctor 2008). EZ treatment either alone or in combination with SV reduced glucose and insulin concentrations following a meal tolerance test (MTT). These results correspond with those of previous studies which have shown that EZ enhances insulin signalling in cultured rat hepatocytes (Deushi et al. 2007). Furthermore, Dueshi et al have shown that EZ treatment improves insulin and glucose response following intraperitoneal injection in Zucker obese rats (Deushi et al. 2007). SV has also been reported to

improve insulin sensitivity in some diabetic patients (Paolisso et al. 2000), which is consistent with combined EZ+SV therapy decreasing plasma insulin concentrations to a greater extent than EZ treatment alone. However, there have been controversial studies showing that SV treatment either does not change or lowered insulin sensitivity in diabetic patients (Farrer et al. 1994, Ohrvall et al. 1995).

The direct mechanisms by which EZ may improve insulin sensitivity have recently been elucidated by Deushi et al (2007) who showed that EZ dramatically enhanced insulin signalling (i.e. phosphorylation of the insulin receptor, IRS-1 and Akt-1) in HepG2 cells *in vitro*. Similarly, *in vitro* studies have revealed that statins can directly stimulate insulin signalling via activation of PI3K and Akt , resulting in translocation of glucose transporters to the cell membrane and enhanced glucose uptake (McFarlane et al. 2002). Combined EZ+SV therapy may indirectly improve insulin sensitivity by lowering circulating TG and possibly free fatty acid (FFA) concentrations, although FFA were not measured in this study (Deushi et al. 2007). FFA cause IR in humans by altering insulin signalling (Deushi et al. 2007).

Ezetimibe treatment either alone or in combination with simvastatin decreases arterial retention of chylomicron remnants and associated cholesterol deposition:

There is now substantial evidence directly implicating CM-r in the pathogenesis of the atherosclerotic process (Proctor 2000, Proctor 2002). This study shows for the first time, the direct effects of EZ treatment either alone or in combination with SV on the retention of CM-r particles in a rodent model of MetS, the

JCR:LA-*cp* rat. The findings revealed that MetS rats have significantly greater retention of apoB48 remnant lipoproteins compared to their lean counterparts. Furthermore, MetS rats demonstrate significantly greater deposition of CM-r-derived cholesterol which corresponds with increased cholesterol content of CM-r particles compared to lean rats. Previous studies have observed increased retention of cholesterol within the intima of Watanabe heritable hyperlipidemic rabbits (Proctor, Mamo 2003). There has been some evidence indicating that binding of lipoproteins to proteoglycans in the arterial wall is partly mediated by particle composition. Alterations in TG, cholesterol, apolipoprotein (apoCIII and apoE), phospholipid and sialic content of CM-r particles have been proposed to modify lipoprotein binding capacity to the arterial wall (Proctor, Mamo 2003, Oorni et al. 1998, Olsson, Camejo & Bondjers 1993, Chauhan et al. 1998). Thus, increased retention of CM-r particles and increased cholesterol deposition in MetS rats may not only be attributed to greater cholesterol content of particles but also to other differences in CM particle composition. In addition, it is speculated that increased CM-r retention in MetS rats may be due to increased arterial wall PG synthesis which has previously been shown to influence the rate at which lipoproteins accumulate in the vessel wall (Vine, Glimm & Proctor 2008, Camejo et al. 2002, Raines, Ferri 2005).

EZ therapy either alone or in combination with SV was observed to decrease apoB48 retention and associated cholesterol deposition. However, analysis of the CM-r biochemical profile revealed that CM-r particles derived from EZ or EZ+SV treated JCR:LA-*cp* rats had greater cholesterol content per particle

compared to control MetS rats. In the present study, the concentration of apoB48 in the perfusate was normalised to 100ug/ml for all experiments to ensure that the same number of particles was delivered in each experimental tissue. Thus, decreased apoB48 arterial retention in MetS rats following either EZ or EZ+SV treatment cannot be attributed to decreased exposure of apoB48 particles. Therefore, EZ may have beneficial effects on both composition of CM particles and the structure of the vessel wall which reduces arterial retention of CM-r. However, there has been no study to date directly examining the effects of EZ on vascular remodelling.

