University of Alberta

The Role of Intestinal Derived Remnant Lipoproteins in the Progression of Atherosclerosis in Animal Models of Type 1 and Type 2 Diabetes.

by

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“There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after.”

-JRR Tolkien (The Hobbit: Or There and Back Again)

Dedication

To my husband Parminder, and my family for supporting me all the way

Abstract

Introduction: Subjects with insulin resistance (IR) and diabetes are at increased risk of cardiovascular disease (CVD) than those without diabetes, however the mechanistic basis remains elusive. Despite LDL-cholesterol lowering by statin therapy, two-thirds of all CVD events remain, constituting a significant 'residual risk' for CVD. This ‘residual risk’ has been found to be greater for patients with diabetes than those without diabetes. This suggests the role for alternative sources of lipoprotein-derived cholesterol in CVD during diabetes. Both type-1 diabetic as well as IR subjects have been found to have increased plasma concentrations of fasting intestinal derived apoB48 containing remnants (CM-r). However it is not known if the diabetic metabolic milieu indeed increases the susceptibility of the arteries to CM-r and if these indeed bind to arterial proteoglycans (PGs).

Objectives: To determine arterial retention of CM-r in type-1 diabetes and IR using *ex vivo* perfusion methodology in a streptozotocin rat model of type 1 diabetes and JCR-LA-*cp* rat model of IR. To determine the direct binding affinity and capacity of CM-r to biglycan using an *in vitro* approach.

Methods and Results: We observed increased arterial CM-R retention in type 1 diabetic vessels as well as in IR vessels when compared to control vessels. The retained CM-r colocalized with arterial biglycan in type 1 diabetic vessels and a direct correlation was observed between the CM-r and the presence of glycated proteins in type I diabetic arteries.

The increased arterial CM-r retention in the IR rats was associated with increased arterial biglycan protein content. We have conclusively demonstrated for the first time that CM-r indeed bind to human biglycan. **Conclusion:** Tight glycemic control in patients with type 1 diabetes can alleviate CVD by reducing hyperglycemia and subsequent retention of CM-r. A significant increase in biglycan protein core content during IR is suggestive of early vascular remodeling and may help to explain how CM-r accumulate more readily during diabetes induced CVD. Based on the results from this study, individuals with IR may be at increased risk for atherogenesis due to increased atherogenicity of the post-prandial CM-r when compared to normal population.

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List of Abbreviations

ABCG	ATP binding Cassette Protein
AGE	Advanced Glycated End Product
Apo	Apolipoprotein
ACAT	Acyl-CoA Cholesterol Acyltransferase
CD36	Cluster of Differentiation 36
CE	Cholesteryl Ester
CETP	Cholesteryl Ester Transfer Protein
CM	Chylomicron
CM-r	Chylomicron Remnants
CSPG	Chondroitin Sulphate Proteoglycan
CVD	Cardiovascular Disease
DCCT	The Diabetes Control and Complications Trial
DGAT	Acyl CoA: Diacylglycerol Acyl Transferase
DSPG	Dermatan Sulphate Proteoglycan
DMMB	Dimethylmethylene Blue
ECM	Extracellular Matrix
EDIC	Epidemiology of Diabetes Interventions and Complications
FA	Fatty Acid
FFA	Free Fatty Acids
FAT	Fatty Acid Transporter
GAG	Glycosaminoglycan
GLP-1	Glucagon Like Peptide 1
HDL	High Density Lipoprotein
HL	Hepatic Lipase
HMG CoA	3-hydroxy-3-methyl-glutaryl-CoA
HSPG	Heparan Sulphate Proteoglycan
IDF	International Diabetes Federation

IR	Insulin Resistance
IRS	Insulin Receptor Substrate
I-FABP	Intestinal Fatty Acid Binding Protein
LDL	Low Density Lipoprotein
LDL-C	Low Density Lipoprotein Cholesterol
LDL-r	Low density Lipoprotein Receptor
L-FABP	Liver-Type Fatty Acid Binding Protein
LPL	Lipoprotein Lipase
MetS	Metabolic Syndrome
MGAT	Acyl CoA: Monoacylglycerol Acyltransferase
MTP	Microsomal Triglyceride Transfer Protein
NPC1L1	Niemannpick C1 Like 1
NEFA	Non-Esterified Fatty Acids
PPAR	Peroxisome Proliferator-Activator Receptor
PGs	Proteoglycans
PI3K	Phosphoinositide 3 Kinase
SAA	Serum Amyloid Alpha
SCAP	SREBP-Cleavage-Activating-Protein
SLRP	Small Leucine Rich Proteoglycan
SRB-1	Scavenger Receptor Class B Type 1
SREBP	Sterol Regulatory Element Binding Protein
TG	Triglyceride
TGF	Transforming Growth Factor
TRLs	Triglyceride Rich Lipoproteins
VLDL	Very Low Density Lipoprotein

Chapter 1 Literature Review

1.1 Cardiovascular Disease (CVD) Pathophysiology

1.1.1 Introduction

Cardiovascular disease is the number one cause of mortality attributing to approximately 29% of all global deaths (WHO). By definition, cardiovascular disease (CVD) includes coronary heart disease, stroke, rheumatoid heart disease, congenital heart disease, peripheral artery disease, deep vein thrombosis, pulmonary embolism and other heart diseases. However amongst these, coronary heart disease and stroke contribute to the highest mortality rates.

Atherosclerosis is a condition in which over a period of time, 'lipid filling' of the arteries leads to plaque formation. This results in the narrowing and hardening of the arteries thus leading to a blockage in the vessels that carry oxygen-rich blood to the heart and brain.

Our current understanding is that the major modifiable risk factors for cardiovascular disease include high blood pressure, abnormal blood lipids, tobacco use, physical inactivity, obesity, unhealthy diets and diabetes mellitus. A major focus for this thesis will be to investigate the early stages of CVD and to determine role of intestinal lipoproteins and vascular matrix changes associated with an increase in cholesterol deposition in the arterial wall. As such, a brief review of these concepts in the etiology of atherogenesis will be discussed in the following introduction.

1.1.2 Etiology of atherogenesis

Over the last three decades, several theories have been proposed to explain the initiation of atherogenesis and the 'lipid-filling' of atherosclerotic plaques. One of the first landmark theories outlining the initiation of atherosclerosis was proposed in 1976 by Ross and coworkers and was called the 'response-to-injury hypothesis' (Ross and Glomset 1976[a.b]). According to Ross,

endothelial desquamation was a key event in atherogenesis, causing an increase in vascular permeability facilitating the entry of cholesterol-rich particles from the blood (Ross and Glomset 1976[a.b]). However, recent evidence has shown quite conclusively that a developing atheromata can be covered by an intact endothelial layer throughout most stages of lesion progression, and therefore is not dependent on endothelial damage *per se* for initiation of atherogenesis (DiCorleto and Soyombo 1993). In 1992, a refined response-to-injury hypothesis was proposed in which endothelial injury that caused functional modifications, but not necessarily gross endothelial denudation, were the key steps in atherogenesis (Ross 1993, Clinton[a] and Libby 1992). Indeed, today we appreciate that a change in endothelial function would affect permeability of the endothelium to atherogenic lipoproteins, and therefore the refined response-to-retention hypothesis led to the lipid infiltration hypothesis (Wissler 1991).

It is also important to note that the literature documents that a normal intact endothelium can transport or 'leak' many molecules, including lipoproteins (Nordestgaard *et al.* 1994, Proctor *et al.* 2002). Therefore suggesting that substantial alterations in endothelial permeability or function are not essential to initiate cholesterol deposition during atherogenesis (Vasile *et al.* 1983, Navab *et al.* 1986, Lin *et al.* 1989, Williams and Tabas 1995). Key studies have shown that the rate of entry of circulating cholesterol containing lipoproteins, for example low density lipoprotein (LDL), into the normal healthy arterial wall vastly exceeds the actual LDL accumulation rate (Carew *et al.* 1984). Schwenke and Carew demonstrated that the early pre-lesional accumulation of atherogenic lipoproteins within the arterial wall can be concentrated in focal sites thought to be prone to the development of atheromata (Schwenke and Carew 1989[a,b]). These seminal studies suggested that it was not endothelial permeability, but lipoprotein retention, that was the key pathological event in atherogenesis. Subsequent studies carried out in several animal models have followed to demonstrate that atherosclerosis-susceptible focal areas can have either increased or

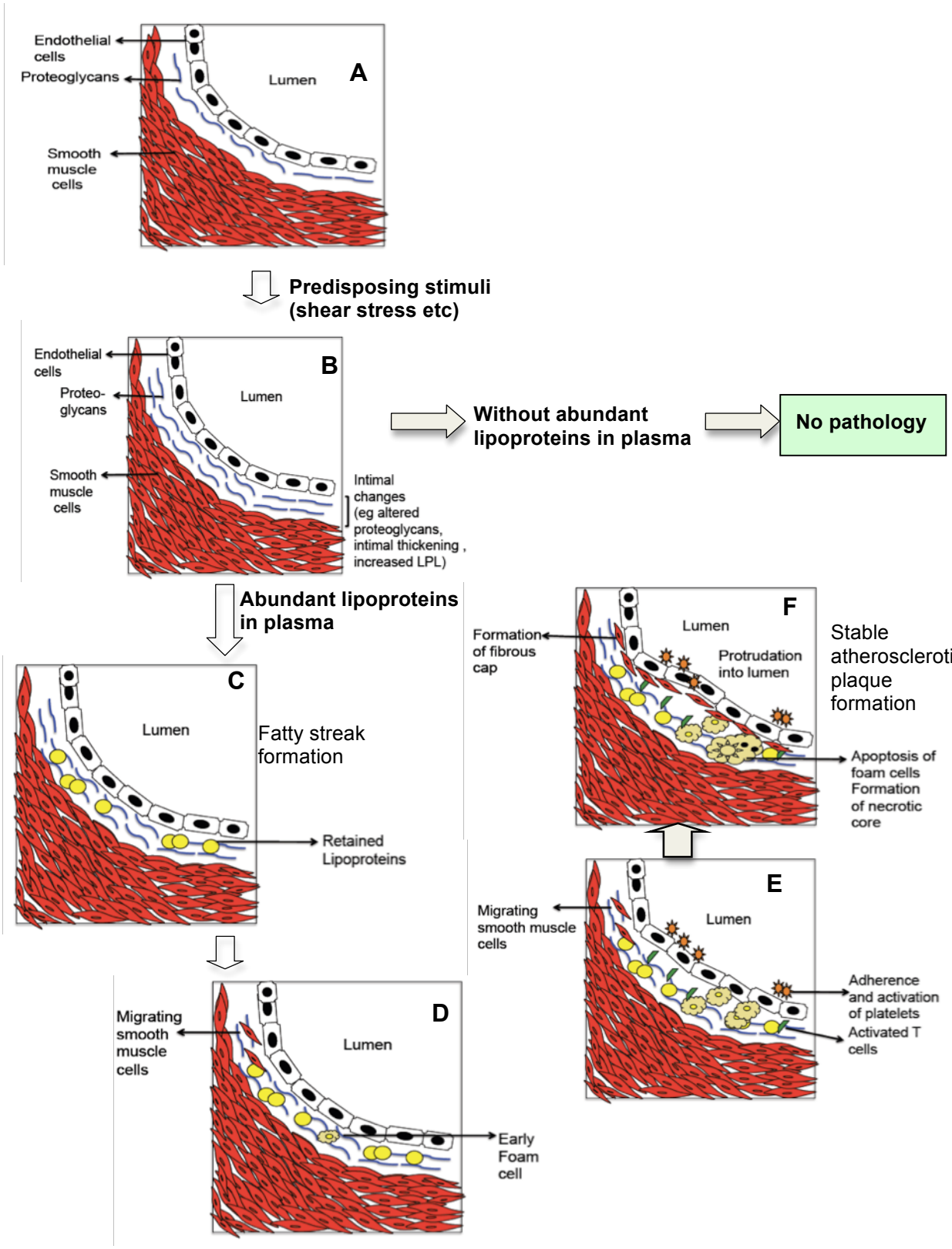
decreased rates of lipoprotein entry (Nielsen *et al.* 1992, Thubrikar *et al.* 1992, Fry *et al.* 1993, and Schwenke *et al.* 1993), suggesting that alterations in endothelial permeability are not essential for atherogenesis. Together with this series of data, it became widely accepted that pre-lesional susceptible focal areas within the arterial wall showed retention of cholesterol carrying lipoproteins, such as apoB-rich lipoproteins (Schwenke and Carew 1989[a]). Shortly thereafter in 1995, Tabas and Williams proposed the response-to-retention hypothesis (Williams and Tabas 1995). According to this hypothesis, lipoprotein *retention* was the critical element in the initiation of atherogenesis (Fry *et al.* 1992, Schwenke and Carew 1989[b]). Figure 1-1 shows the schematic representation of the response to retention hypothesis.

The response-to-retention hypothesis suggests that the deposition and accumulation of atherogenic lipoproteins associated with the extracellular matrix in the arterial intima is the initial event in atherogenesis. It states that the predisposing stimuli (mechanical strain and cytokines) increase local synthesis of proteoglycans (PGs), which in turn bind to lipoproteins entering the arterial intima and as a result they are preferentially retained. These retained lipoproteins are thought to be exposed to reactions that can oxidize or modifiy cholesterol moieties that then further facilitate their uptake by macrophages (Williams and Tabas 1995).

Collectively, together with some convincing epidemiological and clinical intervention data, the understanding of the response-to-retention hypothesis readily accepted that the main source of arterial cholesterol was derived from circulating LDL. Consequently a significant portion of research in this field has been focused on strategies and therapies to reduce the burden of CVD by reducing the circulating concentration of plasma LDL cholesterol (LDL-C). Despite the rapid discovery of new lipid lowering agents with potential for anti-atherosclerotic action, the continued rise in the incidence of CVD suggests that we are yet to completely understand the etiology of the atherosclerotic process.

While this thesis will focus predominantly on the early initiating stages of lipoprotein arterial uptake and retention, the subsequent development of atherosclerotic lesions include the continued accumulation of lipids along with hallmark inflammatory reactions that trigger additional pathology. For example, lipids and lipoproteins retained within the arterial wall lead to an increased expression of vascular cell adhesion molecule-1 (VCAM-1) on the endothelial cells that can bind monocytes and lymphocytes on the endothelial monolayer (Li *et al.* 1993). Further, endothelial cells and smooth muscle cells are thought to increase their production of monocyte chemo-attractant protein-1 (MCP-1) that signals monocytes to bind to the intima at sites of lipid accumulation. Concurrently, it is thought that another class of molecules produced by endothelial cells and vascular smooth muscle cells called 'T cell chemo-attractants' solicit select lymphocytes to the intimal space (Gu *et al.* 1998, Boring *et al.* 1998, Mach *et al.* 1999). The presence of these inflammatory cells in the arterial wall is considered to further stimulate local inflammatory pathways, via the release of macrophage colony stimulating factor (M-CSF) from endothelial cells and smooth muscle cells (Rosenfeld et al. 1992, Clinton[b] et al. 1992) which facilitates monocytes to mature into macrophages. These 'activated' macrophages then begin to engulf lipid and lipoproteins, and the presence of lipid droplets then gives these cells a 'foamy' appearance, which forms the early fatty streak. As the lipid core grows, apoptosis of foam cells can occur, perhaps as a result of endoplasmic reticulum stress, which leads to the formation of a necrotic core and progression to an advanced plaque (Tabas 2010).

Figure 1-1. Schematic representation of response-to-retention hypothesis of atherogenesis (adapted from Tabas and Williams 1995). Predisposing stimuli such as sheer stress makes certain areas of the arterial wall susceptible to lipoprotein retention (B). Without hyperlipidemia, no further pathology is seen. However in the case of hyperlipidemia these susceptible areas lead to fatty streak formation due to retention of cholesterol-dense lipoproteins in these susceptible areas (C). Once significant lipoprotein retention has occurred it leads to changes such as lipoprotein oxidation, aggregation, adherence and activation of platelets and T cell recuitment, eventually leading to formation of an atherosclerotic plaque (D,E,F).


Endothelial cells
Proteoglycans
Lumen
A
Smooth muscle cells
Predisposing stimuli (shear stress etc)
Endothelial cells
Proteo-glycans
Lumen
B
Smooth muscle cells
Intimal changes (eg altered proteoglycans, intimal thickening , increased LPL)
Without abundant lipoproteins in plasma
No pathology
Abundant lipoproteins in plasma
C
Lumen
Fatty streak formation
Retained Lipoproteins
Migrating smooth muscle cells
D
Lumen
Early Foam cell
F
Lumen
Formation of fibrous cap
Protrudation into lumen
Stable atherosclerotic plaque formation
Apoptosis of foam cells Formation of necrotic core
E
Migrating smooth muscle cells
Lumen
Adherence and activation of platelets
Activated T cells

1.1.3 Evidence for response-to-retention hypothesis

There are several studies that highlight the important concepts regarding lipoprotein retention during atherosclerosis, including the role of the extracellular matrix. Nakashima *et al.* investigated the early phases of atherosclerosis using coronary arteries of subjects that died between the ages of 7 and 49 years. They demonstrated that human atherosclerosis appears to begin with the extracellular deposition of apolipoprotein B (apoB) associated lipids in the outer layer surrounding an area of diffuse intimal thickening (Nakashima *et al.* 2007). Indeed, extracellular deposits of lipoproteins in the intima of early human lesions have also been detected in several other studies using immunohistochemistry techniques (Fucuchi *et al.* 2002, Wyler *et al.* 2006, Torzewski *et al.* 1998).

Several studies now support the concept that the accumulation of lipids precedes macrophage infiltration during the early phase of atherosclerosis development. In the intima of fetuses and young adults, accumulation of apoB has been found prior to the infiltration of macrophages (Napoli *et al.* 1997, Babaev *et al.* 1993). A study by Guyton in 1993, recruited young and middle-aged adults and using electron microscopy, identified that the early formation of the atherosclerotic core can arise from lipids derived from within inner-most layer of the intimal extracellular matrix (Guyton *et al.* 1993). It is also noteworthy that Nakashima *et al.* have also confirmed that the extracellular lipid deposition can *occur independently of macrophages* in the initial stages of atherosclerosis.

With respect to the originating source of lipids, Hoff *et al.* have demonstrated lipoproteins containing apoB to be present in grossly normal, as well as atherosclerotic aortas (Hoff *et al.* 1977). Furthermore another study by the same group has shown that lipoproteins extracted from human atherosclerotic plaques by affinity chromatography with anti apoB are the same size (200 angstrom) as plasma LDL, however they did find a small fraction of lipoproteins that were larger in size (larger than 220 angstrom) (Hoff *et al.*

1979). The LDL-like fraction was more electronegative and contained relatively less protein as compared to plasma LDL, and the apoB in this fraction was highly degraded (Hoff *et al.* 1979). The cholesteryl esters (CE) were found to be rich in stearate and oleate, and depleted in linoleate. In a separate study a high percentage of unesterified cholesterol was found associated with the amorphous lipid pool in fibrous plaques and it was concluded that most of cholesterol must be directly derived from plasma LDL (Smith *et al.* 1972).

1.1.4 Development of HMG-CoA reductase inhibitors and CVD risk

In 1978, Merck had produced the first commercial statin (HMG-CoA reductase inhibitor) 'lovastatin', the class of drugs that can effectively lower LDL-C levels. However despite the effective LDL lowering capacity of statin therapy, substantial 'residual risk' remains in subsets of patients (Campbell *et al.* 2007). For instance in the PROVE-IT trial, 22% of the patients receiving intensive lipid-lowering therapy still had a CVD event in the next proceeding 24 months (Cannon *et al.* 2004).

Therefore my research interest lies in the void of appreciation of 'residual risk', particularly the paradox of syndromes or disease states that accelerate CVD during conditions of normolipidemia (i.e. normal concentrations of LDL-C). In particular, during conditions of diabetes the increased risk of CVD is not neccessarily coupled with elevations in LDL-C, suggesting that other factors play a significant role in exacerbating the development of atherosclerosis, including other sources of cholesterol.

To further appreciate the potential contribution of all lipoproteins in atherogenesis and to elaborate the experimental aims of the work presented in this thesis, lipoprotein metabolism will be reviewed.

1.2 Plasma Lipoproteins: Composition and Structure

1.2.1 Classes of lipoproteins

Lipoproteins are macromolecules containing lipid and protein that enables the transport of lipids in the aqueous environment of the blood stream. Triglyceride (TG) and cholesteryl esters (CE) are the hydrophobic lipids that comprise the core of the lipoprotein and are enveloped by a layer of phospholipid, free cholesterol and apolipoproteins (Ginsberg *et al.* 2005). Apolipoproteins are regulators of lipid transport. Several major classes of lipoproteins have been defined on the basis of their physical and chemical characteristics: chylomicrons (CM), very-low density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoproteins (HDL) (Ginsberg *et al*. 2005). Densities and lipid composition of the different lipoprotein classes are shown in (Figure 1-2 and Table 1-1)

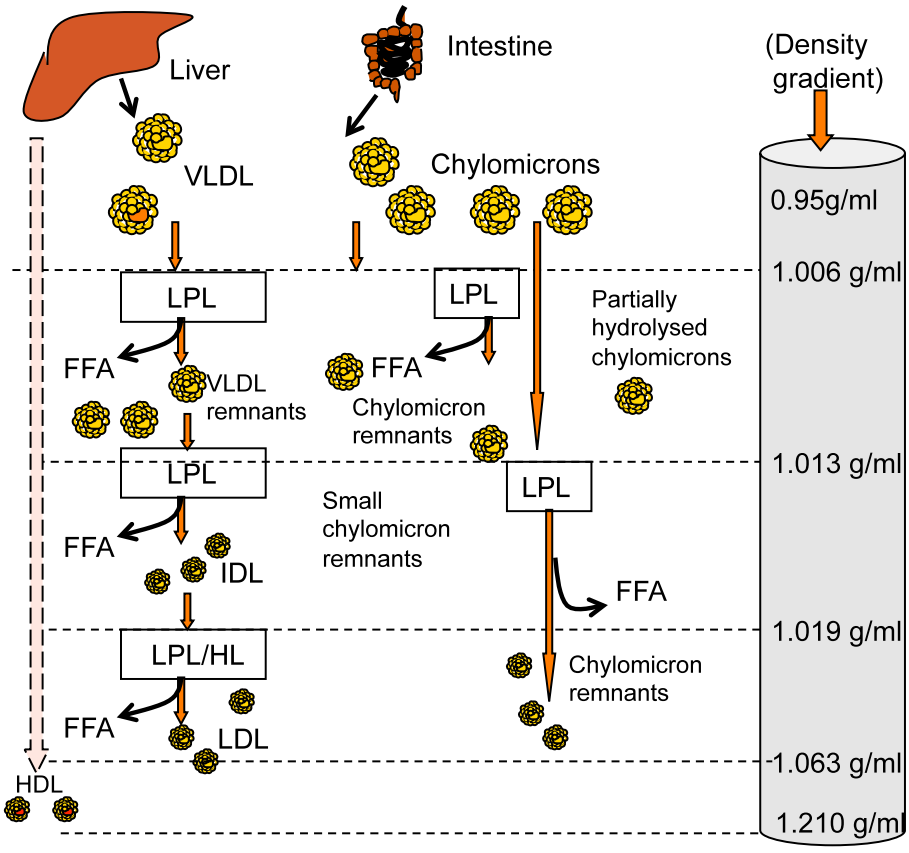


Figure 1-2 Characterization of lipoproteins based on density (adapted from Su et al. 2009). Intestinally derived nascent chylomicrons have a density of <0.95

g/ml. Hepatic derived VLDL particles have a density between 0.95-1.006 g/ml. Hydrolyzed CMs called chylomicron remnants (CM-r) have a density between 1.006-1.013 g/ml, while LDL are found at a higher density between 1.019-1.063 g/ml. However recent studies have shown that dense CM-r can also be found at this higher density i.e 1.019-1.063 g/ml. Therefore to detect CM-r the whole plasma method is recommended instead of using density ultracentrifugation.

Table 1-1. Composition of the classes of lipoproteins (adapted from Ginsberg et al. 2005).

Lipoprotein Classes	**Lipids(%)**		
	Triglycerides	**Cholesterol**	**Phospholipid**
Chylomicrons	80-95	2-7	3-9
VLDL	55-80	5-15	10-20
IDL	20-50	20-40	15-25
LDL	5-15	40-50	20-25
HDL	5-10	15-25	20-30

1.2.2 Apolipoproteins

Table 1-2 shows the characteristics of major the apolipoproteins and their association with each lipoprotein class. Apolipoproteins are found on the surface of lipoproteins and provide structural stability. They play an important role in the transport and redistribution of lipids between lipoproteins and tissues because the delivery of lipids to specific cells involves the recognition of specific apolipoproteins by cell surface lipoprotein receptors (Mahley *et al.* 1984). They also play a crucial role in lipoprotein metabolism since some apolipoproteins act as cofactors for enzymes in lipoprotein lipid catabolism.

For example, lipoprotein lipase, which catalyzes the hydrolysis of CM and VLDL triglycerides to release free fatty acids (FFA) and glycerol, requires the presence of apoC II.

Table 1-2. Characteristics of major apolipoproteins. Adapted from Ginsberg et al. 2005.

Apolipoprotein	**Lipoproteins**	**Location of Secretion**	**Metabolic functions**
Apo AI	HDL, chylomicrons	Liver, Intestine	Structural activator of HDL, LCAT activator
Apo AII	HDL, chylomicrons	Liver, Intestine	2nd most abundant HDL apolipoprotein, Modulates TG metabolism through LPL activity
Apo AIV	HDL, chylomicrons	Intestine (humans), Liver (rats and mice)	Unknown, possibly facilitates transfer of apos between HDL and chylomicrons
Apo AV	HDL	Liver	Associated with lower TG levels, mechanisms unknown
Apo B48	Chylomicrons	Intestine (human, rats and mice), Liver (rats and mice)	Necessary for the secretion and assembly of chylomicrons from the small intestine.

Apolipoprotein	Lipoproteins	Location of Secretion	Metabolic functions
Apo B100	VLDL, IDL, HDL	Liver, Intestine	Necessary for assembly and secretion of VLDL from the liver; Ligand for LDL receptor.
Apo CI	Chylomicrons, VLDL, IDL, HDL	Liver, Intestine	May inhibit hepatic uptake of chylomicrons, VLDL remnants
Apo CII	Chylomicrons, VLDL, IDL, HDL	Liver, Intestine	Activator of Lipoprotein Lipase
Apo CIII	Chylomicrons, VLDL, IDL, HDL	Liver, Intestine	Inhibitor of Lipoprotein Lipase and of uptake of chylomicron and VLDL remnant by the liver.
Apo E	Chylomicrons, VLDL, IDL, HDL	Liver, Intestine, Brain, Adipose, kidney, adrenals, spleen	Ligand for binding of several lipoproteins to the LDL receptor, LRP and PGs
Apo (a)	Lp(a)	Liver	Composed of LDL apoB linked covalently to apo(a): Unknown function but is an independent predictor of CAD.

Apolipoprotein B100

ApoB100 is a hydrophobic protein of 540kD and is synthesized mainly in the liver in humans and to a smaller extent in the intestine. ApoB100 contains an LDL receptor (LDL-r) binding domain, which is involved in the primary uptake of plasma LDL, and to a lesser extent VLDL, and IDL by tissues (Ginsberg *et al*. 2005).

ApoB100 is the protein determinant on LDL that recognizes the hepatic LDL-r (also called apoB/E receptor) that is the first step in the receptor-mediated catabolism of LDL by the liver. ApoB100 is used to determine the *particle number* of LDL.

Apolipoprotein B48

ApoB48 is transcribed from the gene for apoB100 in the small intestine in humans and in the intestine and liver of rats and mice. The mRNA is edited at base 6666 such that a codon for glycine becomes a stop codon and the translation of apoB100 mRNA stops at this codon. The apoB48 is synthesized in the rough endoplasmic reticulum with small amounts of triglyceride, which is transferred to apoB48 by MTP. The resultant particles are called nascent CM that are transferred to the golgi vesicles where they are further lipidated and finally secreted as CM into lymph (Christensen *et al*. 1983). ApoB48 is found in CM and their remnants, and is necessary for intestinal assembly and secretion of these lipoproteins (Davis 1999). Similar to apoB100, apoB48 is used to determine the *particle number* of CM or intestinal derived remnant lipoproteins.

Apolipoprotein E

As compared to apoB, apoE is a smaller molecule that is produced in the liver, intestine, brain, adipose, kidney adrenals and spleen. ApoE mediates the interaction between the apoE containing lipoproteins and the hepatic LDL-r (Mahley *et al.* 1984). LDL-r has a much higher affinity for apoE as compared

to apoB. The higher affinity of apoE as compared to apoB correlates with the rapid rate of plasma clearance of the apoE-containing lipoproteins, such as CM, (clearance in minutes to hours) compared to a much slower clearance of apoB containing LDL (2 to 3 days) (Mahley *et al.* 1984).

Apolipoprotein C

The C apolipoproteins are represented by three low molecular weight apolipoproteins, apoCI, CII, and CIII, that are surface components of chylomicrons, VLDL, and HDL.

During the synthesis of VLDL by the liver and CM in the intestine, the C apolipoproteins are distributed to the surface of these triglyceride-rich lipoproteins (Nestel and Fidge 1982). As the triglyceride core of VLDL and chylomicrons is hydrolyzed and depleted by the action of lipoprotein lipase, excess surface components (phospholipid, unesterified cholesterol, and apolipoproteins) are generated, and the C apolipoproteins along with the other excess surface components are transferred to HDL (Nestel and Fidge 1982).

1.3 LDL Cholesterol Concentration and CVD

The patient populations exhibiting the greatest 'residual risk' for CVD include subjects with type 1 or type 2 diabetes, as well as subjects with metabolic syndrome (Campbell *et al.* 2007). Therefore it is pertinent to investigate the definitive mechanisms or causes underlying this accelerated risk for atherosclerosis during diabetes.

Several large randomized, placebo controlled clinical trials have shown the relationship between LDL-C levels in both primary prevention (WOSCOPS [Shepherd 1995]) and (AFCAPS/TexCAPS [Downs *et al.* 1998]) and secondary prevention of CVD risk trials (4S [Scandinavian Simvastatin Survival Study Group 1994]), (CARE [Sacks *et al.* 1996]), (HPS [HPS study group 2002]) and LIPID ([LIPID study group 1998]). These trials have shown

a reduction of 24 to 37% risk of a major coronary event when LDL-C was reduced by 25-35%. A meta-analysis of 14 prospective trials involving statins and over 90,000 patients found that major coronary events were reduced by 23% per 1 mmol/L reduction in LDL-C. This finding was irrespective of initial lipid profile or other patient characteristics (Cholesterol Treatment Trialists Collaborators 2008).

More recently, mutations in proprotein convertase subtilisin/kexin type 9 (PCSK9) have been shown to cause life long reduction in LDL-C concentration as well as a concomitant reduction in CVD (Benn *et al.* 2010). Therefore the definition of optimal LDL-C concentration is still debated by many though the widely accepted value for optimal LDL-C concentration is <100mg/dl and <70mg/dl in persons with a history of heart disease.

These and several other trials have led to guidelines that target LDL cholesterol lowering.

1.3.1 Role of oxidized LDL in atherogenesis

According to the oxidative hypothesis of atherogenesis, oxidized LDL plays a key role in atherogenesis initiation and progression (Steinberg 2005). Oxidized LDL has been consistently found in human atherosclerotic plaques (Steinberg *et al.* 1989).

Research interest in the role of oxidized LDL in atherogenesis began with two seminal studies. The first study (Hessler *et al.* 1979) showed that oxidized LDL was cytotoxic to endothelial and other vascular cells, and thus could cause damage to arterial cells. The second study showed that the uptake of native LDL by macrophages occurred at a low rate and foam cell formation was prevented, whereas the uptake of oxidized LDL was unregulated and led to macrophage foam cell formation (Heinecke *et al.* 1997, Steinbrecher *et al.*1984). When the apoB100 moiety of the LDL particle is oxidized it results in a more negative charge that leads to an increased affinity for scavenger receptors (non regulated uptake) and a decreased affinity for LDL-r (regulated

by feed back inhibition) (Goldstein *et al.* 1979). Oxidized LDL particles activate endothelial cells, which lead to the expression of vascular adhesion molecule-1 and intercellular adhesion molecule at their surface (Kaplan and Aviram 1999). These adhesion molecules, in addition to MCP-1, promote the adhesion and entry of monocytes into the arterial vessel. Monocytes that have engulfed lipid to become lipid filled macrophages further promote monocyte recruitment and progress to foam cells, contributing to the vicious cycle in the etiology of atherosclerosis.

Plasma concentrations of oxidized LDL have been correlated with intima media thickness and the occurrence of plaque in the carotid and femoral arteries (Wallenfeldt *et al.* 2004, Joensuu *et al.* 1994), and with the progression of atherosclerosis in carotid arteries (Metso *et al.* 2004). Several case control studies have shown that oxidized LDL concentrations are elevated in subjects with coronary heart disease or with risk of coronary heart disease when compared to healthy subjects (Ehara *et al.* 2001, Tsimikas *et al.* 2004). Several studies have shown that plasma oxidized LDL concentration is a more specific and sensitive marker of coronary heart disease risk than total cholesterol, triglycerides, apoB, HDL-C in subjects with established coronary heart disease (Holvoet *et al.* 2004). Plasma oxidized LDL concentrations have been proposed to provide additional information to the Global Additive Risk Scoring System which is based on age, total and HDL-C concentrations, systolic blood pressure, type 2 diabetes and smoking, and this scoring system is used for primary prevention of coronary heart disease (Holvoet *et al.* 2001).

1.4 Accelerated CVD during Diabetes

1.4.1 Accelerated CVD in type 1 diabetes (T1D)

Diabetes mellitus type 1 (T1D, insulin dependent diabetes mellitus, or juvenile diabetes) is a form of diabetes mellitus that is caused by an autoimmune destruction of insulin-producing beta cells of the pancreas (Bach 1994). The

subsequent lack of insulin leads to increased blood and urine glucose. The classical symptoms of polyuria (frequent urination), polydipsia (increased thirst), polyphagia (increased hunger), and weight loss result.

The risk of cardiovascular disease increases by more than 10 fold in subjects with T1D relative to the non-diabetic population (Krolewski *et al.* 1987, Deckert *et al.* 1978, Dorman et al. 1984, Orchard *et al.* 2006). The etiology of atherosclerosis in type 1 subjects is not very well defined.

1.4.1.1 Dyslipidemia in T1D

Type 1 diabetic dyslipidemia is often characterized by marked elevations in the levels of TG-rich lipoproteins, including both CM and VLDL. In subjects with significant insulin deficiency, the concentration of LDL-C can be increased, however this is not a consistent finding (Chahil and Ginsberg 2006). While in patients with well-controlled T1D, LDL-C and VLDL concentration may be normal, while little has been reported on the plasma concentration of intestinal derived remnant lipoproteins. A recent report from our research group has indicated that the fasting concentration of intestinal derived remnant lipoproteins are elevated in type 1 diabetics (Su *et al.* 2009).

1.4.1.2 Role of hyperglycemia in CVD

Current models support the role of hyperglycemia, glycation and oxidation products in initiating endothelial dysfunction which further leads to vasoconstriction, pro-inflammatory and pro-thrombotic changes that contribute to the development of the atherosclerotic plaque, and potential for thrombosis and plaque rupture (Libby *et al.* 2005, Beckman *et al.* 2002). Hyperglycemia is thought to induce profound changes in the endothelium, which include down-regulation of the endogenous vasodilator nitric oxide. Reduced nitric oxide production causes vasoconstriction, thus leading to the increased expression of leukocyte-attracting chemokines and inflammatory cytokines (Beckman *et al.* 2002, Eckel *et al.* 2002). These vascular modifications render the endothelium more susceptible to monocyte

adherance and entry into the arterial wall. It is proposed that these preceding events facilitate the increased formation of foam cells, exacerbate the retention of lipoproteins and the subsequent formation of fatty streaks (Eckel *et al.* 2002).

In spite of such proposed mechanisms and experimental evidence, the lack of epidemiological evidence linking the role of hyperglycemia in the development of T1D and atherosclerosis is debatable. Three large prospective observational studies failed to detect a consistent association between measures of glycemia and cardiovascular disease. In the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR), HbA_{1C} was significantly associated with mortality from heart disease however in univariate analysis it was not associated with myocardial infarction or angina (Klein *et al.* 2004). In EURODIAB, a large multicentre study, HbA_{1C} was not independently related to the incidence of coronary artery disease over a follow-up of 8 years (Soedamah-Muthu *et al.* 2008). When the data from the Pittsburg EDC study was analyzed after a 10-year follow up, neither baseline glycemia nor cumulative glycemic exposure was significantly associated with the incidence of coronary artery disease (Orchard *et al.* 2003, Forrest *et al.* 2000).

Though these studies differed in selection of patient populations, they could not provide evidence for the role of hyperglycemia in the development of atherosclerosis in T1D.

The DCCT (The Diabetes Control and Complications Trial, 1993) was a randomized controlled clinical trial with 1441 patients with T1D, who were assigned to receive either intensive diabetes therapy (frequent blood monitoring with 3 or more insulin injections) or conventional diabetes therapy (one or two insulin injections a day), and these patients were followed for 6.5 years. In this study, the intensive therapy arm showed significantly reduced mean HbA_{1C} levels, but despite a 41% reduction in macrovascular, the results failed to achieve statistical significance. A follow up study from the DCCT

called the EDIC (The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study 1999, Nathan *et al.* 2005) followed the patients from the DCCT study for 17 years and showed that intensive glycemic control in the intensive diabetes therapy group indeed showed a significant 42% reduced risk of all CVD events. Also a reduced risk of 57% was observed for nonfatal myocardial infarction, stroke or cardiovascular mortality. The DCCT study also showed a reduction in the carotid intima media thickness after the 6.5 years follow up period in the intensive diabetes therapy group (Nathan 2005).

Data from animal studies shows a close relationship between glucose and lipids. Studies in pigs have shown that atherosclerosis does not develop in the absence of hyperlipidemia (Howard 1985, Gerrity *et al.* 2001). Studies in mouse models suggest that hyperglycemia may play a role in lesion initiation however hyperlipidemia is needed for lesion progression (Renard *et al.* 2004, Johansson *et al.* 2008).

Therefore the role of hyperglycemia in initiating or exacerbating atherosclerosis in T1D remains inconclusive and needs further investigation.

1.4.2 Accelerated CVD in insulin resistance

The metabolic syndrome is an insidious disease due to the asymptomatic nature of the early stages of its development. A typical clinical history involves a long period of insulin resistance (IR) without obvious underlying disease followed by an apparently sudden development of type 2 diabetes. As the clinical diabetes is diagnosed, proceeding tests indicated established cardiovascular disease (Russell and Proctor 2006). The associated significant damage to the vascular system is thought to develop during the pre-diabetic and early diabetic phases of type 2 diabetes. The focus of this thesis is to investigate the mechanisms of arterial lipid retention in the early progressive stages of type 2 diabetes, that involves IR and the metabolic syndrome.

In metabolic syndrome, the fundamental defect has been postulated to be the resistance of skeletal muscle to insulin mediated glucose uptake. The severity of an individual's IR is marked by the degree of compensatory hyperinsulinaemia that is needed to maintain glucose homeostasis and tissue uptake. The combination of hyperinsulinaemia and IR differentiates the metabolic syndrome from diabetes (Reaven 1988). Type 2 diabetes develops when the compensatory hyperinsulinaemia is not enough to overcome insulin resistance to allow glucose uptake by tissues.

There are several definitions for insulin resistance. See Table 1-3 for some of the established definitions of IR. It is interesting to note that despite the established role of LDL-C as a risk factor for CVD, none of the IR definitions include LDL. Even in subjects with type 2 diabetes, the most common lipid abnormality is the combination of both fasting and postprandial TG levels, and low HDL levels and normal LDL-C levels when compared to non-diabetic subjects. Several trials and observational studies have demonstrated this lipoprotein pattern for type 2 diabetes patients (Howard *et al.* 1998, Siegel *et al.* 1998, Sacks and Campos 2003).

Table 1-3: Definitions of the Metabolic syndrome

World Health Organization **(WHO, Alberti *et al.* 1998)**	**International Diabetes Federation** **(IDF, Day 2007)**	**Adult Treatment Panel** **(ATP III, 2002)**
The patient must have one of the following: *Diabetes mellitus* Fasting plasma glucose 7 mmol/L (126 mg/dL) or 2-h post glucose load 11.1 mmol/L (200 mg/dL) *Impaired glucose tolerance* Fasting plasma glucose 7 mmol/L (126 mg/dL) and 2-h postglucose load 7.8 mmol/L (140 mg/dL) and 11.1 mmol/L (200 mg/dL) *Impaired fasting glucose* Fasting plasma glucose 6.1 mmol/L (110 mg/dL) and 7 mmol/L (126 mg/dL) and (if measured) 2-h postglucose load 7.8 mmol/L (140 mg/dL) *Insulin resistance* Glucose uptake below lowest quartile for background population under investigation under hyperinsulinemic, euglycemic conditions **Plus any 2 of the following:** *Waist-to-hip ratio* 0.9 in men, 0.85 in women; BMI 30; or both *Triglycerides* 1.7 mmol/L (150 mg/dL); HDL cholesterol 0.9 mmol/L (35 mg/dL) in men, 1.0 mmol/L (39 mg/dL) in women; or both *Blood pressure* 140/90 mm Hg (revised from 160/90 mm Hg), *Microalbuminuria*	**The patient must have:** *Central obesity* defined as a waist circumference 94 cm for European men and 80 cm for European women, with ethnicity-specific values for other groups **Plus any 2 of the following 4 factors:** ● *High triglyceride concentration*: 150 mg/dL (1. 7 mmol/L), or specific treatment for this abnormality ● *Low HDL-cholesterol concentration*: 40 mg/dL (1.03 mmol/L) in males and 50 mg/dL (1.29 mmol/L) in females), or specific treatment for this lipid abnormality ● *High blood pressure (BP):* systolic BP 130 mm Hg or diastolic BP 85 mm Hg, or treatment of previously diagnosed hypertension ● *High fasting plasma glucose (FPG) concentration* 100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes. If FPG is above the values stated above, an oral-glucose-tolerance test is strongly recommended but is not necessary to define presence of the syndrome.	**The patient must have any 3 of following:** *Fasting glucose* 6.1 mmol/L (110 mg/dL) Waist circumference Men: 102 cm (40 in) Women: 88 cm (35 in) *Triglycerides* 1.7 mmol/L (150 mg/dL) *HDL cholesterol* Men: 1.036 mmol/L (40 mg/dL) Women: 1.295 mmol/L (50 mg/dL) *Blood pressure* 130/85 mm Hg

1.4.2.1 Dyslipidemia during insulin resistance

An important characteristic of patients with IR is the overproduction of VLDL, with increased secretion of both apoB100 and triglycerides (Ginsberg 2000). Physiologically, it is essential to consider plasma lipoproteins during both the fasting and non-fasted or post-prandial phase.

Humans (and animals) are potentially in the post-prandial state continuously for up to16–20h per day. This contributes to elevated CM-associated TG from the intestine, which contributes to circulating plasma TG levels. Increases in dietary carbohydrate substrate can facilitate increased synthesis of lipid at the site of the liver. Thus hyperphagia and IR collectively generate significant biochemical modulations in the liver including the up-regulation and hypersecretion of VLDL (Russell and Proctor 2006).

Fasting and postprandial lipemia in type 2 diabetics is characterized by elevated concentrations of intestinal CM and their remnants. (Mero *et al.* 2000) The relatively normal LDL-C profile in type 1 and type 2 diabetes patients indicates that lipid in the arterial plaque is possibly derived from alternative sources, such as intestinal remnants. Therefore intestinal derived remnant lipoproteins have been proposed to play a major role in increased CVD risk in both type 1 and type 2 diabetics.

A primary rationale for the research undertaken in this thesis is the unsolved paradox between the apparent normal range of LDL-C during the metabolic syndrome, Type 1 or Type 2 diabetes, and the apparent increase in CVD risk in these patients.

1.4.2.2 Mechanisms for accelerated CVD during insulin resistance

IR often clusters with elevated blood pressure, central obesity, elevated plasma total TG and low HDL cholesterol. Together this clustering of several abnormalities is called the metabolic syndrome and has been shown in several studies to predict CVD (Mottillo *et al.* 2010). However whether

hyperinsulinemia itself is indeed a predictor of CVD has often been debated (Laakso 1996).

An important meta-analysis by Ruige *et al.* showed a weak positive association between high plasma insulin levels and CVD events (Ruige *et al*. 1998). Another meta-analysis (Mottillo *et al.* 2010) involving 87 studies, which included 951,083 patients based on the definitions of metabolic syndrome by the 2001 National Cholesterol Education Program (NCEP) and 2004 revised National Cholesterol Education Program (rNCEP), demonstrated a 2-fold increase in cardiovascular outcomes and a 1.5-fold increase in all causes of mortality. According to the NHANES III data, subjects with the metabolic syndrome but without diabetes proper, had a significantly increased prevalence of CVD (Alexander *et al.* 2003).

However, it is not known whether or not the CVD risk associated with the metabolic syndrome indeed is related to the sum of the individual components or an individual component.

IR has also been shown to be associated with impaired vasodilation, increased oxidative stress, increased plasma concentrations of free fatty acids, vasoconstrictors, cell adhesion molecules, cytokines and several other mediators of low grade inflammation and thrombogenesis (Laakso *et al.* 2009). The CVD risk factors associated with the metabolic syndrome are reviewed in Figure 1-3.

It remains unclear whether accelerated atherosclerosis in IR is simply an accelerated form of underlying atherosclerosis or is indeed different in pathogenesis and is unique to IR.