A further decrease in CM-r retention and associated cholesterol is observed with combined therapy as compared to monotherapy with EZ. The 'statin' class of compounds have been previously shown to reduce production of pro-atherogenic arterial proteoglycans (Siegel-Axel et al. 2003, Riessen et al. 1999), which may partly explain the results observed in this study. Increased entrapment of lipoproteins within the arterial wall in diabetes has been attributed to increased secretion of collagen and proteoglycans by local arterial smooth muscle cells (Little et al. 2002). Therefore, SV may inhibit proteoglycan synthesis reducing remnant lipoprotein binding. Further studies are required to determine whether combined EZ+SV induces vascular wall remodelling.

Ezetimibe treatment reduces frequency of stage 2 myocardial lesions.

The development of end-stage ischemic lesions secondary to vascular damage or dysfunction is one of the major symptomatic end stages of CVD . Russell et al previously determined that MetS JCR:LA-*cp* rats develop atherogenic lesions spontaneously (Russell, Graham & Richardson 1998)(Russell, Graham & Richardson 1998, Russell, Graham & Richardson 1998, Brindley, Russell 2002). The development of lesions is attributed to hyperlipidemia and hyperinsulinemia (Russell, Graham & Richardson 1998). Hyperinsulinemia stimulates proliferation/migration of vascular smooth muscle cells and lipoprotein binding to the arterial wall leading to arterial wall thickening and lesion formation. In addition, hyperinsulinemia can induce vascular smooth muscle cell hypertrophy. Hyperlipidemia (i.e. elvelated circulating LDL and CM plasma concentrations) increases lipoprotein delivery to the arterial wall. In the present study, rats were ended at 14 weeks of age and thus a low frequency of advanced scarred lesions (stage 4) was observed which normally accumulate with age. However, a large number of early inflammatory lesions (stage 2) were observed in control MetS rats. Earlier studies conducted by Russell et al in the JCR:LA-*cp* rat fed a standard chow diet (Lab Diet 5001) have not observed such a high frequency of stage 2 lesions (Russell et al. 1991). Therefore, the increased frequency in the present study may be partly explained by the 1% cholesterol supplementation in the LBD diet.

EZ treatment either alone or in combination with SV was observed to significantly decrease the frequency of lesions in JCR:LA-*cp* rats. The possible mechanisms of EZ regarding improvement of early stage 2 myocardial lesion development may be associated with the hypolipidemic effects of EZ or improvments in the regulation of hyperglycemia and hyperinsulinemia. In the present study, fasting LDL-C and total plasma cholesterol were decreased following EZ+SV treatment. Post-prandial plasma TG, apoB48 and cholesterol were also observed to be decreased following combined treatment. Furthermore, combined EZ+SV treatment improved glucose and insulin response. As a result of improved hyperlipidemia, hyperglycemia and hyperinsulinemia, endothelial dysfunction is likely to be improved resulting in reduced CVD risk and early lesion development.

In conclusion, combined EZ+SV treatment is effective at reducing apoB48 remnant associated cholesterol deposition in the arterial wall, and reduces the frequency of early stage 2 myocardial lesions. These observations may be explained by the combined beneficial effects of EZ and SV on hyperlipidemia, hyperglycemia and hyperinsulinemia. Further studies need to be conducted in order to determine whether the reduced retention of apoB48 remnant lipoproteins in the arterial wall following EZ +SV treatment is a result of changes in CM-r composition (cholesterol, TG, apolipoproteins), vascular remodelling (increased PG synthesis, decreased smooth muscle cell proliferation) or a combination of both.

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Chapter 5. Overall Discussion and Conclusions

5.1 Discussion

The overall aim of this thesis was to investigate the beneficial effects of EZ monotherapy and combined EZ+SV therapy in modulating intestinal cholesterol transport, CM metabolism, both at the site of intestinal synthesis and subsequent uptake of CM-r into the arterial wall in the JCR:LA-*cp* rodent model of MetS. The first phase of my thesis investigated the modulation of intestinal cholesterol transport and lymphatic CM secretion following EZ and SV treatment in the JCR:LA-*cp* rodent model of MetS.