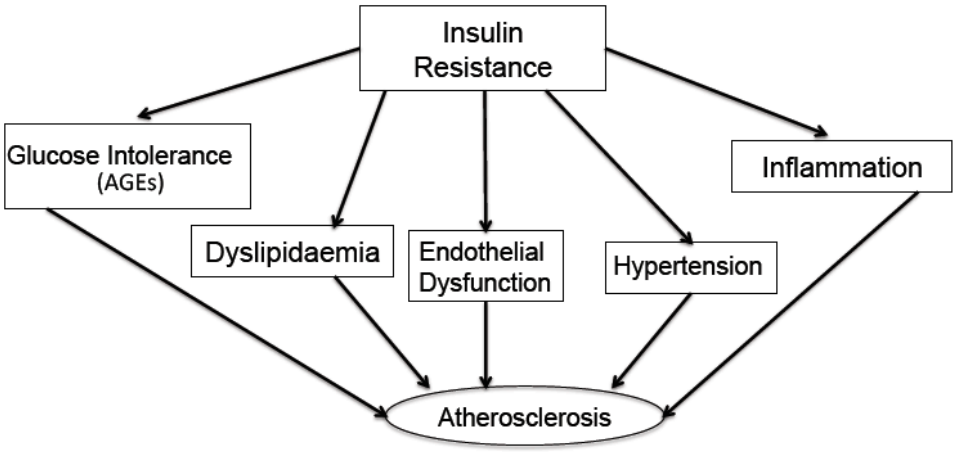


Figure 1-3. Risk factors associated with IR that may lead to atherosclerosis. (Adapted from Rader 2007).

1.5 Lipid Absorption, Synthesis and Metabolism of Chylomicrons

1.5.1 Lipid absorption and synthesis of CM

Under normal physiological conditions, cholesterol and FA, from dietary and biliary sources are presented to the brush border membrane of the enterocyte. A schematic outlining the major steps involved in chylomicron synthesis is presented in Figure 1-4. Absorbed and synthesized FA and monoacylglycerol are chaperoned into the endoplasmic reticulum where they are re-esterifed into TG, and free cholesterol that is absorbed or synthesized in the enterocyte is also esterified. During the biosynthesis and translocation of apoB48 to the lumen of the rough endoplasmic reticulum, co-translational lipidation gives rise to a partially lipidated form of apoB48. The newly formed TG and CE are then incorporated onto a single molecule of apoB48 by the action of microsomal triglyceride transfer protein (MTP) (Mansbach and Gorelick 2007). These core lipids are then coated with a surface layer of phospholipids and other apolipoproteins. However the exact mechanisms of how these other apolipoproteins, unlike apoB48, are synthesized or sourced in the enterocyte, incorporated into the CM and their regulatory mechanisms

are not known in detail. The mechanism that controls the number of chylomicron particles secreted from the intestine is also not completely known (Mansbach and Gorelick 2007). It has also been proposed that the enterocyte may also uptake plasma FA and cholesterol from the basolateral plasma membrane (Duez *et al.* 2008).

There is evidence to suggest that the enterocyte can export lipoproteins via both apoB dependent (CM) and apoB independent (HDL) pathways. Intracellular FA and cholesterol synthesis machinery contribute substantially to the regulation of substrate availability in order to control the lipidation of each apoB particle.

Both FA and cholesterol synthesis machinery can be influenced by insulin signaling pathways and maybe dysfunctional during conditions of IR. Lally *et al.* reported a highly significant increase in the expression of HMGCoA reductase in both liver and intestine of diabetic animals (Lally *et al.* 2007). Additionally IR has been shown to affect the availability of apoB by increasing the activity of microsomal triglyceride transfer protein (MTP) resulting in over-production and excessive particle export (Duez *et al.* 2008). It is not yet clear whether the enterocyte can compensate during IR to adequately maintain intracellular FA and/or cholesterol homeostasis or whether increased dietary (and/or plasma) substrate exacerbates over-production of lipoprotein particles.

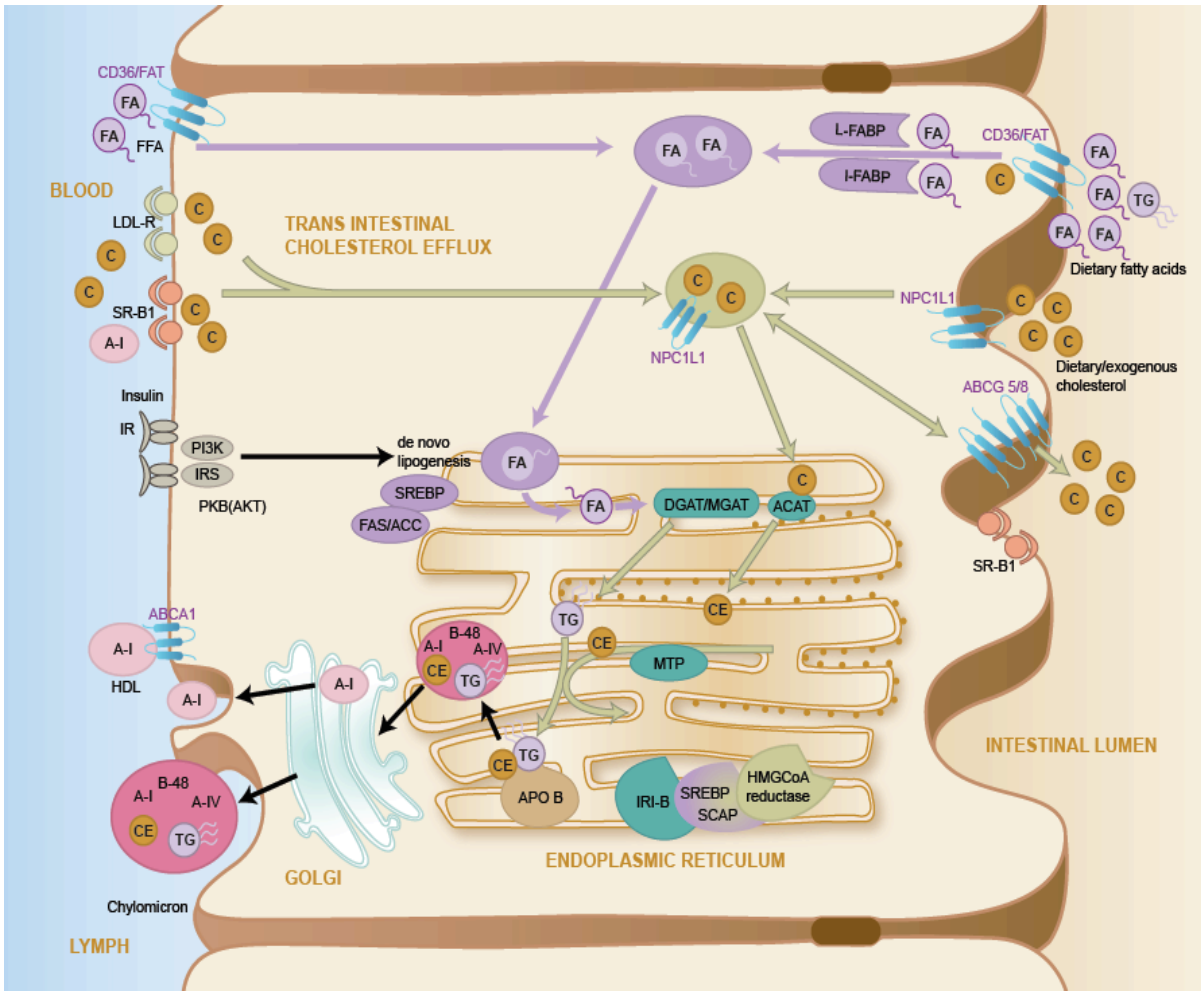


Figure 1-4 Schematic model of cholesterol, fatty acid and triglyceride flux within the intestinal enterocyte. Cholesterol ('C') and triglyceride ('TG' and 'FA') is presented to the brush boarder membrane of the enterocyte (right side of panel) either from dietary or biliary sources to interact with the undisturbed layer and facilitated transporters (i.e. CD36/FAT, NPC1L1/SR-B1). Dietary triglycerides are absorbed into the intestine as mono acyl glycerol or as free fatty acids and are resynthesized into triglycerides in the endoplasmic reticulum. It is likely that both TG and sterols are chaperoned intracellularly to interact with a number of trafficking proteins and/or endosomal-like compartments that regulate homeostasis between absorption, uptake, efflux and lipoprotein secretion within the cell. It is proposed that the

enterocyte may also uptake plasma fatty acids and cholesterol from the basolateral membrane via CD36/FAT and other transporters. Fatty acids are re-packaged into TG prior to their export as lipoproteins. Cholesterol that is lumenally absorbed into the intestine is free cholesterol and it is then esterified in the endoplasmic reticulum by acyl CoA acyl trasferase 1 (ACAT1). Fatty acids and monoacylglycerol are taken to the endoplasmic reticulum by intestinal-fatty acid binding protein (I-FABP) and liver-fatty acid binding protein (L-FABP) and are incorporated into triglycerides by the action of monoacylglycerol acyl transferase (MGAT) and diacylglycerol acyl transferase (DGAT). The newly synthesized triglycerides are then incorporated together with cholesterol and a small amount of cholesteryl esters onto a single molecule of apoB48 by the action of microsomal triglyceride transfer protein (MTP). These are coated with a surface layer of phospholipids and other apolipoproteins, including apoCI,CII,CIII, apoAIV, apoE, apoAI and apoAII.

1.5.2 Metabolism of Chylomicrons

CM are secreted into the intestinal mesenteric lymphatic system and enter the blood stream at the left subclavian vein. The apoAI, apoCII apoCIII, and phospholipids from the CM are transferred to HDL as soon as they enter the blood circulation, while apoE is transferred from HDL to CM (See Figure 1-5). While CM are circulated in the plasma, triglycerides are lipolysed by lipoprotein lipase (LPL) and free fatty acids and glycerol are released. All tissues, including muscle and adipocytes, take up the free fatty acids and glycerol. LPL-mediated triglyceride hydrolysis leads to a reduction in the core volume and surface area of chylomicrons.

The resulting particle, following lipolysis are called CM-r or intestinal derived remnant lipoproteins, and these are rich in CE, apoE, apoB48 and phospholipids. These remnants are then cleared from the circulation by all tissues, but predominately the liver via the apoB100/apoE receptor (Ginsberg

et al. 2005). The apoB100/E receptor has a higher affinity for apoE than for apoB.

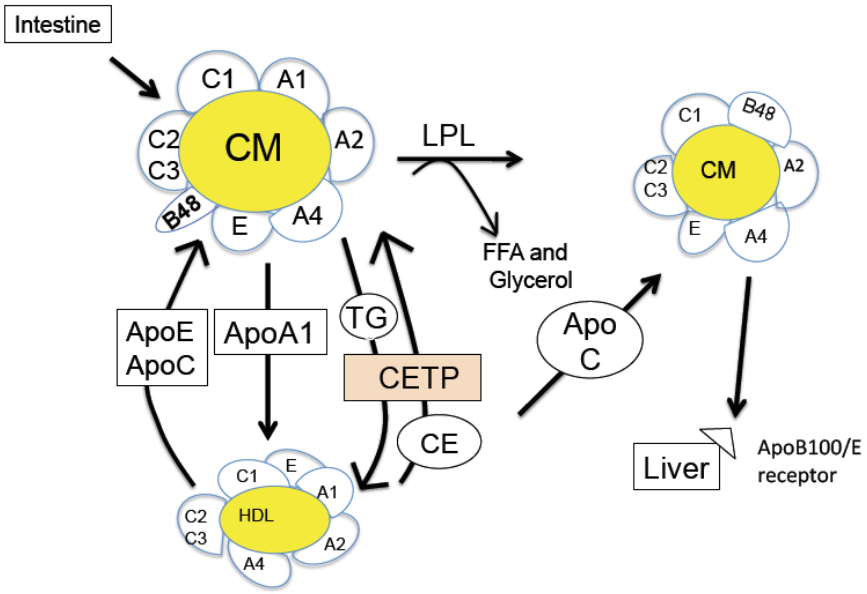


Figure 1-5. Metabolism of Chylomicrons. In the circulation ApoC and ApoE are transferred from the HDL to the nascent chylomicron, while apoA1 is transferred to the HDL. Cholesterol ester transfer protein enables the chylomicron to take up cholesteryl esters (CE) from the HDL while transferring triglycerides (TG) to HDL. As chylomicrons circulate in the body, lipoprotein lipase (LPL) present on the capillary endothelium mediates the lipolysis of chylomicrons and releases free fatty acids and glycerol which are taken up by tissues. Lipolysis converts large nascent chylomicrons to small dense intestinal derived remnant lipoproteins that are then predominately cleared from the liver via the apoB100/E receptor.

1.5.3 Postprandial lipemia and remnant hypothesis

Traditionally the measurements of lipids and lipoproteins in the *fasting* state has been the foundation to detect dyslipidemia and to base hypolipidemic interventions. However we spend significant amounts of time in the

postprandial state which led the hypothesis of the role of postprandial metabolism in atherogenesis (Zilversmit 1979). Thereafter, the study of dyslipidemia now includes postprandial metabolism of lipids and lipoproteins. Several studies have showed that impaired postprandial metabolism is associated with atherogenesis (Kolovou *et al.* 2005). The incremental area under curve (iAUC) for postprandial triaglycerides has been used to assess postprandial response after an oral lipid challenge (Berglund 2002). Figure 1-6 shows a generic postprandial curve in a normal and an insulin resistant individual in response to a meal.

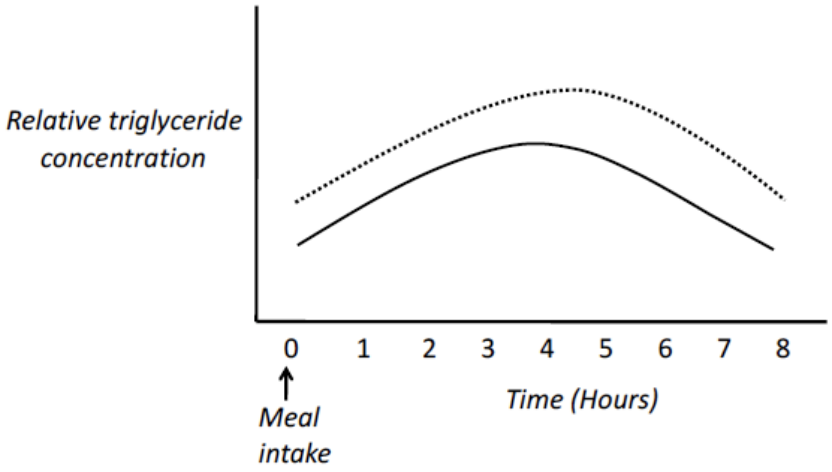


Figure 1-6. Standard postprandial curve showing relative triglyceride concentrations following a meal. Curve for control subjects is shown with a black line and insulin resistant subjects by a dotted line after a single meal intake.

It is evident that postprandial lipemia is prevalent during conditions of IR and may contribute to increased progression of CVD (cardiovascular disease). However, a significant clinical dilemma still exists in diagnosing the early phases of the metabolic syndrome (i.e. pre-diabetes) and how this impacts on

relative risk of CVD. In part, this has been impaired by the continued emphasis on LDL-C, which is often normal during early Type 2 diabetes, leading to the undetected and insidious progression of CVD (Moller and Kaufman 2005, Eckel *et al.* 2005). Indeed it is interesting to note that the recent revision by the IDF (International Diabetes Federation) has defined the metabolic syndrome independent of LDL cholesterol concentration (Zimmet *et al.* 2005).

In the clinic, we continue to recognize the positive effects of LDL-lowering therapy on atherosclerosis and CVD. While these efforts are well documented, increasing evidence supports a causal role between the metabolism of intestinal derived remnant lipoproteins and the development of atherosclerosis (Sullivan *et al.* 2004). CM are TG rich when initially secreted by the enterocyte. Once in circulation, CM particles rapidly undergo hydrolysis to produce cholesterol-dense remnants (van Beek *et al*. 1998, Redgrave 1984). These TG-depleted remnants have been shown to be atherogenic as they are able to penetrate arterial tissue and become entrapped within the subendothelial space (Mamo *et al.* 1994). It has also been demonstrated that intestinal derived remnant lipoproteins can induce macrophage lipid loading, which is a hallmark feature of early atherogenesis (Mamo *et al.* 1997, Tomono *et al.* 1994). Moreover, raised fasting concentrations of apoB48, a specific marker for chylomicrons and their remnants (Phillips *et al.* 1997), have been shown to be elevated in obese, insulin–resistant and Type 2 diabetic subjects (Mamo *et al.* 2001, Cohn *et al.* 1999, Phillips *et al.* 2000, Chan *et al.* 2002).

However, clinical studies have so far failed to provide a definitive association between impaired postprandial metabolism and the very early phases of IR, and corresponding risk indices. Thus animal models offer the potential for further characterize the metabolic syndrome in order to understand the metabolic and post-prandial profile of this condition. Despite a greater emphasis on the study of CVD risk in the metabolic syndrome, there remains

a lack of well established and characterized pre-diabetic animal models in order to investigate the role of postprandial lipoprotein metabolism and the development of atherosclerosis.

Therefore the atherogenicity of apoB48 remnant lipoproteins warrants further investigation to help detect subclinical CVD under conditions of prediabetes and diabetes.

1.6 Effect of Diabetes on Chylomicron Synthesis and Metabolism

1.6.1 Effect of T1D on chylomicron synthesis and metabolism

Insulin is involved in the synthesis of LPL. In subjects with untreated T1D, lipoprotein lipase synthesis is low and therefore hydrolysis of chylomicrons and VLDL is impaired. However in treated T1D, LPL measured in post heparin plasma is normal or elevated, and chylomicron clearance can be normal (Chahil and Ginsberg 2006).

In the study by Levy *et al.* (Levy *et al.* 1985) native CM (C^{14} TG labeled) were collected from streptozotocin induced diabetic rats and these were injected into non-diabetic rats. They observed impaired clearance of CM obtained from diabetic rats when compared to CM obtained from normal rats. They also reported that the CM and remnants obtained from diabetic rats were apoE deficient, and that pre-incubation with HDL increased their apoE content and improved hepatic clearance when compared to CM and remnants obtained from normal rats. The limitation of this study was that the TG content of the lipoprotein was radio-labeled and this is not a representative method to investigate kinetics of CM, as the TG is lipolysed from the CM in the circulation and therefore does not stay with the particle. Another study in type 1 diabetic and control human subjects was not able to detect any differences in the apoE content of postprandial TG rich lipoproteins (Georgopoulos *et al.* 1991). However, this study did demonstrate a delay in the clearance of postprandial TG rich lipoproteins in type 1 diabetic subjects compared to controls.

A study in streptozotocin induced type 1 diabetic rats reported increased intestinal cholesterol absorption in type 1 diabetic rats (Young *et al.* 1988). However this increase was mainly due to increased food intake in the diabetic rats. Another study in human type 1 diabetic subjects concluded that the absorption of cholesterol is higher in type 1 diabetic subjects compared to controls within the range of low absorption, but is similar in those with relatively higher cholesterol absorption (Gylling *et al.* 2007).

Taken together it can be concluded that clearance of chylomicrons may be impaired in subjects with T1D. However it is not known in T1D if CM synthesis is altered or if there are compositional differences in CM that affect their catabolism and clearance from the circulation.

A recent study from our own group has demonstrated post-prandial lipemia and impaired clearance of apoB48 remnant lipoproteins in normolipidemic subjects with T1D (Su *et al.* 2009). In this study the fasting apoB48 plasma concentration was also significantly elevated in these patients compared to control subjects, suggesting fasted apoB48 can predict impaired clearance of these particles in T1D.

ApoB48 is the specific marker for CM and their remnants. However currently there is limited data in human and animal studies that investigates the postprandial metabolism of intestinal derived remnant lipoproteins in T1D, using the specific apoB48 marker of CM metabolism. Previous studies have investigated the metabolism of TRLs in T1D but have not been able to distinguish between fasting and postprandial VLDL (apoB100) or intestinal derived remnant lipoproteins (apoB48) (Georgopoulos *et al.* 1991).

1.6.2 Effect of insulin resistance on CM synthesis and metabolism

CM are synthesized in the enterocyte and deliver endogenous and dietary lipids to the circulation via the lymphatic system. Insulin has been shown to have an acute inhibitory effect on apoB48 production in enterocytes isolated from chow-fed animals, but this effect is not seen in animals that have

hyperinsulinaemia (Haidari *et al.* 2002, Federico *et al.* 2006). Studies in humans have shown that modest delays in postprandial apoB48 peak response may be positively associated with plasma insulin levels in both individuals with fasting hyperinsulinaemia and normal controls (Allister *et al.* 2006). Collectively, under normal physiological conditions it appears that the enterocyte is responsive to postprandial circulating insulin levels which may play a role in down-regulating CM synthesis, but in conditions of chronic hyperinsulinaemia the enterocyte may become resistant to these effects (Federico *et al.* 2006, Allister *et al.* 2006) However, very little is known regarding the effect of insulin on CM synthesis and metabolism, as few laboratories have the expertise to undertake *in vivo* lymphatic isolation of chylomicrons in order to measure CM intestinal synthesis and secretion directly.

Evidence does indicate that intestinal overproduction of apoB48 may be associated with alterations in insulin signaling pathways in the enterocyte (Federico *et al.* 2006). Thus early evidence of CM overproduction during IR provides the impetus to further confirm these findings and also to understand how this may affect intestinal remnant lipoprotein retention in the arterial wall under conditions of IR and diabetes. The proposed mechanisms of lipid transport and CM overproduction in IR is shown in Figure 1-7.

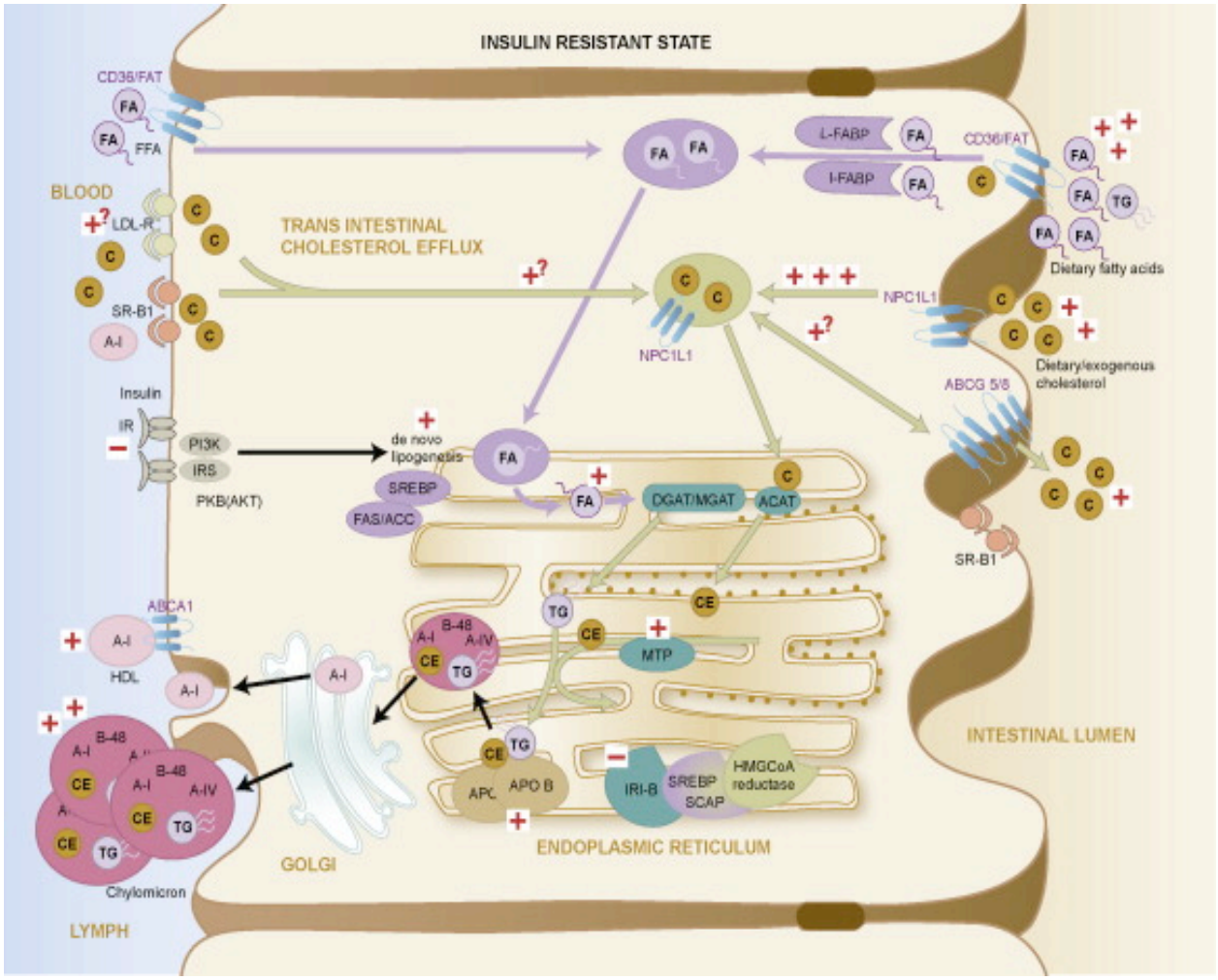


Figure 1-7. Proposed mechanisms of chylomicron overproduction during IR (Mangat et al. 2010). It has been proposed that during IR there is increased de novo lipogenesis in the enterocyte as well as an influx of plasma free fatty acids. This increased lipid availability together with increased expression of microsomal transfer protein (MTP), diacylglycerol acyl transferase (DGAT) and monoacylglycerol acyl transferase (MGAT) have been proposed to lead to increased chylomicron synthesis and secretion. This image is used in accordance with the s.29 provision for fair dealing in the Canadian *Copyright Act*.

The interaction between remnant lipoproteins and PGs in the arterial extracellular matrix forms the key initiating step in atherogenesis. Therefore to

emphasize the findings presented in this thesis, the structure and function of the vascular wall will be reviewed.

1.7 Proteoglycans: Structure and Function in The Normal Vascular Wall

1.7.1 Introduction

The extracellular matrix (ECM) forms the connective tissue between cells or under cells that provides a structural supporting base to cells and connects epithelium to underlying tissues and blood/lymphatic vessels. The ECM also performs various other important functions depending on the type of tissue or vessel.

PGs constitute a major component of the extracellular matrix. These complex and diverse macromolecules are present in all tissues and are synthesized by all cells. Individual functions of PGs can be attributed to either the protein core or the attached glycosaminoglycan (GAG) chain. PGs are constituted by a core protein to which one or more linear negatively charged GAG chains are covalently attached.

Glycosaminoglycans are long unbranched polysaccharides containing a repeating disaccharide unit. The disaccharide units consists of an amino sugar, N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc), and either a hexuronic acid, glucouronic acid (GlcA) or iduronic acid (IdoA) or a galactose (Varki *et al*. 1999 and Wight 1989). These GAGs are covalently attached to the PGs through the O-glycosidic linkage to the serine residues in the protein core. The main families of physiologically significant GAGs are: hyaluronic acid, chondrotin sulphate, heparan sulphate, dermatan sulphate and keratan sulphate. All these GAGs contain sulphate groups except hyaluronic acid. Hyaluronic acid is not found covalently attached to proteins as a PG. It is, however, a component of non-covalently formed complexes with PGs in the extracellular matrix (Wight 1989).

Traditionally PGs were classified on the basis of the type of GAGs. Now it is known that PGs exist as families of related core proteins that share common functions within these families (Nakata and Maeda 2002). Vascular PGs can be found in the interstitial extracellular matrix, as part of the specialized extracellular matrix structures, as part of cell membranes and intracellularly (Wight *et al.* 2005). Every cell (with the exception of red blood cells) that is present in the vascular wall can synthesize PGs, although the arterial smooth muscle cells are the main source of vascular PGs (Wight *et al.* 2005).

1.7.2 Extracellular matrix of the vascular wall

The extracellular matrix in the vascular wall includes collagen, elastic fibers, PGs, hyaluronan, glycoproteins and water. These components impart elastic recoil, tensile strength, compressibility and viscoelastic properties to the vascular wall. The components of the extracellular matrix can also bind to plasma proteins, growth factors, cytokines and enzymes which can mediate the regulation of vascular physiological events including cell migration, adhesion and proliferation. PGs also influence arterial properties such as permeability, lipid metabolism, hemostasis and thrombosis (Wight 1980, Berenson *et al.* 1984).

It has been shown that over 30 different PGs exist or are synthesized by vascular cells. The major PGs in the arterial extracellular matrix are chondroitin sulphate proteoglycans (CSPGs) such as versican (Yao *et al.* 1994), small leucine rich dermatan sulphate proteoglycans (SLRPs) such as decorin (Bianco *et al.* 1990) and biglycan (Stocker G *et al.* 1991, Dreher *et al.* 1990), keratan sulphate proteoglycans (KSPGs) such as lumican (Onda *et al.* 2002), and heparan sulphate proteoglycans (HSPGs) such as perlecan, syndecans and glypicans (Clowes *et al.* 1984). In the vascular extracellular matrix, versican is the predominant PG, and biglycan and decorin are the second most abundant group of extracellular PGs (Nakashima *et al.* 2008). PGs and their GAGs influence different properties of the extracellular matrix.

Decorin, a SLRP, regulates collagen fibrillogenesis i.e modulates collagen fiber diameter and organization (Danielson and Beribault *et al.* 1997). Lumican is also involved in fibrillogenesis (Onda *et al.* 2002) and specifically provides increased strength to the adventitia. Biglycan, another SLRP with similar homology to decorin is responsible for bone development (Goldberg *et al*. 2005). It binds to collagen *in vitro*, however it's exact function in the vascular wall is not known (Wight *et al.* 2005).

Perlecan is a basement membrane PG that interacts with growth factors involved in angiogenesis (Schaefer and Iozzo 2008). Perlecan is a potent inhibitor of vascular smooth muscle cell proliferation, and also influences the transport and retention of lipoproteins across the endothelium (Wight 1989, Tran-Lundmark *et al.* 2008). All Syndecans (syndecan 1 to 4) are normally expressed in the artery. Syndecans are regulators of cell-cell and cell-extracelluar matrix interactions. Syndecan 1 inhibits proliferation of vascular smooth muscle cells in response to arterial injury of factors leading to neointimal hyperplasia (Fukai *et al.* 2009).

Versican, a large CSPG, forms aggregates and binds with hyaluronan and links proteins to provide the vascular tissue with resistance to compression (Wight *et al.* 2005). Versican is found more prominently in the intima and adventitia of both arteries and veins.

Figure 1-8 represents the organization of the more abundant PGs found in the arterial vessel wall.

1.7.3 Proteoglycans secreted by endothelial cells

Vascular endothelial cells are capable of synthesizing and secreting both heparan sulphate and dermatan sulphate PGs (Keller *et al.* 1987, Marcum and Rosenberg 1985). Some heparan sulphate PGs are known to exhibit hydrophobic properties and interact with antithrombin III (Marcum and Rosenberg 1985) and thus play a pivotal role to provide a non-thrombogenic surface on the vascular endothelium. A major portion PGs are synthesized by

the endothelial cells and are deposited as basement membranes. These PGs act as structural frame to interact with other components of the basement membrane such as collagen, laminin and fibronectin (Kleinman *et al.* 1983). The heparan sulphate PGs also contribute to the selective permeability barrier function of basement membranes (Farquhar 2006) and therefore any alteration in the PG content of the basement membranes can compromise this barrier.

1.7.4 Proteoglycans secreted by the vascular smooth muscle cells

Vascular smooth muscle cells mainly synthesize CSPGs, DSPGs, and comparatively smaller amount of HSPGs (Salisbury and Wagner 1981). These PGs make up the bulk of arterial PGs. CSPGs and DSPGs are internalized by arterial smooth muscle cells at different rates. DSPGs are internalized rapidly while CSPGs are taken up more slowly and by a low-affinity endocytic processes (Schmidt and Buddecke 1985). Therefore the turnover of different families of PGs proceeds at different rates.

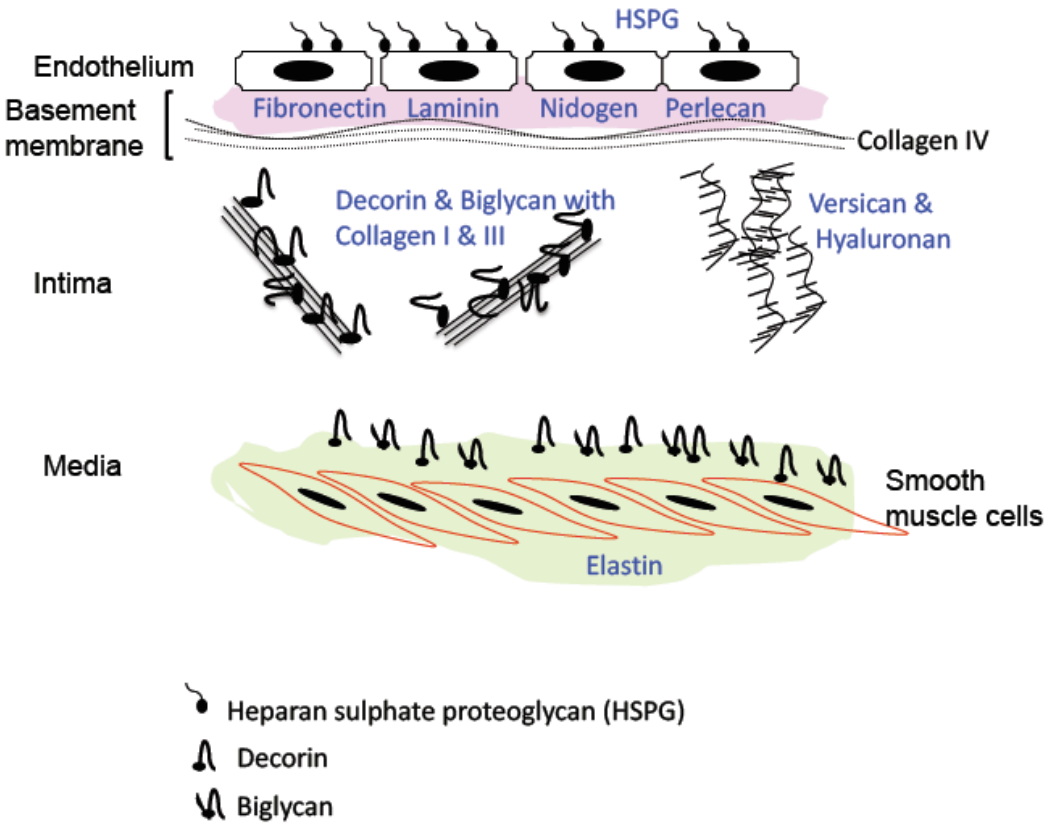


Figure 1-8 Organization of the arterial extracellular matrix (adapted from Pentikainen 1999). Collagen and elastic fibers are embedded in a viscoelastic gel containing PGs, hyaluronan, glycoproteins, and water. In addition, specialized ECM, the basement membrane, is present on the basolateral surface of endothelial cells and around smooth muscle cells. Fibronectin, laminin and nidogen are principal glycoproteins in the basement membrane that interact with other extracellular components and regulate vascular integrity. Quantitatively the major PG (60-70%) of the extracellular matrix is versican that interacts with link proteins and hyaluronan. Biglycan and decorin are the second most abundant PGs in the arterial wall.

1.8 Factors Influencing Proteoglycan-Lipoprotein Interaction

1.8.1 Proteoglycans and the response-to-retention hypothesis

According to the response-to-retention hypothesis, the PG-lipoprotein interaction is the key initiating step in atherosclerosis. The interactions of lipoproteins and PGs are predominantly ionic reactions, whereby, negatively charged sulphate and carboxylic groups on the GAG chains bind with the positively charged amino-acid residues on apoB. However, a protein-protein interaction i.e between apoE and the protein core of the PG has also been shown (Klezovitch *et al.* 2000). This interaction can also be with a bridging molecule such as LPL (Gustafsson *et al.* 2007, Pentikainen *et al.* 2002, Penttikainen *et al.* 2000, Olin *et al.* 1999), apoE (Olin-Lewis *et al.* 2002), SMase (Oorni *et al.* 2000), and sPLA2 (Boyanovsky *et al.* 2005).

1.8.2 Role of proteoglycans in modulating lipoprotein binding

Several studies have demonstrated factors that increase the length and or sulphation of the GAG chains to increase PG binding affinity for lipoproteins (summarized in Table 4). PGs obtained from atherosclerosis susceptible arteries show higher binding to LDL than PGs obtained from non-atherosclerosis susceptible arteries, thus demonstrating that changes in PGs can predispose arteries to atherosclerosis (Cardoso and Maurao 1994).

Despite evidence for up to 2-3 fold increases in CVD risk in subjects with prediabetes and diabetes mellitus as compared to control subjects, it is not known whether the diabetic state leads to changes in arterial PGs that predispose arteries to increased lipoprotein retention and subsequent lipid accumulation.

Table 1-4 [1]Factors affecting the synthesis and degradation of PGs.

Factors or Agents	Effect on core protein	Effect on GAGs/ PG binding	Effect on LDL binding	References
Androgens	ND	↑GAG size and sulphation	Increased binding	Hashimura *et al.* 2005
Angiotensin II	↑ Biglycan, versican, perlecan expression	Elongated GAGs on all secreted PGs	Increased binding	Figueroa *et al.* 2002, Shimizu-Hirota *et al.* 2001
Calcium channel blockers: amlodipine, nefedipine, verapamil	ND	↓GAG sulphation ↓Size (amlodipine)	Decreased binding (amlodipine)	Vijayagopal and Subramaniam 2001
Cadmium	↑Biglycan, ↑ Decorin	No effect	ND	Fujiwara *et al.* 2002
Caloric restriction (LDL isolated after caloric restriction)	ND	Increase binding to PGs	ND	Edwards *et al.* 1998
CYC10424 (pyrido-pyrimidine derivative)	ND	↓GAG size and sulphation	Decreased binding	Ballinger *et al.* 2008
Endothelin 1	ND	↑GAG size and sulphation	Increased binding	Ballinger *et al.* 2008
Fatty acids	↑ Versican, biglycan, perlecan	↑GAG size and sulphation	Increased binding	Olsson *et al.* 1999, Rodríguez-Lee *et al.* 2006
Fenofibrate	ND	↓GAG size and sulphation	Decreased binding	Nigro *et al.* 2004
Glucosamine	ND	↓GAG size and sulphation	Decreased binding	[a]Tannock *et al.* 2002

Factors or Agents	Effect on core protein	Effect on GAGs/PGs binding	Effect on LDL binding	References
Hypoxia/reoxygenation	ND	↑GAG size and sulphation	Increased binding	Figueroa *et al.* 1999
Glucose, insulin and fatty acid combinations	No change	No change	No change	Wilson *et al.* 2007
LDL isolated from fish oil fed monkeys vs lard fed monkeys	ND	↑ PG binding	ND	Edwards *et al.* 1991
Caloric restriction (LDL isolated after caloric restriction)	ND	Increase binding to PGs	ND	Edwards *et al.* 1998
Manganese (supplemented)	ND	↑ non sulphated units on heparan sulphate chains compared to Mn adequate.	ND	Kalea[a] *et al.* 2006
Manganese (deficient)	ND	Tri and disulphated HS chains present but not in Mn supplemented.	ND	Kalea[a] *et al.* 2006
Oxidized LDL	↑Biglycan	↑GAG size and sulphation	Increased binding	Chang *et al.* 2000
PDGF	↑ Versican	↑GAG size and sulphation	Increased binding	Schönherr *et al.* 1993, Schönherr *et al.* 1991, Nigro *et al.* 2004
Proliferating VSMC	ND	↑GAG size and sulphation	Increased binding	Camejo *et al.* 1993

Factors or Agents	Effect on core protein	Effect on GAGs/PG binding	Effect on LDL binding	References
Serum Amyloid A	↑Biglycan	↑GAG size and sulphation	Increased binding	Wilson *et al.* 2008
Statins	ND	↓GAG sulphation but ↑GAG size	Decreased binding	Meyers *et al.* 2003
Thiazolidinediones	ND	↓GAG size and sulphation	Decreased Binding	Tannock *et al.* 2004, de Dios *et al.* 2007
Thrombin	↑Biglycan, ↑ Decorin	↑GAG size and sulphation	ND	Ivey ME 2008
TGF-beta	↑Biglycan, ↑ Versican	↑GAG size and sulphation	Increased binding	Little *et al.* 2002, Schönherr *et al.* 1993, Schönherr *et al.* 1991
Wild Blueberry	ND	↓ GAG sulphation	ND	Kalea[b] *et al.* 2006

[1]This table is derived from Tannock and King (2008).

1.8.3 Role of lipoproteins in modulating proteoglycan binding

Lipoprotein particle, size, number, charge and composition are known to affect binding with PGs. Eight PG binding sites have been identified on intact apoB100. In a normal LDL configuration, only one binding site (site B) is available for interaction with PGs. ApoB48 has an alternative binding site, namely site B-Ib. ApoB48 consists of 48% of the amino acid sequence of apoB100 and contains both site B and site Ib and therefore shows increased PG binding (Olofsson *et al.* 2005, Boren *et al.* 1998).

While apoB100 (LDL particles) has been shown to bind to PGs both in vivo and in vitro it has only been speculated that apoB48 can bind to PGs

(Gustafsson 2007). It has not been shown if apoB48 or apoB48 containing intestinal derived remnant lipoproteins co-localize or bind in vitro or in vivo with PGs.

1.8.3.1 Role of particle composition

It has been shown that apoE containing LDL particles have increased PG binding affinity compared to apoE devoid LDL (Flood *et al.* 2002). Altered phospholipid content of LDL can change the interaction of different PG binding sites on apoB and modulate the binding affinity of lipoprotein particles to PGs (Flood *et al.* 2004). Increased amounts of apoCIII have been shown to change the conformation of LDL, which increases its binding affinity to PGs (Olin-Lewis *et al.* 2002). Modification of the LDL particle, such as acetylation and aggregation, has been shown to strengthen the lipoprotein-PG interaction (Sartipy *et al.* 1998, Hurt-Camejo *et al.* 1992).

The effects of compositional changes on binding between remnant lipoproteins and proteoglycans remain unknown.

1.8.3.2 Role of charge on the lipoprotein particles and proteoglycans

Studies have shown that LDL can be modified by endothelial cells so that it becomes negatively charged, which enables recognition of LDL by the LDL receptor on the macrophage (Henriksen et al. 1983). It is speculated that endothelial derived PGs bind to LDL and in turn modify the surface charge. However it is not known whether the modification induced by endothelial cells can actually increase the affinity of such modified LDL to aortic PGs. It is not known whether endothelial cells can similarly modify the charge on intestinal derived remnant lipoproteins. Electronegative LDL [LDL(-)] is a subfraction of plasma LDL that has increased apoE and apoCIII content and high density and it has been shown to bind to PGs more avidly than normal LDL (Bancells *et al.* 2009).

Intestinal derived remnant lipoproteins are taken up readily by macrophages without any modification (Botham et al. 2007). In addition intestinal remnants have a higher net negative charge as compared to LDL (Proctor and Mamo 2003). *In vitro* studies have suggested that the sialic acid content of LDL is inversely associated with the extent of complex formation with chondroitin sulfate. It has been shown that incubation of LDL with gangliosides results in a decreased interaction between LDL and PGs, whereas incubation with asialogangliosides results in increased binding interactions (Millar 1999). Net sialic acid content of intestinal derived remnant lipoproteins is similar to LDL when expressed per unit of protein. However intestinal derived remnant lipoproteins generally contain less protein than LDL, and therefore net sialic acid content of intestinal derived remnants would be greater per particle, which could be speculated to increase binding to arterial PGs. However, it is not known at this time if the net sialic acid content of intestinal derived remnants affects binding with PGs.

The effect of size, charge and lipid parameters of different lipoproteins (low-density lipoproteins and remnant lipoproteins) on binding to human biglycan remains unknown.

1.9 Arterial Wall Remodeling of Proteoglycans During Vascular Disease and Interaction of Lipoproteins.

1.9.1 Proteoglycans in vascular disease

PGs and hyaluronan increase in the early phases of vascular disease and then decrease as lesions advance and become fibrotic (Wight 2005). There are morphological differences in atherosclerotic lesions in human and animal models (Nakashima *et al.* 2008). Animal models (except non-human primates) do not develop prominent diffuse intimal thickening like humans. In animals, lipids deposit in the narrow subendothelial space that consists of the extracellular matrix and a layer of smooth muscle cells, while in humans lipid deposition occurs in the deep layer of the thickened tunica intima where there

is an abundance of extracellular matrix and smooth muscle cells (Nakashima *et al*. 2008).

1.9.2 Versican

In monkey and human lesions, versican is prominent and diffuse in the intimal thickening during the early stages of human vascular disease (Lin *et al.* 1996, Wight *et al.* 2005). While in the end-stage of disease versican is located at the edge of the necrotic core and is completely absent from the lipid core (O'Brien 1998, Evanko *et al.* 1998, Wight *et al.* 2005). Versican is not colocalized with apoE in atherosclerotic lesions (O'brien 1998). In contrast to the presence of versican in human lesions, versican is absent or detected at very low levels (Kunjathoor *et al.* 2002) in mouse lesions. Platelets bind well to human aorta versican, but weakly to decorin and biglycan (Oorni *et al.* 2000), therefore it has been suggested that versican probably has a role in thrombosis.

1.9.3 Heparan sulphate proteoglycans

Reduced heparan sulphate content has been reported in human arterial atherosclerotic areas compared to non-atherosclerotic areas (Stevens *et al.* 1976, Tran *et al.* 2007). Perlecan is the major heparan sulphate PG in the subendothelial matrix and two experiments have shown the effects of heterozygous perlecan deletion on atherosclerosis. ApoE-/- mice with heterozygous perlecan deletion, showed decreased atherosclerosis in males but not females, and no differences in either gender at 24 weeks of age (Stevens et al. 1976). Another study using LDLR-/- mice with heterozygous perlecan deletion, showed no difference in atherosclerosis at 16 weeks of age (Vikrmadithyan *et al.* 2004). Another murine study has shown the presence of perlecan in both early and advanced atherosclerotic lesions and colocalization with apoAI (Kunjathoor 2002), suggesting that perlecan plays a role in retaining HDL. Another study using apoE-/- mice that were cross-bred with mice expressing HS-deficient perlecan has demonstrated that perlecan heparan sulphate PGs are proatherogenic in mice and these facilitate

increased lipoprotein retention and increase vascular permeability (Tran-Lundmark *et al.* 2008).

Therefore, differences between animal models and humans have added difficulty to the interpretation of the role of heparan sulphate PGs in atherosclerosis.

1.9.4 Decorin

Data regarding the role of decorin in atherosclerosis is hard to interpret because it appears to colocalize with apoB in human atherosclerosis specimens, however it colocalizes with apoB in murine atherosclerosis (Huang *et al.* 2008, Kunjathoor *et al.* 2002). Both biglycan and decorin have been suggested to regulate activity of TGF beta. Both biglycan and decorin can bind to TGF beta and inhibit its activity (Fukushima *et al.* 1993, Ruoslahti *et al.* 1991, Hildebrand *et al.* 1994, Yamaguchi *et al.* 1990). However only decorin inhibits the profibrotic activity of TGF beta *in vivo* (Kolb *et al.* 2001). Therefore it seems that only decorin may be able to regulate the activity of TGF beta. In apoE knock out mice, over expression of decorin by an adenoviral vector insertion resulted in decreased development of atherosclerosis, as well as decreased plaque macrophage and collagen content, and decreased TGF beta levels (Al Haj Zen *et al.* 2006). In this study LDL retention was not measured and the authors attributed the reduction in atherosclerosis to both direct effects of decorin, as well as to decorin-induced reduction of TGF beta levels. Atherosclerotic lesion area was reported to increase in decorin deficient mice (Williams 2001), however no mechanisms were proposed.

A number of studies have evaluated the role of decorin in the kidney. These studies have shown that administration or overexpression of decorin decreases TGF beta levels and pro-fibrotic activity (Border *et al.* 1996, Huijun *et al.* 2005). Decorin deficient mice developed nephropathy when they were made diabetic using streptozotocin compared to wildtype diabetic mice

(Williams *et al.* 2007). Decorin deficient diabetic mice had increased glomerular TGF-beta levels, type 1 collagen and macrophage infiltration (Schaefer *et al.* 2002, Williams *et al.* 2007). Thus most of the literature shows an anti-atherogenic role for decorin associated with regulation of TGF-beta levels independent of it's role in lipoprotein binding.

1.9.5 Biglycan

Biglycan has been shown to bind with LDL, VLDL and apoB (Olin-Lewis *et al.* 2002). When human biglycan was over expressed in rat smooth muscle cells, the resulting extra cellular matrix showed increased high affinity lipoprotein binding (O'Brien *et al.* 2004). Biglycan is found not only in the intima of normal human arteries but also in coronary arteries and saphenous vein (Merrilees *et al.* 2001, Gutierrez *et al.*1997). Immunohistochemical studies have shown that biglycan is localized in the diffuse intimal thickening in human coronary arteries (Nakashima *et al.* 2007). The pre-lesional distribution of biglycan has been found consistent with the distribution of lipids in early lesions suggesting that the presence of biglycan before lipid deposition may be crucial in the initial retention of lipids in the intima (Nakashima *et al.* 2007). This data is also supported by the colocalization of apoB and apoE with biglycan in early as well as advanced human and murine lesions (O'Brien 1998, Nakashima *et al.* 2007, Kunjathoor *et al.* 2002). Biglycan has also been shown to bind to HDL containing higher apoE content (Olin *et al.* 2001).

Lipids deposit eccentrically while biglycan is localized concentrically (Nakashima *et al.* 2008). This could be due to uneven distribution of strain on the arterial wall, uneven plasma lipoprotein concentration, density and permeability of the arterial wall (Nakashima *et al.* 2008).

Biglycan deficient mice are available and they show an osteoporotic phenotype, (Embree *et al.* 2010), however there has been no assessment made on the effect of biglycan deficiency on atherosclerosis. Angiotensin II infusion specifically upregulates vascular biglycan and perlecan content in

LDL-r deficient mice (Huang *et al.* 2008). *Ex vivo* carotid infusion of LDL was increased in these mice thus demonstrating that increased biglycan and perlecan leads to increased LDL retention. Further this study showed that LDL-r deficient mice, with angiotensin II infusions and fed an atherogenic diet, developed atherosclerosis compared to saline infused controls, suggesting that increased biglycan and perlecan preceeded atherosclerosis development.

Recent studies have shown the role of biglycan in inflammation. Biglycan has been shown to play a role in the synthesis and activation of the proinflammatory master cytokine IL-1beta (Babelova *et al.* 2009). Furthermore these authors have shown that reactive oxygen species formation is also involved in biglycan-mediated activation of the inflammasome. Biglycan stimulates the expression of NLRP3 and pro-IL-1beta through the toll like receptors TLR2/4 (Babelova *et al.* 2009). Atherosclerosis is an inflammatory disease and these studies suggest elevated arterial biglycan may lead to an increase in local inflammation and thus may contribute to atherogenesis or accelerated development of atherosclerosis.

Among other functions of biglycan, it plays an important role in cardiac tissue. Biglycan has been shown to be required for the stable collagen matrix formation of infarct scars and for the preservation of cardiac function (Westermann *et al.* 2008). In this study myocardial infarction was induced in the hearts of wild type and biglycan knock out mice by ligation of the coronary artery. It was found that the collagen matrix organization was severely impaired in the biglycan knock out mice. In another study biglycan expression was found to be 2 to 5 times increased in human aortic valve cusps obtained from patients that had a preoperative pressure gradient of more than 100mmHg (Lehmann *et al.* 2009).

It is not known if biglycan null mice show increased atherosclerosis or not. Therefore future studies using biglycan null mice may contribute data on the proposed atherogenic role of biglycan.

The investigations presented in this thesis have focused primarily on biglycan and decorin. The rationale behind this is that these are the second most abundant extracellular PGs, next only to versican, and are the only PGs that have been found to colocalise with apoB in human atherosclerotic lesions.

1.10 The Diabetic Metabolic Milieu and Lipoprotein-Proteoglycan Interaction

1.10.1 Proteoglycans and diabetes

Cardiovascular disease is accelerated in both type I diabetes as well as type 2 diabetes. However the exact mechanisms have not yet been delineated. Studies have shown that thoracic aortae from subjects with type 2 diabetes exhibit a relative decrease in heparan sulphate, a relative increase in dermatan sulphate, and thus a decrease in the ratio of heparan sulphate to dermatan sulphate compared to normal controls (Wasty *et al.* 1993). This suggests that changes in arterial GAGs precede the development of lesions in diabetes and may be important in atherogenesis.

Studies have shown an increase in dermatan sulphate in the tunic intima of arteries that have atheromatous plaques in subjects with T1D, type 2 diabetes and non-diabetic subjects with atherosclerosis, when compared to plaque free areas of arterial tissue isolated from respective subjects. No changes in heparan sulphate but decreased hyaluronic acid content was observed in the arteries containing plaques in subjects with T1D and non-diabetic subjects, compared to the plaque free areas of these subjects. Plaque free areas from subjects with T1D showed an increase in hyaluronic acid when compared with plaque free areas in non-diabetic subjects with atherosclerosis (Heickendorff *et al.* 1994).

Even though there is some inconsistenty in the results of these studies, it can be concluded that both type 1 and type 2 diabetes are characterized by vessel wall remodeling. Some aspects of the remodeling resemble universal changes seen in atherosclerosis, while other changes appear specific to diabetes.

Therefore we can hypothesize that an increased presence of arterial PGs (specifically biglycan and decorin) during early stages of diabetes (both type 1 and type 2) may accelerate the deposition of lipid in arterial vessels due to increased binding of apoB48-containing remnant lipoproteins.

1.10.2 Effect of diabetic metabolic milieu on vascular proteoglycan synthesis

Increased levels of non esterified fatty acids have also been suggested to alter the GAGs on the basement membrane of endothelial cells which may increase permeability (Toborek *et al*. 1998).

In-vitro studies have shown that when non-esterified fatty acids (NEFA) are incubated with human aortic smooth muscle cells, it leads to increased gene expression of extracellular matrix PGs (biglycan, perlecan, decorin and versican), and that these changes increase the binding capacity and affinity of the resultant matrix for LDL (Rodriguez-Lee *et al.* 2006). Furthermore, insulin and the PPAR γ agonist insulin sensitizer darglitazone, dose-dependently reduce the NEFA-induced overexpression of PGs. One mechanism that has been suggested is that NEFA lead to increase in diacylglycerol levels that activates specific PKC kinase isoforms thus leading to impaired insulin signaling causing an overexpression of PGs (Griffin *et al.* 1999).

Whilst other studies have shown no effect of glucose, insulin or fatty acids on PG synthesis by smooth muscle cells, endothelial cells or macrophages (Wilson *et al.* 2007). However in this study it was found that TGF-β altered PG synthesis as well as the LDL binding affinity of the matrix. TGF-β is known to be elevated in subjects with both type 1 as well as type 2 diabetes (Flores *et al.* 2004, Pfeiffer *et al.* 1996, Sharma *et al.* 1997).

In both of these studies, the cells used were normal cells. It would be interesting to determine if cells isolated from type 1 and/or type 2 diabetic animals would have different PG synthesis due to the metabolic factors associated with diabetes.

Serum amyloid alpha (SAA) has been shown to be predictive of cardiac disease risk, and is elevated in obese subjects (Yang *et al.* 2006), as well as during IR and type 2 diabetes (Vettor *et al.* 2005). Apart form SAA's inflammatory effects it also stimulates proatherogenic vascular PG synthesis in mice overexpressing SAA (Wilson *et al.* 2008). This was associated with increased TGF-β levels, therefore the protherogenic effects of SAA have been suggested to be mediated via TGF-β.

It can be speculated that aortic smooth muscle cells may increase arterial PG-secretion during T1D and insulin-resistance, specifically in response to local increases in TGF-β.

1.10.3 Effect of advanced glycated end products on the extracellular matrix

Advanced glycated endproducts (AGEs) refers to a multitude of non-enzymatically glycosylated proteins and lipids that have succumbed to Maillard reactions (Price *et al.* 2007). Hyperglycemia in diabetes mellitus leads to formation of AGEs and these have emerged as a major factor in diabetic vascular remodeling which results in impaired extracellular matrix flexibility and an increase in matrix area due to cross-linking of matrix proteins. Collectively, it has been proposed that these changes leads to the enhanced entrapment of LDL (Spinetti *et al.* 2008). Collagen and other long-lived matrix proteins get glycated with AGEs and help facilitate binding of LDL (Ichida *et al.* 1968). Another study has shown that glycated collagen inhibits binding of LDL (Kalant *et al.* 1993). The conflicting results described between these two studies are likely due to the source of collagen used and other technical aspects in the study design.

Another mechanism by which AGEs increase the matrix area is by increasing the proliferation of smooth muscle cells and by reducing the activity of matrix metalloproteases: a family of endopeptidases involved in matrix degradation (Galis *et al*. 2002, Newby 2006).

However, at present there have been no investigations into the role of advanced glycation end products in arterial remnant retention.

1.10.4 Effect of diabetes on lipoprotein modification: Implication for increased retention.

In a primate model of streptozotocin-induced T1D, LDL was found to be significantly glycated in diabetic animals when compared to controls (Edwards *et al.* 1999). Another study by the same group demonstrated that LDL isolated from type 2 diabetic subjects, following improved glucose control with glipizide GITS/metformin therapy, showed less binding to arterial PGs compared to LDL isolated from subjects with significantly lesser glucose control (using monotherapy with either agent) (Edwards *et al.* 2002).

Diabetic dyslipidemia is associated with small dense LDL particles and these particles (LDL-III, d = 1.044-1.063 g/ml) have been shown to be associated with increased complex formation with arterial PGs, as compared to large buoyant LDL (LDL I, d = 1.019-1.033 g/ml) (Anber *et al.* 1996). Small dense LDL particles isolated from subjects with type 2 diabetes are enriched in apoCIII and a significant correlation has been observed between the apoCIII content in small dense LDL from type 2 diabetic subjects and their affinity for arterial versican (Davidsson *et al.* 2005). ApoCIII enriched LDL has also been shown to demonstrate increased affinity for biglycan (Hiukka *et al.* 2009 and Olin-Lewis *et al.* 2002).

Fasting TG-rich lipoproteins (density <1.019 g/ml) isolated from subjects with type 2 diabetes show no difference in binding to biglycan ([b]Tannock *et al.* 2002) compared with TG-rich lipoproteins isolated from control subjects. This study found no difference in the apoE content per particle between the

diabetic and control subjects, and this study did not measure apoE glycation. At present we do not understand how the composition or glycation of intestinal derived remnants isolated from diabetic or insulin resistant subjects may alter their binding to arterial PGs.

1.11 Experimental Approaches to Study the Role of lipoproteins in CVD

1.11.1 Animal and human studies of CVD development

Traditionally studies investigating the role of lipoproteins in CVD have used the approach of determining the plasma levels of various lipoprotein fractions and then correlating these with surrogate markers of CVD, such as carotid intima media thickness or other biomarkers. Experimental approaches used in the literature also include animal models of CVD. In these studies plasma levels are correlated with hard end points such as aortic lipid staining to determine the extent of atherosclerosis. We know that CVD is multifactorial and therefore the main disadvantage of these studies is that these study designs do not provide direct proof of principle.

1.11.2 History of in vivo imaging of lipoproteins

Stender and Zilversmit (Stender and Zilversmit 1982) were one of the first groups to study the arterial uptake of CM derived CE and the arterial uptake of intact remnant lipoproteins by using ^{3}H or ^{14}C CE. These studies concluded that in a rabbit model, CM contribute only a neglible amount of CE to the intima-media layer of the thoracic aorta. These conclusions further strengthened the hypothesis that LDL was the predominant lipoprotein involved in atherosclerosis. However the main drawback of this method was that the CE radiolabel used equilibrated or transferred to other plasma lipoproteins, and therefore the results using this technique were confounded.

Pittman and coworkers (Yedgar *et al.* 1983, Pittman *et al.* 1983, Carew *et al.* 1984) developed a radiolabeled tyramine cellobiose technique to study the metabolic degradation of LDL. This technique allowed for the labeling of the

protein moieties of the lipoproteins. This was an improved modification over the technique used conventionally that involved labeling protein moieties with iodotyrosine. When a protein labeled with iodotyrosine enters the lysosome of a cell it gets degraded to the constituent amino acids, including labeled iodotyrosine. Since iodotyrosine is not reused, it rapidly escapes from the cell and is excreted, predominantly in urine. Therefore this method did not allow for measuring degradation of a protein. Pittman and coworkers developed the iodinated tyramine-cellobiose label for the protein moiety, which is also degraded upon entering the lysosome to the constituent amino acids and to iodinated tyramine-cellobiose. However, iodinated tyramine-cellobiose remains attached to short peptide fragments and does not escape the cell and remains trapped intracellularly enabling the measurement of lipoprotein degradation. Using this method Pittman and coworkers concluded that 40% to 50% of LDL degradation in the intima, predominantly endothelial cells, was mediated by LDL-r. These studies raised other important questions. Does LDL-r on the endothelial cells contribute to trans-endothelial transport of LDL? Can intestinal derived remnants be taken up by the intima is this mediated via LDL-r and trans-endothelial transport of these particles?

Nordestgaardt and coworkers (Nordestgaardt *et al.* 1992) performed studies to directly compare the arterial uptake of VLDL, IDL, HDL and LDL. These studies used the modified tyramine cellobiose technique developed by Pittman and coworkers, which labeled the protein moieties of the lipoproteins with different isotopes of iodine. To reduce the amount of exchangeable iodine label, i.e. label not attached to apoB in labeled VLDL or IDL, each of these lipoprotein fractions were incubated with a rabbit plasma fraction containing unlabeled HDL and plasma lipid transfer protein for 48 hours at 37°C. The lipoprotein fractions were subsequently re-isolated by ultracentrifugation at their upper density limits. The results from this study demonstrated that the arterial influx and permeability of lipoproteins was inversely correlated with the particle size. Smaller particles such as HDL and albumin had the greatest influx while larger particles such as IDL and VLDL

particles had significantly lesser influx. These studies demonstrated that arterial permeability was primarily dependent on particle size and therefore larger VLDL particles and native TG rich CM could not permeate the arterial vessel wall.

The main advantage of the modified tyramine cellobiose technique used by Nordestgaard *et al.* was that this method used the radioactivity associated with apoB as an indicator of lipoprotein uptake. The main disadvantage of this procedure was that it involved large amounts of homogenized arterial tissue in order to determine the radioactivity associated with apoB, and complex mathematical modeling was used to estimate the distribution of lipoproteins in the intima and media.

In 1994, for the first time Mamo and Wheeler (Mamo and Wheeler 1994) studied the arterial uptake of CM and their remnants. Mamo and Wheeler used a slightly modified technique to label CM with radioiodinated tyramine cellobiose. They performed kinetic analysis and established that neither lipolysis of the particle nor uptake was affected due to tyramine cellobiose labeling. Hepatic uptake of tyramine cellobiose labeled CM was similar to that of non-labeled particles. In this study Mamo *et al.* established that despite the fact that the radiolabeled product was equally distributed between the apoB48 and the 'soluble' apolipoproteins (40-50% on apoB48, 35-50% on non-apoB apolipoproteins), transfer of radiolabeled chylomicron apolipoproteins to HDL did not occur *in-vitro* after prolonged incubation. This study demonstrated that in rabbit thoracic aorta, a substantial 'uptake' of intestinal derived remnants could occur. This was the first direct evidence that intestinal derived remnants could be delivered to the arterial vessel wall. However the *in vivo* technique still required the use of mathematical 'normalization' due to differences in lipoprotein particle kinetics. This study expressed arterial uptake as a measure of lipoprotein clearance in order to provide comparable data with equivalent arterial exposure. Mamo and Wheeler established that the size of CM-r was approximately 50-60 nm, which was consistent with the particle

transcytosis studies done by Siminionescu *et al.* (Siminionescu and Siminionescu 1991).

1.11.3 Development of fluorescent labeling technique for remnant lipoproteins

There are still several disadvantages when using the modified method to radiolabel CM. When using radioisotopes, it is extremely difficult to visualize the arterial distribution of lipoproteins. The labeling of native lymph CM also requires a large amount of radioisotopes. Furthermore the visualization of isotopes via photographic development is long (4-6 weeks incubation with emulsion) and provides inaccurate results. Therefore to overcome the limitations of using radioisotopes, a fluorescent method was developed by Proctor and Mamo (1998) to label CM and CM-r.

Use of fluorescence as a means to trace lipoprotein particles removes the necessity to use radioisotopes and therefore allows for significantly enhanced visual assessment. Fluorescent microscopy can give minute anatomical details that autoradiography cannot provide.

Similar to the problems of labeling lipid or the phospholipid part of the lipoprotein, it became clear from early studies that the lipid portions of lipoproteins are continuously being transferred to other lipoprotein fractions. Labeling the lipid or phospholipid part of lipoproteins is a good tool when studying the traffic of these molecules between lipoproteins and/or intracellular uptake and degradation. However since these probes do not remain with the original particle this is not a useful method to study arterial uptake. Labeling of the apoB48 with fluorescent dyes was another alternative to trace remnant lipoproteins and provided a better option for visualization and imaging. Proctor and Mamo (1998) explored the kinetics of CM labeled with tyramine cellobiose radio-iodination vs. the CM labeled with rhodamine and concluded that there was no difference in the kinetic properties of the two labels.

The advantage of the fluorescent labeling technique is that it provides the

means to attach the fluorescence to non-transferable protein portions of lipoproteins. The labeling procedure developed by Proctor *et al.* was designed to attach the fluorescent probe to native CM particles prior to the formation of smaller remnants. Labeling the native CMs allows the label to bind to all the proteins in the CM. In the next step the labeled CM are injected into a hepatectomised rabbit for ~4 hours. This second step hydrolyzes the CM to remnants, and more importantly the transferable apolipoproteins (apoA and apoC) are mostly removed by the exchange with other lipoproteins in the circulation while the non-transferable protein, apoB48, remains with the particle during lipolysis. Proctor et al. further established that this technique does not alter the size of the labeled intestinal derived remnants and that these particles are metabolically identical to non-labeled intestinal derived remnants. Furthermore these fluorescently labeled particles can be visualized using confocal microscopy thus enabling visualization of lipoprotein distribution in arterial vessels.

1.11.4 Development of ex vivo perfusion methodology

In vivo perfusion of fluorescently labeled remnant lipoproteins could only provide an estimate of arterial tissue uptake (using mathematical modeling) due to differences in the *in vivo* clearance of lipoproteins. This problem was even more complex when low-density lipoproteins were simultaneously perfused with remnant lipoproteins. The *in vivo* system did not allow standardizing for lipoprotein numbers, and comparing and contrasting lipoprotein uptake by the arterial tissue without the effects of lipoprotein lipase. Rutledge and coworkers (Rutledge and Goldberg 1994) had developed an in-situ perfusion system for perfusion of LDL in frogs, rats and hamsters. This was a closed loop perfusion system that re-circulates the perfusate at physiological conditions of temperature and pressure. Proctor et al. (Proctor and Mamo 1998) used in-situ rabbit arterial perfusion system for the first time to study the arterial uptake of fluorescently labeled remnant lipoproteins.

Due to the advantage and relevance of the ex vivo perfusion method over other existing methods, this was chosen as a major tool in the investigations of arterial uptake of lipoproteins in this thesis.

1.12 Rat Models of Diabetes

1.12.1 Rat models of T1D

Animal models for T1D can be divided into two groups: spontaneous genetic models of diabetes or experimentally induced models of diabetes. Evidence from several studies including the twin studies has suggested that (Kyvik *et al.* 1995) the cause of T1D is due to nongenetic environmental factors operating in a genetically susceptible host that initiates a destructive immune process (Akerblom *et al.* 2002). It has been suggested that viral infection is the most important perturbant specifically in populations in which the incidence of diabetes is on the rise (Laron 2002). These findings have led to the development of inbred animal model systems that are thought to be representative of how children develop auto-immune diabetes in response to environmental interactions. This had further led to the development of models of induced T1D. Susceptibility to induced autoimmune disease by rat virus infections (BBDR rat and LEW.1WR1 rat) or thymectomy followed by irradiation (PVG/c rat and $RT1^{U}$ strains of rat) can induce T1D spontaneously in rodents.

Another type of induced type 1 diabete animal model without the autoimmune component of the disease, includes the streptozotocin treated young adult ratsor low-dose streptozotocin treated mouse. Streptozotocin is an agent that induces beta cell necrosis in the pancreas and induces severe insulinopaenia, hyperglycaemia, glycosuria, polydipsia and muscle wasting (features associated with T1D). Streptozotocin induced mouse fed a high fat diet also exhibit atherosclerosis. Since rodents are naturally resistant to atherosclerosis, the streptozotocin induced rat model fails to develop atherosclerosis although it does exhibit high plasma triglyceride and

cholesterol levels, and develops endothelial dysfunction (features similar to clinical manifestation of T1D). Another agent sometimes used to cause beta cell death is alloxan, but some studies have shown that it is not specific to beta cells and has some non-specific side effects as well (Bracken *et al.* 2003). Therefore the streptozotocin-induced models are beneficial in studying microvascular and macrovascular disease in T1D, since these models do not involve the autoimmune components in other models that may confound observations.

1.12.2 Rat models of insulin resistance

Rats are highly resistant to developing atherosclerosis. Even dietary cholesterol as high as 10%w/w is not sufficient to create significant hypercholesterolemia. However a few genetically modified strains of rats that mimic important aspects of abnormal lipid metabolism and CVD similar to humans have been developed.

1.12.2.1 Fatty Zucker rats

Zucker was the first genetically unique rat strain to study obesity and hypertension. This strain consists of a spontaneous mutant gene (*fa* or fatty) that affects the action of the adipocyte peptide hormone leptin which is a key regulator of food intake through the inhibition of the release of hypothalamic neuropeptide Y (Denis *et al.* 2004, Kalra *et al.* 2004). When the Zucker rat is homozygous for the *fa* gene (*fa/fa*), it develops the metabolic syndrome, becomes obese, moderately insulin resistant and hypertriglyceridemic but with no progression to diabetes or cardiovascular complications (Amy *et al.* 1988). The heterozygous or the homozygous normal animals are lean and metabolically normal. The Zucker rat provides an excellent model for obesity and IR but is not suitable for studies pertaining to CVD.

1.12.2.2 Zucker diabetic fatty rats (ZDF)

The onset of diabetes in the obese Zucker male rats led to the isolation of a

new strain of rats called the Zucker Diabetic Fatty (ZDF) strain. Development of diabetes in this strain is related to the loss of GLUT-2 glucose transporters in pancreatic beta cells and the concomitant loss of muscle GLUT-4 transporters (Friedman *et al.* 1991). Therefore diabetes in this strain is associated with impaired insulin secretion and peripheral glucose transporter function. Diabetic ZDF exhibit many of the complications of the hyperglycemic diabetic state, particularly the microvascular damage (Schafer *et al.* 2004). However there are no reports of atherosclerosis or macrovascular disease reported in this model.

1.12.2.3 Sand rat

The sand rat *psammomomys obesus* is a small rodent found in deserts in the Middle East and North Africa. The sand rat is metabolically normal when it subsists on its native food, salt brush. However when placed on an energy-rich diet (laboratory chow), it becomes obese and insulin-resistant and exhibits hyperlipidemia and eventually develops type 2 diabetes (Shafrir *et al.* 1999*,* Zoltowska *et al.* 2001). The sand rat has been used to study the metabolic aspects of the insulin-resistant hyperlipidemic state. However it has been reported that even in the presence of cholesterol feeding, the sand rat fails to develop advanced atherosclerotic lesions resembling those seen in humans, but does develop 'cholesterol overload lesions'.

1.12.2.4 Corpulent rats

A new mutation was isolated from a cross between Sprague-Dawley and spontaneously hypertensive rats (SHR). Animals of this strain became highly obese, hyperlipidemic, hyperinsulinaemic and developed CVD and atherosclerosis (Koletsky *et al.* 1975[a,b]). Initially the mutation was designated as *f* by Koletsky and later as *cp* by Hansen. Rats that are homozygous for the autosomal recessive *cp* gene (*cp/cp*) develop obesity, hyperlipidemia and IR with ensuing hyperinsulinaemia. While animals that are heterozygous (*cp/*+) or homozygous normal (+/+) are metabolically normal. Hansen developed a

number of congenic strains on various inbred backgrounds. However these fully congenic strains have lost the atherosclerosis/CVD prone characteristic. The JCR:LA-*cp* strain has been maintained as a closed outbred colony from the early stages of development. This has maintained the highly insulin-resistant trait and the atherosclerotic prone trait that has been lost in the SHROB, SHR/N-*cp* and LA/N-*cp* strains.

1.12.2.4.1 JCR:LA-cp rat as a model of atherosclerosis associated with metabolic syndrome.

Inspite of the high rate of insulin secretion (Pederson *et al.* 1991), the cp/cp rats do not develop pancreatic β cell failure and the resultant development of insulin-dependent diabetes. The prediabetic, insulin resistant state is strongly associated with cardiovascular disease as seen in humans, in which the *cp/cp* rats spontaneously develop marked vasculopathy, atherosclerosis and ischemic lesions. Early atherosclerotic changes are evident in rats at 12 weeks of age and by 9 months of age most cp/cp males have advanced intimal lesions of the aortic arch. Thrombi of different sizes and ages are found on the arterial surface and sometimes these thrombi are found to occlude the coronary artery. The *cp/cp* rats have increased levels of plasminogen activator Inhibitor-1 and this may be a cause of thrombi formation (Schneider *et al.* 1998). Adherent macrophages are also found to be present in the areas where endothelium is abnormal (Russell and Proctor 2006).

Intimal lesions were found to be very similar to human atherosclerotic lesions as these lesions contained lipids, PG, collagen, macrophages and vascular smooth muscle cells (Russell and Proctor 2006). The endothelium overlying the lesions is usually observed to be intact and appears normal while the vascular smooth muscle cells of the media were observed to be activated and migratory, and moving through breaks in the internal elastic intimal space (Russell and Proctor 2006). According to a study by Absher *et al*. the aortic vascular smooth muscle cells of the cp/cp rat are hyper-proliferative and

hyper-responsive to various cytokines, specifically insulin (Absher *et al.* 1997). When hyperinsulinaemia was experimentally reduced in the *cp/cp* males, a concomitant reduction in hyperactivity of vascular smooth muscle cells was observed (Absher *et al.* 1997).

1.12.2.4.2 JCR:LA-cp rat as a model of vascular dysfunction associated with metabolic syndrome

Along with the presence of the vascular lesions, and medial smooth muscle cell abnormalities, vascular dysfunction is present in the form of enhanced contractile response to noradrenaline or phenylephrine and thus impaired nitric oxide-mediated relaxation (O'Brien SF *et al.* 1997, 1999). These effects were observed in resistance vessels in both the aortae as well as mesenteric resistance vessels, while no such dysfunction was observed in the lean *+/?* rats.

In the *cp/cp* rats there is no impairment observed in the relaxation of phenylephrine pre-contracted vessels in response to the direct nitric oxide donor sodium nitroprusside, therefore indicating that the response of vascular smooth muscle cells *per se* is not compromised (Russell and Proctor 2006).

1.12.2.4.3 JCR:LA-cp rat as a model of post prandial lipemia

The plasma lipid profile of the JCR:LA-cp (*cp*/*cp*) rat has been characterized extensively over recent years (Russell and Proctor 2006). The *cp*/*cp* phenotype has mildly higher total plasma cholesterol levels compared with lean (+/?) counterparts (Russell and Proctor 2006). The observed increase in total cholesterol has been associated with the VLDL (very-low–density lipoprotein), and not the LDL fraction, similar to that observed in the pre-diabetic state in humans. As a result, the JCR:LA-cp model provides a unique opportunity to study dyslipidemia in the pre-diabetic state.

Recently, the metabolism of post-prandial CM was assessed in the JCR:LA-

cp rat (Vine *et al.* 2007). The approach for these studies has been to develop a novel, oral fat challenge test for the JCR-LA rodents analogous to the existing approach used in clinical studies (Taggart *et al.* 1997, Smith *et al.* 1999). After an overnight fast (16h), animals are offered a 5g food pellet supplemented with dairy fat (approx. 30% carbohydrate, 60% fat, 10% protein w/w). This methodology uses a well-established standardized conscious, non-restraint protocol (Vine *et al.* 2007). Given that the post-prandial phase (0–4h) predominantly represents plasma apoB48 derived from the intestine, this was utilized as a guide to assess CM metabolism in the JCR:LA-cp rat (Vine *et al.* 2007). In addition, our research group has developed techniques to accurately quantitate apoB48 in plasma using a highly sensitive Western blotting/enhanced chemiluminescent procedure (Vine *et al*. 2007, Smith *et al.* 1997, Jackson and Williams 2004). Using this approach in the JCRL:LA-*cp* rat, significant postprandial lipemia associated with apoB48-containing particles has been observed. Importantly, it has been determined that fasting concentrations of apoB48 in this model, despite contributions from both intestinal (95%) and hepatic (5%) (Vine *et al.* 2007, Teng *et al.* 1994, Liu *et al.* 1991) can be used to predict the impairment in post-prandial response (Vine *et al.* 2007). These results are consistent with reports in human subjects that also show elevated levels of fasting apoB48 in individuals at risk of CVD (Mamo *et al.* 2001,Chan *et al.* 2002). Thus an oral fat load (and/or challenge) in these animals is likely to contribute to the pro-atherogenic processes by either facilitating the saturation of lipolytic pathways, reducing the clearance capacity of cholesterol-rich lipoproteins, or exacerbating the permeability and retention of cholesterol in arterial vessels (Mamo *et al.* 2001,Chan *et al.* 2002).

Preliminary data from our laboratory has shown that in the hyperinsulinaemic JCR:LA-cp phenotype, lymphatic apoB48 concentration is doubled following a lipid meal compared with control animals. This is associated further with a reduced triglyceride/apoB48 ratio (indicative of the size of particles) in the hyperinsulinaemic phenotype, suggesting that dietary lipids may play a key

role in the regulation of post-prandial chylomicron composition and synthesis. Indeed high levels of insulin may exacerbate an overproduction of apoB48 in the post-prandial phase following a lipid-containing meal.

Consistent with observation in humans, the JCR:LA-cp model also presents with classic hypertriglyceridaemia and VLDL oversecretion which appears to develop in response to several lipogenic factors, including sterol regulatory element-binding protein-1c (SREBP-1c) regulation (Russell and Proctor 2006, Vine *et al.* 2007). SREBPs are a family of transcription factors that regulate cholesterogenesis and lipogenesis. *In vivo* studies have shown that the SREBP-1c isoform is primarily regulated by insulin (Eberle *et al.* 2004). SREBP-1c levels are increased in the liver of obese, insulin-resistant and hyperinsulinaemic *ob*/*ob* mice (Shimomura *et al.* 1999, 2000), and the over secretion of VLDL in JCR:LA-cp animals has been attributed to the dysregulation of endogenous FA synthesis via increased expression of SREBP-1c (Russell and Proctor 2006). Further contributions to increased plasma TG concentrations may be due to a down-regulation of muscle LPL activity and increased adipose LPL activity in the JCR: LA-cp rat (Russell and Proctor 2006).

Consequently, evidence from these studies supports studies that use the JCR:LA-cp model to investigate the pathological role of post-prandial lipemia in atherosclerosis, particularly in the pre-diabetic state.

1.13 Proposed Mechanisms of Increased Atherogenicity of ApoB48 Remnants During T1D and Metabolic Syndrome

1.13.1 Response-to-retention hypothesis

An important study reinforcing the response-to-retention hypothesis was from Skalen *et al.* who used a site-directed mutagenesis approach which targeted the length of the apoB molecule in different LDL species isolated from mice (Skalen *et al.* 2002). By reducing the length of apoB100 molecule they were able to identify a novel epitope on LDL that binds to PGs. In a second study

they created a transgenic mice that expressed this PG-binding defective LDL or LDL-r binding defective LDL (Flood *et al.* 2004). Following an atherogenic diet, mice with the PG-binding defective LDL developed less atherosclerosis compared to mice with LDL-r binding defective LDL or wildtype LDL.

It is very curious from the perspective of this thesis that studies up until now had only ever demonstrated the *'response-to-retention of LDL'*. However it has been suggested in the literature that the affinity of recombinant apoB48 may be greater for PGs as compared to recombinant apoB100 due to the potential masking of epitope sites on the larger B100 molecule (Flood *et al.* 2002).

According to the response-to-retention hypothesis and Figure 1-9, there are several factors that influence the initiation of atherosclerosis. However the effect of IR and diabetes on all these factors has not completely been defined.

1.13.2 *Number of lipoprotein particles and their clearance*

Lipoproteins (LDL and intestinal derived remnants) carry CE into the vessel wall, therefore a higher number of plasma lipoproteins in the circulation can potentially deliver more cholesterol to the vessel wall.

There is evidence from several studies (using both animal models and humans) that an equivalent fat load (challenge) will result in a very divergent response of the intestine when comparing normal and insulin-resistant conditions (Duez *et al.* 2008) (Figure 1-8). The exaggerated secretion of CM from an 'insulin-resistant' intestine is now thought to contribute to the accumulating presence of TG rich particles in plasma. LPL hydrolyses the native CM to form remnants, and these are predominately cleared by the liver. The rate of intestinal CM production, the level of LPL activity and the hepatic LDL-r receptor activity determine the number of CM and remnants in circulation (Figure 1-8).

Despite the presence of a higher number of circulating CM-r during diabetes,

it remains unknown whether these lipoproteins would deliver more cholesterol to the arterial vessel wall when it is exposed to *the same number of lipoprotein particles.*

1.13.3 *Permeability of the arterial vessel wall*

A smaller size of lipoproteins and damage/endothelial dysfunction of the arterial vessel wall can increase the permeability of cholesterol carrying lipoproteins. However, substantive alterations in permeability or endothelium function are not required to initiate cholesterol deposition during atherogenesis (Vasile *et al.* 1983, Navab *et al.* 1986, Lin *et al.* 1989, Williams and Tabas 1995).

Since the role of endothelial permeability in atherogenesis is not considered a necessary factor it was not investigated in this thesis.

1.13.4 *Composition of lipoprotein particles: Cholesterol per particle*

Intestinal derived remnants contain significantly more *cholesterol per particle* compared to LDL. It has been shown that despite lower delivery of intestinal derived remnants to the arterial tissue, they deliver significantly more cholesterol than LDL (Proctor *et al.* 2004). Therefore particle number as well as *cholesterol per particle* is important in determining the net cholesterol delivery to the arterial tissue.

IR may alter the *composition of particles,* either apolipoprotein or cholesterol content. Preliminary data from our laboratory have shown that in the hyperinsulinaemic JCR:LA-cp phenotype, lymphatic apoB48 concentration is doubled following a lipid meal compared with normo-insulinaemic control animals. This is associated further with a reduced TG/apoB48 ratio (indicative of the size of particles) in the hyperinsulinaemic phenotype.

In this thesis the altered composition of intestinal derived remnants during IR will be examined to determine if it is a factor that influences the binding capacity and/or affinity of intestinal remnants to arterial PGs.

1.13.5 *Lipoprotein-proteoglycan interaction: Role of bridging molecules*

Irreversible ionic bonding between lipoproteins and PGs forms the basis of the response-to-retention hypothesis. Interaction of lipoproteins with arterial PGs is dependent on the number of binding sites available, as well as on molecules such as LPL acting as bridging molecules. During diabetes, hyperglycemia leads to non-enzymatic glycation on several proteins including the arterial vessel wall. It has been shown that hyperglycemia leads to CVD however the role of non-enzymatic glycation on proteins as a bridging factor in lipoprotein-retention remains unknown. Therefore in this thesis the relationship between non-enzymatic glycation of the arterial matrix and its role in intestinal derived remnant retention will be investigated.

1.13.6 *Arterial proteoglycans*

Due to the role of PGs in PG-lipoprotein interactions, an increase in arterial PGs or their GAG chain length can lead to an increase in the retention of lipoproteins. In *in-vitro* conditions several factors during diabetes such as NEFA, TGF-beta and hyperinsulinaemia have been shown to increase the amount of PGs and the length of GAGs produced.

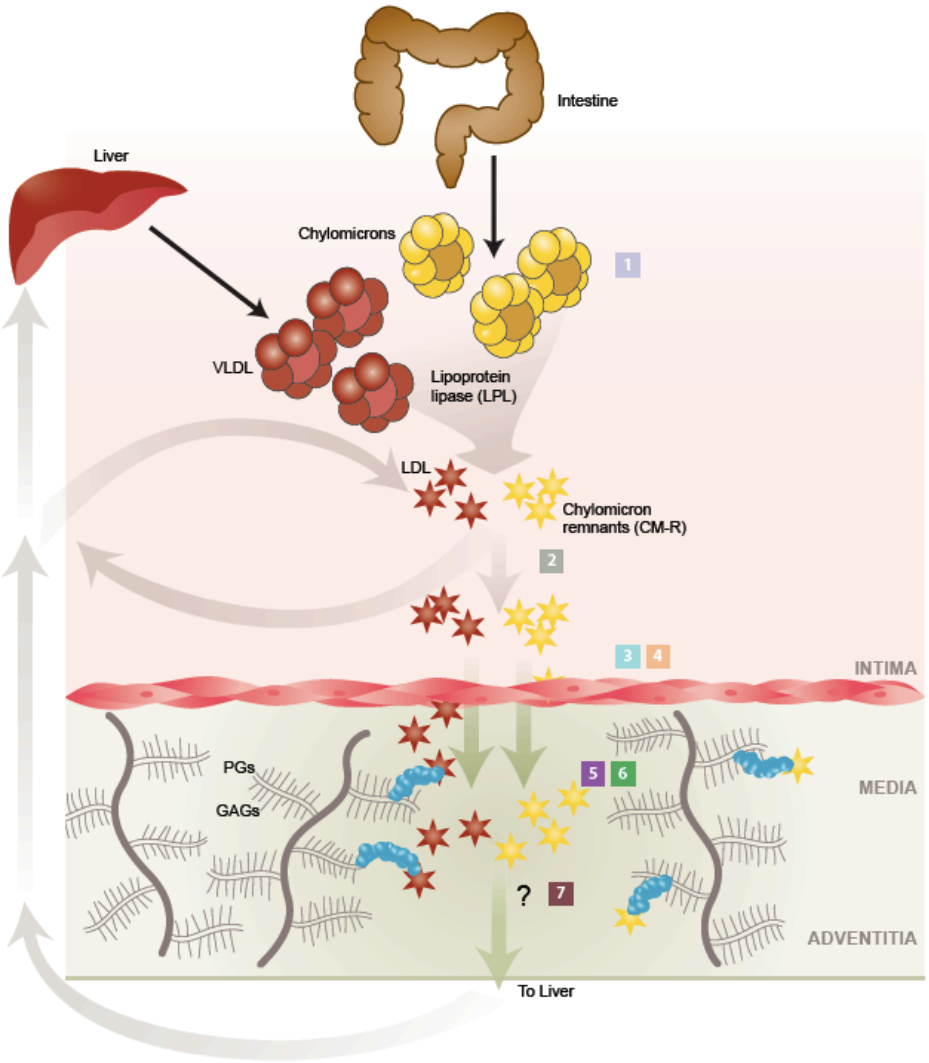
One of the primary objectives of this thesis is to determine whether there is increased presence of atherogenic PGs in the arterial vessels during IR. Although experiments using confocal microscopy have shown intestinal derived remnants are preferentially retained in the arterial wall, it remains to be investigated if intestinal remnants bind to arterial proteoglycans *per se as a mechanism of retention*. In this thesis, diabetic and pre-diabetic arterial vessels will be used to investigate if they have increased retention of intestinal derived remnant lipoproteins.

1.13.7 Efflux of lipoproteins

Lipoproteins that permeate the vessel wall can either form irreversible ionic interaction or they can efflux out from the vessel wall. Using *ex vivo* perfusion

studies in rabbits, it has been shown that, relative to apoB48 containing intestinal derived remnants, LDL is preferentially retained (Proctor *et al*. 2002, Proctor and Mamo 2003). However due to it's small size or due to the nature of binding interactions within the arterial intima, it is effluxed more over time as compared to intestinal derived remnants. However the effect of diabetic conditions on efflux of intestinal derived remnants and LDL is not known.

Consequently, the current model proposed by our laboratory is that *the response-to-retention hypothesis of atherosclerosis is not just dependent on circulating concentrations of low-density lipoprotein cholesterol but is equally dependent on intestinal remnant lipoprotein concentrations and perturbations in the arterial vessel wall that influence the rate of arterial lipoprotein retention.*



1 **Number of particles**-Particle production is increased from intestine due to insulin resistance

2 **Clearance**- Influenced by LPL activity or receptor mediated uptake and/or glycation. Delayed clearance in both IR as well as T1D.

3 **Permeability**- Increased by small size and inflammation.

4 **Composition of particles**- IR may increase cholesterol per particle and decrease size.

5 **Interaction**-LPL may act as bridge molecule, depends on number of binding sites and/or glycation.

6 **Proteoglycans**- TGF- beta, free fatty acids may result in elevated PG secretion and longer GAGs and more binding sites. May be affected by both IR and T1D

7 **Efflux**- Chylomicron remnants and LDL may efflux due to reversible binding.

Figure 1-9 Summary of factors involved in response-to-retention hypothesis of CVD (Warnakula et al. 2011). This image is used in accordance with the s.29 provision for fair dealing in the Canadian Copyright Act. Secretion of lipoproteins derived from either the liver (VLDL) or intestine (chylomicrons) may be upregulated during conditions of IR. Lipoprotein lipase (LPL) hydrolyses both native chylomicrons to chylomicron remnants and VLDL to LDL respectively. Circulating LDL and chylomicron remnants are delivered back to the liver by receptor-mediated processes. Clearance of lipoproteins can be delayed due to reduced LPL activity or defective receptor mediated processes (including lipoprotein glycation). Arterial exposure is dependent on the number of particles as well as their clearance and associated cholesterol mass. Arterial permeability is increased when particles are smaller in size or under conditions of damage (dysfunction or IR). Insulin resistance may alter the composition of particles either via apolipoprotein or cholesterol content. As lipoproteins permeate the arterial wall and come in contact with the GAGs (GAG) of the arterial extracellular PG, they form ionic bonds and may become entrapped. Some particles may efflux due to reversible binding. Interaction with arterial PG is dependent on the number of binding sites available, as well as LPL (or other factors such as glycation) acting as bridging molecules. Factors associated with IR such as free fatty acids and/or transforming growth factor (TGF)-beta may result in elevated secretion of PGs and/or with longer GAG side-chains. Collectively, these factors influence the overall contribution to lipoprotein- cholesterol accumulation in arterial vessels and/ diabetes associated atherosclerosis.

1.14 Working Hypothesis and General Objectives of the Thesis

The review presented in this chapter led us to the following working hypothesis and three main objectives for this thesis:

The overall general working hypothesis for this thesis:

Accelerated CVD during clinical conditions of IR and diabetes, where LDL-C concentrations are normal, is dependent on the circulating levels of intestinal derived remnant lipoproteins and phenotypical perturbations in the arterial vessel wall that modulate the rate of arterial lipoprotein retention.

General Objective 1. To determine whether the diabetic metabolic milieu that includes, but is not limited to, hyperglycemia as well as arterial vessel remodeling leads to increased *ex vivo* arterial retention of intestinal derived remnants in a rodent model of T1D.