5.1.1 Modulation of intestinal cholesterol transport and chylomicron secretion during insulin resistance following ezetimibe and simvastatin therapy

Alterations in intestinal cholesterol metabolism in diabetes and IR (Gylling et al. 2004, Gylling, Miettinen 1996) have been observed and may be responsible for the increase in CM-associated cholesterol observed in MetS JCR:LA-*cp* rats. Indeed, studies have demonstrated net cholesterol absorption to be up-regulated in the diabetic state which has been proposed to be a result of altered expression of intestinal cholesterol transporters (Lally et al. 2006, Lally, Owens & Tomkin 2007). The JCR:LA-*cp* rat has been established as a model of intestinal CM over-production during MetS (Vine, Glimm & Proctor 2008). The model presents with intestinal hypertrophy and altered transport of cholesterol across the mucosal membrane (Vine, Glimm & Proctor 2008). In addition, there is an increased number of CM derived from the mesenteric lymph duct of MetS rats, and these

particles are higher in cholesterol content compared to CM isolated from lean controls (Vine, Glimm & Proctor 2008).

In the present study, combined EZ+SV treatment reduced intestinal net cholesterol influx and normalized net cholesterol efflux in MetS rats. More specifically, EZ+SV decreased M-S cholesterol transport compared to control MetS rats whilst normalizing S-M cholesterol transport. EZ decreases intestinal cholesterol absorption via NPC1L1, FAT/CD36 and/or SR-B1 transporters (Hui, Labonte & Howles 2008). To date there has been no study examining the direct effects of EZ on intestinal efflux transporters such as ABCG5 and ABCG8. There is some evidence that HMG-CoA reductase inhibitors, such as SV, increase cholesterol absorption as a compensatory response to decreased hepatic cholesterol synthesis. *In-vitro* studies have further revealed that the apparent increase in intestinal cholesterol absorption following statin therapy is accompanied by an increase in efflux transporters such as ABCG5 and ABCG8 in an attempt to limit the increased sterol absorption load (Lally et al. 2006). Here, the reduction in net cholesterol transport mediated by EZ+SV is consistent with the beneficial effects of EZ on intestinal cholesterol absorption transporters. Although previous studies have shown cholesterol efflux to be up-regulated following SV therapy, in combination with EZ, SV may not up-regulate cholesterol efflux transporters in an attempt to maintain cholesterol levels within the enterocyte. The additive effect of SV in combination with EZ on decreasing M-S cholesterol transport compared to EZ monotherapy suggests that SV can also

mediate M-S cholesterol transport. There is currently limited evidence on the actions of SV on M-S transporters.

Intriguingly, combined EZ+SV therapy ameliorated an EZ induced increase in cholesterol:B48 in the post-prandial state via up-regulation of apoB48 production. Lipidation of apoB48 by MTP prevents apoB48 proteosomal degradation (Hussain et al. 2005). Thus, we speculate that the increased intracellular stability of apoB48 may be a consequence of EZ+SV ability to up-regulate MTP.

In addition, it has been suggested that lipid availability is important for the translocation of apoB48 across the ER to prevent apoB48 degradation (Luchoomun, Hussain 1999). We speculate that EZ+SV may enhance *de novo* lipogenesis within the enterocyte via up-regulation of intestinal lipogenic enzymes: SREBP-1c, monoacylglycerol transferase (MGAT) and diacylglycerol transferase (DGAT).

Collectively, findings of the first phase of this thesis suggest that combined EZ+SV treatment has beneficial effects on the modulation of intestinal cholesterol transport. However, this may initiate a compensatory mechanism in which apoB48 production is up-regulated. The precise mechanisms by which apoB48 production is up-regulated are unknown and further studies to measure the effects of EZ+SV on MTP, SREBP-1c MGAT and DGAT expression are required to help validate these findings.

5.1.2. The effect of combined ezetimibe and simvastatin therapy on arterial retention of chylomicron remnants and myocardial lesion development in MetS.

The second phase of this thesis examined the effects of EZ and combined EZ+SV therapy on arterial uptake of CM-r and subsequent development of myocardial lesions in the JCR:LA-*cp* rat. Briefly, EZ and combined EZ+SV therapy decreased the arterial retention of apoB48 remnant lipoproteins and associated cholesterol, compared to untreated control MetS rats. However, biochemical analysis of the CM-r profile revealed that remnants derived from combined EZ+SV treatment had significantly greater cholesterol content compared to CM-r isolated from control MetS rats. Previous studies have implicated particle composition and characteristics such as lipid, apolipoprotein, phospholipid and sialic content in mediating lipoprotein binding to the arterial wall (Proctor, Mamo 2003, Oorni et al. 1998, Olsson, Camejo & Bondjers 1993, Chauhan et al. 1998). CM-r contain apoB48, apoE and some apoC. ApoE and apoCIII have been shown to bind or exacerbate binding to extracellular arterial proteoglycans and their components, such as glycosaminoglycan side chain and biglycan core proteoglycan structures (van Barlingen et al. 1996). We speculate that EZ+SV treatment may alter apolipoprotein composition of CM-r particle, thus reducing their arterial retention. In addition, EZ+SV may increase the phospholipid composition of CM-r particles. Surface phospholipids of CM-r may mask apoB epitopes that serve as binding ligands to proteoglycans (Oorni et al. 1998, Olsson, Camejo & Bondjers 1993, Chauhan et al. 1998). Alternatively, these findings may also suggest that combined EZ+SV therapy has effects on vascular wall remodelling. Previous studies have shown that increased proteoglycan synthesis

during IR is associated with greater lipoprotein binding (Tannock et al. 2002, Little et al. 2002). Furthermore, TGF-beta-1 has been identified in atherosclerotic vessels and stimulates the synthesis of proteoglycans by arterial smooth muscle cells (Little et al. 2002). Studies in our own laboratory have revealed that MetS JCR:LA-*cp* have increased circulating levels of TGF-beta-1 and a greater mass of biglycan in the aorta compared to lean rats (Vine, Glimm & Proctor 2008). The potential effects of EZ+SV on TGF-beta-1 plasma concentrations and proteoglycan synthesis were not measured in this study but future studies could address these factors to determine their role in modulating arterial retention of CM-r.

CVD is strongly associated with hyperinsulinemia and hyperlipidemia (Russell, Graham & Richardson 1998). In this thesis, combined EZ+SV treatment reduced the frequency of stage 2 myocardial lesions in MetS rats suggesting that EZ+SV are able to modulate the development of early lesions, but have no significant effect on late lesions. Correspondingly, EZ+SV improved hyperinsulinemia, hyperglycemia and hyperlipidemia in MetS rats. More specifically, the improvements in hyperlipidemia included : reduced cholesterol content of CM in lymph (in chapter 3), lowered fasting and post-prandial cholesterol and lowered post-prandial TG and apoB48 concentrations in plasma (in chapter 4). The potential mechanisms for reduced post-prandial apoB48, cholesterol and TG may include enhanced clearance of CM-r and LDL via up-regulation of hepatic receptors (LDL-receptor and the LRP) and increased LPL activity. As a result of decreased plasma lipid levels following EZ+SV treatment, there is reduced

potential of lipoprotein delivery to the arterial wall, decreasing potential lesion development. In addition, the improved insulin sensitivity may be a result of the direct actions of EZ and SV on insulin signalling (McFarlane et al. 2002, Deushi et al. 2007). Improved insulin sensitivity decreases vascular smooth muscle cell proliferation, proteoglycan synthesis, subsequent lipoprotein binding to the arterial wall and lesion formation.