General Objective 2. To determine whether remnant lipoproteins (containing predominantly apoB48-intestinal derived CM-r) bind directly to recombinant human biglycan *in vitro*. Secondly to assess the impact that glycated biglycan may have on the putative binding potential of intestinal derived remnant lipoproteins during conditions of Type-1 diabetes.

General Objective 3: To determine whether the prediabetic metabolic factors (e.g. hyperinsulinemia/IR) lead to increased arterial retention of intestinal derived remnant lipoproteins *ex vivo* in a rodent model of the metabolic syndrome (MetS) (the JCR:LA-*cp* rat).

These general objectives are explained in further detail with specific hypotheses and objectives in chapter 2.

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Chapter 2 Rationale: Objectives and Hypotheses

2.1 Executive Summary and Introduction to Research Hypothesis

Atherosclerosis and cardiovascular disease (CVD) are the leading causes of death and disability in the Western World today (WHO 2010). Moreover we know that vascular disease is accelerated under conditions of IR and diabetes (Caballero 2005, Eckel 2005). Despite the discovery of new lipid lowering agents with potential for anti-atherosclerotic action, the continued rise in the incidence of CVD suggests that we are yet to completely understand the etiology of the atherosclerotic process. The etiology of the response-to-retention hypothesis for atherosclerosis suggests that lipid and cholesterol accumulate in arterial vessels, and this lipid is derived from circulating lipoproteins (primarily apoB-containing particles) (Williams *et al.* 1995, Steinberg *et al.* 2005). The literature documents a significant epidemiological association between raised plasma concentrations of circulating LDL-C and CVD risk (LIPID study group 2002, Mediene-Benchekor *et al.* 2001, Colhoun *et al.* 2004). However, up to 40% of subjects diagnosed with vascular disease are normolipidemic without elevations in LDL, suggesting that during the development of atherosclerosis cholesterol may be derived from alternative sources (Caballero 2005, Eckel 2005).

2.2 'Residual risk' of CVD after LDL Cholesterol Lowering

Based on results from large clinical trials, the lipid management for reducing CVD risk has been typically focused on reducing LDL-C by statin therapy (Sacks *et al.* 1996, Downs *et al.* 1998, Marschner *et al.* 2001, LIPID study group, MRC/BHC Heart protection Study 2003). Despite the lowering of LDL-C by statin therapy CVD and ischemic events continue to occur. Indeed it is estimated that approximately two-thirds of all CVD events take place despite a lowering of LDL-cholesterol in patients at risk (Fazio 2007). These 'residual' events appear to be independent of the

LDL-C relationship and in recent years have gained momentum as a concept of *'residual risk'* of CVD. Interestingly, this 'residual risk' has been found to be greater for statin-treated patients with diabetes or the metabolic syndrome compared to untreated patients without these conditions (Fazio 2007, Deedwania *et al.* 2006). It could be interpreted by these observations that statin therapy (LDL-C lowering) does not necessarily reduce the relative CVD risk in patients with diabetes and the metabolic syndrome, and therefore does not lower the overall risk as compared to patients without these diseases.

2.3 Role of Intestinal Derived Remnant Lipoproteins in Response-to-Retention Hypothesis of Atherogenesis

There is now strong and convincing evidence that intestinal derived remnant lipoproteins (intestinal apoB48-containing remnant lipoproteins) can contribute to atherogenesis and are a significant risk factor for CVD (Zilversmit 1995, Cabezas *et al.* 2005, Proctor *et al.* 2002, Huff 2003, Karpe *et al.* 1994, Twickler *et al.* 2005). Moreover, numerous studies have shown that fasting levels of intestinal CM (measured as plasma apoB48) can predict impaired metabolism of CM, particularly in those at risk of CVD (Cabezas *et al.* 1998, Dane-Stewart et al. 2001[a], 2002[b], 2003[c]). Under experimental conditions, studies have shown that LDL particles have a higher rate of delivery but these particles also efflux more readily from arterial tissue compared to intestinal derived remnants (Proctor and Mamo 2002, Proctor *et al.* 2004). Collectively, experiments in this field to date highlight that intestinal derived remnants permeate through arterial tissue differently. Moreover the arterial 'flux' of particles may be dependent on the individual's metabolic state which may modulate potential interactions with extracellular matrix components, such as arterial PGs. For the purposes of this thesis, these factors may be particularly relevant in the development of CVD, particularly during the early phases of insulin resistance and diabetes proper (in pre-diabetes and the metabolic

syndrome before the onset of Type-2 and in Type-1 diabetes). Consequently, the current model proposed in this thesis is *the re-defining of the response-to-retention hypothesis of atherosclerosis to propose that it is not only dependent on circulating concentrations of LDL-C but is equally dependent on apoB-remnant lipoprotein concentrations and perturbations in the arterial vessel wall that influence the rate of arterial lipoprotein retention.*

The potential impact of this working hypothesis is perhaps most appreciated during conditions of increased atherosclerotic risk. For example, subjects with IR, type-1 and type-2 diabetes have 5 times greater risk of CVD than those without IR or diabetes, despite the fact that circulating concentrations of LDL are not elevated in these patients (Caballero 2005, Eckel 2005). In addition, both type-1 and type-2 diabetic subjects have been found to have raised plasma concentrations of fasting apoB48-containing remnant lipoproteins, and an impairment in post-prandial CM metabolism (Su *et al*. 2009, Hogue *et al.* 2007).

Critical Note: *Despite evidence for significant increases in CVD risk in subjects with IR and diabetes compared to control subjects, it is not unequivocally known whether the metabolic factors associated with these conditions predispose the arteries to increased intestinal derived remnant lipoprotein cholesterol uptake and accumulation.*

<u>The overall general working hypothesis for this thesis is:</u>

Accelerated CVD during clinical conditions of IR and diabetes, where LDL-cholesterol concentrations are normal, is dependent on the circulating levels of intestinal derived remnant lipoproteins and phenotypical perturbations in the arterial vessel wall that modulate the rate of arterial lipoprotein retention.

2.4 Vessel Wall Remodeling During Pre- and Overt Diabetes

Metabolic syndrome and IR can pre-dispose individuals to the development of type-2 diabetes with the associated sequelae of atherosclerosis and CVD risk (Despres *et al.* 1994, Nzekwu et al. 2007, Vine *et al.* 2007, Tomkin 2001). While lack of glycemic control in type-1 diabetes accelerates the progression to CVD (The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study 1999, Nathan *et al.* 2005), we are yet to delineate whether diabetic-atherosclerosis simply represents an accelerated form or a specific genesis unique to diabetes.

Arterial PGs and GAGs have been shown to have elevated mass and distribution in both type-1 diabetes as well as type-2 diabetes (Heickendorff *et al.* 1994). Chronic IR has also been shown to re-model extracellular arterial PGs *in-vitro* (Rodríguez-Lee *et al.* 2006, Camejo *et al.* 2002). However arterial PG content during various stages of IR have not been investigated.

We know that the proliferation of vascular smooth muscle cells can stimulate the secretion of arterial PGs, which in turn can increase the capacity of lipoprotein binding (*in-vitro*) (Tannock *et al.* 2002, Little *et al.* 2002). Transforming growth factor (TGF)-beta-1 has been identified in atherosclerotic vessels and has been shown to stimulate the synthesis of chondroitin sulphate- and dermatan sulphate-containing proteoglycans by arterial smooth muscle cells (Little *et al.* 2002). Furthermore, subjects with IR have been shown to have raised circulating levels of TGF-beta which may be responsible for the observed increase in vascular intimal medial thickness in these individuals (Panday *et al.* 2005).

Critical Note: *It is proposed that an increase in arterial biglycan during pre-diabetes and IR (both type 1 and type 2) may accelerate the deposition of lipid, specifically cholesterol, in arterial vessels due to*

increased binding and retention of apoB48-containing remnant lipoproteins. It is speculated that aortic smooth muscle cells may increase arterial PG secretion during IR.

2.5 ApoB48 Remnants-Proteoglycan Interaction

There have been several studies that have investigated the capacity for arterial PGs to bind apoB100-containing lipoproteins *in-vitro,* particularly due to their electrostatic properties (Camejo *et al.* 1998, Olsson *et al.* 2001). However, the translation of these studies to the *'in vivo'* potential of arterial PGs to bind apoB-containing lipoproteins remains limited. Experiments by O'Brien and Chait have shown a high degree of co-localization of apoB, apoE and biglycan deposits in human atherosclerotic lesions (O'Brien *et al.* 1998). Unfortunately, these studies were unable to determine if the specific source of apoE was from apoB100 or apoB48-containing lipoproteins.

Critical Note: *Proteoglycan-lipoprotein interaction is a fundamental principle in the response-to-retention hypothesis, however to date there remains no data regarding the binding affinity of apoB48-containing lipoproteins to proteoglycans.*

2.6 Composition of Post-prandial Chylomicrons During Lipid Load and During Conditions of Insulin Resistance and/or Hyper-insulinaemia.

Recent data from hamsters (Fedrico *et al.* 2006) and humans (Duez *et al.* 2008) has demonstrated that IR leads to overproduction of intestinal CM i.e increased number of particles being synthesized. The impact of particle number, apoprotein, lipid and cholesterol composition of CM collected in the fed and fasted state under conditions of hyperinsulinemia forms the rationale of this thesis: to test under these different conditions the capacity of CM to bind to arterial PGs.

Critical Note: *It is proposed that the solid-binding assay technique will be used to assess the direct binding affinity/capacity of native apoB48 containing CM particles isolated under different physiological conditions. CM will be isolated in the fasted or fed state, and in conditions with or without hyper-insulinemia to ascertain the relative binding capacity of PGs to chylomicrons.*

Clinical data from our research group has shown a significantly higher number of fasting apoB48-containing lipoproteins in subjects with T1D (Su *et al.* 2009 and Chapter three). In addition, this study also showed impaired post-prandial metabolism of apoB48-containing CM lipoproteins in type 1 diabetic subjects. Another study from our research group has shown increased arterial retention of apoB48-containing remnant lipoproteins in insulin deficient rats and rabbits when compared to controls (Proctor *et al.* 2000). Therefore the natural extension to the objectives of this thesis are to investigate the mechanisms underlying the exacerbated retention of intestinal derived remnants in T1D and to determine the extent of arterial binding of these lipoproteins during conditions of insulin resistance.

2.7 General Working Hypothesis

Accelerated cardiovascular disease during early phases of type 1 and type 2 diabetes (where LDL-C concentrations are normal), is dependent on the circulating levels of intestinal derived remnant lipoproteins and phenotypical perturbations in the expression of proteoglycans in the arterial vessel wall that collectively increase the rate of arterial lipoprotein retention and cholesterol accumulation.

General Approach to Study the Working Hypothesis

In the *first* study (experimental chapter 3) the arterial retention of remnant lipoproteins was investigated in T1D using a streptozotocin induced rat model and an *ex vivo* perfusion model of intestinal derived remnant retention. Furthermore the role of arterial PGs (biglycan and decorin) and

advanced glycated end products in *ex vivo* retention of intestinal derived remnant lipoproteins was investigated.

In the *second* study (experimental chapter 3), the direct binding affinity and capacity of CM, intestinal remnant lipoproteins and LDL to recombinant human biglycan was determined using an *in-vitro* solid phase assay. The direct binding affinity and capacity of intestinal remnant lipoproteins to biglycan was also determined under experimental conditions of a 'glycated' extracelluar matrix. This *in-vitro* approach enabled the standardization of the arterial matrix expression of PG, and provided a model to test if intestinal derived remnants indeed bind to biglycan *per se,* and if there is differential binding of lipoprotein fractions. Furthermore the binding of remnant lipoproteins to recombinant human biglycan and bovine biglycan was also investigated by the *in vitro* binding assay. At the time these experiments were conducted there was no known source for human biglycan and the biglycan used in binding assays was the bovine biglycan. Therefore the comparison of human recombinant biglycan to bovine biglycan was done to determine whether remnant lipoprotein binding from both these sources was similar or differential.

The *third* study (experimental chapter 4) determined the arterial retention of intestinal derived remnant lipoproteins in IR using an *ex vivo* perfusion model in the JCR:LA-*cp* rodent model of the metabolic syndrome. Furthermore, the *ex vivo* arterial 'delivery' and 'retention' of intestinal derived remnant lipoproteins vs. human LDL in the JCR:LA-*cp* rodent model of the metabolic syndrome was also explored. Rodent models are generally more resistant to atherosclerosis however the JCR-LA-cp rat model develops atherosclerosis. Therefore the JCR:LA-*cp* rat model was chosen because focus of this study was to study the early initiating stages of atherosclerosis.

The final and *fourth* study (experimental chapter 4) was designed to compare and contrast the direct binding affinity and capacity of native

TRLs isolated under fasted and fed conditions during IR compared to the non-IR state using the *in-vitro* approach developed in study two.

Currently there is no evidence to show that there is CM overproduction or compositional changes in CM in T1D, therefore a study similar to the *fourth* study design in a type 1 diabetic animal model was not conducted as part of this thesis herein.

Endothelial dysfunction can exacerbate lipoprotein retention however it is not a prerequisite for lipoprotein retention. Previous studies in the JCR:LA-*cp* rat model have documented endothelial dysfunction in this rat model (O'Brien *et al.* 1999).

The rationale for considering models of both type 1 and type 2 diabetes in the working hypothesis is following: We are interested in the possibility that exacerbated remnant lipoproteins in both type 1 and type 2 diabetes can accelerate CVD with either normoglycemia or with differential dyslipidemia. The understanding of remnant lipoproteins in T1D is less understood. Hyperglycemia and the vascular complications of hyperglycemia may exacerbate lipoprotein retention during type 1 diabetes. For type 2 diabetes, the rationale is somewhat more well developed as some of the metabolic causes of insulin resistance and increases in systemic and local factors can give rise to increased intimal thickening and may exacerbate remnant lipoprotein retention. Therefore we believe that inclusion of both type 1 and type 2 diabetic states in the study hypothesis is valuable.

2.8 Thesis Objectives and Hypotheses

Study 1: Arterial Retention of Remnant lipoproteins in Type-1 Diabetes Ex vivo

General Objective 1. To determine whether the diabetic metabolic milieu that includes, but is not limited to, hyperglycemia as well as arterial vessel remodeling leads to increased *ex vivo* arterial retention of intestinal derived remnants in a rodent model of T1D.

Hypothesis: T1D is associated with an increase in the susceptibility of arteries to retain intestinal derived remnant lipoproteins and positively correlates with extracellular arterial matrix remodeling; including increased PGs (biglycan and decorin) and advanced glycated end products.

Specific objectives

1(i) To determine if arteries isolated from type 1 diabetic rats (streptozotocin induced Wistar rat model of T1D) have greater ex vivo retention of intestinal derived remnant lipoproteins compared to non-diabetic rat arteries.

Hypothesis: T1D increases the susceptibility of diabetic arteries to intestinal derived remnant lipoprotein retention.

Expected Outcome: After ex vivo arterial perfusion with intestinal derived remnant lipoproteins, it is expected that the carotid vessels isolated from type 1 diabetic rats will exhibit increased retention of intestinal derived remnants compared to control carotid vessels.

1(ii) To assess the extent to which arterial PGs (biglycan, and decorin) co-localize with the retention of intestinal derived remnant lipoproteins in the steptozotocin-induced rat model of T1D.

Hypothesis: Intestinal derived remnant lipoproteins bind to arterial PGs and therefore co-localize with arterial PGs (biglycan and decorin) in the

streptozotocin-induced rat model of T1D.

Expected Outcome: Intestinal derived remnant lipoproteins retained in the type 1 diabetic carotid vessels after ex vivo perfusion will co-localize to a significant extent with arterial PGs, specifically biglycan and decorin.

1(iii) Assess the putative role of advanced glycated end products in the arterial retention of intestinal derived remnant lipoproteins ex vivo in type 1 diabetic arteries isolated from the streptozotocin induced rodent model of T1D.

Hypothesis: Advanced glycated end products provide additional putative binding sites for lipoprotein binding and therefore exacerbate the arterial retention of intestinal derived remnant lipoproteins in the streptozotocin induced rat model of T1D.

Expected Outcome: Intestinal derived remnant lipoproteins retained in the carotid vessels of type 1 diabetic rats after ex vivo perfusion will co-localize significantly with advanced glycated end products, and this co-localization will be positively correlated with cholesterol deposition in the arterial wall.

The results from these series of experiments are presented in Chapter 3. Portions of these data have been published (See Mangat et al. 2011).

Study 2: Compare and contrast the direct binding affinity and capacity of intestinal derived remnant lipoproteins, relative to other lipoprotein fractions, to biglycan using an in-vitro approach under experimental conditions of a glycated matrix.

General Objective 2. To determine whether remnant lipoproteins (containing predominantly apoB48-intestinal derived CM-r) bind directly to recombinant human biglycan *in vitro*. Secondly to assess the impact that glycated biglycan may have on the putative binding potential of intestinal derived remnant lipoproteins during conditions of Type-1 diabetes.

Hypothesis: The putative binding site on apoB48 facilitates the binding of intestinal derived remnant lipoproteins to recombinant human biglycan. Hyperglycemia and subsequent glycation of biglycan further increases the binding of intestinal derived apoB48 remnant lipoproteins possibly by providing additional binding sites.

Specific objectives Study 2:

2(i) To establish the methodology and assess the affinity of apo-B48 containing remnant lipoproteins isolated from control rats to both bovine and recombinant human biglycan using in-vitro solid-phase binding assay methodology.

Hypothesis: Intestinal derived apoB48 remnant lipoproteins bind to both bovine biglycan and recombinant human biglycan in the in-vitro solid phase binding assay via putative apoB48 binding sites (described by Flood et al. 2002).

Expected Outcome: It is expected that the longer GAG chains of bovine biglycan may bind intestinal derived remnant lipoproteins with a higher binding capacity and affinity as compared to recombinant biglycan.

2(ii) Compare both the binding affinity and capacity of hepatic derived human LDL, native TRL chylomicrons and intestinal derived chylomicron-remnants isolated from control rats with recombinant human biglycan using an in-vitro solid phase binding assay.

Hypothesis: Due to the differences in size and lipid characteristics, the low-density lipoproteins, native TRL CM and CM-r bind to recombinant human biglycan with variable binding affinities.

Expected Outcome: Since the putative apoB48 binding has been suggested to bind to biglycan with higher affinity compared to the apoB100 binding site on low-density lipoproteins, it is expected that intestinal derived remnant lipoproteins will bind to biglycan with higher capacity and affinity relative to low-density lipoproteins.

2(iii) Assess the impact of glycated versus non-glycated biglycan on the retention of remnant lipoproteins isolated from control rats using an in-vitro solid phase binding assay.

Hypothesis: Additional binding sites for intestinal derived remnant lipoproteins provided by glycation of biglycan leads to increased binding of intestinal derived remnant lipoproteins in an in-vitro solid phase binding assay.

Expected Outcome: It is expected that the glycated biglycan increases retention of intestinal derived remnant lipoproteins in an in-vitro binding assay when compared to non-glycated biglycan.

The results from these series of experiments are presented in Chapter 3. Portions of these data have been published (See Mangat et al. 2011).

Study 3: Ex vivo Arterial Retention of Remnant lipoproteins in pre-diabetes (MetS).

General Objective 3: To determine whether the prediabetic metabolic factors (e.g. hyperinsulinemia/IR) lead to increased arterial retention of intestinal derived remnant lipoproteins *ex vivo* in a rodent model of the MetS (the JCR:LA-*cp* rat).

Hypothesis: In the IR state there is increased production of atherogenic PGs that is associated with an increase in the accumulation of intestinal derived remnant lipoproteins in the arterial wall.

Specific objectives Study 3:

3(i) To determine if arteries isolated from the JCR:LA-cp IR rat are more susceptible to arterial retention of intestinal derived remnant lipoproteins ex vivo as compared to non-IR control JCR:LA-cp rats.

Hypothesis: The IR state is associated with an increase in the arterial retention of remnant lipoproteins in the JCR:LA-cp model of MetS.

Expected Outcome: It is expected that arteries from IR rats will retain more intestinal derived remnant lipoproteins and this will be associated with increased cholesterol retention as compared to arteries from control non-IR animals using an ex vivo arterial perfusion model.

3(ii) Contrast the arterial 'delivery' and 'retention' of intestinal derived remnant lipoproteins compared to human low-density lipoproteins ex vivo in order to directly assess rates of lipoprotein 'efflux' from arterial tissue in the JCR:LA-cp model of MetS.

Hypothesis: Intestinal derived remnant lipoproteins 'efflux' from arterial tissue at a slower rate and have increased net retention compared to low-density lipoproteins.

Expected Outcome: It is expected that the intestinal derived remnant

lipoproteins will have increased net arterial retention and this will be associated with an increase in cholesterol deposition, compared to low-density lipoproteins.

3(iii) To determine the mass of both decorin and biglycan in aortic tissue in the JCR:LA-cp rodent model of MetS.

Hypothesis: IR animals have an increase in the mass of arterial PGs, biglycan and decorin, compared to control non-IR animals.

Expected Outcome: It is expected there will be an increase in total arterial biglycan and decorin, and an increase in total arterial GAGs in the prediabetic, hyperinsulinemic animal compared to the control non-IR JCR:LA-cp rat.

The results from these series of experiments are presented in chapter 4 and currently being prepared for publication.

Study 4: Compare and contrast the direct binding affinity and capacity of remnant lipoproteins isolated during conditions of insulin resistance using an in-vitro binding assay approach.

General Objective 4: To determine whether the native triglyceride rich lipoproteins (TRLs) produced during the fed and fasted states in IR bind differently to human recombinant biglycan compared to TRLs isolated under control non-IR conditions.

Hypothesis: The compositional differences and size of intestinal derived remnant lipoproteins synthesized during fasting and fed states in IR alter the binding of TRLs to arterial biglycan.

Specific objectives Study 4:

4(i) Compare the binding capacities of native TRLs isolated in the fasted state from JCR:LA-cp IR rats and non-IR JCR:LA-cp rats to human biglycan.

Hypothesis: In IR, native TRLs isolated during the fasting state (relative to lipoproteins produced during non-IR, fasted condition) are associated with an increase in binding capacity to arterial biglycan.

Expected Outcome: It is expected that native TRLs isolated in the fasted state from IR animals will have increased binding capacity for biglycan in an in-vitro assay, when compared to native TRLs isolated in the fasted state from control non-IR rats.

4(ii) Compare the binding capacities of native TRLs isolated in the fed state from JCR:LA-cp insulin resistant rats and non-IR rats to human biglycan.

Hypothesis: Changes in size and/or compositional differences in the TRLs produced in the fed state will result in increased binding capacity of TRLs isolated from IR animals to arterial biglycan.

Expected Outcome: It is expected that native TRLs produced during the fed state and under IR conditions will have increased binding capacity to biglycan compared to TRLs isolated from control non-IR rats.

The results from these series of experiments are presented in chapter 4 and are currently being prepared for a publication.

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Chapter 3. Increased risk of cardiovascular disease in type 1 diabetes: Arterial exposure to remnant lipoproteins leads to enhanced deposition of cholesterol and binding to glycated extracellular matrix proteoglycans.

This chapter has been published as a manuscript (Please see Appendix A).

3.1 INTRODUCTION

Type 1 diabetes (T1D) is associated with accelerated atherosclerosis and a 3 to 4 fold increase in cardiovascular disease (CVD) risk as compared with the non-diabetic population (Kannel and McGee 1979). The paradox of T1D is that despite increased CVD risk, classic fasting lipid indices (elevated low density lipoprotein cholesterol, LDL-C or low concentration of high density lipoprotein cholesterol, HDL-C), are often comparable to those of a healthy population (Howard 1987). Furthermore, there is no associated increase in other cardiovascular risk factors such as obesity, smoking or hypertension in T1D. Thus, our current understanding of the etiology of increased atherosclerotic risk in T1D remains limited.

Accumulating evidence has implicated a role for remnant lipoproteins in the development of atherosclerosis among populations at risk of CVD including; normolipidemic subjects with coronary heart disease; obesity, metabolic syndrome, type 2 diabetes, and familial hypercholesterolemia (Kannel and McGee 1979, Howard 1987, Tomkin *et al.* 2001, Nordestgaard *et al.* 2007 and Meyer *et al.* 1996). However the role of remnant lipoproteins and their metabolism in T1D atherosclerotic risk remains unclear. To date, no studies have examined apolipoprotein (apo) B48, a specific marker of intestinal remnant lipoprotein particle metabolism, in a human population with T1D. Nor is it known whether apoB48-remnant lipoproteins and associated cholesterol accumulate at a

greater propensity in the arterial vessels of subjects with T1D (Proctor *et al.* 2002). A recent human study conducted by our research group suggests that the metabolism of remnant lipoproteins is impaired in normolipidemic subjects with T1D (Su *et al.* 2009). Indeed, others have proposed that the increased CVD risk associated with T1D may result from a unique diabetic dyslipidemic profile that is predominantly attributable to changes in the remnant lipoprotein cholesterol fraction (Georgopoulos *et al.* 1991).

Together with the findings presented in this chapter, the human study on T1D subjects has been published as a manuscript (Please see appendix A, Mangat *et al.* 2011). The main findings of this human study are summarized below as these findings led us to the objectives of the *first* and *second* studies outlined in *chapter 2* and that are further presented in detail in this chapter.

3.1.1 Impaired ApoB48-Remnant Lipoprotein Metabolism in T1D

The human study done by our research group on subjects with TID has reported a significantly higher concentration of fasting remnant lipoproteins (measured as apoB48) among normolipidemic T1D subjects with longstanding diabetes. Importantly, fasting plasma apoB48 concentration was the predominant lipoprotein-associated difference between T1D subjects and matched controls. Furthermore, T1D participants demonstrated a postprandial deficit in the clearance of remnant lipoproteins from plasma, with a greater total plasma apoB48-AUC relative to controls. While the corresponding total iAUC in T1D subjects was not different from controls following the initial meal (breakfast), the second meal (lunch) appeared to exacerbate the inability of T1D subjects to efficiently metabolize remnant lipoproteins. We wish to highlight that net AUC is an important indicator of total cholesterol associated with remnant lipoproteins when considering exposure to the arterial wall. It is also important to note that individuals are in the post-absorptive state

repeatedly throughout the day, and typically, individuals would ingest a third meal. Thus, we propose that impaired remnant metabolism under these conditions may contribute to increased CVD risk in T1D, and this may be independent of other risk factors such as fasting lipid profile, body weight, and age. It is important to note here that there are no studies that have investigated the apoB48 remnant lipoproteins in T1D subjects. The patient population studied here had HbA1c of 8.9%. Therefore it is not known whether elevated HbA1c is required for postprandial lipemia of remnant lipoproteins. We can speculate that post prandial lipemia in T1D subjects is independent of elevated HbA1c.

3.1.2 Mechanisms of Impaired Remnant Lipoprotein Metabolism in T1D

Mechanisms underlying increased and prolonged residence time of apoB48-remnant lipoproteins in T1D *per se* have been associated with decreased lipase activity in patients with diabetes (De Man *et al*. 1996). However, if lipase activity was decreased in our study subjects, we would have expected to observe a corresponding increase in either fasting and post-prandial AUC response for TG and/or TRL apoB48 (density < 1.006 g/mL). Other investigators have suggested that remnant particle clearance may be impaired in T1D due to altered particle composition affecting high affinity uptake by receptors (Georgopoulos *et al.* 1991, Taskinen 1992, and Geogopoulos *et al.* 1989), and/or increased competition with VLDL for receptor-mediated clearance from plasma (Jensen *et al.* 1989). A similar conclusion was reached by Georgopoulos and Phair (Georgopoulos et al. 1991) using low-dose ^{125}I labeling of apoB particles in T1D. Another possible and more novel mechanism is the finding of increased lipid synthesis and/or secretion from the intestine *per se* which contributes to the total systemic pool of cholesterol. Overproduction of intestinal chylomicrons has recently been demonstrated in animals and humans with insulin resistance (Duez *et al.* 2006) as well as type 2 diabetes (Hogue *et al.* 2007, Federico *et al.* 2006). Studies in streptozotocin-induced diabetic

rats have also shown both greater intestinal absorption (Young *et al.* 1983) and increased synthesis of cholesterol (Young *et al.* 1988).

Furthermore in T1D, the arterial wall is characterized by intimal thickening, smooth muscle cell proliferation, increased expression of extracellular proteoglycans and the accumulation of glycated proteins (Nakamura *et al.* 1993, Rabago Rodriguez *et al.* 2007, and Genuth *et al.* 2005). Recent data supports the hypothesis that hyperglycaemia and insulin deficiency facilitate the entrapment of apoB and apoE-containing atherogenic lipoproteins in the sub-endothelial matrix of the vessel wall (Camejo *et al.* 2002). However, direct evidence has been lacking to determine if remnant lipoproteins avidly bind with arterial extracellular matrix components or if there is preferential binding and accumulation of these particles during T1D.

Consequently, the main objective of the study presented in this chapter was to assess the consequence of remnant lipoprotein (and associated cholesterol) accumulation in the arterial vessels *ex vivo* during experimental diabetes. We hypothesized that remnant lipoprotein metabolism would be impaired in T1D and that arterial vessels from diabetic animals would accumulate cholesterol to a greater extent compared to controls.

3.2 RESEARCH DESIGN AND METHODS

3.2.1 Biochemical Analysis

Total as well as newly synthesized intestinal-derived remnant lipoprotein concentration was determined by measuring apoB48 from whole plasma and the TRL fraction (density <1.006 g/mL), respectively. TRL fractions were separated at each time point by ultracentrifugation at 25000 rpm for 25 mins at 20°C and were immediately frozen and stored at -80°C.

ApoB48 was quantified using a previously described sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and modified western blotting technique (Vine *et al.* 2007).

Glucose, total plasma cholesterol, LDL-C, High density Lipoprotein cholesterol and TG were all determined by the University of Alberta Hospital laboratory, using standard enzymatic colorimetric assays. Insulin was measured using a radioimmunoassay highly specific for human insulin and analogues (Linco Research).

3.2.2 Experimental Diabetes

Diabetes was induced in male Sprague Dawley rats (200–250 g body weight) by tail vein injection of streptozocin (50 mg/kg) following an overnight fast. Animals with plasma glucose concentration >15 mmol/L one week post-induction of diabetes were included in the study as diabetic. Diabetes was confirmed by glycosuria and elevated plasma glucose concentration (Boehringer Mannheim). *Ex vivo* perfusion experiments were completed 8 weeks following the induction of diabetes. Sham-injected control rats (sodium citrate buffer pH 4.5) were used concurrently. All animal procedures were in accordance with guidelines set by respective Institutional Ethics Committees. Plasma triglyceride concentration was measured at the end of the study by an enzymatic procedure and corrected for free glycerol (Wako) and cholesterol was determined colorimetrically (Wako). Fluorescent glycated proteins present

in rat serum were assayed using on-line spectrofluorometric detection in a flow system (Forbes *et al.* 2003). Samples were run in triplicate and the area under the curve was used for signal measurements. The assay was calibrated against glycated proteins obtained from enzymatic hydrolysis of glycated BSA (10 g/L). Total peptide content was estimated using an on-line spectrophotometric detector set to a wavelength of 280 nm (Forbes *et al.* 2003).

3.2.3 Lipoprotein Isolation and Labeling

Intestinal remnant lipoproteins were isolated and labeled with fluorophores as described previously (Proctor *et al.* 2003 and 2004). Briefly, nascent chylomicron particles were collected from superior mesenteric lymph duct cannulated donor rats given Intralipid™ via a gastric cannula. Remnant lipoproteins were generated endogenously *in vivo* via a surgical rabbit hepatectomy procedure (Proctor *et al.* 2003 and 2004). Lipoproteins were isolated by ultracentrifugation and then conjugated with Cy5 (Amersham). Cy5 is conjugated to all the proteins on the chylomicron. Importantly, we have established that this fluorophore does not exchange with other plasma macromolecules, is protein bound and does not have altered clearance from plasma, or organ uptake (Proctor *et al.* 2003 and 2004). All fluorescent lipoprotein preparations are routinely screened for potential aggregation or modification by confirming particle size (Brookhaven).

3.2.4 Recombinant biglycan

For the production of human recombinant biglycan, cDNA from Origene was used to transfect host FS293 cells which were cultured in sterile 250ml Erlenmeyer flasks with 10×10^7 cells per flask, in FSEM medium (Invitrogen). Each flask was transfected with 76 μg human biglycan DNA using 293fectin reagent. Cell media containing biglycan was separated by SDS-PAGE and dimethylmethylene blue (DMMB) positive fractions were pooled and dialyzed. Biglycan glycosaminoglycan chains were

characterized on a DMMB stained gel and the size of protein core was validated after ABCase (Chondroitinase) digestion on a coomasie stained gel. To modify biglycan for glycation, human recombinant biglycan was incubated in 50mM glucose for 15 days at 37°C.

3.2.5 Characterization of recombinant human biglycan

The recombinant biglycan that was produced had smaller glycosaminoglycan (GAG) chains when compared with the GAGs isolated from the rat thoracic aorta because the recombinant expression system cannot usually produce GAG chains very effectively (Figure 3-1). However the protein core was of the size corresponding to 45 kDa as seen in the coomassie stained gel (3-1B).

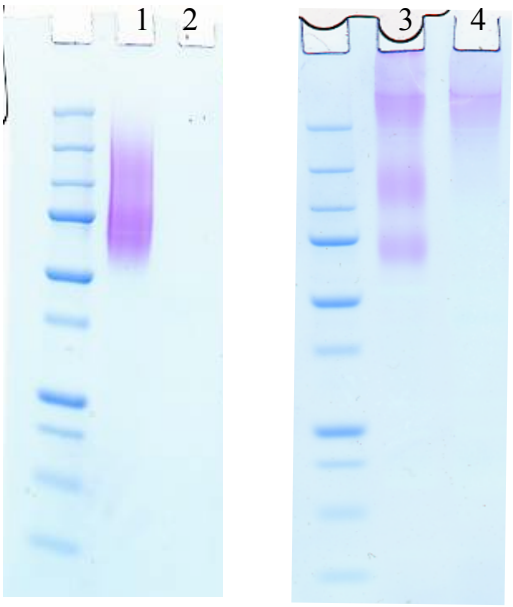
As observed in lane 4 of figure 3-1A, we found that some residual sulphated GAGs remained on the DMMB gel following chondroitinase digestion of the aortic extract. These could be hyaluronic acid or heparan sulphate proteoglycans.

(A) DMMB stained gel
Lane 1: Recombinant human biglycan

Lane 2: Recombinant human biglycan digested by chondroitinase

Lane 3:
Total proteoglycans isolated from rat thoracic aorta

Lane 4:
Total proteoglycans from rat thoracic aorta, digested by chondroitinase



(B) Coomassie stained gel
Lane 1: Recombinant human biglycan

Lane 2: Recombinant human biglycan digested by chondroitinase

Lane 3: Total proteoglycans isolated from rat thoracic aorta

Lane 4: Total proteoglycans from rat thoracic aorta, digested by chondroitinase

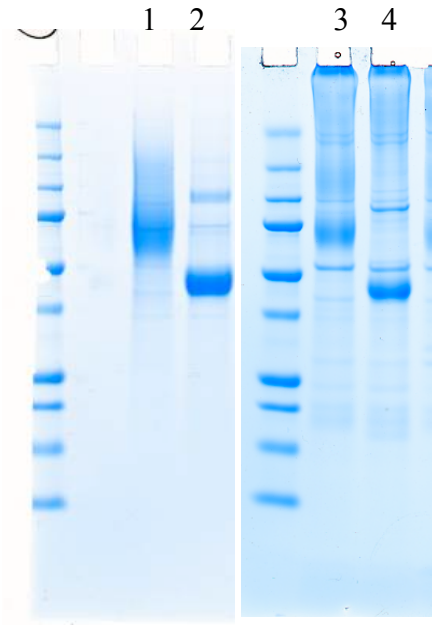


Figure 3-1 Characterization of recombinant human biglycan and the proteoglycans isolated from rat thoracic aorta: (A) DMMB stain showing the glycosaminoglycans, (B) Coomassie stain showing protein cores respectively.

3.2.6 Solid phase binding assay

Human biglycan was diluted in HBS (HEPES buffered saline) and 2ug/well was added to a Maxisorp immunoplate (Nunc) and incubated at room temperature overnight. To determine non-specific binding additional control wells were coated with only HBS. All wells were blocked with BSA for an hour and following that lipoprotein samples diluted in sample buffer (20mg/L, 10mg/L, 7.5mg/L, 5mg/L, 2.5mg/L, 1.25mg/L and 0.625mg/L in triplicate) were added to wells and incubated for 1h at room temperature. Human LDL was purchased from Chemicon (catalog number:LP2).

The number of intestinal remnant lipoproteins in each well was standardized to apoB48 and the number of LDL was standardized to apoB100 concentration. To detect lipoproteins bound, HRP-conjugated anti-human/rodent apoB (R&D BioDesign) diluted 1/750 in HBS-Tween with 0.1% BSA was used for incubation for 1h 30 min at room temperature and washed. Finally 1-Step^{TM} Turbo TMB-ELISA (Pierce) was used for approximately 5mins to allow for color development. Absorbance was measured at 450 nm.

3.2.7 Ex vivo arterial perfusion

Carotid artery segments were cannulated at the proximal and distal ends to create a closed circuit. Carotid vessels from insulin deficient (n=10 vessels) and control rats (n=7 vessels) were perfused *ex vivo* with equivalent and physiological concentrations of remnant lipoproteins. The labeled remnants were diluted in PBS to achieve a final concentration of $8.8x10^{13}$-$1.2x10^{14}$ lipoprotein particles per mL perfusate, which was chosen to reflect remnant concentration in the post-absorptive state ($5x10^{13}$ mL plasma) (Proctor *et al.* 2003 and 2004). The perfusate (Cy5 labeled remnant lipoproteins diluted in PBS) was oxygenated and recirculated. The carotid vessels were perfused with Cy5 labeled remnant lipoproteins for 25 minutes followed by a washout period of only PBS perfusion for 20 minutes.

We have shown previously that efflux of non-bound arterial lipoproteins is complete within 20-30 minutes and that perfusion of non-lipoprotein buffer for as long as 60 minutes fails to clear sites of focal accumulation (Proctor *et al.* 2003 and 2004). Vessels are removed and fixed in 2% paraformaldehyde for exactly 30min. Carotid segments are frozen in liquid nitrogen and sectioned (approximately 10-15μm) by cryostat for digital analysis and immuno-histochemistry.

3.2.8 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 was traced on the carotid sections 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 volume of each section was calculated as the product of: area, number of slices, and the interval between the slices. The total intensity and the volume were used to calculate the intensity per unit volume of each carotid section. Data from n=10 insulin deficient rats (n=20 z-stack files used for 3D analysis from a total of n=10 perfused vessels) and control rats n=7 (n=15 z-stack files used for 3D analysis from a total of n=7 perfused vessels).

3.2.9 Retention of CM-r 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) and total cholesterol (WAKO Chemicals USA). ApoB48 was quantified using an adapted western immune-blot technique previously described.

The fluorescence intensity of the perfusate (Cy5 labeled remnant

lipoproteins) 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 (mg/ml) or cholesterol (mg/ml) in the perfusate / intensity per unit volume of the perfusate * intensity per unit volume of the tissue.

3.2.10 Immuno-histochemistry

Sequential sections of perfused arterial tissue were immuno-stained for the proteoglycans biglycan and decorin (primary antibodies kindly provided by Dr. Larry Fisher). Anti-sera directed against the core proteins of biglycan and decorin aggregates were used on sections pre-treated with Chondroitinase-ABC (Sigma) in order to remove dermatan sulphate chains. Biglycan and decorin anti-serum was incubated at a titre of 1:600 and 1:800 respectively for one hour at room temperature. Following PBS wash, fluorescent detection was with standard pre-conjugated fluorescent secondary antibody (horse anti-rabbit IgG conjugated to Cy3, Chemicon). Immuno-staining with the secondary antibody (1:5000 with 1% horse serum) alone was used as the negative control.

Advanced glycated endproducts (AGE) detection in the vessel wall was assessed by immunoperoxidase technique using the polyclonal anti-AGE antibody which detects carboxymethylysine containing proteins but not pentosidine (Cooper et al. 2000).

3.2.11 Statistical Analysis

Results were considered significant at a p value < 0.05. All analyses were performed with Graph Pad Prizm 4. B_{max} and K_d curves were generated using Graph Pad Prizm 4 for increasing lipoprotein concentrations using non-linear regression curve and one site binding analysis.

3.2.12 Image Analysis

Digital images of fluorescence associated with remnant lipoproteins and proteoglycans in arterial sections were generated utilizing confocal microscopy (Proctor et al. 2003 and 2004). Three dimensional z-series images containing both fluorescent lipoproteins and proteoglycans were subjected to analysis by Laser Pix® software, in order to calculate co-localization co-efficient(s). The calculations are based on Pearson's correlation analysis enabling accurate co-efficient values with vastly different signal intensities.

3.3 RESULTS

3.3.1 Experimental Animal Model of T1D-Physiological Parameters

Insulin deficient (n=10) rats had a reduced body weight, elevated plasma glucose and total plasma cholesterol, as well as an increase in arterial intimal thickness compared to control (n=7) rats (Table 3-1). Furthermore insulin deficient rats had a 1.5 fold increase in circulating serum glycated peptides, compared to control rats indicative of mature diabetes (Table 3-1).

Table 3-1. Biochemical profile of control and insulin deficient animals[1].

Parameter	Control (n=7)	Insulin Deficient (n=10)
Weight (grams)	415.6 ± 16.3	252.6 ± 61.4 *
Triglyceride (mmol/L)	1.23 ± 0.24	1.44 ± 0.17
Cholesterol (mmol/L)	1.74 ± 0.061	2.11 ± 0.14 *
Glucose (mmol/L)	7.36 ± 0.19	34.1 ± 1.54 *
Serum AGE Peptide (units/ml)	4.73 ± 0.77	8.35 ± 1.37*
Arterial Intimal Thickness (um^2)	13,150 ± 3,011	26,730 ± 9,269 *

[1]Wistar rats were made diabetic by STZ injection. Values represent mean ± SD. Significant differences between control and diabetic groups was determined by non-paired students t-test analysis. Significant differences ($p<0.0001$) between control and diabetic animals associated with animal body weight, plasma cholesterol concentration, plasma glucose concentration and arterial intimal thickness (measured as absolute volume μm^3, $p=0.01$).

3.3.2 Arterial Uptake of Remnant Lipoproteins

Insulin deficient rats were observed to have a more extensive and diffuse distribution of fluorescence associated with remnant lipoproteins in the arterial tunica compared to control animals (shown in Figure 3-2A). Whilst both groups displayed focal retention of remnant lipoproteins in the tunica intima, diabetic rats appeared to have increased particle retention in the intima. Consistent with this, the mass of cholesterol derived from remnant lipoproteins was elevated 7-fold higher in the intima of vessels from the diabetic rats compared to controls (Figure 3-2B).

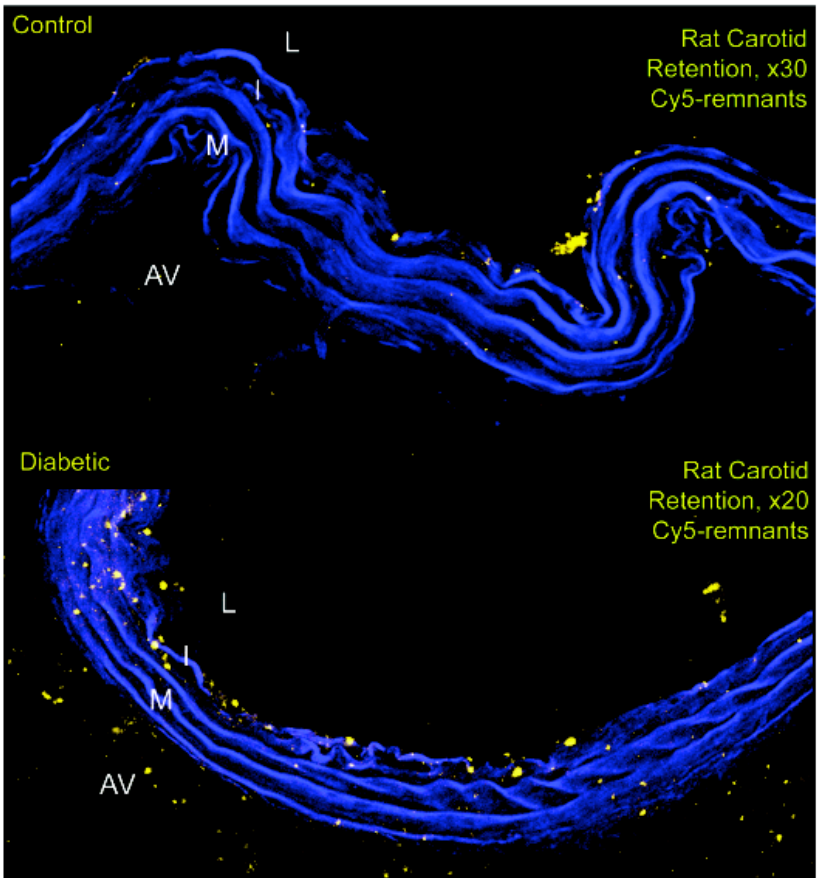


Figure 3-2A Fluorescence associated with the retention of remnant lipoproteins shown for control (upper panel x30) and insulin deficient (lower panel x20) following in-situ arterial perfusion. Representative images from n=10 insulin deficient rats (n=20 z-stack files used for 3D analysis from a total of n=10 perfused vessels) and control rats n=7 (n=15 z-stack files used for 3D analysis from a total of n=7 perfused vessels). Morphology of the vessel wall is shown in blue and fluorescence associated with remnant lipoproteins is shown in yellow. L, lumen; I, intima; M, media; AV, adventitia

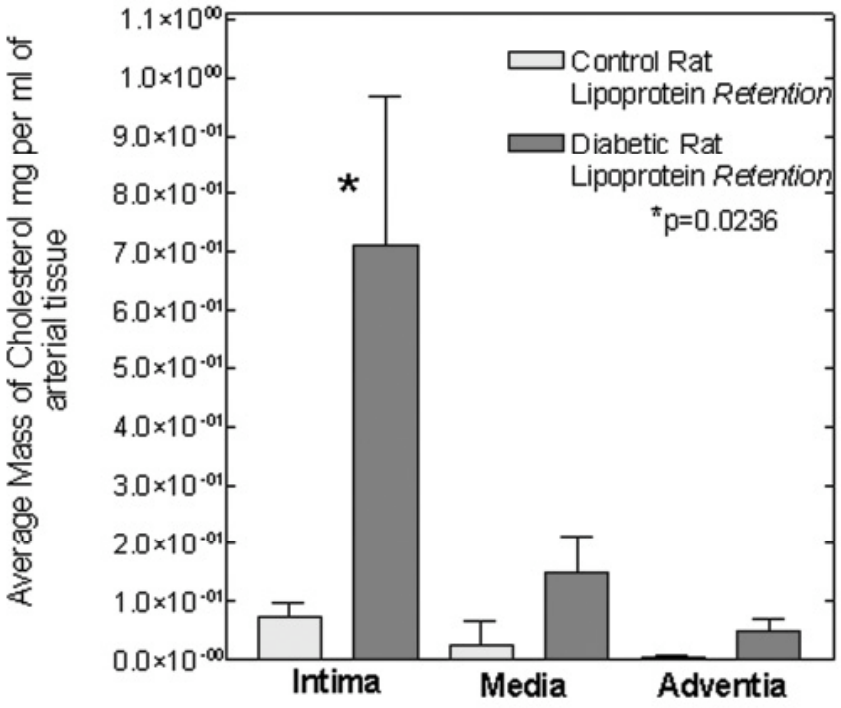


Figure 3-2B Quantification of average mass of cholesterol associated with remnant lipoproteins retained in carotid vessels of both insulin deficient (grey bars, n=20 z-stack files used for 3D analysis from a total of n=10 perfused vessels) and control rats (white bars, n=15 z-stack files used for 3D analysis from a total of n=7 perfused vessels). Values indicate the average of three-dimensional 'stacks' of z-sectioned images assessed for fluorescence with corresponding SEM. Statistical difference was found between the retention of cholesterol in control intima versus retention of cholesterol in diabetic intima (p=0.0236).

3.3.3 In vitro Binding of Remnant Lipoproteins to Human Biglycan

In order to demonstrate direct binding of remnant lipoproteins to biglycan, we employed an *in vitro* solid phase binding assay. Interestingly, despite differences in lipid content and size (Table 3-2), remnants, LDL and lymph chylomicrons, bound recombinant human biglycan with equal *affinity* as assessed by K_D values 0.66, 0.63 and 0.63 respectively (Table 3-3 and Figure 3-3A). In contrast, LDL had a significantly greater B_{max} as compared to either that of lymph chylomicrons ($p<0.05$) or remnants ($p<0.05$) (Figure 3-3A). Conversely, remnant lipoproteins contained 2.5-fold greater cholesterol per particle as compared to LDL (Figure 3-3B). Consequently, the binding of remnant-associated cholesterol at B_{max} was significantly greater (1.9 fold; $p<0.001$) as compared to LDL-associated cholesterol (Figure 3-3C). Modification of biglycan (pre-incubation with glucose) resulted in a 2.3 fold increase in B_{max} ($P<0.0001$) and 1.7 fold increase in K_D ($p<0.01$) for remnant lipoproteins, suggestive of preferential binding to glycated matrix components (Figure 3-3D).

Table 3-2 Particle characteristics of lipoproteins used in binding experiments. (*$p<0.001$) when compared to native lymph and LDL (n=3).

Particle characteristics	Lymph chylomicrons	ApoB48 remnant lipoproteins	Low density lipoprotein
Triglyceride (TG) (mg/ml)	76.08±0.09	*3.10±0.007	1.80±0.004
Cholesterol (Chol) (mg/ml)	3.48±0.01	*1.36±0.01	7.56±0.02
TG/Chol	21.8	2.2	0.23
ApoB (mg/ml)	1.698±0.008	*0.260±0.007	4.931±0.030
TG/ApoB	44.8	11.92	0.365
Particle Size (nm)	165	54	30
Chol/apoB	2.04	5.23	1.53

Table 3-3 Binding constants for the binding assay of lipoprotein fractions to recombinant human biglycan. # $p<0.05$ when compared to B_{max} for lymph chylomicrons (n=3), * $p<0.01$, when compared to B_{max} for apoB48 remnants (n=3).

Binding constants	Lymph	ApoB48 remnants	LDL
B_{max}	0.076±0.001	0.067±0.001	0.119±0.003*#
K_D	0.636±0.061	0.669±0.086	0.638±0.080

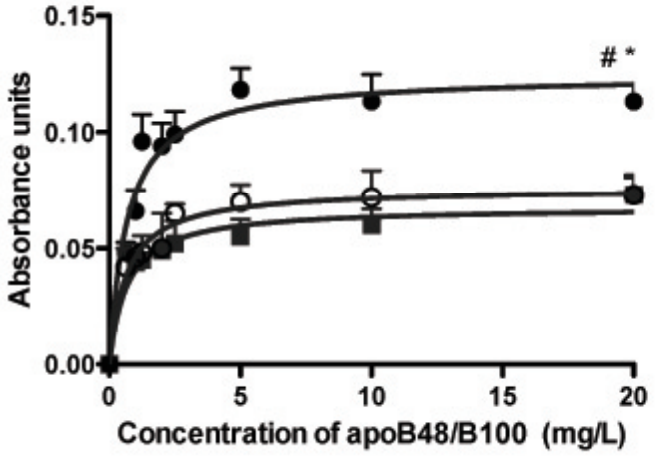


Figure 3-3A Binding of recombinant human biglycan to lipoprotein fractions. # $p<0.05$, where Bmax for LDL (1.02-1.063g/mL) (black circles) was compared to native TRL (white circles) (n=3). * $p<0.05$, where LDL (black circles) was compared to Bmax for remnant lipoproteins (<1.006g/mL) (black squares) (n=3). Nonlinear regression analysis using one site binding (Graphpad Prizm) hyperbola was used.

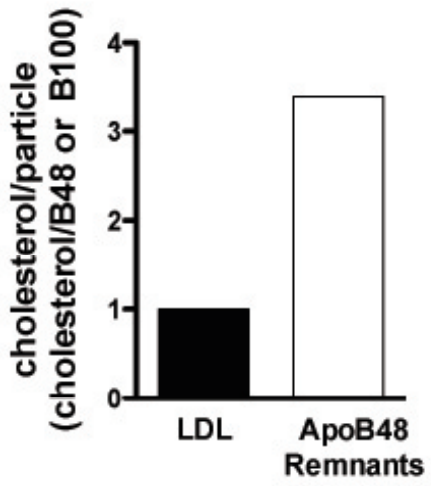


Figure 3-3B Fold difference in cholesterol per particle (cholesterol/ApoB, from particle characteristics).

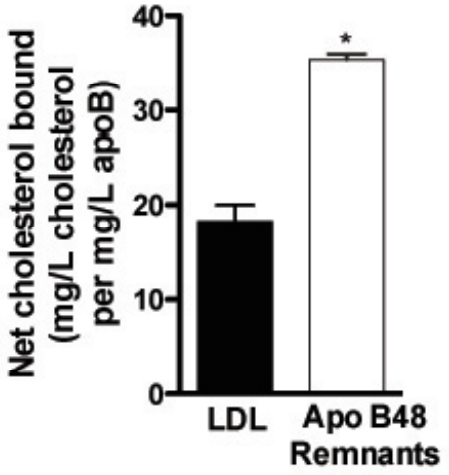


Figure 3-3C Calculated net cholesterol bound (mg/L cholesterol per mg/L apoB) associated with remnant lipoproteins and LDL at Bmax. (* p<0.001, n=3).

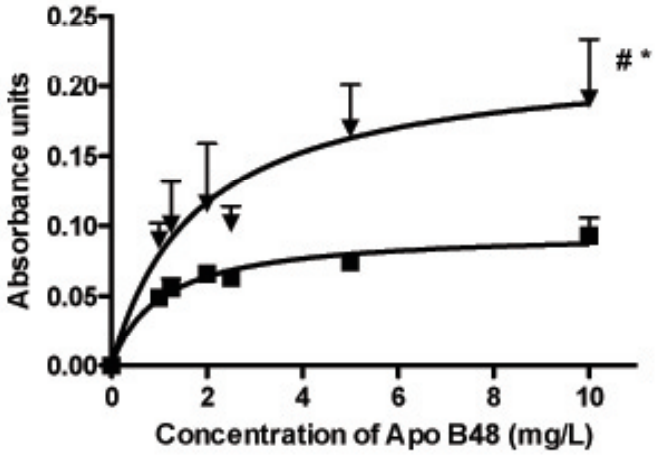


Figure 3-3D Binding of glucose modified biglycan (black triangles) with remnant lipoproteins. * p=0.01, when compared to K_D of binding with normal biglycan (black squares) (n-3). # p<0.0001, when compared to Bmax of binding with biglycan (n=3).

3.3.4 Arterial Expression of Proteoglycans and Arterial Glycated Proteins

Immunohistochemical imaging of vessels from rats with diabetes displayed an increased accumulation of biglycan relative to control rats (Figure 3-4). Our findings are consistent with previous studies (McDonald *et al.* 2007) that suggest T1D causes arterial remodelling and can increase vascular proteoglycan expression. Moreover, we observed focal accumulation of biglycan and a high degree of remnant lipoprotein retention in the tunica intima (Figure 3-5). Image analysis also revealed a significant association between remnant lipoproteins and biglycan (co-localization coefficient =0.71 ± 0.15; $p<0.01$). In addition, there was a high degree of association between remnant lipoproteins and glycated protein in focal clusters (co-localization co-efficient =0.78 ± 0.13; $p<0.01$) (Figure 3-6). These findings are consistent with the concentration of relative serum AGE (advanced glycation end products) in diabetic versus control rats (Table 3-1). The arterial expression of proteoglycan decorin was not different between the control and diabetic rats (Figure 3-7), and minimal co-localization was observed for decorin with remnant lipoproteins (data not shown).

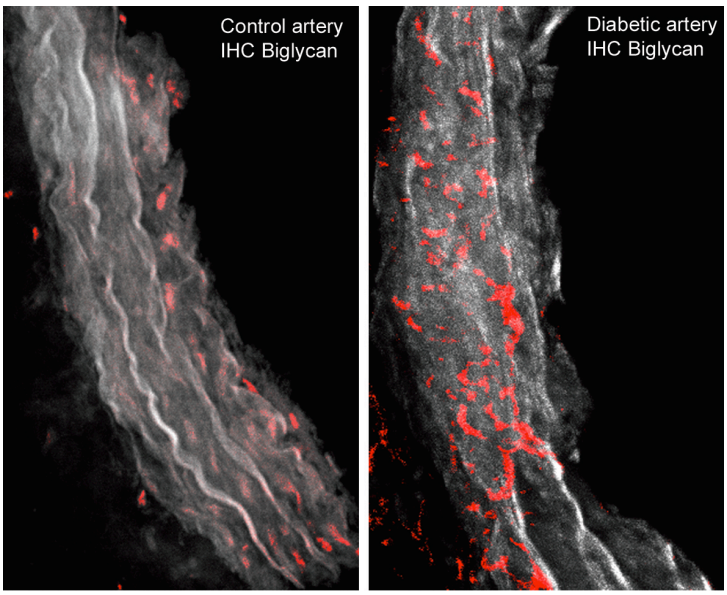


Figure 3-4 Immunohistochemistry for biglycan in control and diabetic arteries. Representative images from n=10 insulin deficient rats (n=20 z-stack files used for 3D analysis from a total of n=10 perfused vessels) and control rats n=7 (n=15 z-stack files used for 3D analysis from a total of n=7 perfused vessels).

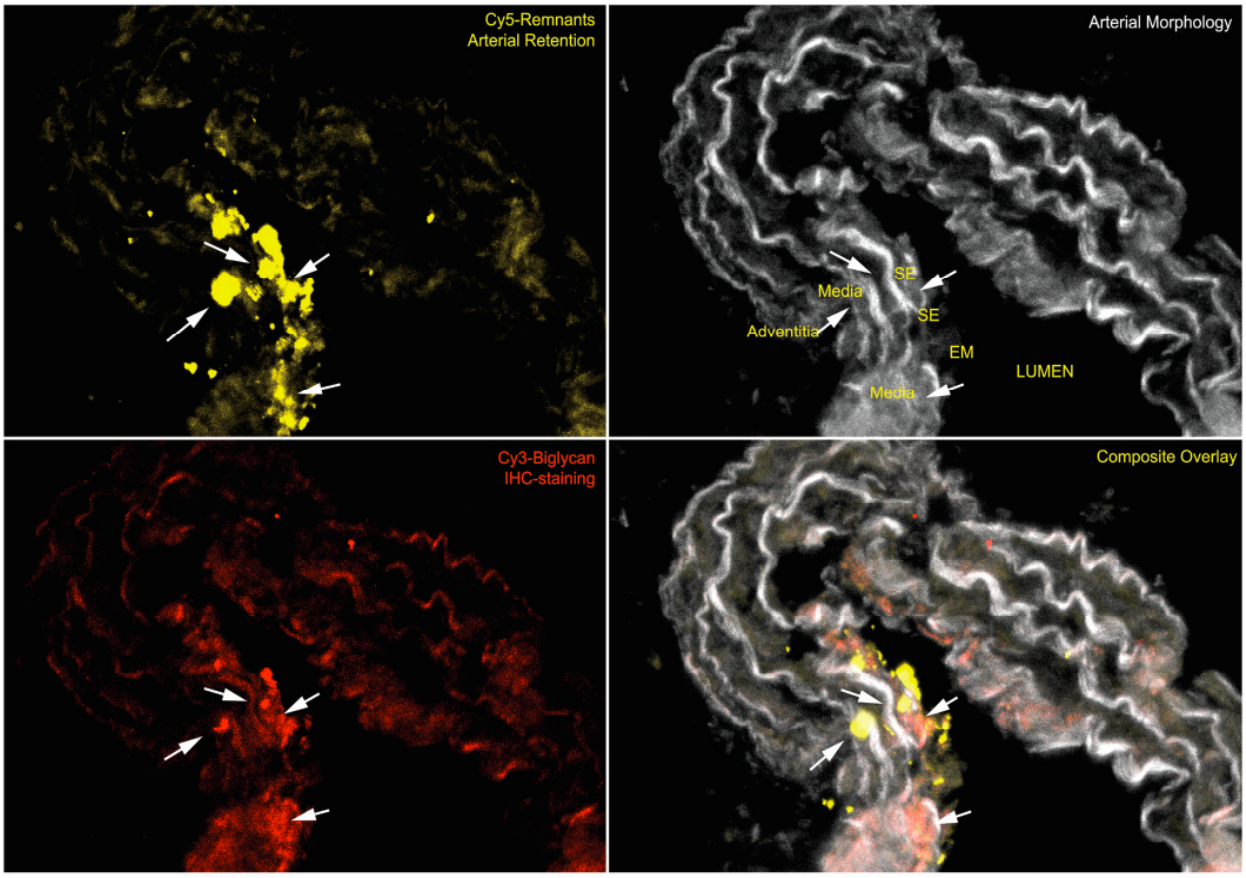


Figure 3-5 Co-localization of proteoglycans and remnant lipoproteins in arterial vessels of insulin deficient rats. Fluorescent-lipoproteins were perfused through carotid arteries under physiological conditions (yellow). Select arterial proteoglycans (Biglycan) were simultaneously stained via IHC (red). Sequential fluorescent emissions, including arterial morphology (greyscale) were collected as a z-series stack via confocal microscopy and digitally overlayed (composite panel). Pearson's co-localization coefficient was 0.71±0.15. Arrows indicate regions of co-localization of both lipoprotein retention and PG IHC. Representative images from n=10 insulin deficient rats (n=20 z-stack files used for 3D analysis from a total of n=10 perfused vessels). SE; Sub-Endothelial layer, EM; Endothelial Monolayer Magnification x40.

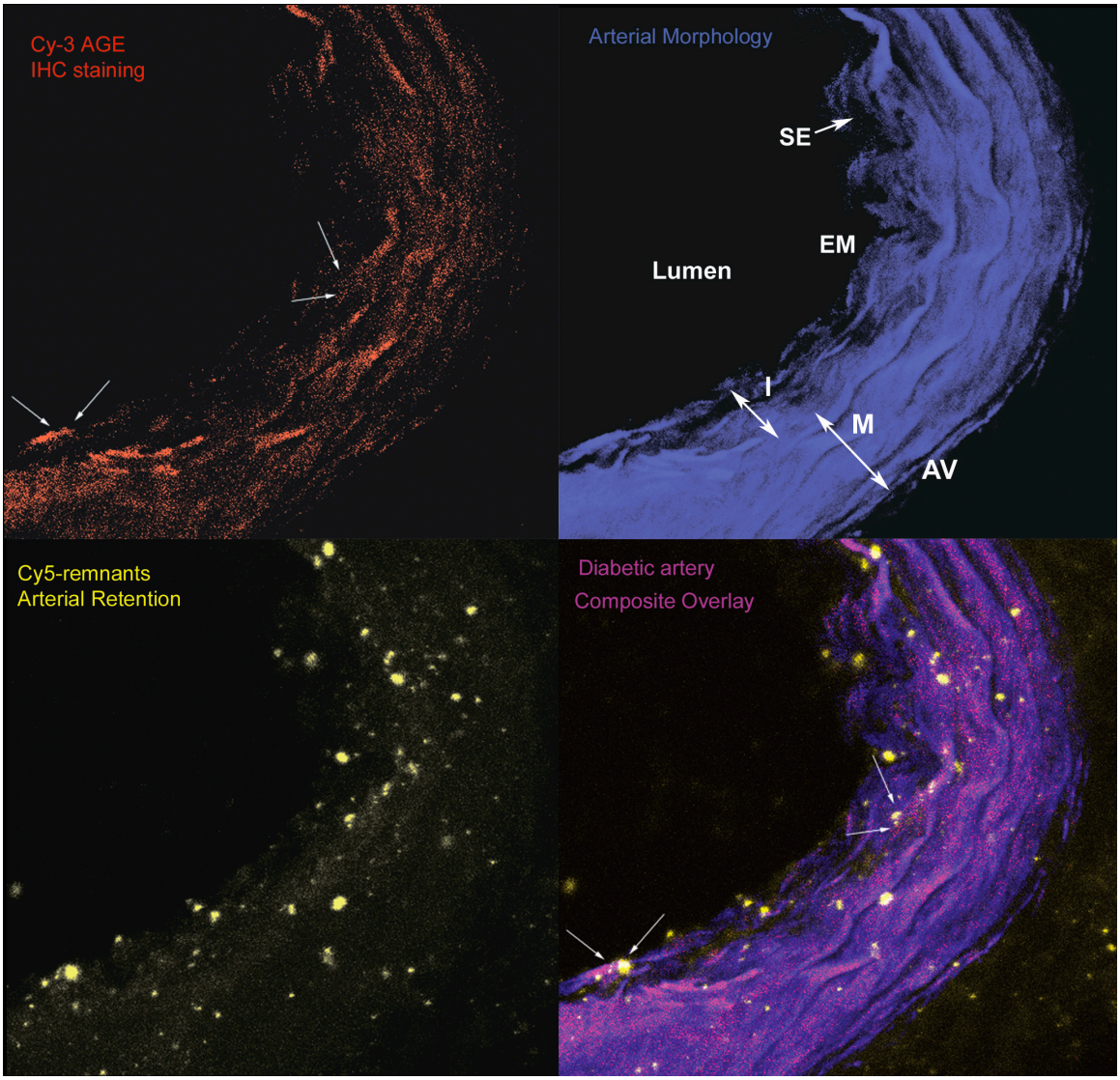


Figure 3-6 Focal points of co-localization of fluorescence associated with remnant lipoproteins and glycated proteins in carotid arterial tissue is shown for insulin deficient animals (x40 magnification) in composite overlay. Morphology of the vessel wall and fluorescence associated with remnant lipoproteins and glycated proteins is shown in blue, yellow and red at x40 magnification respectively. Pearson's colocalizaton coefficient was 0.78 ± 0.13. Representative images from n=10 insulin deficient rats (n=20 z-stack files used for 3D analysis from a total of n=10 perfused vessels). I, intima; M, media; AV, adventitia; SE, Sub-Endothelial layer; EM, Endothelial Monolayer.

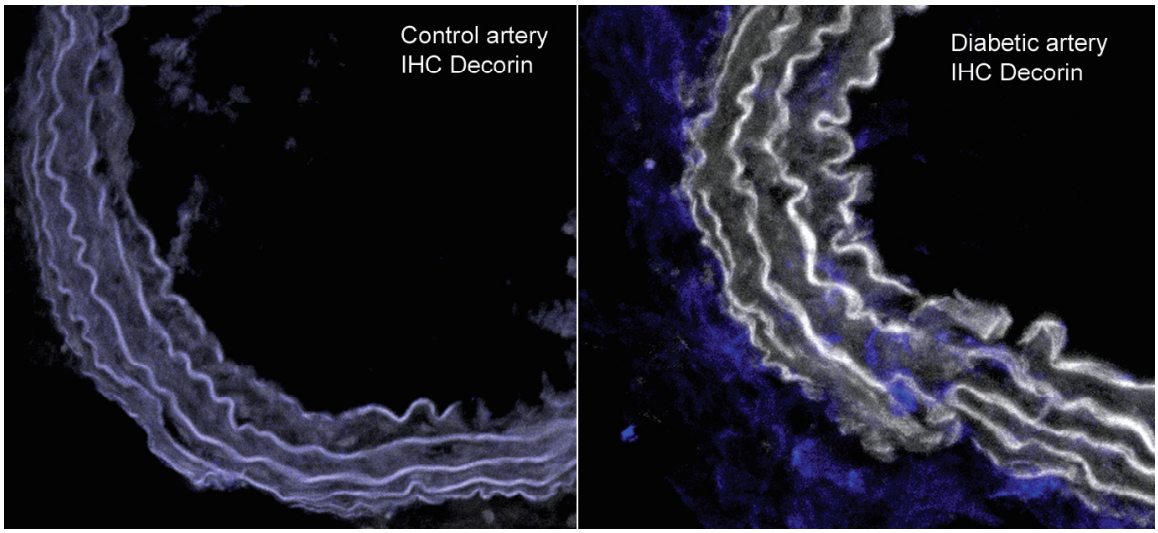


Figure 3-7 Immunohistochemistry for decorin in control and diabetic arteries. Representative images from n=10 insulin deficient rats (n=20 z-stack files used for 3D analysis from a total of n=10 perfused vessels) and control rats n=7 (n=15 z-stack files used for 3D analysis from a total of n=7 perfused vessels).

3.3.5 Relationship of Glycated Proteins and Proteoglycans with Remnant Lipoprotein Derived Arterial Cholesterol Retention

The co-localization of biglycan and remnant lipoproteins was shown to have an inverse relationship with the mass of cholesterol retained in the arterial wall (r^2=-0.6236, n=12) (Figure 3-8). Therefore, increasing retention of remnant cholesterol could not be fully explained by the association with biglycan alone, suggesting that additional binding factors are involved. Interestingly, this relationship was reversed for glycated proteins, in which there was a positive linear association with increasing mass of cholesterol retained in the arterial wall (r^2=+0.77, n=14) (Figure 3-9).

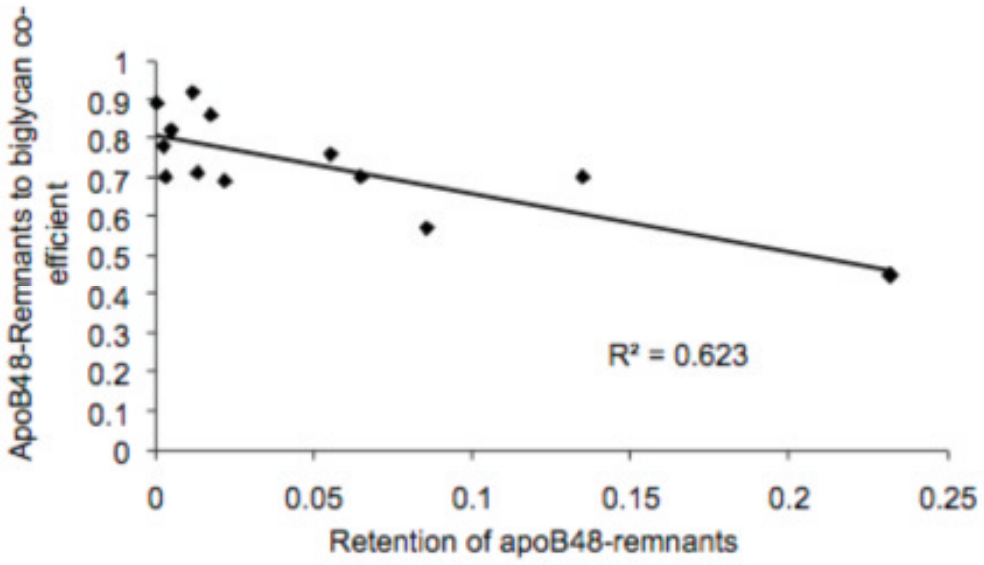


Figure 3-8 Relationship of Pearson's co-localization (remnant lipoproteins to biglycan) to the amount of fluorescence associated with cholesterol in arterial vessels in-situ. Data from n=10 insulin deficient rats (n=20 z-stack files used for 3D analysis from a total of n=10 perfused vessels).

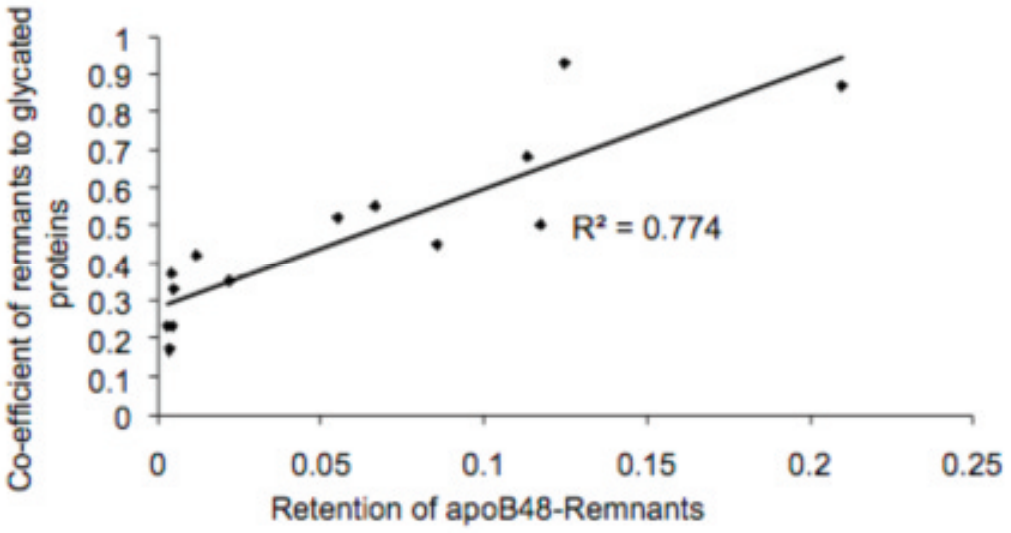


Figure 3-9 Relationship of Pearson's co-localization (remnant lipoproteins to glycated proteins) to the amount of fluorescence associated with cholesterol in arterial vessels in-situ. Data from n=10 insulin deficient rats (n=20 z-stack files used for 3D analysis from a total of n=10 perfused vessels).

3.4 DISCUSSION

3.4.1 Retention of Remnant Lipoproteins with Biglycan in Arterial Vessels.

In the human study done by our research group on T1D subjects (Mangat *et al.* 2010), all T1D subjects had significant clinical vascular disease, however whether elevated plasma remnant concentration and/or the impaired postprandial metabolism of remnant particles is casually associated with the development of atherosclerosis remains unknown. Using radiolabeled techniques, we have previously reported that arterial retention of remnant lipoproteins is greater in arteries of diabetic rodents when compared to controls (Proctor *et al.* 2000). In these current studies the relationship of remnant lipoproteins with arterial proteoglycans was further explored. It was shown that the retention of remnant lipoproteins in arterial tissue was increased and was co-localized with biglycan in focal sites in the tunica intima (Figures 3-2B and Figure 3-5). Indeed, our findings in the insulin deficient rat model are congruent with an increased content of arterial proteoglycan as observed in diabetic subjects (Olin-Lewis *et al.* 2002, Heickendorff *et al.* 1994, and Wasty *et al.* 1993). Our results are also consistent with the co-localization of biglycan with apoB/E and lipid rich areas within human atherosclerotic lesions (Nakashima *et al.* 2007, O'Brien *et al.* 1998). Long standing work by Wight *et al.* has shown that glycosaminoglycan chains of the proteoglycan molecule are negatively charged and bind irreversibly with apoB/apoE-containing lipoproteins, as well as inflammatory proteins (Chait *et al.* 2000, Skalen *et al.* 2002 and Flood *et al.* 2004). Importantly, the co-localization of biglycan with apoE and/or apoB has been shown to be predominant with macrophage infiltrated regions of arterial vessels (Nakashima *et al.* 2007, O'Brien *et al.* 1998). Further, it has been demonstrated that apoB48-remnant lipoproteins *specifically* do not require pre-oxidation (or any chemical modification) in order to induce foam cell formation by human monocyte-derived macrophages (Batt *et al.* 2004[a,b], Botham *et al.* 2007).

Collectively, these results support the hypothesis that accelerated atherogenesis observed in diabetes may involve the direct association of remnant lipoproteins with biglycan in the extracellular matrices of the tunica intima.

3.4.2 Direct Binding of Remnant Lipoproteins to Biglycan

Remnant lipoprotein preparations were shown to have significant binding affinity for recombinant human biglycan *in vitro* (Figure 3-3A). Despite differences in lipid composition and/or size between native CM, remnant and LDL preparations, we did not observe any difference in the binding *affinity* between these three lipoprotein classes. These results suggest that other factors such as the apolipoprotein content (e.g. apoE, apoCIII), charge and/or density of remnant lipoproteins may be more important in determining binding affinity with biglycan (Olin-Lewis *et al.* 2002). We have previously shown that small-dense remnant lipoproteins, rather than larger TRL, readily penetrate the arterial wall (Proctor SD *et al.* 2003). Collectively therefore, it appears that the physical size of a lipoprotein is critical for vascular permeability, but is not a major factor in determining extracellular attachment to proteoglycans, in particular biglycan *per se.*

3.4.3 Accelerated Atherogenesis during T1D due to Glycation

It is well established that one of the primary consequences of prolonged hyperglycemia during T1D is the glycation of proteins and/or formation of advanced glycated end products (AGEs). Glycated proteins are thought to contribute to diabetic complications by interacting with their receptors (RAGE) on the cell surface and by formation of cross-links in the extracellular matrix (Negre-Salvayre *et al.* 2009, Jandeleit-Dahm *et al.* 2008). Formation of glycated proteins in the matrix has been shown to lead to arterial stiffness, altered properties of matrix proteins and increased trapping of LDL (Jandeleit-Dahm *et al.* 2008, Brownlee *et al.* 1985). Indeed, we observed increased binding of remnant lipoproteins with

glycated forms of biglycan (Figure 3-3D). Consistent with this, remnant lipoprotein co-localization with glycated proteins in focal clusters within the arterial wall is suggestive of causality (Figure 3-6). It would now be of interest to determine whether the glycated proteins in the vessel wall form as a result of glycation of collagen, proteoglycans and/or due to accumulation of glycated proteins via endocytosis. Biglycan is known to interact with both glycated and non-glycated collagen (Reigle *et al.* 2008). It is also noteworthy that there was a simultaneous increase in the retention of cholesterol derived from remnant lipoproteins associated with increased glycation, a relationship that was not observed for non-glycated biglycan (Figure 3-9). Therefore we speculate that binding of biglycan to glycated collagen may provide additional cross-links that may facilitate further remnant lipoprotein binding to the matrix in conditions of hyperglycemia.

The complexity of hyperglycemia and it's potential consequence to vascular disease is multi-factorial. Glycated proteins appear to have independent pro-inflammatory effects (Vlassara *et al.* 2002[a,b]). One could speculate that glycation within the arterial wall may not only exacerbate the rate of lipoprotein retention, but extenuate the subsequent inflammatory response including enhancing macrophage recruitment mediating accelerated atherogenesis in diabetes (Bucala *et al.* 1995). We also know that hyperglycemia can lead to glycation or glycoxidation of the lipids within the lipoprotein particle itself, which in turn could further accelerate atherosclerosis (Bucala *et al.* 1995). In the present study, all remnant lipoprotein preparations were obtained from healthy non-diabetic donor rats. Future studies are needed to determine the potential effect of insulin deficiency and/or hyperglycemia on modifications of remnant lipoproteins *per se* and how these factors may relate to diabetic atherosclerosis.

In conclusion, the results from this study suggest that a pro-atherogenic lipoprotein profile with elevated fasting and impaired postprandial metabolism of remnant lipoproteins in T1D may pre-dispose these subjects to the development of CVD. We propose that apoB48 remnant lipoproteins bind directly to known atherogenic arterial proteoglycans involved in atherogenesis and that remnant retention by the arterial wall in diabetes is exacerbated. Evidence from these studies supports the hypothesis that increase CVD risk in T1D may result from increased exposure of remnant lipoproteins to the vasculature and that glycation in the arterial wall may facilitate the retention of remnant lipoprotein-derived cholesterol directly. Collectively, impaired metabolism of remnant lipoproteins in T1D may contribute to the deposition of cholesterol in arterial tissue, and at least in part, the accelerated progression of atherosclerosis in the hyperglycemic and insulin-deficient state.

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Chapter 4. Arterial retention of remnant lipoprotein cholesterol is increased during insulin resistance by biglycan in a model of the metabolic syndrome, the JCR:LA-*cp* rat.

This chapter is presented in the format of manuscript that has been prepared for publication.

4.1 INTRODUCTION

Population-based studies have consistently demonstrated that the metabolic syndrome (MetS), is significantly associated with 1.5 to 3 fold increased risk for atherosclerotic cardiovascular disease (CVD), and a 3 to 5 fold higher risk for type 2 diabetes (Eckel 2007, Hu *et al.* 2005, Eckel 2005). However, the mechanisms that accelerate arterial cholesterol deposition during conditions of insulin resistance and early MetS remain unclear.

The etiology of the 'response-to-retention' hypothesis for atherosclerosis suggests that lipid and cholesterol that accumulate in arterial vessels is sourced primarily from apoB containing lipoproteins (Olofsson and Boren 2005, Williams and Tabas 1998). The literature documents a significant epidemiological association between raised levels of LDL-C and CVD risk (Ferrieres 2009). However, the definition of MetS (as defined by the International Diabetes Federation and a number of other well-recognized societies including the American Heart Association), remain independent of LDL-C, suggesting that other risk factors may be involved (Day 2007). Moreover, there is now accumulating evidence that remnant lipoproteins (including non-fasting post-prandial remnants) are significantly elevated during insulin resistance; either due to overproduction (Duez *et al.* 2008), impaired clearance (Brunzell *et al.* 1973), or both, and may promote CVD risk (Nzekwu *et al.* 2007).

The focal retention of apoB containing lipoproteins bound to arterial proteoglycans within the sub-endothelial matrix of the arterial wall is

considered the key-initiating step in atherogenesis (Gustafsson and Boren 2004). Biglycan and decorin are members of SLRP family of proteoglycans, which are characterized by the presence of leucine rich repeat sequences (Fisher *et al.* 1989). Several studies have found that biglycan and/or decorin co-localize with apoB in human atherosclerotic and restenotic lesions (Nakashima *et al.* 2007). Remnant lipoproteins have been shown to co-localize with arterial biglycan in an insulin deficient rat model of Type-1 diabetes *ex vivo* (Chapter 3). In addition, it has been demonstrated that remnant lipoproteins can bind directly to human biglycan *in vitro* (Chapter 3).

During the pre-diabetic phase, we know that chronic insulin resistance can lead to vascular dysfunction (including vascular endothelial and arterial smooth muscle cells) as well as remodeling of arterial proteoglycans (Rodriguez-Lee *et al.* 2006). Further, it has been documented that proliferation of vascular smooth muscle cells can stimulate the secretion of arterial proteoglycans, increasing the capacity of lipoprotein binding *in-vitro* (Rodriguez-Lee *et al.* 2006). However, whether conditions of insulin resistance can increase the propensity for arterial deposition of remnant lipoproteins *per se* is unknown. Moreover, while it has been shown that as compared to LDL, remnant lipoproteins can contribute physiologically significant quantities of cholesterol to the arterial wall, the relative contributions during insulin resistance remain less clear (Chapter 3, Proctor *et al.* 2003).

In the present study, we employed the JCR:LA-*cp* rat model of MetS to (i) determine the extent of arterial accumulation of remnant lipoproteins and LDL *ex vivo*; (ii) assess the remodeling of arterial proteoglycans during IR and (iii) measure the relative binding capacity of native TRL to biglycan during IR.

4.2 METHODS

4.2.1 Animal model

Male rats of the JCR:LA-*cp* strain that are homozygous for the corpulent trait (*cp/cp*) develop profound IR, which becomes developed by 12 weeks of age and results in a complete absence of insulin-mediated glucose uptake (Russell *et al.* 2006). Heterozygous or wildtype rats (+/?) from the same strain are lean and metabolically normal and were used as the control. For proteoglycan analysis, rats were sacrificed at 6, 12 and 32 weeks of age (n=6 at each time point) to represent early, middle and late IR and thoracic aortae harvested. For perfusion experiments, rats were chosen at 12 weeks of age (to represent mid-stage IR) and were fed 1% cholesterol diet to accelerate CVD pathology. 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.

4.2.2 Generation of lymph chylomicrons and remnant lipoproteins

The superior mesenteric lymphatic duct was cannulated and lymph collected from rats (n=4 control and n=4 IR, 12 weeks of age) following intralipid (20%), and glucose (4%) infusion as described previously (Proctor *et al.* 2004). Cy5-labeled small dense remnant lipoproteins were generated by endogenous hydrolysis via hepatechomized rabbit procedure described elsewhere (Proctor *et al.* 2004). For comparative experiments, human LDL was obtained from Millipore and was labeled with Cy3 as previously described (Proctor *et al.* 2003).

4.2.3 Ex vivo perfusion of labeled remnants

Carotid vessels from rats (n=3, of each phenotype, incorporating both carotids per rat) were exposed to Cy5-labeled remnant lipoproteins. In additional experiments, carotid vessels from IR rats (n=2, incorporating both carotids per rat) were perfused simultaneously with Cy5-labeled remnants and Cy3-labeled human LDL. '*Delivery*' of lipoproteins was

defined and quantified as 20 minutes of lipoprotein perfusion only while '*retention*' of lipoproteins was defined as 20 minutes of lipoprotein perfusion with an additional 60 minutes of washout with non-labeled and liporpotein-free physiological buffer. Carotid vessels were perfused under physiological conditions of temperature and pressure (70mmHg) using a Harvard Apparatus model UNIPER UP-100 as previously described (Proctor et al. 2003) and standardized for apoB (100ug/ml) for all experiments. It has been shown in the past that LDL and remnants labeled with Cy5 and Cy3 dyes are comparable and that 60 minutes of washout time is adequate (Proctor *et al.* 2004). Arterial macrophages and smooth muscle cells can metabolize these particles during the washout period, however the flux through this pathway will be very minor and therefore will not be a potential confounder.

4.2.4 Image quantification

Fluorescent lipoproteins were visualized by the Zeiss 510 confocal laser (Kr/Ar) scanning microscope or Perkin Elmer spinning disk microscope and images were viewed by Zeiss LSM Image Browser Version 4.2.0.121 or Volocity as previously described (Proctor *et al.* 2004). The fluorescent intensity associated with remnants was determined by digital analysis in three dimensions using Image J software (version 1.36b) and the plug-in LSM reader. The biochemical profile for each remnant lipoprotein preparation was determined prior to each experiment. Direct enzymatic colorimetric assays were used to measure TG and total cholesterol (Vine *et al.* 2007). ApoB from remnants was quantified using an adapted western immuno-blot technique *(*Vine *et al.* 2007). The fluorescent intensity of the perfusate per unit volume was also collected for every experiment, under identical conditions in Z-series. These data were subsequently used to calculate the corresponding mass of cholesterol as previously described (Proctor *et al.* 2004).

4.2.5 Extraction of small leucine rich proteoglycans from rat aortae

Proteoglycans were extracted from aortae of rats of differing age (as described above) using the 4M Guanidine hydrochloride containing extraction solution as previously described (Kuwabara *et al.* 2002). The proteoglycan extract was further concentrated by eluting on a DEAE column (Kuwabara *et al.* 2002) and the DMMB positive fractions were pooled. The final pooled fraction was digested using ABCase (Sigma) and separated on a 4-12% Bis-Tris gel. Western blots were probed by LF106 antibody (kind gift from Dr. Larry Fischer, National Institutes of Health, Bethesda) for murine biglycan (1:1000) and 6D6 (1:5000) antibody for decorin (kind gift from Dr. Paul Scott, University of Alberta). For detailed protocol see appendix 'D'.

4.2.6 Recombinant biglycan

For the production of human recombinant biglycan, cDNA from Origene was used to transfect host FS293 cells as described elsewhere (Chapter 3). The culture medium was semi-purified by DEAE Sephacel column. The DMMB positive fractions were pooled and dialyzed. GAG chains were characterized on a DMMB stained gel and the size of protein core was validated after ABCase digestion followed by a western blot.

4.2.7 Solid phase binding assay

Human biglycan was diluted in HBS and 2ug/well was added to a Maxisorp immunoplate (Nunc) and incubated at room temperature overnight as according to *Boren et al* (Chapter 3, Flood *et al.* 2004). The number of lipoproteins in each well was standardized to apoB concentration. To detect lipoproteins bound, HRP-conjugated anti-human/rodent apoB (R&D BioDesign) diluted 1/750 in HBS-Tween with 0.1% BSA was used. Finally 1-Step^{TM} Turbo TMB-ELISA (Pierce) was used to allow for colour development. Absorbance was measured at 450 nm.

4.2.8 Statistical analysis

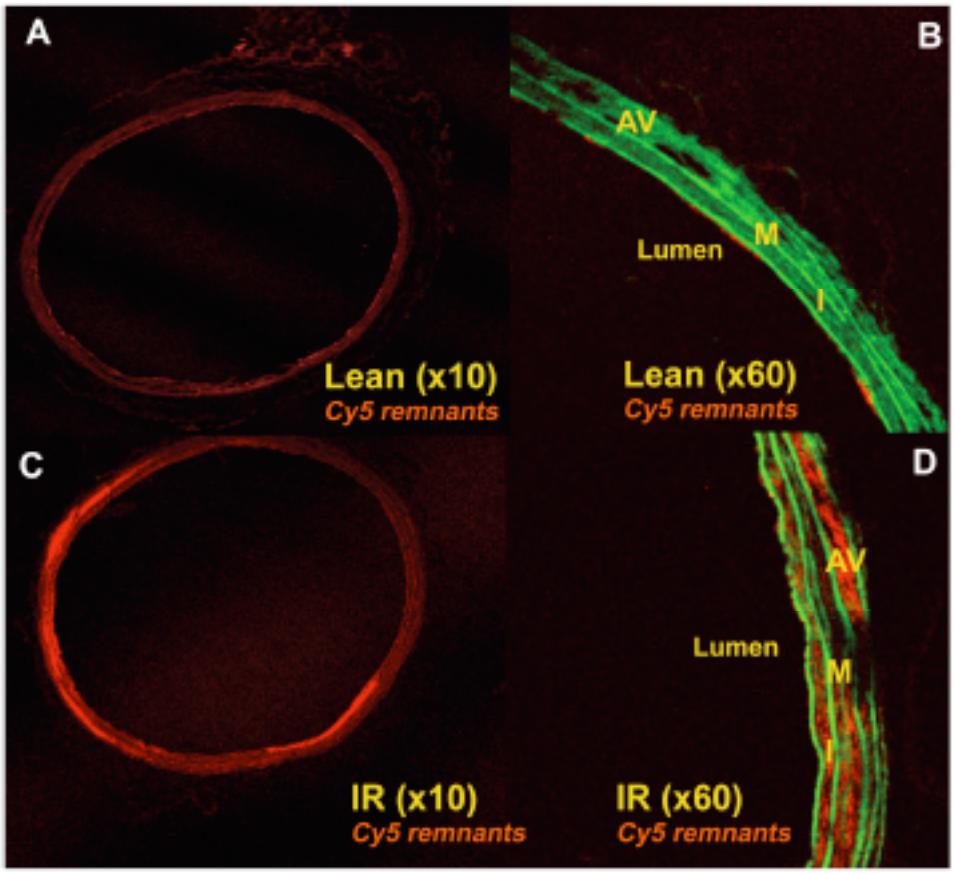
Graphpad prism was used for statistical analysis. Unpaired t test, two-way ANOVA and One-way ANOVA with Bonferroni post hoc analysis was used. Non-linear regression analysis was used to generate binding curves.

4.3 RESULTS

4.3.1 Ex vivo retention of labeled remnants in carotid vessels of JCR:LA-cp rats.

We observed 76% greater retention of remnant lipoproteins (number of particles) in carotid vessels from IR rats (0.51±0.02 x 10^{-12} mg/um^3 tissue) relative to control (0.29 ± 0.01 x 10^{-12} mg/um^3 tissue) (Figure 4-1A to 4-1E). In addition, there was a greater than 2.5-fold increase in the accumulation of remnant-derived cholesterol in IR rats (1.73 ± 0.08 x 10^{-12} mg/um^3) compared to control (0.45 ± 0.02 x 10^{-12} mg/um^3, $p<0.001$) (Figure 4-1F).