5.1.3 Collective Discussion

The first phase of this study demonstrated that combined EZ+SV therapy decreased net cholesterol absorption. Furthermore, we revealed that EZ+SV therapy up-regulated the number of CM particles secreted into lymph and subsequently decreased cholesterol content of CM particles in MetS rats. In the second phase of this thesis we revealed that combined therapy of EZ+SV reduced CM-r arterial retention and associated cholesterol. In addition, combined therapy reduced the frequency of myocardial lesion in JCR:LA-*cp* rat. The conflicting results between chapter 3, which showed that combined EZ+SV therapy up-regulated apoB48 secretion into lymph in the fed state, and chapter 4, which demonstrated that combined therapy reduced post-prandial apoB48 concentration, may in part be explained by enhanced clearance of CM-r. Both monotherapy of EZ and SV have been shown to up-regulate hepatic LDL-receptor and LRP expression (Bays, Stein 2003, Telford et al. 2007, Repa et al. 2005). Thus, combined therapy may enhance CM-r clearance via these pathways reducing total apoB48 concentrations in plasma. Furthermore, hepatic clearance of CM-r is

mediated by lipolysis and the expression of lipoprotein lipase. The effects of combined EZ+SV therapy on these pathways was not investigated in this study.

In addition, in chapter 3 we observed decreased CM cholesterol:B48 ratio in lymph from MetS rats following EZ+SV treatment and in chapter 4 we noted that combined treatment decreased plasma cholesterol and TG concentrations in the post-prandial state. However, intriguingly, analysis of the CM-r biochemical profile revealed that remnants derived from EZ+SV treated rats were far greater in their cholesterol enrichment than from control MetS rats. The discrepancy in results may be partly explained by alterations in particle composition as CM are hydrolyzed in circulation. CM are hydrolyzed via the actions of LPL and to a lesser extent hepatic lipase thus liberating TG and forming cholesterol rich CM-r particles. We speculate that combined EZ+SV therapy may alter CM hydrolysis in plasma which may affect CM particle composition, including lipid and apolipoprotein content. The results of this thesis are summarized in figure 5-1.

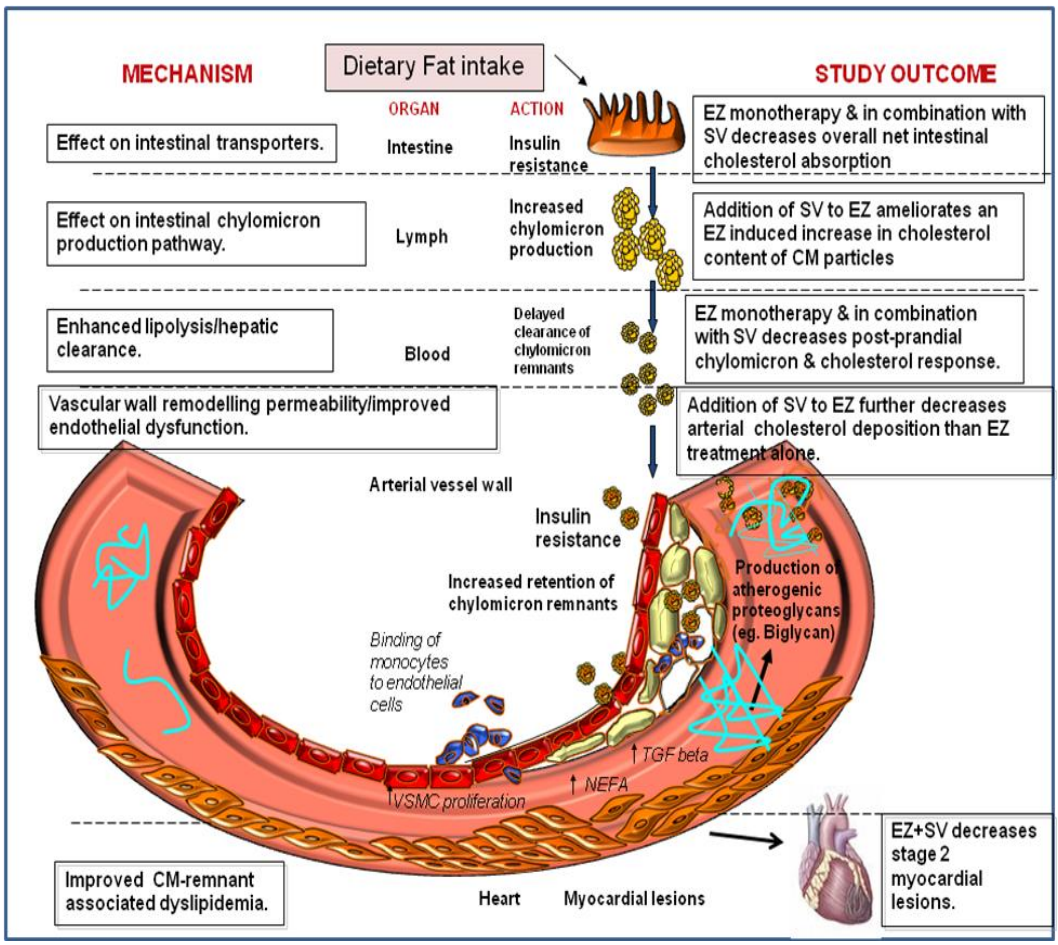

Figure 5-1. Summary of combined effects of ezetimibe and simvastatin therapy on intestinal cholesterol flux, post-prandial metabolism, arterial retention of chylomicron remnants and myocardial lesion frequency.

5.2 Study Limitations

On reflection of the outcomes of the study, a number of experimental limitations of the work were identified. Notably, differences between rodents and humans will impose limitations on the direct translation of findings to humans. The JCR:LA-*cp* rodent model is leptin resistant and thus has a genetic pre-disposition to obesity and MetS rather than a predominantly nutrition influence (i.e. over

exposure to food), as is seen in the obesity epidemic observed in the present human population.

In humans, apoB48 is unique to the CM particle. However, in rats and mice, apoB48 in synthesized in both the intestine and liver and is therefore associated with both intestinal and hepatic derived lipoproteins (i.e. CM and VLDL) (Liu, Fan & Redinger 1991). Thus, it is important to discriminate between the metabolism of apoB48 in humans vs. rodents. Furthermore, EZ is known to primarily induce its cholesterol lowering properties through NPC1L1 (Garcia-Calvo et al. 2005). In rodents, NPC1L1 is specifically expressed in the intestine but in humans, NPC1L1 is highly expressed in both the liver and intestine (Altmann et al. 2004, Davies et al. 2005). Thus EZ may have differential effects on lipid metabolism in humans to the outcomes observed in this study.

In chapter 3, the effects of EZ+SV on intestinal cholesterol transport were determined using [3H]-cholesterol and the USSING chamber technique. However, *in vivo,* cholesterol is packaged into micelles to aid transport across the unstirred water layer and subsequent absorption into the small intestine. Thus, the differences between a physiological situation, whereby cholesterol absorption is aided by its packaging into micelles, versus the method used in the present study, where free cholesterol absorption was measured may cause discrepancies in the application of these findings to an *in vivo* situation. However, further studies examining the differences in intestinal cholesterol absorption between micellular cholesterol and free cholesterol would need to be conducted.

Further limitations in the methodology used in chapter three include not stripping the intestinal tissue mounted in the USSING chambers. Un-stripped tissue is thought to be reflective of whole tissue permeability and prevents inflammation induced by the stripping process. However, as a result of not stripping intestinal tissue, radiolabelled cholesterol may become trapped in the muscularis layer, preventing cholesterol flux through the intestinal segment.

Findings of chapter 4 revealed carotid retention of apoB48 and associated cholesterol deposition to be reduced following EZ+SV therapy. However, carotid retention does not necessarily equate to retention in other arterial vessels, such as the abdominal aorta or femoral arteries. Furthermore, the present study was unable to address whether other alterations in CM-r composition, such as lipid and apolipoprotein content, vascular wall remodelling or indeed both explain the reduced of arterial CM-r. Finally, it must be acknowledged that although amelioration of CM-r and associated cholesterol retention in the JCR:LA-*cp* model of MetS via EZ+SV treatment has beneficial implications, the effect of these treatments in a rodent model may not be translatable to a reduction in the development of progression of atherosclerosis in the clinical setting.