Interestingly, for comparative perfusion experiments (Cy5-labeled remnants simultaneously with Cy3-labeled LDL with equivalent B100 and B48), we observed a significantly greater number of LDL particles *delivered* to the vessel (4.5 ± 1 x 10^{-9}ug/um^2 tissue) as compared to remnants (0.48 ± 0.15 x 10^{-9}ug/um^2 tissue) (Figure 4-2 A to 4-2F). However, after extensive washout (a further 60 mins) with lipoprotein free buffer (i.e *retention*) we observed significantly fewer (55% decrease) LDL particles remaining in the tissue (2.9 ± 0.42 x 10^{-9}ug/um^2 'retention' vs. 4.5 ± 1 x 10^{-9}ug/um^2 'delivery', $p<0.05$). In contrast, we did not observe any difference in the retention of remnants (following 60 minutes of washout) relative to delivery experiments (no washout). Furthermore, despite the *greater number of LDL particles* retained compared to remnant particles, we observed a greater than 1.5-fold increase in total *mass of cholesterol* associated with remnant particles (1.606 ± 0.09 x 10^{-9}ug/um^2 tissue) as compared to cholesterol associated with LDL (0.610 ± 0.05 x 10^{-9}ug/um^2 tissue) (Figure 4-2H).


A
B
AV
Lumen
M
I
Lean (x10)
Cy5 remnants
Lean (x60)
Cy5 remnants
C
D
AV
Lumen
M
I
IR (x10)
Cy5 remnants
IR (x60)
Cy5 remnants

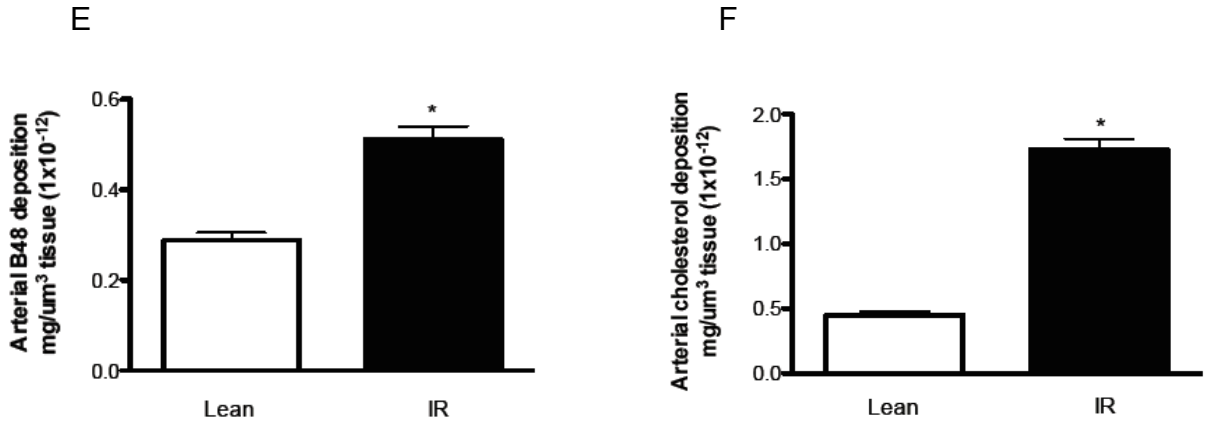


Figure 4-1. *Ex vivo* retention of Cy5 labeled remnant lipoproteins in carotid arteries of the lean and insulin resistant JCR:LA-*cp* rats.

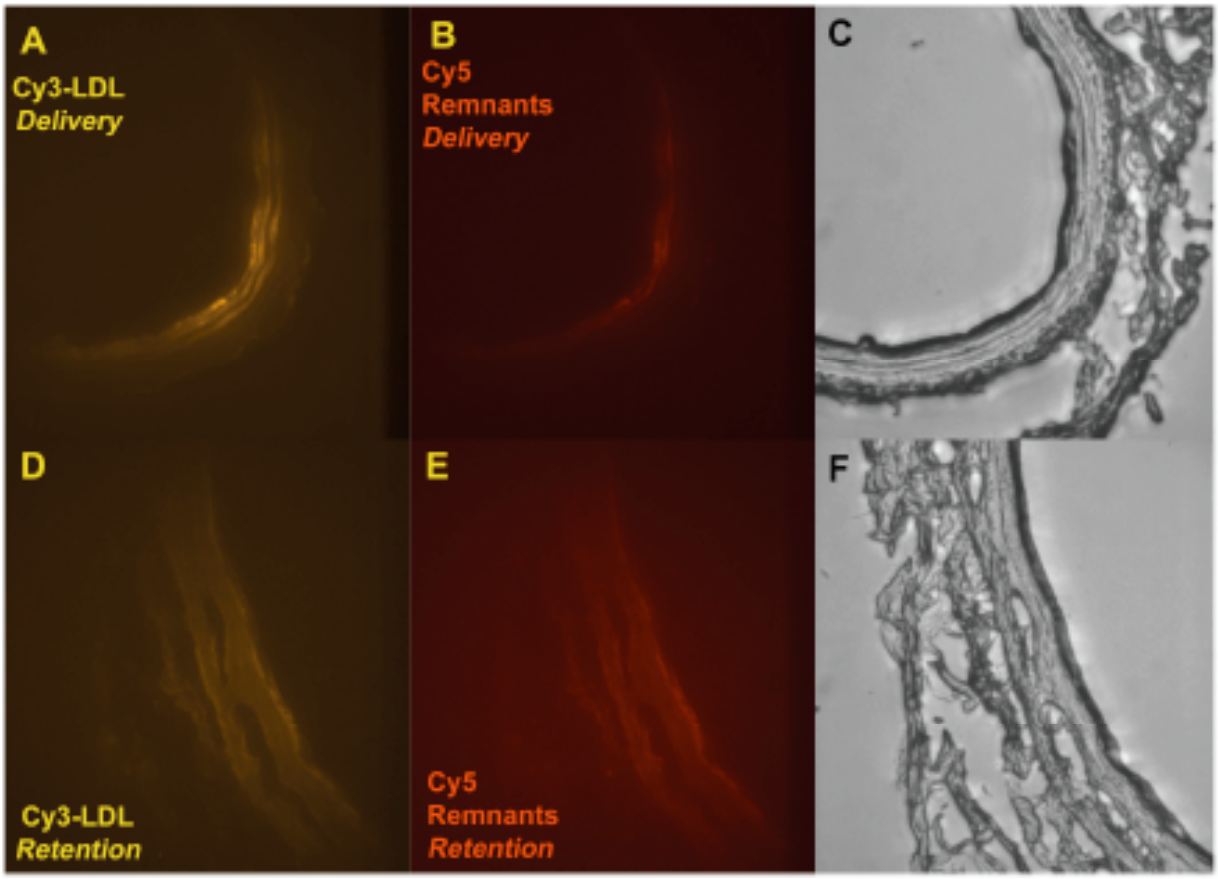
Representative images of the retention of Cy5 labeled remnants in carotid vessels of lean and IR rats Panels (A) and (C) respectively (x10 magnification) and Panels (B) and (D) respectively (x60 magnification).

Morphology of the vessel is shown in green and labeled remnants in red. I=Intima, M=Media, A= Adventitia.

An autologous study design was used in these experiments. Cy5 labeled remnants were obtained from lean and obese rats (after lymph cannulation and rabbit hepatectomy) that were then perfused into lean and obese carotid vessels respectively.

Ex vivo retention of remnants in carotid vessels of lean and IR rats was further quantified by measuring the fluorescent intensity in the linear range and calculating the corresponding number of apoB particles (using known concentration of apoB in the perfusate) Panel (E) as well as the corresponding concentration of cholesterol (Panel F), for each remnant preparation * $p<0.0001$ (n=3, 2-3 sections from each vessel, complete cross-sections at x10 magnification from each rat).

Images were collected in three dimensions for each arterial section. Fluorescent lipoproteins were visualized by the Zeiss 510 confocal laser (Kr/Ar) scanning microscope and the images were viewed by Zeiss LSM Image Browser Version 4.2.0.121.


A
Cy3-LDL
Delivery
B
Cy5
Remnants
Delivery
C
D
Cy3-LDL
Retention
E
Cy5
Remnants
Retention
F

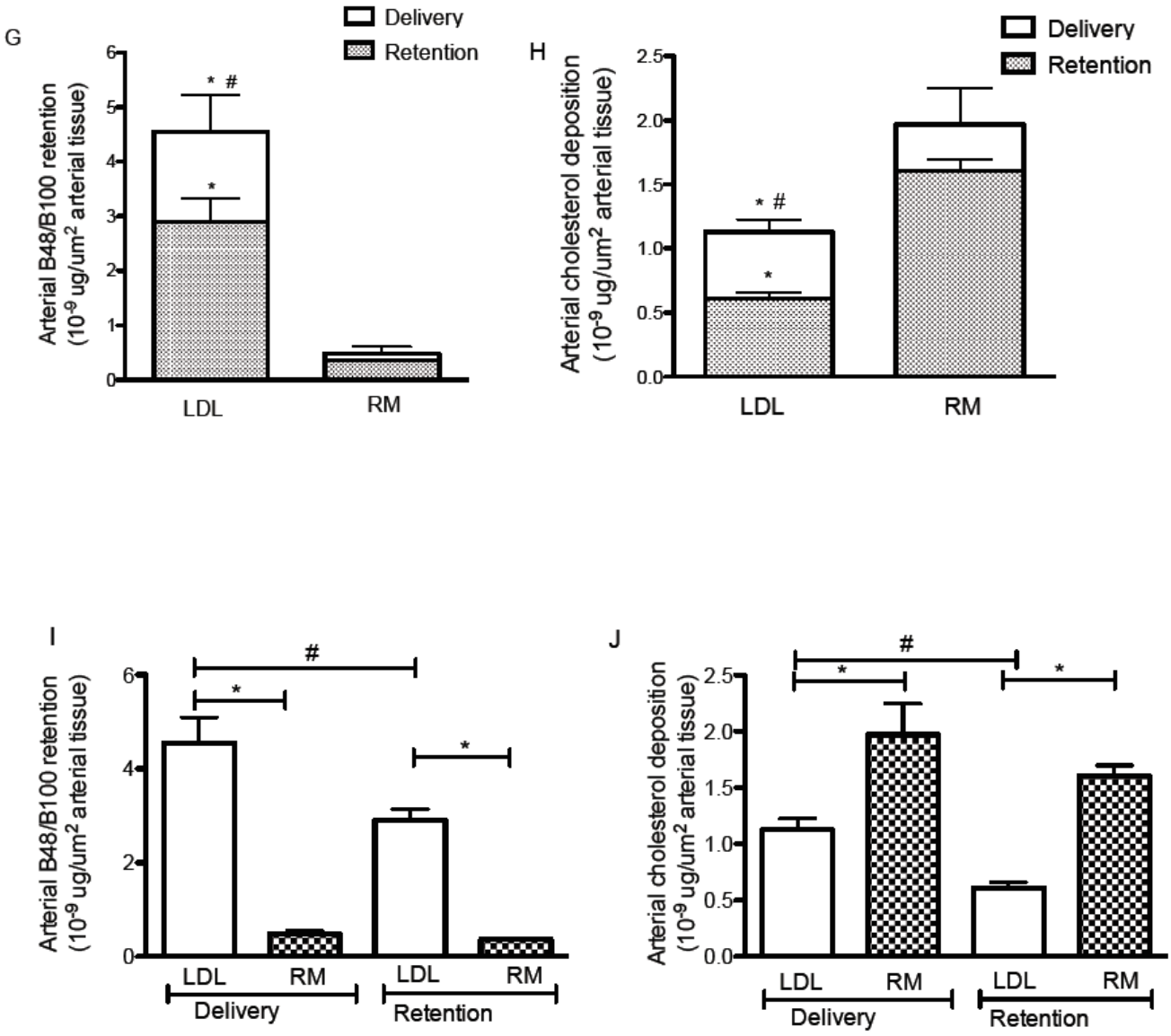

G
Delivery
Retention
Arterial B48/B100 retention
(10^{-9} ug/um^2 arterial tissue)
* #
*
LDL
RM
H
Delivery
Retention
Arterial cholesterol deposition
(10^{-9} ug/um^2 arterial tissue)
* #
*
LDL
RM
I
Arterial B48/B100 retention
(10^{-9} ug/um^2 arterial tissue)
#
*
*
LDL
RM
LDL
RM
Delivery
Retention
J
Arterial cholesterol deposition
(10^{-9} ug/um^2 arterial tissue)
#
*
*
LDL
RM
LDL
RM
Delivery
Retention

Figure 4-2. *Ex vivo* retention of Cy5 labeled apoB48 remnants and Cy3 labeled LDL in the carotid arteries of the obese insulin resistant JCR:LA-*cp* rats.

Representative images of '*delivery*' (20 minutes perfusion only) panels (A) and (B) and '*retention*' (20 minutes of perfusion with an additional 60 minutes washout) panels (D) and (E) of LDL (yellow) and remnants (red) respectively (x20 magnification).

Morphology of the vessel is shown by the respective bright field images panels (C) and (F).

Ex vivo '*delivery*' and '*retention*' of remnants and LDL in carotid vessels IR rats was further quantified by measuring the fluorescent intensity in the linear range and calculating the corresponding number of apoB48/100 particles (using known concentration of apoB48/100 in the respective perfusate) as shown in Panel (G) as well as the corresponding concentration of cholesterol (Panel H), for each LDL and remnant preparation. Statistical significance (*) is ($p<0.01$) (n=2, 2-3 sections from each vessel) for '*delivery*' between LDL and remnants and '*retention*' between LDL and remnants using one-way ANOVA. Statistical significance (#) is ($p<0.01$) between '*delivery*' and '*retention*' of LDL using one-way ANOVA.

Data presented in panels G and H are further illustrated in a different graphical form in panels I and J. This further demonstrates the difference between '*delivery*' and '*retention*' of LDL and remnants.

Images were collected in two dimensions for each arterial section. Fluorescent lipoproteins were visualized by the Perkin Elmer spinning disk microscope and the images were viewed by Volocity software.

4.3.2 Effect of insulin resistance on the remodeling of aortic biglycan, decorin protein core and sulphated glycosaminoglycans.

In order to better understand the expression pattern of arterial proteoglycans in JCR:LA-*cp* rats *in vivo* we assessed the abundance of biglycan and decorin in aorta. The fold difference of biglycan protein mass extracted from aorta of IR rats significantly increased with age (1.53 fold ± 0.12, 2.19 fold ± 0.17 and 2.83 fold ± 0.26 at 6, 12 and 32 weeks respectively, $p<0.05$, (Figure 4-3). In contrast, the mass of decorin protein extracted from the aorta did not differ between the phenotypes at any time point (Figure 4-4). Decorin protein core isolated from rats at 12 and 32 weeks of age was significantly greater ($p<0.05$) than at 6 weeks of age (Figure 4-4). Interestingly, aortic biglycan protein core isolated from IR rats of increasing age had a significant correlation ($r^2=0.60$, $p=0.003$) with fasting plasma insulin concentration (Figure 4-5). We also note that there was no change in total aortic sulphated glycosaminoglycans from rats of any age or between phenotype (Figure 4-6).

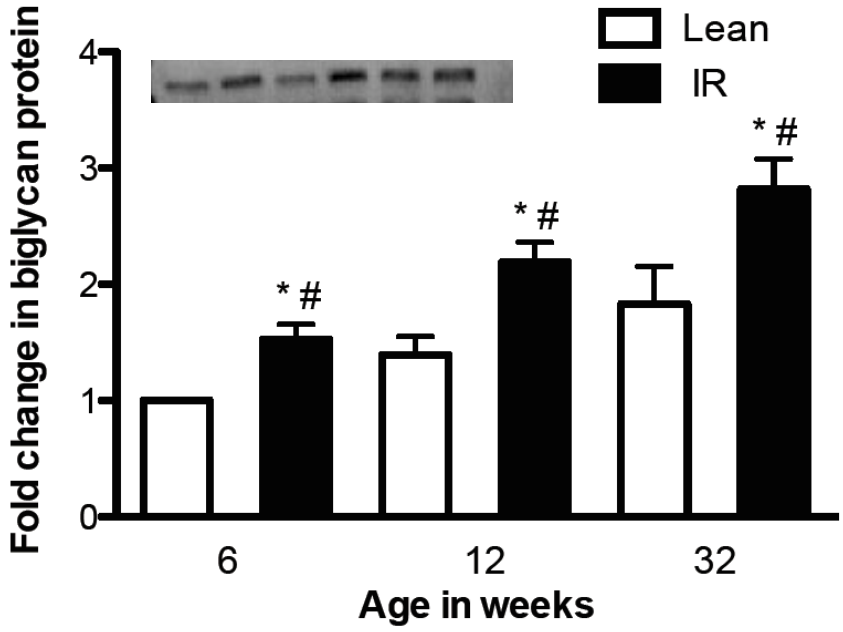


Figure 4-3. Fold change in biglycan protein content in aorta from both lean and insulin resistant JCR:LA-*cp* rats.

Pooled rat samples of JCR:LA-*cp* rats of increasing age (n=3, 2-3 rats each) and measured by densitometry (average ± SD). Fold differences compared to lean phenotype at 6 weeks of age. Significance (*) is for statistical difference ($p<0.01$) between phenotype at each age point using 2-way ANOVA and significance (#) is ($p<0.01$) for increasing content of biglycan with increasing age in IR animals using one-way ANOVA. Blots show mass of biglycan extracted from equivalent weights of aorta.

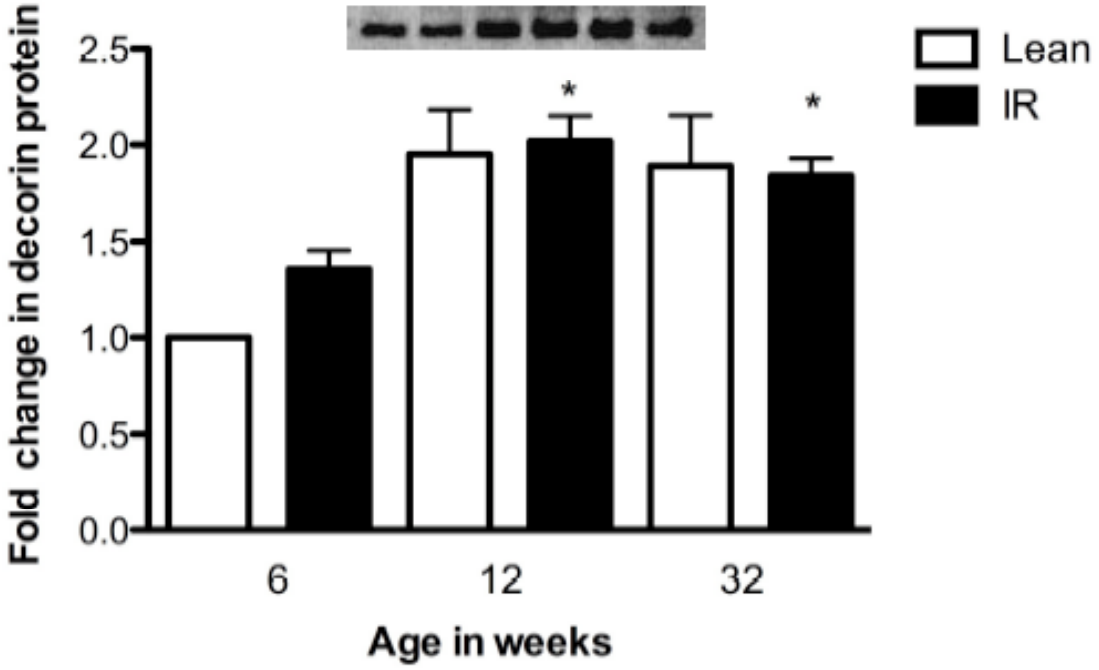


Figure 4-4. Fold change in decorin protein content in aorta from both lean and insulin resistant JCR:LA-*cp* rats.

Pooled rat aorta samples (n=3, 2 rats each) and measured by densitometry (average ± SD). Fold differences compared to lean phenotype at 6 weeks of age. There was no difference in fold change decorin protein between the phenotypes at any age point as assessed by two-way ANOVA. Significance (*) is for statistical difference ($p<0.05$) when IR phenotype at 12 and 32 weeks is compared to the IR phenotype at 6 weeks time point using one-way ANOVA. Blots show mass of decorin extracted from equivalent weights of aorta.

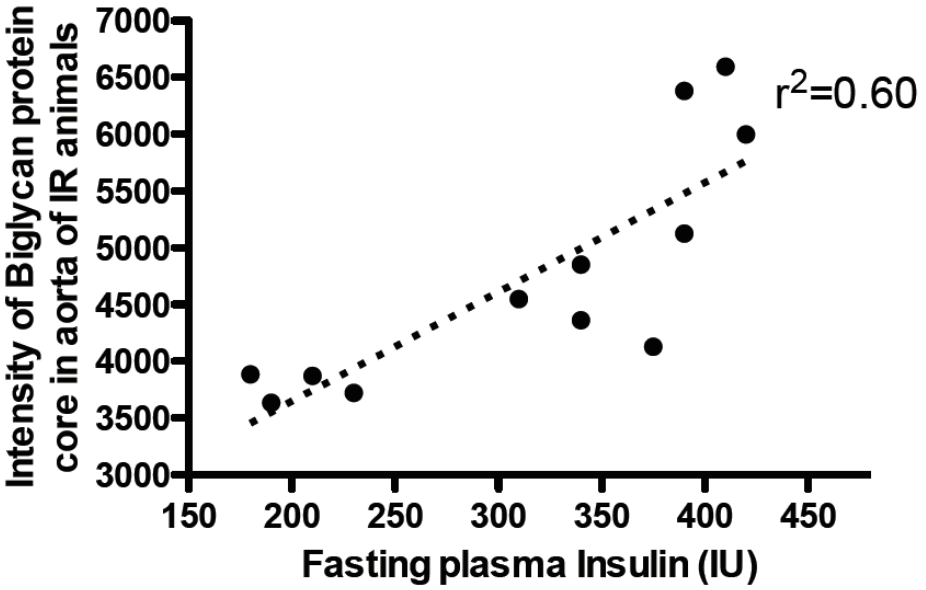


Figure 4-5. Relationship of aortic content of biglycan from JCR:LA-*cp* insulin resistant rats with fasting concentrations of insulin.

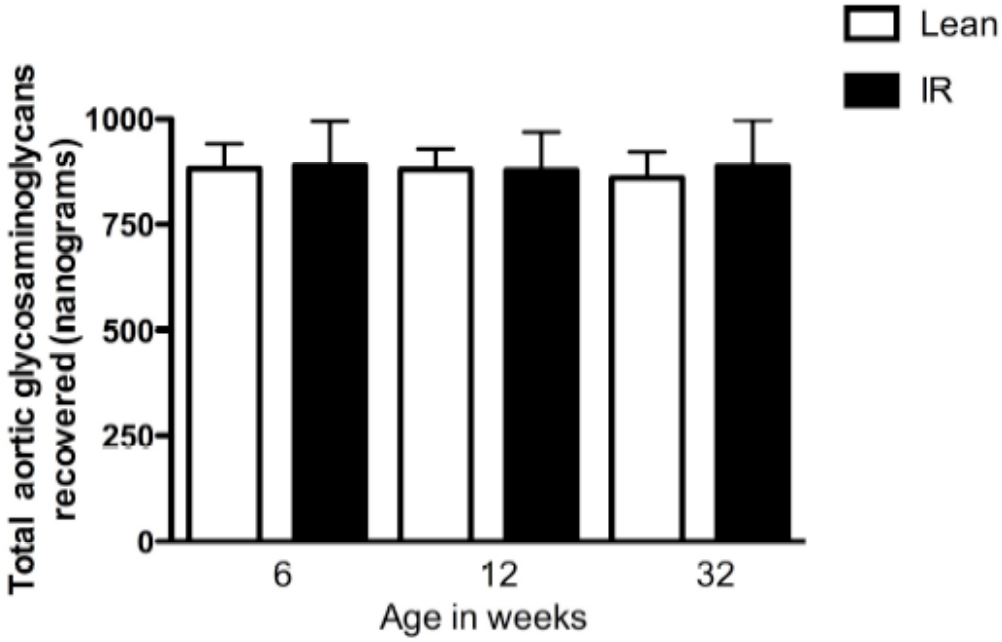


Figure 4-6A. Total sulphated glycosaminoglycans recovered from lean and insulin resistant JCR:LA-*cp* rat aorta. Please see more detailed description in methods section.

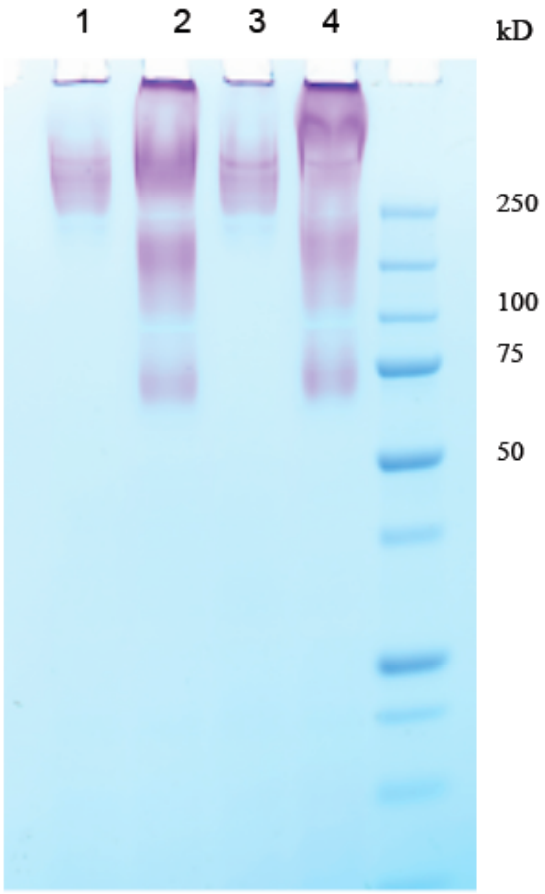


Figure 4-6B. Total sulphated glycosaminoglycans (GAGs) isolated from lean and insulin resistant JCR:LA-*cp* rat aorta at 12 weeks of age.

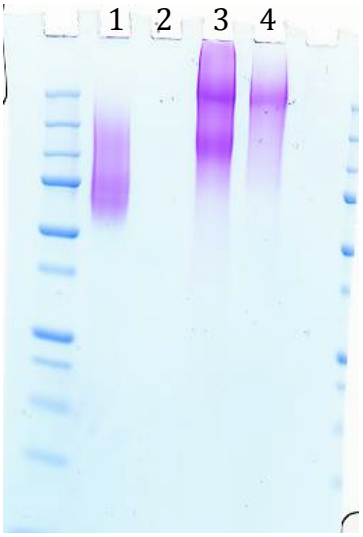
DMMB stained gel shows GAGs isolated from lean (lane 2) and insulin resistant (lane 4) JCR:LA-*cp* rat aorta.

Lanes (1) and (3) show chondroitin ABCase digested samples of lean and insulin resistant total sulphated GAGs repectively.

Please see more detailed description in methods section.

4.3.3 Lipoprotein binding to bovine biglycan vs. recombinant human biglycan

We used recombinant human biglycan (short glycosaminoglycans and more core protein than bovine biglycan) and bovine biglycan (longer glycosaminoglycan chains and less core protein than recombinant human biglycan.) for a solid phase binding assay. Figures 4-7A and 4-7B show the glycosaminoglycans and the protein cores respectively of bovine and recombinant human biglycan.



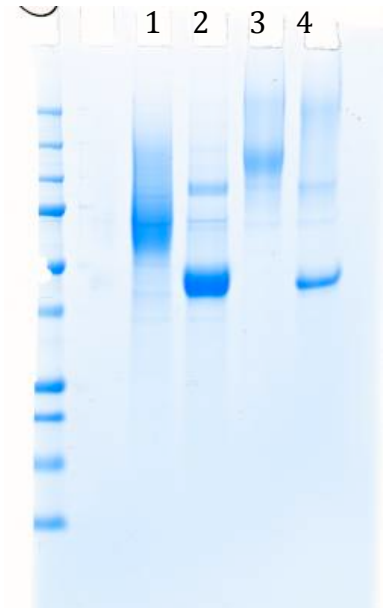
Lane 1: Intact Recombinant human biglycan

Lane 2: Digested recombinant human biglycan

Lane 3: Intact Bovine biglycan

Lane 4:Digested bovine biglycan

Figure 4-7A DMMB stained gel showing glycosaminoglycans of the recombinant human biglycan and bovine biglycan.



Lane 1: Intact Recombinant human biglycan

Lane 2: Digested recombinant human biglycan

Lane 3: Intact Bovine biglycan

Lane 4:Digested bovine biglycan

Figure 4-7B Coomassie stained gel showing the protein cores of recombinant human biglycan and bovine biglycan.

For the binding assay, *equal weighed amounts of bovine and recombinant human biglycan* was used to coat the wells (2ug/well) and binding was determined with increasing amounts of LDL. There was no difference in the binding affinity or the binding capacity between these two types of biglycan with LDL. The recombinant human biglycan used has a lot more protein core (0.43ug/ul) as compared to the bovine biglycan (0.075ug/ul).

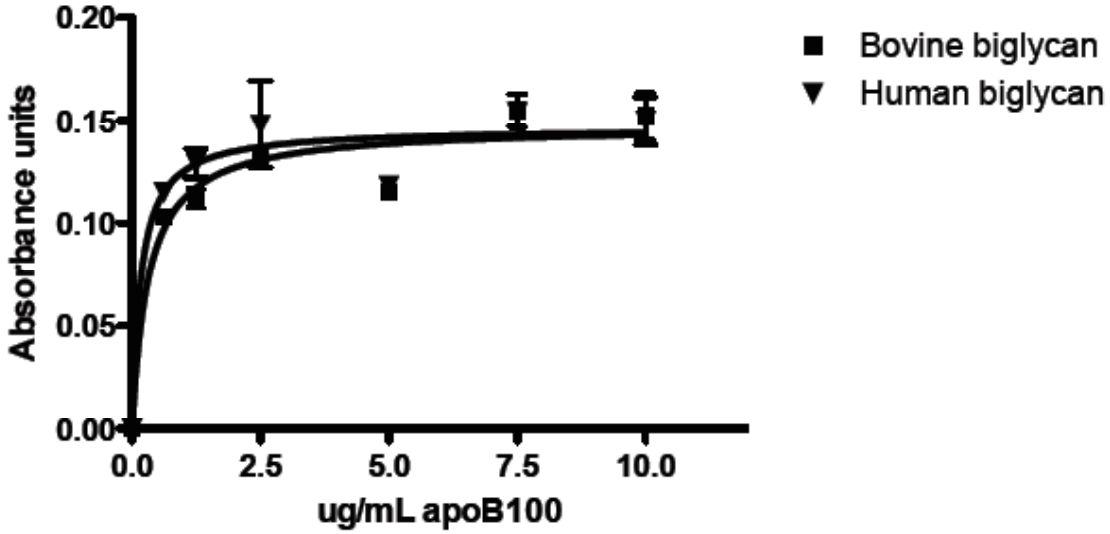


Figure 4-7C. Solid phase binding assay showing binding of bovine biglycan and human biglycan with human LDL.

4.3.4 Binding of native TRL isolated from insulin resistant rats to human biglycan during the fasted and fed state.

Binding of native TRL isolated from lean control and IR rats during the *fasted* (intra-gastric saline infusion) and *fed conditions* (intra-gastric intra-lipid infusion) to recombinant human biglycan was evaluated by a solid phase binding assay. We did not observe any difference in the binding affinity (K_D) to biglycan for lipoproteins collected from control or fasted IR

rats (Figure 4-8A). In contrast, native TRL from IR rats collected during the *fed state* was shown to have 1.26 fold higher binding capacity (B_{max}) to biglycan (0.215 ± 0.008 absorbance units) (Figure 4-8B) when compared to the binding capacity of TRL from the lean rats (0.160±0.009 absorbance units, $p<0.01$).

In order to appreciate the contribution of cholesterol associated with lipoproteins bound to biglycan *in vitro*, we calculated the mass of cholesterol bound at the corresponding B_{max} for each of the solid phase binding assay experiments. Notably, native TRL derived cholesterol bound was significantly higher (55%) for particles isolated during the *fed state* regardless of phenotype (43.23 ± 10 vs. 67.34 ± 8 ug/ml per ug/ml apoB for lean fasted and fed respectively $p<0.001$ and 27.52 ±7 vs. 148 ± 10 ug/ml per ug/ml apoB for IR rats in the fasted and fed state respectively, $p<0.001$) (Figure 4-9). However, it was also interesting that native TRL derived cholesterol bound to biglycan from particles collected from IR rats under fed conditions was 2-fold that of control ($p=0.01$) (Figure 4-9).

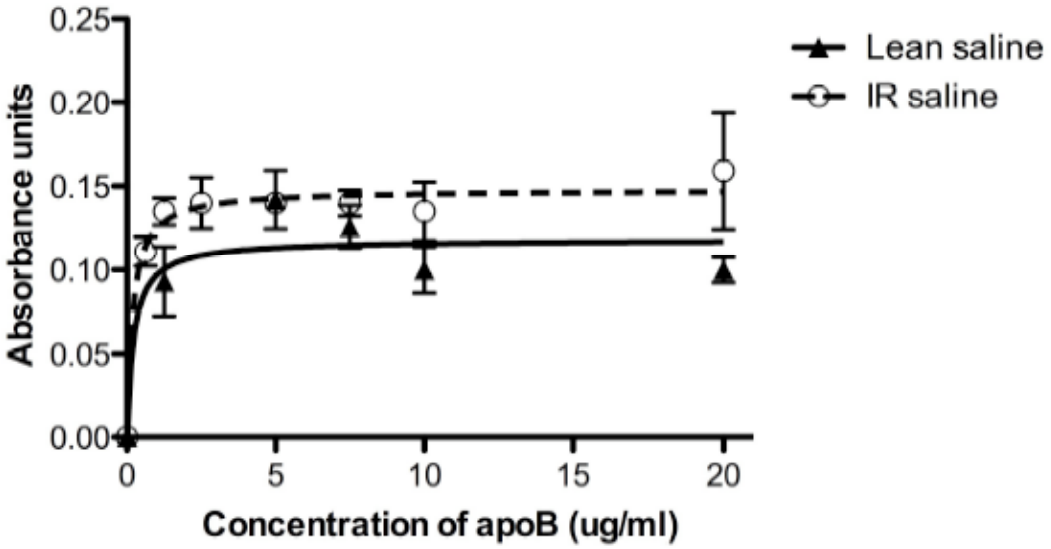


Figure 4-8A. Binding of native TRL, collected from lean and insulin resistant JCR:LA-*cp* rats under fasted condition, to human biglycan.

The superior mesenteric lymphatic duct was cannulated and native TRL was collected from lean and insulin resistant rats (12 weeks of age) following saline and glucose (4%) infusion as described previously.

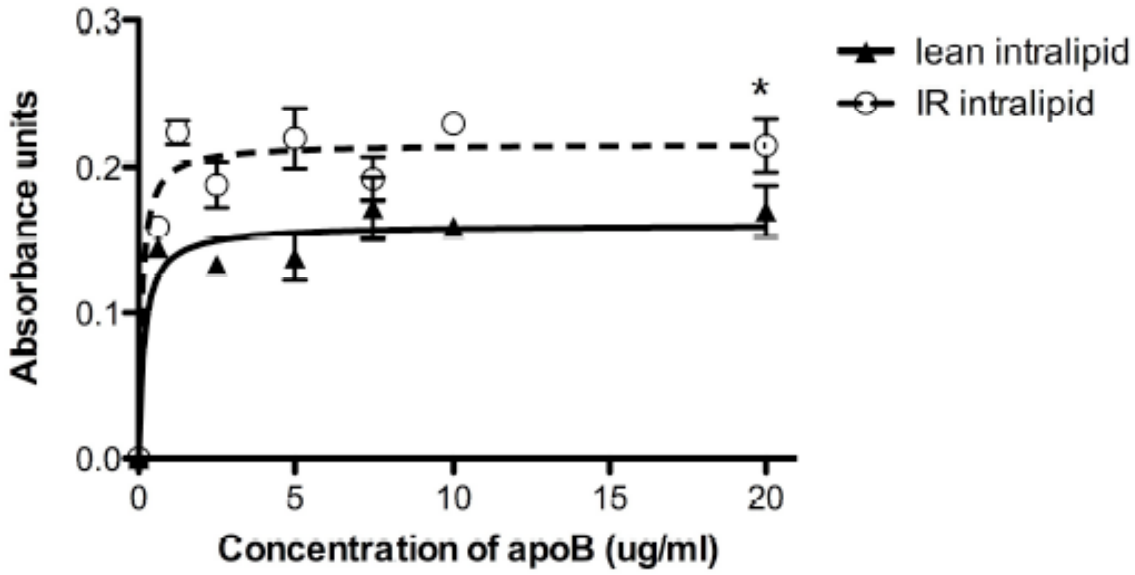


Figure 4-8B. Binding of native TRL, collected from lean and insulin resistant JCR:LA-*cp* rats under fed condition, to human biglycan.

The superior mesenteric lymphatic duct was cannulated and native TRL was collected from lean and insulin resistant rats (12 weeks of age) following intralipid and glucose (4%) infusion. Significance (*) is equal to statistical significance p=0.01 (n=3), when Bmax is compared using unpaired t test.

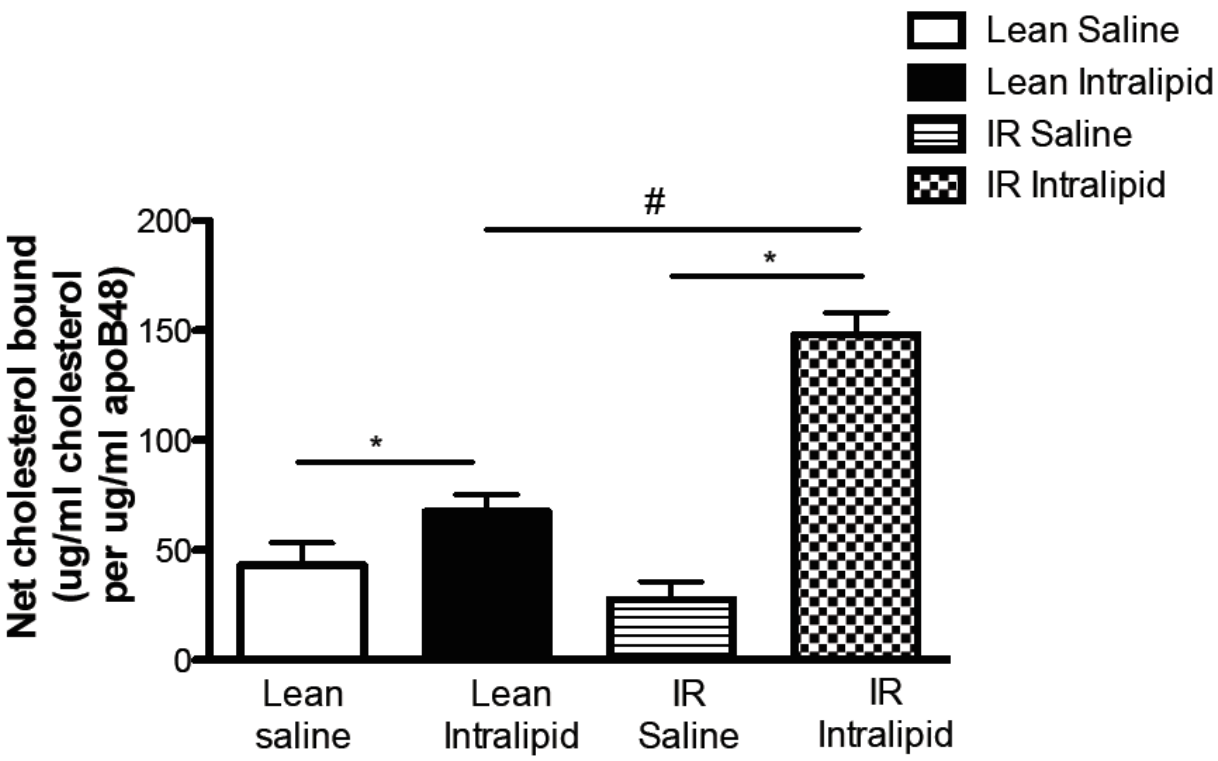


Figure 4-9. Net cholesterol bound (associated with native TRL) at B_{max} as assessed from the solid phase binding assay.

Native TRL were isolated following saline or intralipid infusion in the lean and insulin resistant JCR:LA-*cp* rats. B_{max} values (maximal binding capacity) were calculated from a solid phase binding assay, which was standardized to the apoB48 (particle number) concentration.

Net cholesterol bound was calculated as the product of the B_{max} value for the respective TRL fraction and its cholesterol concentration.

Significance (#) is for statistical difference ($p<0.001$) when saline and intralipid treatments were compared between lean and IR phenotypes using 2-way ANOVA. While significance (*) is for statistical difference ($p<0.05$) when lean and IR phenotypes are compared within their respective groups for saline or intralipid treatment using an unpaired t-test.

4.4 DISCUSSION

4.4.1 Increased arterial retention of remnant lipoproteins during insulin resistance

Increased deposition of remnant lipoproteins and associated cholesterol in IR rats (Figure 4-1 and Figure 4-2) may be the result of a number of potential differences that can occur during insulin resistance and/or MetS including; particle composition, permeability and/or vessel wall remodeling. There is evidence of vascular endothelial dysfunction and damage in the obese phenotype of the JCR:LA-*cp* rat after the onset of hyperinsulinemia (Flood *et al.* 2004) and this in turn may render the vessel more permeable. Indeed, we have previously shown that in a rat model of T1D, remnant lipoproteins also co-localize with biglycan in focal clusters (Chapter 3), suggestive of vessel wall remodeling. We note that the design of studies from these current experiments (where arteries are exposed to similar concentrations of apoB particles derived from the analogous phenotype) support the hypothesis that arterial modeling is a major determinant of particle accumulation.

Increased *net retention* of remnant-associated cholesterol as compared to LDL-associated cholesterol in IR vessels is consistent with previous findings in hyperlipidemic rabbits (Proctor *et al.* 2003) as well as in *in-vitro* experiments (Chapter 3). These data further support the notion that lipoprotein composition (i.e. cholesterol per particle) and size are major factors that can influence net arterial cholesterol accumulation. In particular, we would propose that these factors may become more prominent during the non-fasted state. Data from other studies also indicate that under conditions of insulin resistance, not only do the number of particles increase during the lipemic period (fed-state), but lipid and protein composition may also be altered (Russell *et al.* 2010).

It is curious that remnant lipoproteins appear to have reduced 'efflux' (i.e removal) from arterial tissue relative to LDL (Figure 4-2). Indeed these observations are consistent with experiments in rabbits (Proctor *et al.* 2003), rats (Chapter 3) and mice (Tran-Lundmark *et al.* 2008). The impact of this finding is that clinically, over long periods of exposure (years), remnant dyslipidemia may account for an increased cholesterol deposition during conditions of MetS and insulin resistance.

4.4.2 Aortic remodeling of biglycan and decorin during insulin resistance

McDonald et al reported that a high-fat fed swine model of T1D had a 4-fold increase in biglycan content in the coronary artery lesions (McDonald *et al.* 2007). Indeed, we demonstrate here in the JCR:LA-*cp* rat, that under the pre-diabetic milieu, aortic biglycan protein core content also increases significantly with age and correlates linearly with increasing hyper-insulinemia (Figure 4-3 & 4-5). We know that the expression of biglycan protein core has been shown to be increased with FA (Rodriguez-Lee *et al.* 2007, Olsson *et al.* 1999), angiotensin II (Figueroa *et al.* 2002), oxidized LDL (Chang *et al.* 2000), TGF-ß (Little *et al.* 2002, Schonherr *et al.* 1993), as well as serum amyloid alpha (SAA)(Wilson *et al.* 2008). Consistent with this, obese JCR:LA-*cp* rats have been shown to have elevated concentrations of TGF-ß (Bauer *et al.* 2004) and non-esterified free FA (Riessen *et al.* 1994). Therefore an increase in TGF-ß concentration and free FA concentration in the JCR:LA-*cp* rats with increasing insulin concentration may have caused the concomitant rise in biglycan protein core content. Furthermore, it has been shown that TGF-ß is expressed in primary and restenotic atherosclerotic lesions (Riessen *et al.* 1994) and that its concentration is commonly elevated in diabetes (Flores *et al.* 2004) and MetS (Gomez-Fernandez *et al.* 2004). In vitro studies, using cell culture and smooth muscle cells have demonstrated that TGF-ß can upregulate biglycan expression but can simultaneously down-regulate decorin expression (Schonherr *et al.* 1993).

Decorin has been shown to co-localize with apoB in human atherosclerotic specimens (Nakashima *et al.* 2007) but not in murine atherosclerotic lesions (Huang *et al.* 2008, Kunjathoor *et al.* 2002). Curiously, over-expression of decorin in apoE knockout mice results in reduced development of atherosclerosis (Al Haj Zen *et al.* 2006). We found that decorin protein core increased significantly with age up to 12 weeks, yet did not correlate with increasing hyper-insulinemia (Figure 4-4). Data from our study are consistent with the proposal by *Al Haj Zen et al*, and do not support the involvement of decorin protein with atherosclerotic risk associated during conditions of insulin resistance.

4.4.3 Effect of insulin resistance on arterial sulphated glycosaminoglycans (GAGs)

We did not observe any age-related changes in total sulphated GAGs between the lean and obese thoracic aorta (Figure 4-6A). However *Tovar et al.* has shown that total sulphated GAG content can increase from zero to 40 years of age in humans, thought to be due to an increase in chondroitin sulphate (Tovar *et al.* 1998). The difference in our results as compared to that of *Tovar et al.* may be due to the narrow age range of rats chosen for our study. In our study, the comparative age range of rats would be equivalent to ~20 to 40 human years of age and it is interesting that the study by *Tovar et al.* did not report any observable difference in total sulphated GAG content in humans during this same period.