An overall limitation of the study is the selected dosage of EZ and SV. JCR:LA-*cp* rats were treated with 3mg/day of EZ whilst in a human clinical setting, current guidelines suggest administering 10mg/day of EZ to hypercholesterolemic subjects (Bays et al. 2008). Thus, although the selected EZ dose with which JCR:LA-*cp* rats were treated with is equivalent to a human clinical setting, we do

not know whether this a high enough dose to see maximal effects, specifically on CM production.

5.3 Future Directions

The preliminary findings of this thesis provides the background necessary to further explore the mechanistic pathways by which EZ and combined EZ+SV treatment alters intestinal cholesterol transport, and CM metabolism. Based on the findings of this thesis, it would be beneficial to investigate the effects of combined EZ+SV treatment on the expression of intestinal cholesterol transporters: NPC1L1, FAT/CD36, SR-B1, ABCG5 and ABCG5, to further explain the results obtained for intestinal cholesterol transport. Furthermore, it would be compelling to examine the effect of EZ+SV treatment on the expression of enzymes (i.e.MTP) involved in CM assembly in the enterocyte. In addition, examining the theory of increased enterocyte lipid synthesis by investigating the expression of key lipogenic enzymes: FAS, ACC, SREBP ,MGAT and DGAT would also be beneficial. The study design for chapter 3 was not able to determine whether findings for M-S and S-M intestinal cholesterol flux were a result of uni-directional or bi-directional cholesterol flux. In order to address this question, future studies would need to inhibit the activity of intestinal cholesterol absorption transporters (NPC1L1, SR-BI and FAT/CD36) to determine whether S-M cholesterol flux findings are a result of uni-directional or bi-directional cholesterol flow. Similarly, inhibition of cholesterol efflux transporters (ABCA1,

ABCG5 and ABCG8) would help determine whether M-S cholesterol flux findings are a result of uni-directional or bi-directional cholesterol flow.

The present study provides the first evidence to date, to show that EZ+SV treatment reduces arterial retention of CM-r particles. However, this thesis was not able to clarify whether EZ+SV treatment reduces arterial retention of CM-r by inducing changes in composition of CM-r particles, vascular wall remodelling or indeed both. Thus, it would be intriguing to observe whether there are differences in the arterial retention of CM-r particles derived from control MetS rats and those treated with EZ or EZ+SV perfused through carotid arteries of control MetS rats. In addition, the effects of EZ+SV on vascular wall remodelling through assessment of alterations in proteoglycan synthesis would further explain the results observed in the present study. The proliferation of smooth muscle cells can stimulate the secretion of arterial proteoglycans which in turn can increase the capacity of lipoprotein binding (Tannock et al. 2002, Little et al. 2002). More specifically, TGF-beta-1 has been shown to increase proteoglycan synthesis by smooth muscle cells (Little et al. 2002). Therefore, determining the effects of EZ+SV treatment on TGF-beta-1 circulating plasma concentrations and proteoglycan synthesis may help clarify whether these factors explain why combined therapy reduces arterial retention of CM-r. In addition, the combined effects of EZ+SV on inflammatory markers such as CRP are unknown. Further studies examining the effects of these drugs on inflammatory cytokines, chemokines and adhesion molecules in the JCR:LA-*cp* rat would provide valueable results.

5.4 Conclusions

Combined EZ+SV therapy reduces net intestinal cholesterol absorption, through its potential effects on intestinal transporters. Furthermore, EZ+SV therapy reduces arterial retention of apoB48 remnant particle and associated cholesterol possibly via effects on CM-r composition and vascular wall remodelling. Combined EZ+SV therapy reduces frequency of early myocardial lesions which may be mediated by improvements in insulin sensitivity and CM-associated dyslipidemia.

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