Dalferes et al. (1989) have shown that fatty streak lesions can contain an increased content of GAGs, due to increased levels of chondroitin sulphate A, B and C. In our study there was no difference in the amount of Chondroitin ABCase digestible GAGs (isolated from the whole aorta) (Figure 4-6B) neither between phenotype nor with age. Due to limited mass of tissue available to us using the current extraction techniques, we could not separate the different types of sulphated GAGs (specifically biglycan associated GAGs), and this may be one reason for data variance.

We know that atherogenesis begins in small focal regions and therefore we cannot exclude the possibility of increased total sulphated GAGs in those specific areas prior to or during lesion formation.

Perlecan is a heparan sulphate proteoglycan and has been shown to have conflicting pro-atherogenic (Vikramadithyan *et al.* 2004) as well as anti-atherogenic effects (Edwards *et al.* 2004). The heparan sulphate GAGs of perlecan have recently been shown to be atherogenic in a mouse model (Tran-Lundmark *et al.* 2008). We did not measure the perlecan core protein however we did not observe any notable differences in the heparan sulphate chains in our Chondroitin ABCase digested samples (Figure 4-6B).

4.4.4. Bovine biglycan and recombinant human biglycan bind to low-density lipoprotein with similar binding capacity and affinity

Earlier *in-vitro* binding experiments in literature have used bovine biglycan. So we decided to compare the binding affinities and capacities of these two types of biglycan. There was no difference in the binding affinity or binding capacity between human recombinant biglycan and the bovine biglycan to low-density lipoprotein (Figure 4-7C). Therefore from this we can suggest that when *equivalent mass* of bovine biglycan and recombinant human biglycan are used for the solid phase binding assay, the length of glycosaminoglycans or the amount of protein core does not make a difference in binding. From this we can conclude that may be the protein core of the proteoglycans also contribute to lipoprotein binding. It is not known if apoB can bind to the protein core of the proteoglycans *per se*, however apoE has been shown to bind to the protein core of biglycan (Klezovitch and Scanu 2001).

We did not perform an experiment by normalizing either the protein cores or the glycosaminoglycans of the bovine biglycan and the recombinant human biglycan. There may be a difference in lipoprotein binding to

bovine biglycan vs. recombinant human biglycan, if equivalent mass of protein cores is used because then the only difference between the two would be the length of the glycosaminoglycans.

4.4.5 Increased binding of native TRL isolated from insulin resistant rats to human biglycan

We have previously shown that binding capacity between preparations of native TRL and apoB remnants to human biglycan in a solid phase binding assay are similar (Chapter 3). However, increased retention of native TRL and associated-cholesterol to human biglycan in an *in-vitro* binding assay from our current experiments suggest that insulin resistance may induce compositional differences, specifically during the fed state. Among these differences, conformation of apoCIII (Olin-Lewis *et al.* 2002) or the amount of apoE (Klezovitch and Scanu 2001) in native TRL are important. Indeed, preliminary data from 2D gel anlaysis of lymph from JCR:LA-cp IR rats supports this concept (Russell *et al.* 2010). Indeed we observed an abundance of apoCIII in the lymph obtained from IR rats as compared to control rats during the fed state (data not shown). However we were not able to quantify the amount of apoCIII per particle in this experiment. We also note that previous studies that have investigated the comparative interaction of arterial proteoglycans with different lipoproteins have utilized either TRL or very low density lipoprotein fractions from plasma, which both contain a large ratio of apoB100 to apoB48 (Olin-Lewis *et al.* 2002, van Barlingen *et al.* 1996, Tannock *et al.* 2002). In contrast, newly secreted lymph chylomicrons or their remnants do not contain apoB100, have increased amounts of apoE and have an increased particle charge (measured as zeta potential) 10-fold greater than LDL (Proctor *et al.* 2003). The impact of subtle differences in the use of remnant preparations *per se* may have a substantial relevance to the interpretation of existing studies.

Based on the results of this study and other current literature, we propose that the existing 'response to retention' hypothesis can be further refined as the sum of (a) Exposure of lipoproteins to arteries (i.e particle number/concentration vs time/clearance) (b) Affinity of the particle to bind arterial tissue (i.e permeability/size, binding/dissociation and efflux) (c) Available number of particle binding sites (i.e proteoglycan expression and/or lipoprotein lipase) and (d) Capacity of lipoproteins to 'carry' cholesterol (i.e variations in size and net cholesterol composition).

Collectively, this study supports the hypothesis that insulin resistance leads to an acute exacerbated retention of remnant lipoprotein and associated cholesterol. Further, that increased entrapment of remnant particles maybe a result of increased arterial biglycan expression and/or increased propensity of binding by particles during the non-fasted state. We conclude that increased exposure and retention of remnant lipoproteins to arterial proteoglycans in the non-fasted state may in part explain increased progression of atherosclerotic CVD in insulin resistance.

4.5 Literature Cited

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Chapter 5: Final Discussion

Parts of this chapter are published (Please see Mangat et al. 2007 and 2010)

5.1 Executive Summary of Findings

5.1.1 Glycation of the extracellular matrix proteins in the arterial vessel wall exacerbates the retention of intestinal derived remnant lipoproteins

In the *first* study (experimental chapter 3) using *ex vivo* perfusion techniques in carotid vessels it was shown that intestinal derived remnants have increased retention in type 1 diabetic vessels compared to controls. The retained remnant lipoproteins were shown to be co-localized with arterial biglycan. A direct positive correlation was observed between the extent of remnant lipoprotein retention and the presence of glycated proteins in type I diabetic arteries.

Increased intestinal remnant retention during type 1 diabetes may contribute to the initiation and progression of atherosclerosis in patients with type 1 diabetes as compared to those without diabetes. This study has provided direct evidence for the first time that hyperglycemia and subsequent non-enzymatic glycation of the matrix components is a potential mechanism which exacerbates arterial remnant retention and the development of CVD. Tight glycemic control in patients with type 1 diabetes may potentially reduce CVD risk by reducing the generation of circulating advanced glycated end products, and therefore reduce the susceptibility of arterial retention of remnants and associated cholesterol delivered in these particles.

5.1.2 Intestinal derived remnants bind to human biglycan in a solid phase binding assay

In the *second* study (experimental chapter 3) it was conclusively demonstrated that intestinal derived remnants indeed bind to human

biglycan. Despite differences in lipid content and size, different lipoprotein fractions (intestinal derived remnants, LDL and lymph chylomicrons) bound to recombinant human biglycan with equal *affinity*. In contrast, LDL had significantly greater binding *capacity* to human biglycan compared to lymph chylomicrons and intestinal remnants. Interestingly, the intestinal remnant lipoproteins contained 2.5-fold cholesterol per particle as compared to LDL. Consistently, the binding of intestinal remnant-associated cholesterol at maximal binding *capacity* was also significantly greater (2-fold) compared to LDL-associated cholesterol. Furthermore glycation of biglycan, resulted in a 2.3 fold increase in binding capacity and 1.7 fold increase in binding affinity for intestinal derived remnant lipoproteins. These results show increased atherogenicity of intestinal derived remnant lipoproteins as compared to LDL and the preferential binding of intestinal derived remnants to glycated matrix components.

These results reinforce the response-to-retention hypothesis of intestinal derived remnant lipoproteins in atherogenesis. When the arterial matrix is presented with *equal numbers of* LDL and intestinal derived remnant lipoproteins, the net remnant lipoprotein derived cholesterol retained in the matrix is significantly more than the net cholesterol derived from LDL. This may explain the 'residual risk' phenomenon of CVD that occurs despite the lowering of plasma LDL-cholesterol (Fruchart *et al.* 2008). The 'residual risk' of CVD appears to be independent of the LDL-cholesterol relationship (Fruchart *et al.* 2008). LDL-cholesterol lowering is remains the cornerstone target of lipid for treatment in subjects at risk for CVD, whilst the concentration of intestinal derived remnants is not measured in these subjects (Fruchart *et al.* 2008). The findings presented in this thesis show intestinal derived remnants have increased atherogenicity compared to LDL, and therefore suggests intestinal remnants are a cause for 'residual risk' of CVD in these subjects. Additionally this data suggests the need to measure the concentration of intestinal derived remnants in subjects at

risk for CVD, specifically those subjects that present with normal LDL concentrations.

5.1.3 Insulin resistance leads to exacerbated arterial intestinal remnant lipoprotein retention

In the *third* study (experimental chapter 4) it was shown that the *ex vivo* perfusion of intestinal derived remnant lipoproteins in the JCR:LA-*cp* rat model of IR leads to a 76% greater arterial remnant lipoprotein retention when compared to control non-IR rats, and a 2.5-fold increase in arterial retention of remnant derived cholesterol in IR rats, as compared to control non-IR rats. Furthermore, the increased arterial remnant retention in the IR rats was associated with increased arterial biglycan protein content. During comparative perfusion experiments in the obese IR carotid vessels, we observed that a significantly greater number of LDL particles were *delivered* to the vessel as compared to intestinal remnants. However after extensive washout *significantly fewer* (55%) LDL particles remained in the tissue while there was no difference, *i.e* no lowering, in the retention of intestinal remnants (following 60 minutes of washout) relative to delivery experiments (no washout). In addition, despite the *greater number of LDL particles* retained compared to intestinal derived remnant particles, there was a 1.5-fold greater increase in the total *mass of cholesterol* associated with intestinal remnant particles as compared to cholesterol associated with LDL.

The results from this study may explain (at least in part) the mechanism for accelerated atherosclerosis associated with IR: increased arterial biglycan in the matrix of IR vessels, and increased intestinal remnant and associated cholesterol retention. Of note, these results have also shown that intestinal derived remnant lipoproteins appear to have increased atherogenicity compared to LDL, and this may further contribute to the *elevated* 'residual risk' of CVD during diabetes and IR.

5.1.4 Increased atherogenicity of triglyceride rich lipoproteins produced during the fed state in insulin resistance

In the *fourth* study (experimental chapter 4) native triglyceride rich lipoproteins isolated under fed conditions during IR, were observed to bind to biglycan with greater capacity compared to triglyceride rich lipoproteins isolated under fed conditions from control non-IR rats. No difference was found in the binding between native triglyceride rich lipoproteins isolated during the fasted state in control or IR conditions. Additionally In the *second* study (experimental chapter 3) there was no difference in binding affinity or binding capacity between native triglyceride rich lipoproteins and apoB48 containing intestinal remnant lipoproteins. Taken together these results demonstrate the atherogenicity of intestinal derived remnants during IR, specifically those produced in the fed state. Figure 5-1 summarized the proposed mechanisms for the increased atherogenicity of intestinal derived remnant lipoproteins during IR.

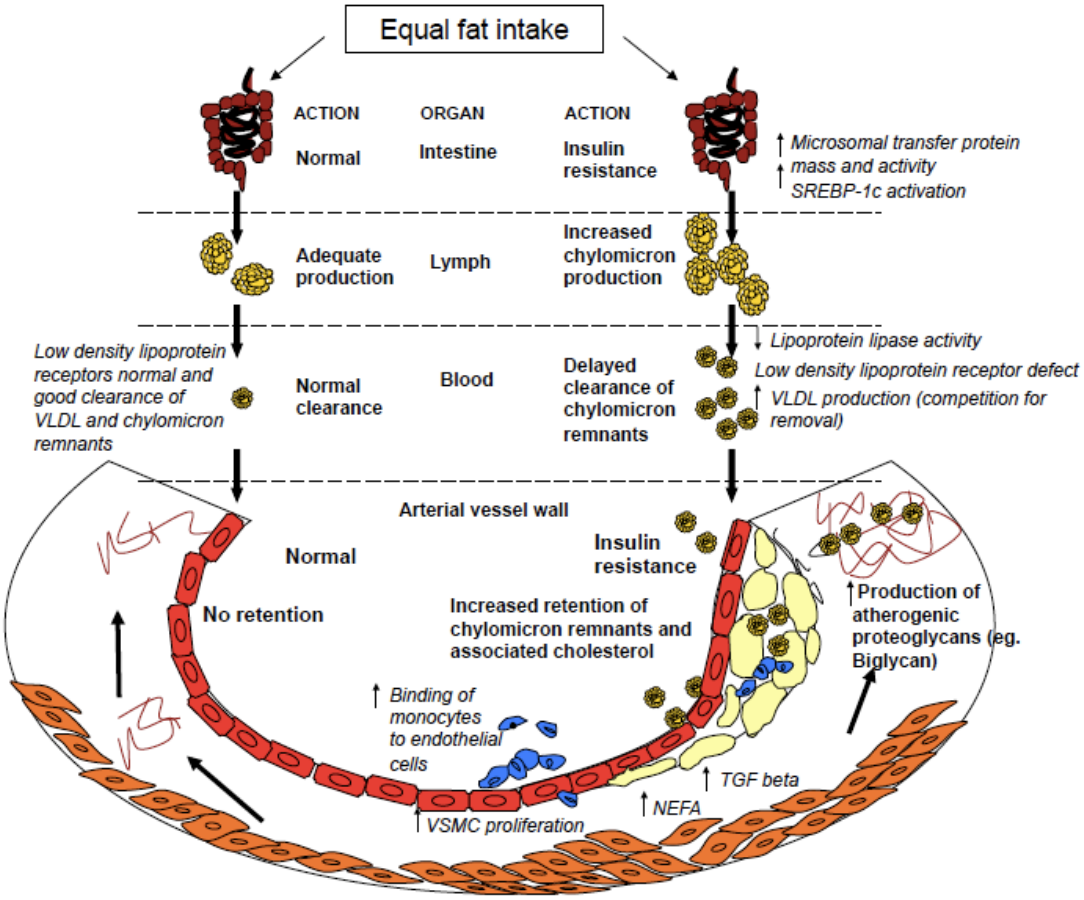

Figure 5-1 Proposed mechanism of increased atherogenicity due to overproduction and increased plasma circulating concentrations of intestinal derived remnant lipoproteins during IR (Mangat et al. 2007). This image is used in accordance with the s.29 provision for fair dealing in the Canadian Copyright Act. Despite equivalent food intake, IR leads to overproduction of chylomicrons as compared to control non-IR conditions. Increased numbers of chylomicrons in the plasma compete with VLDL for lipolysis and clearance. IR also results in increased production of biglycan protein, proliferation of vascular smooth muscle cells, production of TGF beta and non-esterified fatty acids. These factors lead to an increased binding capacity in the vessel wall for remnant lipoproteins and associated cholesterol. Collectively, an increased number of intestinal remnants and

number of binding sites within the vessel wall results in exacerbated arterial remnant-cholesterol retention.

The significance and impact of the findings of these studies is that we spend a majority of our time, up 10 to 16 hours a day, in the postprandial state (Lopez-Miranda *et al.* 2007). The relevance of this is the potential of the arterial wall to be exposed to increased circulating concentrations of intestinal remnant lipoproteins. In IR individuals this may be exacerbated, and therefore contributes to an increased risk of CVD. Indeed these subjects have been shown to have impaired postprandial clearance of apoB48 containing lipoproteins, in addition to increased fasting concentrations of intestinal remnants (Otokozawa *et al.* 2009). The findings of this thesis are consistent with the literature where individuals with IR or the metabolic syndrome are at increased risk for atherogenesis, and the studies herein may provide the contributing physiological mechanisms for this observation. In IR individuals there is increased intestinal production of chylomicrons in the fed state or post-prandially which leads to an increase in circulating intestinal derived remnants. While the diabetic metabolic milieu leads to an increase in arterial biglycan production that appears to provide an increased number of binding sites for intestinal derived remnants. Thereafter, avid binding of intestinal remnants to biglycan contributes to an increase in arterial cholesterol accumulation.

In conclusion, these collective experiments presented in this thesis have provided the first direct *ex vivo* evidence of the atherogenicity of intestinal derived remnant lipoproteins specifically under conditions of type 1 diabetes and insulin resistance. The mechanistic findings of these studies have contributed to the growing literature that supports the need for clinical guidelines for assessing intestinal derived remnant lipoprotein concentrations in otherwise normolipidemic patients, and in particular IR

and diabetic populations who are at increased risk for developing CVD (Kinoshita *et al.* 2009, Yamamoto *et al.* 2008, Su *et al.* 2009).

5.2 Factors Affecting Retention of Remnant Lipoproteins and Progression to CVD

The response-to-retention hypothesis of atherogenesis was oringinally dependent on the circulating concentration of LDL. Based on previous studies and the data presented in this thesis a re-defined response-to-retention hypothesis has been proposed below in Fig 5-2. It is proposed that the deposition of cholesterol in arteries can be defined as a sum of: (i) Exposure of lipoprotein particles to arteries (i.e. particle number/concentration vs. time/clearance), (ii) Affinity of the particle to bind arterial tissue (i.e. permeability/size, binding/dissociation and efflux), (iii) Available number or extent of particle binding sites (i.e. proteoglycan expression and/or lipoprotein lipase) and (iv) Capacity of lipoproteins to 'carry' cholesterol (i.e. variations in size and net cholesterol composition). The relationship and conceptual mathematical interplay of these factors are shown in Figure 5-2.

$$\text{Net accumulation of cholesterol} = \left\{ \text{Arterial exposure} \left(\frac{\text{Number of particles}}{\text{Clearance}} \right) \times \text{Cholesterol} \left(\text{Size} \times \text{Mass} \right) \right\} \times \left\{ \frac{\text{Permeability} \left[\frac{1}{\text{size}} \right] \times \text{Interaction} \times \text{Proteoglycan}}{\text{Efflux}} \right\}$$

Figure 5-2. Determinants of net arterial accumulation of cholesterol.

Circulating plasma apoB lipoproteins carry cholesterol into the arterial vessels (Olofsson *et al.* 2005). Therefore net accumulation of arterial cholesterol depends directly on the amount of cholesterol carried by each lipoprotein particle as well as the number of cholesterol carrying lipoproteins in the circulation. Results from this thesis and previous studies

have shown that intestinal derived remnant lipoproteins carry significantly more, cholesterol than the LDL (Proctor *et al.* 2004). The number of intestinal derived remnant lipoproteins fluctuates in circulation throughout the day as they are increased postprandially and decreased during fasting, while the number of LDL particles in the circulation remains constant. In IR subjects there is increased production of intestinal derived remnant lipoproteins that can potentially deliver more cholesterol to the vessel wall compared to normal subjects (Qin *et al.* 2007).

The number of cholesterol carrying lipoproteins in circulation is inversely proportional to the rate of clearance of these lipoproteins from the plasma. ApoB containing lipoproteins are cleared from the plasma by several hepatic receptors. The major pathway is via the LDL receptor (LDL-r) and via heparan sulphate proteoglycans (HSPG) (Stanford *et al.* 2009, Brown *et al.* 1986). The minor pathways are via scavenger receptor type B class I (SR-B1) and via the LDL related protein (LRP) (Out *et al.* 2005, Koval *et al.* 1989). The affinity of intestinal derived remnants for LDL-r is higher than for LDL, therefore intestinal remnants are cleared from the circulation at a faster rate compared to LDL (Chappell and Medh 1998). As a consequence, over a 24 hour period the intestinal remnant lipoproteins actually transport approximately 42 times more cholesterol than LDL (Proctor *et al.* 2002, Mamo *et al.* 2002). In Type 1 Diabetes, HSPG assembly is suppressed, and in IR and Type 2 Diabetes there is decreased sulphation of HSPG, which may lead to the accumulation of remnant lipoproteins. To date, the literature suggests that during diabetes, remnant lipoproteins not LDL, are raised in plasma (Hogue *et al.* 2007, Williams *et al.* 2008, Chan *et al.* 2006, Mooradian 2009). While during LDL-r deficiency, intestinal remnants do not accumulate in plasma, but LDL does (Hoeg *et al.* 1985, Rubinsztein *et al.* 1990). These observations suggest that the LDL-r pathway may not be compromised during diabetes.

Furthermore, the net arterial accumulation of lipoproteins is directly dependent on the permeability of the endothelium to cholesterol-dense remnant lipoproteins, which is increased as the size of the lipoproteins become smaller. Several studies have shown that >85% of arterial lipoprotein delivery occurs by transcytosis and particles greater than 70 nm cannot traverse the endothelium because of the size limitation of transcytotic vesicles (Simionescu and Simionescu 1991, Mamo and Wheeler 1994, Proctor and Mamo 1996). The action of LPL is required for the hydrolysis of native chylomicrons and VLDL particles to the size range required for transcytosis (70 nm) (Pentikainen et al. 2002, Proctor and Mamo 1998). At this stage, LPL is a rate-limiting regulator for the arterial delivery of pro-atherogenic lipoproteins. Evidence for this concept is highlighted in individuals with type 1 hyperlipoproteinaemia, who are deficient in LPL, but these subjects are not at increased risk for CVD (Goldberg and Merkel 2001, Mamo 1995).

Once the lipoproteins have permeated the arterial vessel wall their retention is dependent on the types and amounts of proteoglycans present in the arterial vessel wall and factors such as LPL or glycation that can facilitate their binding interactions. Results from this thesis demonstrate that increased glycation in the arterial matrix leads to increased retention of intestinal derived remnant lipoproteins. Also results presented in this thesis show that increased amounts of arterial proteoglycans during IR lead to increased retention of intestinal derived remnant lipoproteins.

The net retention of remnant lipoproteins is inversely proportional to the differential efflux of intestinal remnants and LDL from the vessel wall. Results presented in this thesis show that during IR, more LDL particles are delivered to the vessel wall as compared to intestinal derived remnants. However almost half of those LDL particles efflux out over time, while there is no difference in the delivery and efflux of the intestinal derived remnants during IR. It is not known why intestinal remnant

particles do not efflux out of the tissue to the same extent as LDL particles during IR. However, it could be suggested that during IR the binding between apoB48 and the arterial matrix involves different interactions, which preferentially retain apoB48-remnants compared to apoB100-LDL, thus leading to reduced efflux of intestinal remnants. Furthermore the particle composition of intestinal remnant lipoproteins during IR may be associated with irreverible binding of intestinal remnants to arterial proteoglycans during IR.

Taken together we can suggest that the response to retention hypothesis is not just dependent on the number of circulating cholesterol carrying lipoproteins but also on a number of other factors involving lipoproteins and the arterial wall .

5.3 Atherogenic Effects of Intestinal Derived Remnant Lipoprotein Retention

5.3.1 Retention of Intestinal Derived Remnant Lipoproteins and Progression to Foam Cell Formation

The data presented in this thesis did not investigate the fate of intestinal remnant lipoproteins retained in the vessel wall *per se*. However Brown *et al.* (2000) have cloned and purified an apoE independent receptor for apoB48 that is primarily expressed by reticuloendothelial cells: monocytes, macrophages, and endothelial cells. This apoB48 receptor has been found associated with foam cells isolated from human atherosclerotic lesions (Brown *et al.* 2000). The apoB48 receptor is thought to facilitate rapid high-affinity uptake of chylomicrons as well as VLDL particles rich in triglyceride, but not low triglyceride containing VLDL or LDL, which results in lipid engorgement in both murine and human macrophages (Gianturco *et al.* 1988, Gianturco *et al.* 1994). Therefore one of the major implications of the data collected from this thesis is that increased retention of intestinal remnant lipoproteins may enhance foam cell formation and atherogenesis.

5.3.2 Presence of Intestinal Remnant Lipoproteins in Human Atherosclerotic Plaque

The results of this thesis have shown that under conditions of IR at least, apoB48 containing remnants are effluxed to a lesser extent than LDL. This raises several unanswered questions that include; is apoB48 present in human atherosclerotic lesions and more importantly, is there a difference in the relative abundance of apoB100 (LDL) and apoB48 (intestinal derived remnants) containing lipoproteins in human atherosclerotic lesions?

ApoB48 has been shown to be present in human atherosclerotic plaques (Nakano *et al.* 2008, Pal *et al.* 2003). It has also been shown that in type 2 diabetic subjects, with normal concentrations of plasma LDL, that apoB48 remnant concentration (fasting as well as diurnal variation) correlates significantly with the presence of carotid lesions (Tanimura *et al.* 2008). Furthermore, a significantly higher concentration of apoB48 relative to apoB100 has been found in human atherosclerotic plaques (Pal *et al.* 2003, Voros personal communications). However it remains to be determined whether plaques from IR or diabetic subjects also show a similar distribution of lipoproteins.

5.3.3 Pleiotropic Effects of Intestinal Remnant Lipoproteins

In addition to inducing foam cell formation, retained intestinal remnant lipoproteins appear to also contribute to the progression of atherosclerotic development by exerting effects on several other key pathways.

It has been shown that intestinal remnant lipoproteins induce smooth muscle cell proliferation, regardless of the oxidative state of the lipids in these particles, (Kawakami *et al.* 2001, Kawakami *et al.* 2003). In organ chamber experiments, intestinal remnant lipoproteins have been shown to inhibit relaxation of rabbit aortic rings (Doi *et al.* 1999). While another study in human subjects reported an association between high serum

remnant lipoproteins and coronary vascular endothelial cell dysfunction (Inoue *et al.* 1998). In addition remnant lipoproteins have been shown to induce apoptosis in endothelial cells by NAD(P)H oxidase-mediated superoxide and cytokine production (Shin *et al.* 2004). Therefore intestinal remnant lipoproteins may potentially contribute to CVD by inducing endothelial dysfunction, proliferation of vascular smooth muscle cells, as well as by inducing foam cell formation.

5.4 Study Limitations

In chapter 4, autologous perfusion experiments were designed. According to this autologous study design, the LDL and intestinal remnant lipoproteins were generated and perfused into the same phenotype. Intestinal remnant lipoproteins derived from the lymph of control non-IR rats were perfused into the carotid arteries of control non-IR animals. Similarly intestinal remnant lipoproteins generated from the lymph from IR rats was perfused into the carotid arteries of IR animals. This study design did not differentiate between the effects of chylomicron *composition (apo-proteins, lipid)* and *intrinsic changes in the arterial wall matrix between animal phenotypes: control and IR animals.*

A future study (crossover study design) involving perfusion of intestinal remnants from control non-IR rats into IR rats, and *vice versa* would be able to differentiate the effects of chylomicron composition and arterial matrix remodeling caused as a direct result of IR. However, from the collective evidence in this thesis, insulin resistance may independently exert proatherogenic effects on both lipoprotein, as well as arterial matrix composition. The *in vitro* binding study using fed and fasted native triglyceride rich lipoproteins directly demonstrated the proatherogenic effects of insulin resistance on *particle composition* under fed conditions. In addition, arterial biglycan protein expression was greater in insulin

resistant animals, suggesting the role of matrix remodeling in contributing to the atherogenicity of intestinal remnant lipoproteins. Therefore, a crossover study design would help in determining the contribution of changes in both lipoprotein composition and the arterial matrix that are involved in the retention of intestinal remnants.

The increased arterial biglycan protein content observed in IR rats (chapter 4) may be only one of the alterations associated with matrix remodeling in this phenotype that is associated with an increase in intestinal remnant retention. Future studies could investigate the role of lipoprotein lipase during IR and modification of elastin and collagen during IR and diabetes and the role of perlecan in facilitating lipoprotein retention during IR.

LDL used in these studies was commercially obtained and therefore modifications during preparative procedures might have caused oxidation or aggregation of LDL which could have impacted the binding pf LDL to the matrix.

We acknowledge that the solid phase binding assay used in the studies shows high background binding which makes it difficult to detect binding at lower concentrations and therefore there is a paucity of data points on the steep parts of the slope.

5.5 Future Directions

5.5.1 Lipoprotein retention in a biglycan knock out mice model

The increased presence of biglycan protein during IR could confer the use of a biglycan knock out mice model to confirm the role of biglycan in arterial retention of lipoproteins. Furthermore, if a biglycan knock out mouse model exhibits less atherosclerosis compared to the wild type mouse then pharmaceutical approaches to target arterial biglycan could potentially be developed. In addition, pre-perfusion with biglycan

antibodies or other compounds that block biglycan binding studies would also contribute to our understanding of the involvement of biglycan-associated binding of intestinal derived remnant lipoproteins.

5.5.2 Effect of insulin resistance and diabetes on lipoprotein composition: lipid and apolipoproteins

In future studies, the particle composition of remnant lipoproteins could be characterized in greater detail i.e charge, glycation, phospholipid, composition and other apolipoprotein content (A1, E, CI, CII, CIII) in apoB48 containing remnants. Since low-density lipoproteins have a longer half-life, these lipoprotein particles have a higher propensity for glycation in subjects with hyperglycemia (Shiu *et al.* 2009, Chao *et al.* 2010). Intestinal remnant lipoproteins have a relatively shorter half-life therefore it is likely that remnant lipoproteins may not become glycated to the extent of LDL, however this has yet to be investigated.

5.5.3 The effect of remnant fatty acid composition of intestinal derived remnants and their atherogenicity

Recently it has been shown that saturated fatty acids can cause endoplasmic reticulum stress and apoptosis in macrophages (Seimon *et al.* 2010). However the effects of different dietary fats on the atherogenicity of intestinal derived remnant lipoproteins inclusive of arterial binding, macrophage uptake and apoptosis remains unknown. A follow up of these types of studies to examine dietary fatty acids and intestinal remnant properties would be particularly useful, and may contribute to specific dietary intake recommendations, particularly for patients with IR, type 1 and type 2 diabetes.

5.5.4 Increased arterial biglycan production during insulin resistance verses reduced biglycan turnover

The aortic mass of biglycan protein in the insulin resistant rats was investigated in this thesis, however the mRNA expression of biglycan was not measured. Future studies could also measure biglycan mRNA expression, which may provide further insight into understanding whether the increased biglycan mass is a direct result of increased production or decreased turnover of biglycan.

5.5.5 Role of diabetic metabolic factors on the production of proteoglycans in vascular smooth muscle cells

In vitro studies provide conflicting evidence that arterial smooth muscle cells in the presence of fatty acids and glucose can secrete proteoglycans that have increased binding to LDL (Tannock et al. 2002 and Rodriguéz-Lee *et al.* 2007). These studies use *in vitro* conditions to mimic diabetic metabolic milieu while the smooth muscle cells used in these studies are not isolated from insulin resistant or diabetic animals. Therefore isolating and culturing vascular smooth muscle cells isolated from diabetic animals in serum obtained from similar diabetic animals, and then analyzing the proteoglycans produced from these cells may be able to further test the hypothesis: that proteoglycans produced from smooth muscle cells in the IR or diabetic state indeed exhibit more lipoprotein binding *per se.*

5.5.6 Role of bridging molecules in intestinal derived remnant retention

It is known that C-reactive protein (CRP) is an independent risk factor for CVD (Devaraj *et al.* 2009). Several studies have shown that elevated CRP levels prevail in subjects with the metabolic syndrome (Devaraj *et al.* 2009). There is evidence that CRP can act as a bridging molecule to bind with aggregated LDL and that this CRP-aggregated LDL complex is readily taken up by macrophages (Fu *et al.* 2002). It remains to be investigated if intestinal remnant lipoproteins are also capable of forming a complex with CRP and if this facilitates macrophage uptake and formation of foam cells. In addition, perhaps intestinal remnant lipoproteins use CRP

as a bridging molecule to bind with arterial proteoglycans. Studies analyzing the co-localization of CRP and apoB48 could be conducted in both human tissue and animal models of the arterial wall, and at different stages of atherosclerotic lesion development to answer these questions.

5.5.7 Role of secretory sphingomyelinase in intestinal derived remnant retention

Basolateral secretion of secretory-sphingomyelinase from endothelial cells acts on LDL bound sphingomyelin and yields ceramide enriched LDL, which is prone to aggregation and leads to subendothelial retention of aggregated LDL (Tabas 1999). Apical secretion of secretory-sphingomyelinase by endothelial cells has been suggested to account for the elevated secretory-sphingomyelinase activity in serum that has been reported in patients with inflammatory conditions, type II diabetes (Górska *et al.* 2003) and chronic heart failure (Doehner *et al.* 2007). However it is not known whether the activity of endothelial secretory-sphingomyelinase is increased during IR and if its action on remnants can cause aggregation that facilitates retention during IR. Alternatively the relative concentration of sphingomyelin in intestinal remnants and LDL is also not known.

5.6 Therapeutic Interventions to Reduce intestinal derived Remnant Retention

Therapeutic interventions to reduce remnant lipoprotein retention could be designed to target the intestinal production, plasma clearance and composition of intestinal remnants. Also interventions to reduce the production of atherogenic arterial proteoglycans or that modulate proteoglycan-remnant binding could be potential target therapies.

5.6.1 Potential therapeutic effects of AGE inhibitors and AGE breakers

Therapeutic approaches targeted at AGEs include compounds that inhibit the formation of AGEs (eg. aminoguanidine) and compounds that break

the already existing AGE-protein crosslinks (eg. ALT-711, Alagebrium chloride) (Vasan *et al.* 2003). The therapeutic effects of AGE inhibitor aminoguanidine has been extensively investigated in animal models and is currently in phase 3 clinical trials (Cooper 2004). Alagebrium chloride as well as ALT-711 have been shown to reduce atherosclerosis, inflammation, collagen deposition, and improve cardiac function indices and markers of renal disease (Cooper 2004).

In this thesis it was shown that there is a direct correlation of remnant lipoprotein retention with glycated matrix components. Therefore it can be speculated that AGE inhibitors or AGE-breaker compounds may reduce arterial remnant retention. However the effects of these compounds is yet to be determined on the arterial retention of lipoprotein remnants and associated cholesterol deposition.

5.6.2 N-3 PUFA intervention to reduce production of atherogenic proteoglycans

It has been reported that a diet high in saturated fat increases biglycan gene expression in the white adipose tissue of insulin resistant animals, whilst a diet rich in n-3 PUFA decreases biglycan expression compared to a low fat diet (Huber *et al.* 2007). The effect of different dietary fats on arterial proteoglycan expression remains unknown. N-3 PUFAs are peroxysome proliferator activated-receptor γ (PPAR γ) ligands and have been shown to inhibit proliferation of vascular smooth muscle cells (Diep *et al.* 2000). Proteoglycans synthesized in the presence of PPAR ligands, such as thiazolidinedione, show reduced binding to LDL (Nigro *et al.* 2008). Therefore the effect of acute or chronic n-3 PUFA feeding on arterial binding of remnant lipoproteins warrants further investigation.

5.6.3 Effects of combined Ezetimibe and HMG-CoA reductase inhibition on chylomicron production during insulin resistance

Several compounds are under investigation that target intestinal physiology to reduce the secretion of remnant lipoproteins, reduce cholesterol absorption and improve postprandial lipid metabolism. Some of these compounds are reviewed in Table 5-1.

Ezetimibe is a novel pharmaceutical compound that reduces intestinal cholesterol absorption (Davis *et al.* 2007). Ezetimibe either alone, or in combination with statins can decrease plasma LDL-cholesterol and intestinal remnant concentration in humans (Tremblay *et al.* 2006, 2009). However, the combined effects of Ezetimibe and statins on post-prandial dyslipidemia and/or arterial retention of intestinal derived remnant lipoproteins, specifically under conditions of IR has remained unclear.

Recent preliminary results from studies in the JCR:LA-*cp* rat suggest that combination therapy of Ezetimibe and Simvastatin may actually upregulate intestinal chylomicron (apo-B48) production and this is associated with a decrease in the cholesterol:apo-B48 ratio (Mangat *et al.* 2010). Consequently, it could be suggested that under conditions of established IR, whereby chylomicron production is upregulated, the current clinical recommended dosage of Ezetimibe (with or without Simvastatin) may not be sufficient to correct for these 'atherogenic' alterations in chylomicron synthesis and composition.

5.6.4 Effects of Ezetimibe (alone or in combination) on arterial remnant retention

A preliminary report indicates that in the JCR:LA-*cp* model of the MetS, Ezetimibe is able to decrease the *number* of remnant particles (measured as apoB48), as well as the associated mass of cholesterol retained in carotid vessels compared to non-treated animals (Mangat *et al.* 2010). Moreover, in combination with Simvastatin, Ezetimibe was able to further reduce arterial cholesterol deposition in IR JCR:LA-*cp* rats, compared to monotherapy alone. These observations support a positive association of

these compounds to reduce early stages of arterial intestinal remnant accumulation and cholesterol deposition. The interpretation from these studies is that combination therapy of Ezetimibe with a Statin may have additive benefits to not only cholesterol absorption and metabolism, but also to other factors that modulate the susceptibility of the vascular matrix to lipoprotein-remnant cholesterol accumulation. Consistent with these findings an *in vitro* study has shown that vascular smooth muscle cells pre-incubated with a statin leads to the production of proteoglycans that reduce LDL binding. Indeed, the recent VYCTOR study has shown that in subjects with exacerbated coronary disease risk, treatment with a combination of Ezetimibe with Simvastatin/Pravastatin results in a significant reduction in carotid intima medial thickness (Meaney *et al.* 2009).

Collectively, emerging data from animal models of IR and the metabolic syndrome support the concept that the combination of Ezetimibe with Statins may confer synergistic effects on the following: cholesterol metabolism, chylomicron production and clearance, and vascular factors, compared with Ezetimibe treatment alone. Clinical studies are now warranted to further address both regressive and preventative anti-atherosclerotic strategies using a combination of these lipid-lowering targets. Currently Ezetimibe is given to patients who are already on a Statin to further help reduce LDL-cholesterol. However emerging data shows that the combination therapy may be beneficial in reducing atherosclerosis risk in patients with normal LDL-cholesterol but high intestinal derived remnant concentrations.

Table 5-1. Summary of drug/compounds in development that target intestinal physiology to decrease cholesterol absorption and improve post-prandial lipid metabolism.

Drug/Compound	**Reference**	**Mode of action**	**Main findings**	**Outlook**
MTP Inhibitor (AEGR-733, CP-346086)	(Cuchel *et al.* 2007, Samaha *et al.* 2008, Chandler *et al.* 2003)	Inhibition of microsomal triglyceride transfer protein (in intestine and liver).	Reduction in LDL and triglycerides.	AEGR-733 is currently in phase III clinical trial for evaluation of long-term safety and efficacy.
Pluronic F-68,	(Au *et al.* 2009)	Inhibits cholesterol absorption.	Inhibits absorption of dietary lipids and secretion of VLDL.	Ongoing phase I clinical trial to determine it's effect on cholesterol absorption.
FM-VP4 (Disodium ascorbyl phytostanol phosphate)	(Vissers *et al.* 2008)	Decreases cholesterol absorption.	Safe, tolerable and reduces LDL-C.	A Phase 2, multicenter clinical trial on subjects with primary hypercholesterolemia has been completed.
Sitagliptin, Vidagliptin (DPP IV inhibitor)	(Matikainen *et al.* 2006)	Reduces intestinal TG absorption, reduces post-prandial apoB48 and increases chylomicron catabolism.	Vidagliptin reduces postprandial intestinal triglyceride-rich lipoprotein particles in patients with type 2 diabetes.	Ongoing phase III clinical trial on the effects of Sitagliptin on postprandial lipemia in men with type 2 diabetes.
Exenatide (GLP-1 receptor agonist)	(Buse *et al.* 2010, Bunck *et al.* 2010)	Exhibits the same mechanism of action as native GLP-1.	Body weight regulation, glycemic control and improves blood lipids.	Ongoing clinical trial on the regulation of intestinal and hepatic lipoprotein production

Drug/Compound	Reference	Mode of action	Main findings	Outlook
LXR agonists (Intestinal specific GW6340, systemic agonist GW3965)	(Yasuda *et al.* 2010)	Promotes fecal sterol excretion and inhibits intestinal sterol absorption.	Promotes reverse cholesterol transport.	No human studies to date.
Pactimibe (ACAT inhibitor)	(Meuwese *et al.* 2009)	Inhibits esterification of free cholesterol in intestine, liver and other cell types, thus inhibiting the incorporation of cholesterol into chylomicrons and VLDL.	In patients with familial hypercholesterolemia, pactimibe was associated with increased incidence of major cardiovascular events.	CAPTIVATE trial was terminated because major CVD events occurred more often in patients treated with pactimibe.

5.7 Need for Clinical Guidelines for Intestinal Derived Remnants

It is now known that LDL has to be oxidized before these particles can be taken up by macrophages and this is an unregulated process leading to foam cell formation (Miller *et al.* 2010). Intestinal derived remnants do not need to be modified for unregulated uptake by macrophages (Botham *et al.* 2007). In addition, based on our findings and previous findings (Proctor *et al.* 2002), intestinal derived remnants contain significantly more cholesterol as compared to LDL and efflux less readily from the sub-endothelial space. Taken together it can be suggested that intestinal derived remnants are more atherogenic than low-density lipoproteins.

Therefore under conditions where low-density lipoprotein concentrations are normal, there is a warranted need to determine chylomicron-remnant concentrations in order to fully assess atherogenic risk.

In light of these new findings and other accumulating evidence regarding the atherogenicity of intestinal remnant lipoproteins (Cohn 2008), there is an urgent need to develop clinical guidelines for assessing remnant concentrations that are age, gender and ethnicity specific. In the past this has not been possible as the methods to detect intestinal remnants were not specific due to antibodies cross-reacting with both apoB100 and apoB48. While western blot methods are able to quantitate differences in these apo-lipoproteins, these methods are time consuming and tedious, and therefore not suitable for high throughput diagnosis of large numbers of clinical samples (Cohn 2008). Recently ELISA (Enzyme linked Immunosorbent Assay) kits have been developed for detection of apoB48 by using monoclonal antibodies (Kinoshita *et al.* 2005). One limitation with the ELISA kit is that the storage and preparation of samples for the ELISA technique can affect the exposure of immunogenic sites on apo-B48 (Jackson *et al.* 2004). Furthermore, the use of monoclonal antibodies in ELISA has made this approach less sensitive, due to fewer epitope binding sites (relative to immunoblotting that uses polyclonal antibodies). It has been reported that lipid enrichment of sample and/or standard may also increase the potential for the masking of epitope-binding sites, particularly with protocols that utilize monoclonal antibodies. As ELISA method(s) are yet to be standardized, literature has reported a wide range of results (Jackson *et al.* 2004). However, due to the potential advantages of the ELISA method, overcoming methodological limitations would allow apo-B48 ELISA to be routinely applied in clinical practice. For example, it may be feasible to identify the most robust ELISA method in an attempt to induce a standardized approach, especially when a large number of samples have to be analyzed.

5.8 Conclusion

In conclusion the results of this thesis have provided direct evidence of the atherogenicity of intestinal remnants and the potential mechanisms associated with increased arterial retention of these particles in Type 1 diabetes as well as IR. Early detection of impairments in intestinal remnant metabolism and potential biomarkers of altered arterial matrix proteoglycans are pivotal to the further development and implementation of dietary and pharmaceutical interventions that target the prevention of CVD. In addition the early diagnosis of IR, and other associated risk factors may contribute to our global efforts to reduce the incidence of the morbidity and mortality associated with this chronic disease (WHO2010).

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Appendix A

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Article: Pathophysiology

Increased risk of cardiovascular disease in Type 1 diabetes: arterial exposure to remnant lipoproteins leads to enhanced deposition of cholesterol and binding to glycated extracellular matrix proteoglycans

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Abstract

Aims To determine fasting and postprandial metabolism of apolipoprotein B48 (apoB48) remnant lipoproteins in subjects with Type 1 diabetes and the relationship to progressive cardiovascular disease, and to investigate the impact of remnant lipoprotein cholesterol accumulation associated with arterial wall biglycan using a rodent model of Type 1 diabetes.

Methods Normolipidaemic subjects (n = 9) with long-standing Type 1 diabetes (and advanced cardiovascular disease) and seven healthy control subjects were studied. Fasting and postprandial apoB48 concentration was determined following a sequential meal challenge. A rodent model of streptozotocin-induced diabetes was used to investigate the *ex vivo* retention of fluorescent-conjugated remnants. Binding of remnant lipoproteins to human recombinant biglycan was assessed *in vitro*.

Results A significantly higher concentration of fasting plasma apoB48 remnants was observed in patients with Type 1 diabetes compared with control subjects. Patients with Type 1 diabetes exhibited a greater total plasma apoB48 area under the curve (AUC) and an increased incremental AUC following a second sequential meal compared with control subjects. The arterial retention of remnants *ex vivo* and associated cholesterol was increased sevenfold in Type 1 diabetes rats relative to controls. Remnants were shown to bind with significant affinity to human biglycan *in vitro* and a further 2.3-fold increased binding capacity was observed with glycated biglycan. Remnants were shown to colocalize with both arterial biglycan and glycated matrix proteins in the Type 1 diabetes rodent model.

Conclusion Impaired metabolism of remnant lipoproteins associated with enhanced binding to proteoglycans appears to contribute to the arterial cholesterol deposition in Type 1 diabetes. Our findings support the hypothesis that impaired remnant metabolism may contribute to accelerated progression of atherosclerosis in the hyperglycaemic and insulin-deficient state.

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Keywords atherosclerosis, chylomicrons, diabetes, lipoproteins

Abbreviations apoB48, apolipoprotein B48; AUC, area under the curve; iAUC, incremental area under the curve

Introduction

Type 1 diabetes is associated with accelerated atherosclerosis and a three- to four-fold increase in cardiovascular disease risk as compared with the non-diabetic population [1]. The paradox of Type 1 diabetes is that, despite increased cardiovascular disease risk, classic fasting lipid indices (elevated LDL cholesterol or low concentration of HDL cholesterol) are often comparable with those of a healthy population [2]. Furthermore, there is no associated increase in other cardiovascular risk factors such as obesity, smoking or hypertension in Type 1 diabetes. Thus, our current understanding of the aetiology of increased atherosclerotic risk in Type 1 diabetes remains limited.

Accumulating evidence has implicated a role for remnant lipoproteins in the development of atherosclerosis among populations at risk of cardiovascular disease, including:

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normolipidaemic subjects with coronary heart disease; obesity, metabolic syndrome, Type 2 diabetes and familial hypercholesterolaemia [1–5]. However, the role of remnant lipoproteins and their metabolism in Type 1 diabetes atherosclerotic risk remains unclear. To date, no studies have examined apolipoprotein B48 (apoB48), a specific marker of intestinal remnant lipoprotein particle metabolism, in a human population with Type 1 diabetes. Nor is it known whether apoB48-remnant lipoproteins and associated cholesterol accumulate at a greater propensity in the arterial vessels of subjects with Type 1 diabetes [6]. Preliminary evidence from our laboratory suggests that the metabolism of remnant lipoproteins is impaired in normolipidaemic subjects with Type 1 diabetes [7]. Indeed, others have proposed that the increased cardiovascular disease risk associated with Type 1 diabetes may result from a unique diabetic dyslipidaemic profile that is predominantly attributable to changes in the remnant lipoprotein cholesterol fraction [8]. Moreover, in Type 1 diabetes, the arterial wall is characterized by intimal thickening, smooth muscle cell proliferation, increased expression of extracellular proteoglycans and the accumulation of glycated proteins [9–11]. Recent data supports the hypothesis that hyperglycaemia and insulin deficiency facilitate the entrapment of apoB-and apoE-containing atherogenic lipoproteins in the sub-endothelial matrix of the vessel wall [12]. However, direct evidence has been lacking to determine if remnant lipoproteins avidly bind with arterial extracellular matrix components or if there is preferential binding and accumulation of these particles during the course of Type 1 diabetes.

Consequently, the objectives of this study were twofold. Firstly, remnant lipoprotein metabolism in subjects with Type 1 diabetes was assessed to define the extent of postprandial dyslipidaemia. Secondly, the consequence of remnant lipoprotein (and associated cholesterol) accumulation was assessed in the arterial vessels *ex vivo* during experimental diabetes. We hypothesized that remnant lipoprotein metabolism would be impaired in Type 1 diabetes and that arterial vessels from diabetic animals would accumulate cholesterol to a greater extent compared with controls.

Research design and methods

Study subjects

Nine normolipidaemic subjects with long-standing Type 1 diabetes (five males, four females) and seven healthy control subjects (two males, five females) were studied. Subjects were matched based on gender, age (53.3 ± 3.3 vs. 46.5 ± 6.3 years; $P = 0.31$) and BMI (24.9 ± 1.2 vs. 23.7 ± 0.80 kg/m^2; $P = 0.44$). Subjects with Type 1 diabetes were recruited from a patient population with unstable glycaemic control and under consideration for islet transplantation at the University of Alberta, Edmonton, Alberta. The duration of diabetes in subjects enrolled was 41.6 ± 3.3 years (range 20–45 years) and HbA_{1c} was 8.9 +/- 0.5% (74 +/- 6 mmol/mol). Cholesterol-lowering medications were discontinued 1 week prior to the study, as cholesterol-lowering agents have been shown to alter the clearance of intestinally derived triglyceride-rich lipoproteins and their remnants [13]. All subjects with Type 1 diabetes were treated with an intensive insulin regimen using combinations of short-acting and basal insulin, which included conventional and analogue derivatives. Control subjects were recruited via advertisements throughout Edmonton and the University of Alberta regions. Control subjects were not on any medication and exclusion criteria included smoking, hypertension (> 140/90 mmHg), elevated LDL cholesterol (> 3.4 mmol/l), low HDL cholesterol (< 0.90 mmol/l) and/or a family history of premature cardiovascular disease. All subjects gave informed consent to participate in the study and the research protocol was approved by the Health Research Ethics Board at the University of Alberta.

Cardiovascular disease risk in study subjects

Eligible subjects with Type 1 diabetes underwent coronary angiography and were found to have one or more lesions, which narrowed the lumen diameter by at least 50%. Additional minor disease in multiple vessels was also common among subjects with Type 1 diabetes. The angiogram findings were consistent with accelerated vascular disease associated with diabetes. The control group was not eligible for cardiac catheterization.

Study protocol

All subjects were asked to follow their regular diet and exercise regime leading up to the study. Dietary evaluations, as assessed by 3-day food recalls, revealed no significant differences in total calories or per cent of daily calories from fat, carbohydrate or protein between the study groups (see also Supporting Information, Table S1).

Subjects were fasted overnight prior to the test day and a baseline blood sample was obtained from an intravenous catheter inserted into the forearm vein at approximately 08.00 h (0 h). Breakfast (0.5 h) and lunch (4.5 h) were subsequently provided to all subjects. Postprandial blood samples were drawn at 2, 4, 6 and 8 h into vacutainer tubes containing 0.1% EDTA.

Both breakfast and lunch meals provided approximately one third of daily caloric requirements, with 20%, 30% and 50% of total energy as protein, fat and carbohydrate, respectively. The two meals were provided sequentially to represent food consumption in a free-living situation and to elicit a typical postprandial response. Subjects with Type 1 diabetes self-administered their insulin doses before each meal as prescribed by their physician.

Biochemical analysis

Total as well as newly synthesized intestinally derived remnant lipoprotein concentration was determined by measuring apoB48

from whole plasma and triglyceride-rich lipoprotein fraction (density < 1.006 g/ml), respectively. Triglyceride-rich lipoprotein fractions were separated at each time point by ultracentrifugation at 25 000 rpm for 25 min at 20 °C and were immediately frozen and stored at –80 °C.

ApoB48 was quantified using a previously described SDS-PAGE and modified western blotting technique [14].

Glucose, total plasma cholesterol, LDL cholesterol, HDL cholesterol and triglyceride were all determined by the University of Alberta Hospital laboratory, using standard enzymatic colorimetric assays. Insulin was measured using a radioimmunoassay highly specific for human insulin and analogues (Linco Research, St Charles, MO, USA).

Experimental diabetes

Diabetes was induced in male Sprague Dawley rats (200–250 g body weight) by tail vein injection of streptozocin (50 mg/kg) following an overnight fast. Animals with plasma glucose concentration > 15 mmol/l 1 week post-induction of diabetes were included in the study as diabetic. Diabetes was confirmed by glycosuria and elevated plasma glucose concentration (Boehringer, Mannheim, Germany). *Ex vivo* perfusion experiments were completed 8 weeks following the induction of diabetes. Sham-injected control rats (sodium citrate buffer pH 4.5) were used concurrently. All animal procedures were in accordance with guidelines set by respective Institutional Ethics Committees. Plasma triglyceride concentration was measured at the end of the study by an enzymatic procedure and corrected for free glycerol (Wako Chemicals, Richmond, Virginia, USA) and cholesterol was determined colorimetrically (Wako Chemicals). Fluorescent glycated proteins present in rat serum were assayed using online spectrofluorometric detection in a flow system [15]. Samples were run in triplicate and the area under the curve was used for signal measurements. The assay was calibrated against glycated proteins obtained from enzymatic hydrolysis of glycated BSA (10 g/l). Total peptide content was estimated using an online spectrophotometric detector set to a wavelength of 280 nm [15].

Lipoprotein isolation and labelling

Intestinal remnant lipoproteins were isolated and labelled with fluorophores as described previously [16,17]. Briefly, nascent chylomicron particles were collected from superior mesenteric lymph duct cannulated donor rats given Intralipid™ (Fresenius Kabi, Bad Homburg, Germany) via a gastric cannula. Remnant lipoproteins were generated endogenously *in vivo* via a surgical rabbit hepatectomy procedure [16,17]. Lipoproteins were isolated by ultracentrifugation and then conjugated with Cy5 (GE Healthcare, Piscataway, NJ, USA). Importantly, we have established that this fluorophore does not exchange with other plasma macromolecules, is protein bound and does not have altered clearance from plasma or organ uptake [16,17]. All fluorescent lipoprotein preparations are routinely screened for potential aggregation or modification by confirming particle size (Brookhaven, Holtsville, NY, USA).

Recombinant biglycan

For the production of human recombinant biglycan, cDNA from Origene (Rockville, MD, USA) was used to transfect host FS293 cells which were cultured in sterile 250-ml Erlenmeyer flasks with 10×10^7 cells per flask, in FreeStyle 293 Expression Medium (Invitrogen, Carlsbad, CA, USA). Each flask was transfected with 76 μg of human biglycan DNA using 293 fectin reagent. Cell media containing biglycan was separated by SDS-PAGE and dimethylmethylene blue-positive fractions were pooled and dialysed. Biglycan glycosaminoglycan chains were characterized on a dimethylmethylene blue stained gel and the size of protein core was validated after ABCase digestion on a coomassie stained gel. To modify biglycan for glycation, human recombinant biglycan was incubated in 50 mM glucose for 15 days at 37 °C.

Solid-phase binding assay

Human biglycan was diluted in HEPES buffered saline (HBS) and 2 μg/well was added to a Maxisorp immunoplate (Nunc, Naperville, IL, USA) and incubated at room temperature (21C) overnight. To determine non-specific binding, additional control wells were coated with only HBS. All wells were blocked with BSA for 1 h and, following that, lipoprotein samples diluted in sample buffer (20, 10, 7.5, 5, 2.5, 1.25 and 0.625 mg/l in triplicate) were added to wells and incubated for 1 h at room temperature. Human LDL was purchased from Chemicon (Temecula, CA, USA) (catalogue no. LP2).

The number of intestinal remnant lipoproteins in each well was standardized to apoB48 and the number of LDL particles was standardized to apoB100 concentration. To detect lipoproteins, bound, HRP-conjugated Goat anti apoB48/B100 (Biodesign International, Saco, ME, USA) diluted 1/750 in HBS-Tween with 0.1% BSA was used for incubation for 1 h 30 min at room temperature and washed. Finally, 1-Step™ Turbo TMB-ELISA (Pierce, Rockford, IL, USA) was used for approximately 5 min to allow for colour development. Absorbance was measured at 450 nm.

Ex vivo arterial perfusion

Carotid artery segments were cannulated at the proximal and distal ends to create a closed circuit. Carotid vessels from insulin-deficient ($n = 10$ vessels) and control rats ($n = 7$ vessels) were perfused *ex vivo* with equivalent and physiological concentrations of remnant lipoproteins. The labelled remnants were diluted in PBS to achieve a final concentration of 8.8×10^{13}–1.2×10^{14} lipoprotein particles per ml perfusate, which was chosen to reflect remnant concentration in the post-absorptive state (5×10^{13} ml plasma) [16,17]. The perfusate (Cy5-labelled remnant lipoproteins diluted in PBS) was

oxygenated and recirculated. The carotid vessels were perfused with Cy5-labelled remnant lipoproteins for 25 min, followed by a washout period of only PBS perfusion for 20 min. We have shown previously that efflux of non-bound arterial lipoproteins is complete within 20–30 min and that perfusion of non-lipoprotein buffer for as long as 60 min fails to clear sites of focal accumulation [16,17]. Vessels are removed and fixed in 2% paraformaldehyde for exactly 30 min. Carotid segments are frozen in liquid nitrogen and sectioned (approximately 10–15 μm) by cryostat for digital analysis and immuno-histochemistry.

Quantification of fluorescence

The fluorescence intensity associated with chylomicron-remnant particles was determined by digital analysis using Image J software (version 1.36b) (http://rsbweb.nih.gov/ij/) and the plug-in LSM reader. An area of interest was traced on the carotid sections 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 volume of each section was calculated as the product of area, number of slices and the interval between the slices. The total intensity and the volume were used to calculate the intensity per unit volume of each carotid section. Data from $n = 10$ insulin-deficient rats ($n = 20$ z-stack files used for 3-D analysis from a total of $n = 10$ perfused vessels) and control rats $n = 7$ ($n = 15$ z-stack files used for 3-D analysis from a total of $n = 7$ perfused vessels).

Retention of chylomicron-remnant particles and cholesterol *ex vivo*

The biochemical profile for chylomicron-remnant particles was determined prior to each experiment. Direct enzymatic colorimetric assays were used to measure triglyceride (Wako Chemicals) and total cholesterol (Wako Chemicals). ApoB48 was quantified using an adapted western immune blot technique as previously described (14).

The fluorescence intensity of the perfusate (Cy5-labelled remnant lipoproteins) per unit volume was also collected for every experiment, under identical conditions in the Z-series. The following formula was used to calculate the retention of apoB48 and cholesterol in the arterial tissue: concentration of apoB48 (mg/ml) or cholesterol (mg/ml) in the perfusate/intensity per unit volume of the perfusate × intensity per unit volume of the tissue.

Immuno-histochemistry

Sequential sections of perfused arterial tissue were immuno-stained for the proteoglycans biglycan and decorin (primary antibodies kindly provided by Dr Larry Fisher, Craniofacial and Skeletal Disease Branch, National Institutes of Dental Research, NIH, Bethesda, Maryland). Anti-sera directed against the core proteins of biglycan and decorin aggregates were used on sections pretreated with Chondroitinase-ABC (Sigma, St Louis, MO, USA) in order to remove dermatan sulphate chains. Biglycan and decorin antiserum were incubated at a titre of 1:600 and 1:800, respectively, for 1 h at room temperature. Following a PBS wash, fluorescent detection was with standard pre-conjugated fluorescent secondary antibody (horse anti-rabbit IgG conjugated to Cy3; Chemicon). Immuno-staining with the secondary antibody (1:5000 with 1% horse serum) alone was used as the negative control. Advanced glycated end product (AGE) detection in the vessel wall was assessed by immunoperoxidase technique using the polyclonal anti-advanced glycated end product antibody which detects carboxymethylysine-containing proteins, but not pentosidine [18].

Statistical analysis

All results are presented as mean ± SEM. Absolute postprandial total cholesterol, triglyceride, insulin, glucose and apoB48 responses were calculated as postprandial area under the curve (AUC) (0–8 h). Incremental postprandial variations in apoB48 were determined by subtracting the fasting baseline concentration from the AUC. Statistical comparisons for area and baseline measurements were determined by unpaired *t*-tests and Spearman's correlation coefficients were calculated to study associations. Results were considered significant at a *P*-value < 0.05. All analyses were performed with GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). B_{max} and K_d curves were generated using GraphPad Prism 4 for increasing lipoprotein concentrations using a non-linear regression curve and one site binding analysis.

Image analysis

Digital images of fluorescence associated with remnant lipoproteins and proteoglycans in arterial sections were generated utilizing confocal microscopy [16,17]. Three-dimensional z-series images containing both fluorescent lipoproteins and proteoglycans were subjected to analysis by Laser Pix® software (BIORAD, UK), in order to calculate colocalization coefficient(s). The calculations are based on Pearson's correlation analysis enabling accurate coefficient values with vastly different signal intensities.

Results

Subject characteristics

Fasting biochemical characteristics of Type 1 diabetes and control subjects are shown (Table 1). Fasting plasma glucose and insulin concentrations were modestly elevated in participants with Type 1 diabetes compared with control

Table 1 Fasting plasma metabolic characteristics of control subjects and subjects with Type 1 diabetes

Variable	Control subjects (n = 7)	Subjects with Type 1 diabetes (n = 9)	P-value
Glucose (mmol/l)	5.0 ± 0.2	7.1 ± 1.1	0.12
Insulin (pmol/l)	6.8 ± 1.9	16.4 ± 11.1	0.06
Total cholesterol (mmol/l)	4.9 ± 0.2	4.8 ± 0.3	0.58
LDL cholesterol (mmol/l)	3.0 ± 0.2	2.9 ± 0.3	0.64
HDL cholesterol (mmol/l)	1.5 ± 0.1	1.5 ± 0.1	0.82
Triglyceride (mmol/l)	0.9 ± 0.1	0.9 ± 0.1	0.69
ApoB48 (mg/l)	12.3 ± 1.0	22.8 ± 2.5	< 0.01
Subjects with a history of cardiovascular events	None	All subjects	

Values are given as means ± SEM.

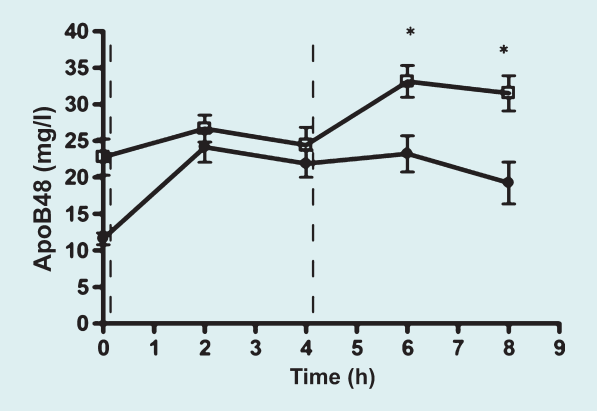

FIGURE 1 The postprandial-associated plasma apoB48 response (area under the curve; AUC) following the intake of sequential meals (represented by dotted lines). Data are shown for control subjects (black circles) and subjects with Type 1 diabetes (white squares) as mean ± SEM. ApoB48 total AUC (control subjects 169.8 ± 15.9 area units vs. subjects with Type 1 diabetes 222.9 ± 11.3 area units; $P < 0.01$) and time points 6 and 8 h (*P < 0.01 for both) $P < 0.01$ for both) are significantly elevated in subjects with Type 1 diabetes compared with the control group.

subjects, but these values did not reach statistical significance. The fasting lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol and triglyceride) for all subjects was within the normal range and did not significantly differ between the two groups. In contrast, fasting apoB48 was the only lipid-associated variable found to be higher in patients with Type 1 diabetes relative to control subjects (22.8 ± 2.5 and 12.3 ± 1.0 mg/l, respectively; $P < 0.01$).

Postprandial response of plasma insulin, glucose, triglyceride and cholesterol

Postprandial insulin and glucose concentrations are presented in the Supporting Information (Fig. S1). When compared with control subjects, consistent with the nature of long-standing diabetes, subjects with Type 1 diabetes showed a significantly elevated postprandial glucose AUC (64.4 ± 8.4 vs. 39.9 ± 1.8 area units; $P < 0.01$) and greater glucose concentration at time points 6 and 8 h ($P = 0.03$ and $P < 0.01$, respectively) following the lunch meal. The postprandial AUCs for cholesterol and triglyceride concentrations were not different between subjects with Type 1 diabetes and control subjects (see also Supporting Information, Fig. S2).

Postprandial response of plasma apoB48

The postprandial AUC response of plasma apoB48 from whole plasma demonstrated that subjects with Type 1 diabetes had a progressive and significant delay in the clearance of remnant particles over the 8-h time period (Fig. 1). Total plasma apoB48-AUC indicated that circulating concentrations of apoB48 were elevated by 31% in subjects with Type 1 diabetes compared with control subjects (AUC, 222.9 ± 11.3 and 169.8 ± 15.9 area unit, respectively; $P < 0.01$). Ingestion of sequential meals, consistent with a free-living situation, resulted in a biphasic response of plasma apoB48 in both patients with Type 1 diabetes and control subjects, which peaked at 2 h following the initial meal (i.e. breakfast) (patients with Type 1 diabetes, 26.1 ± 1.9 mg/l; control subjects, 25.5 ± 1.7 mg/l) and then at 6–8 h following the second meal (i.e. lunch) (patients with Type 1 diabetes, 33.1 ± 2.2 mg/l; control subjects 22.8 ± 2.2 mg/l). However, in subjects with Type 1 diabetes, plasma circulating apoB48 concentration was 45% greater at 6 hours ($P < 0.01$), which progressively increased to 69% at 8 h ($P < 0.01$) following a second meal (i.e. lunch) relative to controls. Corresponding incremental area under the curve (iAUC) analysis showed that following intake of a second meal (i.e. lunch), iAUC was 5-fold higher in subjects with Type 1 diabetes compared with control subjects (iAUC 4–8 h, 25.0 ± 2.2 and 5.0 ± 1.4 area units; $P = 0.044$) (data not shown).

ApoB48 measurements in triglyceride-rich lipoprotein fractions

ApoB48 was also quantified from the fasting triglyceride-rich lipoprotein fraction (density < 1.006 g/ml) to represent newly synthesized intestinally derived particles. ApoB48 concentration in triglyceride-rich lipoproteins (6.0 ± 1.5 vs. 2.3 ± 1.2 mg/l; $P = 0.19$) and for triglyceride-rich lipoproteins-AUC (59.2 ± 14.3 vs. 41.1 ± 4.1 area units; $P = 0.58$) tended to be higher in subjects with Type 1 diabetes compared with control subjects, but these differences did not reach statistical significance. These observations are consistent with the notion that measuring apoB48 from whole plasma is more reflective of systemic remnant particle metabolism per se [19].

Table 2 Biochemical profile of control and insulin-deficient animals

Variable	Controls (n = 7)	Insulin deficient (n = 10)
Weight (g)	415.6 ± 16.3	252.6 ± 61.4*
Triglyceride (mmol/l)	1.23 ± 0.24	1.44 ± 0.17
Cholesterol (mmol/l)	1.74 ± 0.061	2.11 ± 0.14*
Glucose (mmol/l)	7.36 ± 0.19	34.1 ± 1.54*
Serum AGE peptide (units/ml)	4.73 ± 0.77	8.35 ± 1.37*
Arterial intimal thickness (μm_2)	13 150 ± 3011	26 730 ± 9269†

Wistar rats were made diabetic by streptozotocin (STZ) injection.
Values represent mean ± SD.
Significant differences between control and diabetic groups were determined by non-paired Student's t-test analysis.
Significant differences (*P < 0.0001) between control and diabetic animals associated with animal body weight, plasma cholesterol concentration, plasma glucose concentration and arterial intimal thickness (measured as absolute volume μm^3, †P = 0.01).
AGE, advanced glycated end product.

Experimental animal model of Type 1 diabetes—physiological variables

Insulin-deficient (n = 10) rats had a reduced body weight, elevated plasma glucose and total plasma cholesterol, as well as an increase in arterial intimal thickness compared with control (n = 7) rats (Table 2). Furthermore, insulin-deficient rats had a 1.5-fold increase in circulating serum glycated peptides, compared with control rats indicative of mature diabetes (Table 2).

Arterial uptake of remnant lipoproteins

Insulin-deficient rats were observed to have a more extensive and diffuse distribution of fluorescence associated with remnant lipoproteins in the arterial tunica compared with control animals (Fig. 2a). Whilst both groups displayed focal retention of remnant lipoproteins in the tunica intima, diabetic rats appeared to have increased particle retention in the intima. Consistent with this, the mass of cholesterol derived from remnant lipoproteins was elevated seven-fold higher in the intima of vessels from the diabetic rats compared with controls (Fig. 2b).

In vitro binding of remnant lipoproteins to human biglycan

In order to demonstrate direct binding of remnant lipoproteins to biglycan, we employed an *in vitro* solid-phase binding assay. Interestingly, despite differences in lipid content and size (see also Supporting Information, Table S2), remnants, LDL and lymph chylomicrons, bound recombinant human biglycan with equal affinity, as assessed by K_D values 0.66, 0.63 and 0.63,

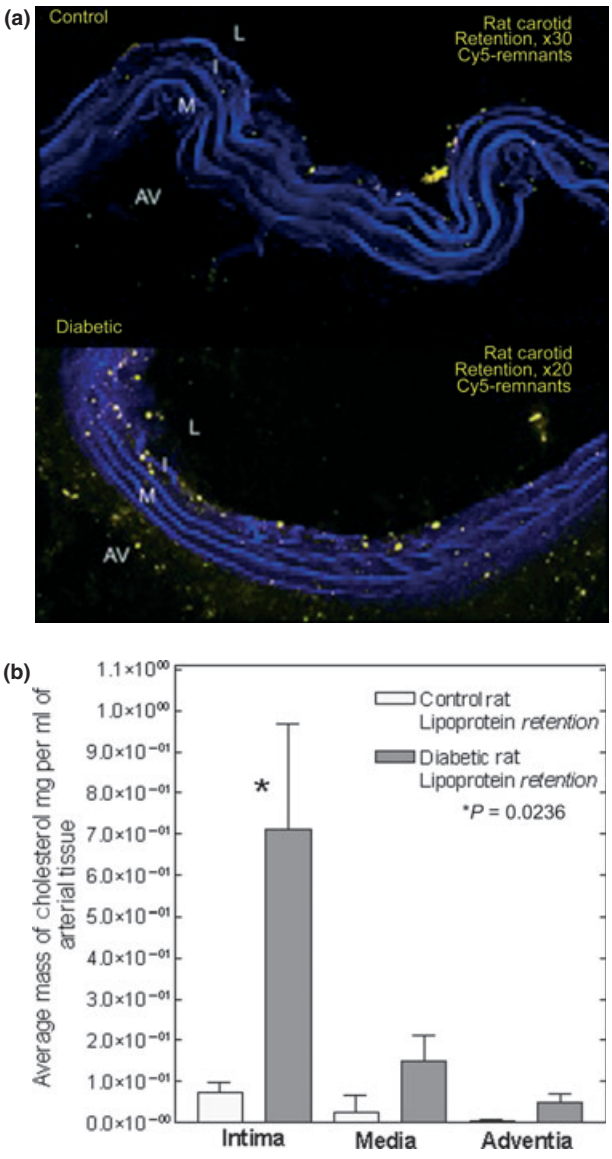

FIGURE 2 (a) Fluorescence associated with the retention of remnant lipoproteins shown for control (upper panel × 30) and insulin-deficient rats (lower panel × 20) following *in situ* arterial perfusion. Representative images from n = 10 insulin-deficient rats (n = 20 z-stack files used for 3-D analysis from a total of n = 10 perfused vessels) and control rats n = 7 (n = 15 z-stack files used for 3-D analysis from a total of n = 7 perfused vessels). Morphology of the vessel wall is shown in blue and fluorescence associated with remnant lipoproteins is shown in yellow. AV, adventitia; I, intima; L, lumen; M, media. (b) Quantification of average mass of cholesterol associated with remnant lipoproteins retained in carotid vessels of both insulin-deficient (grey bars, n = 20 z-stack files used for 3-D analysis from a total of n = 10 perfused vessels) and control rats (white bars, n = 15 z-stack files used for 3-D analysis from a total of n = 7 perfused vessels). Values indicate the average of three-dimensional 'stacks' of z-sectioned images assessed for fluorescence with corresponding SEM. Statistical difference was found between the retention of cholesterol in control intima vs. retention of cholesterol in diabetic intima (*P = 0.0236).

respectively (Fig. 3a, and see also Supporting Information, Table S3). In contrast, LDL had a significantly greater B_{max} as compared with either that of lymph chylomicrons (P < 0.05) or

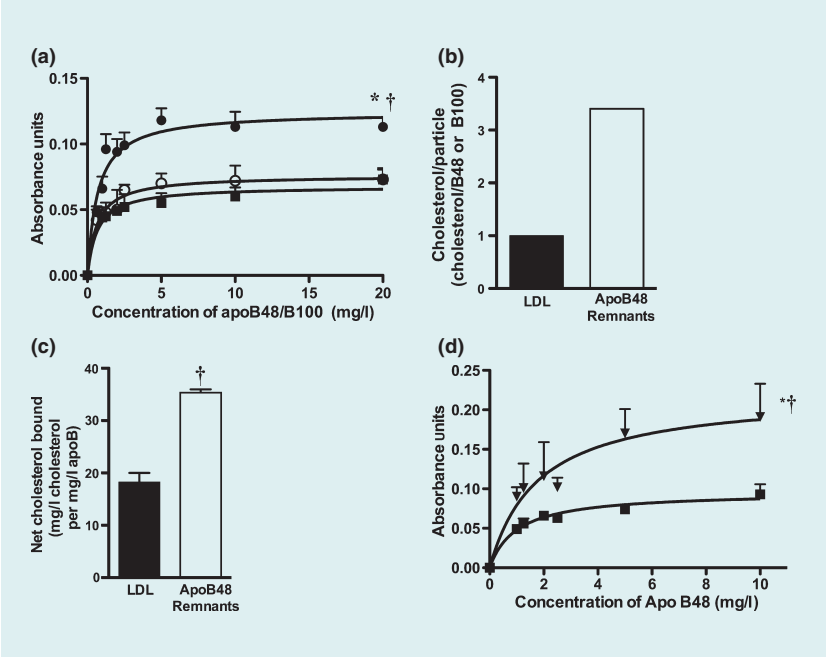

FIGURE 3 (a) Binding of recombinant human biglycan to lipoprotein fractions. *$P < 0.05$, where B_{max} for LDL (1.02–1.063 g/ml) (black circles) was compared with native triglyceride-rich lipoproteins (white circles) ($n = 3$). †$P < 0.05$, where LDL (black circles) was compared with B_{max} for remnant lipoproteins (< 1.006 g/ml) (black squares) ($n = 3$). Non-linear regression analysis using one-site binding (GraphPad Prism) hyperbola was used. (b) Fold difference in cholesterol per particle (cholesterol/ApoB, from particle characteristics). (c) Calculated net cholesterol bound (mg/l cholesterol per mg/l apoB) associated with remnant lipoproteins and LDL at B_{max} †$P < 0.001$ and $n = 3$. (d) Binding of glucose-modified biglycan (black triangles) with remnant lipoproteins. †$P = 0.01$, when compared with K_D of binding with normal biglycan (black squares) ($n = 3$). *$P < 0.0001$, when compared with B_{max} of binding with biglycan ($n = 3$).

remnants ($P < 0.05$) (Fig. 3a). Conversely, remnant lipoproteins contained 2.5-fold greater cholesterol per particle as compared with LDL (Fig. 3b). Consequently, the binding of remnant-associated cholesterol at B_{max} was significantly greater (1.9-fold; $P < 0.001$) as compared with LDL-associated cholesterol (Fig. 3c). Modification of biglycan (pre-incubation with glucose) resulted in a 2.3-fold increase in B_{max} ($P < 0.0001$) and a 1.7-fold increase in K_D ($P < 0.01$) for remnant lipoproteins, suggestive of preferential binding to glycated matrix components (Fig. 3d).

Arterial expression of proteoglycans and arterial glycated proteins

Immunohistochemical imaging of vessels from rats with diabetes displayed an increased accumulation of biglycan relative to control rats (see also Supporting Information, Fig. S3). Our findings are consistent with previous studies [20] that suggest Type 1 diabetes causes arterial remodelling and can increase vascular proteoglycan expression. Moreover, we observed focal accumulation of biglycan and a high degree of remnant lipoprotein retention in the tunica intima (Fig. 4). Image analysis also revealed a significant association between remnant lipoproteins and biglycan (colocalization coefficient =0.71 ± 0.15;p<0.01). In addition, there was a high degree of association between remnant lipoproteins and glycated protein in focal clusters (colocalization coefficient = 0.78 ± 0.13; $P < 0.01$) (Fig. 5). These findings are consistent with the concentration of relative serum advanced glycation end products in diabetic vs. control rats (Table 2). The arterial expression of proteoglycan decorin was not different between the control and diabetic rats (see also Supporting Information, Fig. S4) and minimal colocalization was observed for decorin with remnant lipoproteins (data not shown).

Relationship of glycated proteins and proteoglycans with remnant lipoprotein-derived arterial cholesterol retention

The colocalization of biglycan and remnant lipoproteins was shown to have an inverse relationship with the mass of cholesterol retained in the arterial wall ($r^2 = –0.6236$, $n = 12$) (Fig. 6a). Therefore, increasing retention of remnant cholesterol could not be fully explained by the association with biglycan alone, suggesting that additional binding factors are involved. Interestingly, this relationship was reversed for glycated proteins, in which there was a positive linear association with increasing mass of cholesterol retained in the arterial wall ($r^2 = 0.77$, $n = 14$) (Fig. 6b).

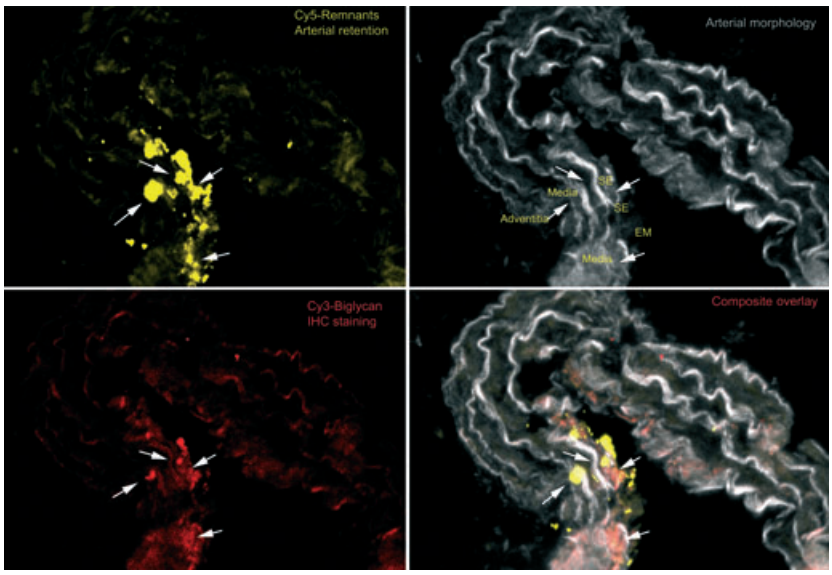

FIGURE 4 Colocalization of proteoglycans and remnant lipoproteins in arterial vessels of insulin-deficient rats. Fluorescent lipoproteins were perfused through carotid arteries under physiological conditions (yellow panel). Select arterial proteoglycans (biglycan) were simultaneously stained via immunohistochemistry (IHC) (red panel). Sequential fluorescent emissions, including arterial morphology (greyscale) were collected as a z-series stack via confocal microscopy and digitally overlaid (composite panel). Pearson's colocalization coefficient was 0.71 ± 0.15. Arrows indicate regions of colocalization of both lipoprotein retention and proteoglycan immunohistochemistry. Representative images from n = 10 insulin-deficient rats (n = 20 z-stack files used for 3-D analysis from a total of n = 10 perfused vessels). EM, endothelial monolayer; SE, sub-endothelial layer. Magnification × 40.

Discussion

Impaired ApoB48-remnant lipoprotein metabolism in Type 1 diabetes

We report a significantly higher concentration of fasting remnant lipoproteins (measured as apoB48) among normolipidaemic subjects with long-standing Type 1 diabetes (Table 1). Importantly, fasting plasma apoB48 concentration was the predominant lipoprotein-associated difference between subjects with Type 1 diabetes and matched control subjects. Furthermore, participants with Type 1 diabetes demonstrated a postprandial deficit in the clearance of remnant lipoproteins from plasma, with a greater total plasma apoB48-AUC relative to control subjects. While the corresponding total iAUC in subjects with Type 1 diabetes was not different from control subjects following the initial meal (breakfast), the second meal (lunch) appeared to exacerbate the inability of subjects with Type 1 diabetes to efficiently metabolize remnant lipoproteins (Fig. 1). We wish to highlight that net AUC is an important indicator of total cholesterol associated with remnant lipoproteins when considering exposure to the arterial wall. It is also important to note that individuals are in the post-absorptive state repeatedly throughout the day and, typically, individuals would ingest a third meal. Thus, we propose that impaired remnant metabolism under these conditions may contribute to increased cardiovascular disease risk in Type 1 diabetes and this may be independent of other risk factors such as fasting lipid profile, body weight and age.

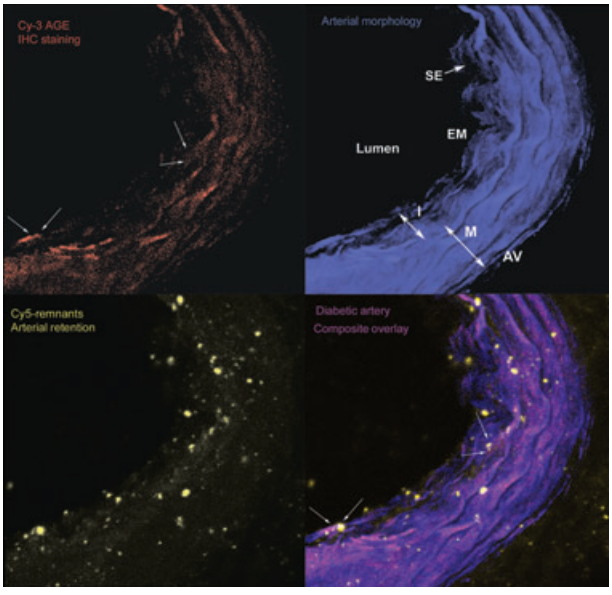

FIGURE 5 Focal points of colocalization of fluorescence associated with remnant lipoproteins and glycated proteins in carotid arterial tissue is shown for insulin-deficient animals (× 40 magnification) in composite overlay. Morphology of the vessel wall and fluorescence associated with remnant lipoproteins and glycated proteins is shown in blue, yellow and red at × 40 magnification, respectively. Pearson's colocalizaton coefficient was 0.78 ± 0.13. Representative images from n = 10 insulin-deficient rats (n = 20 z-stack files used for 3-D analysis from a total of n = 10 perfused vessels). AGE, advanced glycated end product; AV, adventitia; IHC, immunohistochemistry; EM, endothelial monolayer; I, intima; M, media; SE, sub-endothelial layer.

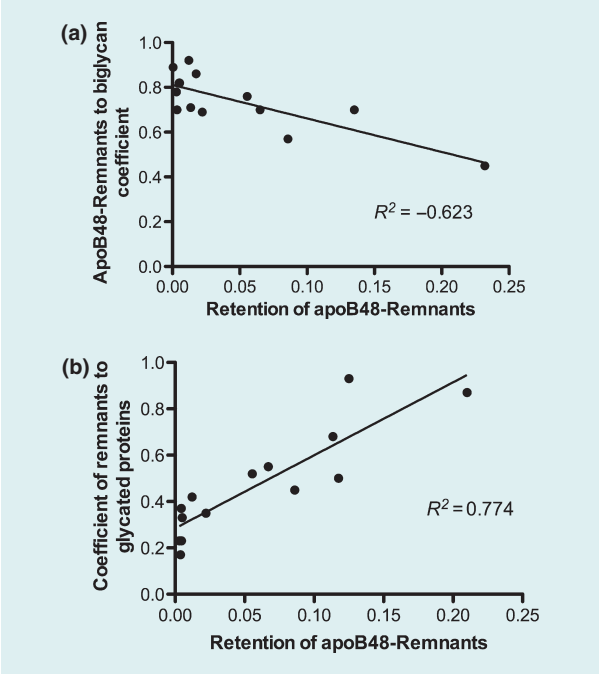

FIGURE 6 (a) Relationship of Pearson's colocalization (remnant lipoproteins to biglycan) to the amount of fluorescence associated with cholesterol in arterial vessels *in situ*. Data from n = 10 insulin-deficient rats (n = 20 z-stack files used for 3-D analysis from a total of n = 10 perfused vessels). (b) Relationship of Pearson's colocalization (remnant lipoproteins to glycated proteins) to the amount of fluorescence associated with cholesterol in arterial vessels *in situ*. Data from n = 10 insulin-deficient rats (n = 20 z-stack files used for 3-D analysis from a total of n = 10 perfused vessels).

Mechanisms of impaired remnant lipoprotein metabolism in Type 1 diabetes

Although not addressed in the present study, mechanisms underlying increased and prolonged residence time of apoB48-remnant lipoproteins in Type 1 diabetes *per se* have been associated with decreased lipase activity in patients with diabetes [21]. However, if lipase activity was increased in our subjects, we would have expected to observe a corresponding increase in either fasting or postprandial AUC response for triglyceride and/or triglyceride-rich lipoprotein apoB48 (density < 1.006 g/ml). Other investigators have suggested that remnant particle clearance may be impaired in Type 1 diabetes because of altered particle composition affecting high-affinity uptake by receptors [8,22,23] and/or increased competition with VLDL for receptor-mediated clearance from plasma [24]. A similar conclusion was reached by Georgopoulos and Phair [8] using low-dose ^{125}I-labelling of apoB particles in Type 1 diabetes. Another possible and more novel mechanism is the finding of increased lipid synthesis and/or secretion from the intestine *per se*, which contributes to the total systemic pool of cholesterol. Overproduction of intestinal chylomicrons has recently been demonstrated in animals and humans with insulin resistance [25] as well as Type 2 diabetes [26,27]. Studies in streptozotocin-induced diabetic rats have also shown both greater intestinal absorption [28] and increased synthesis of cholesterol [29].

Retention of remnant lipoproteins with biglycan in arterial vessels

In this study, all subjects with Type 1 diabetes had significant clinical vascular disease; however, whether elevated plasma remnant concentration and/or the impaired postprandial metabolism of remnant particles is casually associated with the development of atherosclerosis remains unknown. Using radiolabelled techniques, we have previously reported that arterial retention of remnant lipoproteins is greater in arteries of diabetic rodents when compared with controls [30]. To further address the relationship with arterial proteoglycans, we show in this study that the retention of remnant lipoproteins in arterial tissue was increased and found to colocalize with biglycan in focal sites in the tunica intima (Figs 2b and 4). Indeed, our findings in the insulin-deficient rat model are congruent with an increased content of arterial proteoglycan as observed in subjects with diabetes [31–33]. Our results are also consistent with the colocalization of biglycan with apoB/E and lipid-rich areas within human atherosclerotic lesions [34,35]. Long-standing work by Wight and co-workers has shown that glycosaminoglycan chains of the proteoglycan molecule are negatively charged and bind irreversibly with apoB/apoE-containing lipoproteins, as well as inflammatory proteins [36–38]. Importantly, the colocalization of biglycan with apoE and/or apoB has been shown to be predominant with macrophage infiltrated regions of arterial vessels [34,35]. Further, it has been demonstrated that apoB48-remnant lipoproteins specifically do not require pre-oxidation (or any chemical modification) in order to induce foam cell formation by human monocyte-derived macrophages [39–41]. Collectively, these results support the hypothesis that accelerated atherogenesis observed in diabetes may involve the direct association of remnant lipoproteins with biglycan in the extracellular matrices of the tunica intima.

Direct binding of remnant lipoproteins to biglycan

Remnant lipoprotein preparations were shown to have significant binding affinity for recombinant human biglycan *in vitro* (Fig. 3a). Despite differences in lipid composition and/or size between native chylomicron, remnant and LDL preparations, we did not observe any difference in the binding affinity between these three lipoprotein classes. These results suggest that other factors such as the apolipoprotein content (e.g. apoE, apoCIII), charge and/or density of remnant lipoproteins may be more important in determining binding affinity with biglycan [31]. We have previously shown that small dense remnant lipoproteins, rather than larger triglyceride-rich lipoproteins, readily penetrate the arterial wall [16]. Collectively, therefore, it appears that the physical size of a lipoprotein is critical for vascular permeability, but is not a major

factor in determining extracellular attachment to proteoglycans, in particular biglycan *per se*.

Accelerated atherogenesis during Type 1 diabetes because of glycation

It is well established that one of the primary consequences of prolonged hyperglycaemia during Type 1 diabetes is the glycation of proteins and/or formation of advanced glycated end products. Glycated proteins are thought to contribute to diabetic complications by interacting with their receptors (RAGE) on the cell surface and by formation of cross-links in the extracellular matrix [42,43]. Formation of glycated proteins in the matrix has been shown to lead to arterial stiffness, altered properties of matrix proteins and increased trapping of LDL [43,44]. Indeed, we observed increased binding of remnant lipoproteins with glycated forms of biglycan (Fig. 3d). Consistent with this, remnant lipoprotein colocalization with glycated proteins in focal clusters within the arterial wall is suggestive of causality (Fig. 5). It would now be of interest to determine whether the glycated proteins in the vessel wall form as a result of glycation of collagen, proteoglycans and/or because of accumulation of glycated proteins via endocytosis. Biglycan is known to interact with both glycated and non-glycated collagen [45]. It is also noteworthy that there was a simultaneous increase in the retention of cholesterol derived from remnant lipoproteins associated with increased glycation, a relationship that was not observed for non-glycated biglycan (Fig. 6b). Therefore, we speculate that binding of biglycan to glycated collagen may provide additional cross-links which may facilitate further remnant lipoprotein binding to the matrix in conditions of hyperglycaemia.

The complexity of hyperglycaemia and its potential consequence to vascular disease is multifactorial. Glycated proteins appear to have independent pro-inflammatory effects [46,47]. One could speculate that glycation within the arterial wall may not only exacerbate the rate of lipoprotein retention, but extenuate the subsequent inflammatory response, including enhancing macrophage recruitment mediating accelerated atherogenesis in diabetes [48]. We also know that hyperglycaemia can lead to glycation or glycoxidation of the lipids within the lipoprotein particle itself, which in turn could further accelerate atherosclerosis [48]. In the present study, all remnant lipoprotein preparations were obtained from healthy non-diabetic donor rats. Future studies are needed to determine the potential effect of insulin deficiency and/or hyperglycaemia on modifications of remnant lipoproteins *per se* and how these factors may relate to diabetic atherosclerosis.

In conclusion, the results from this study demonstrate that subjects with Type 1 diabetes have a pro-atherogenic lipoprotein profile with elevated fasting and impaired postprandial metabolism of remnant lipoproteins, which may predispose these subjects to the development of cardiovascular disease. We propose that apoB48-remnant lipoproteins bind directly to known atherogenic arterial proteoglycans involved in atherogenesis and that remnant retention by the arterial wall in diabetes is exacerbated. Evidence from these studies supports the hypothesis that increased cardiovascular disease risk in Type 1 diabetes may result from increased exposure of remnant lipoproteins to the vasculature and that glycation in the arterial wall may facilitate the retention of remnant lipoprotein-derived cholesterol directly. Collectively, impaired metabolism of remnant lipoproteins in Type 1 diabetes may contribute to the deposition of cholesterol in arterial tissue and, at least in part, the accelerated progression of atherosclerosis in the hyperglycaemic and insulin-deficient state.

Competing interests

Nothing to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The postprandial response in plasma insulin (a) and glucose (b) (AUC) for subjects with Type 1 diabetes and control subjects following the intake of sequential meals.

Figure S2. The postprandial response in plasma triglyceride (a) and cholesterol (b) (AUC) for subjects with Type 1 diabetes and control subjects following intake of sequential meals.

Figure S3. Immunohistochemistry for biglycan in control and diabetic arteries.

Figure S4. Immunohistochemistry for decorin in control and diabetic arteries.

Table S1. Three-day food records of subjects with Type 1 diabetes and matched control subjects.

Table S2. Particle characteristics of lipoproteins used in binding experiments.

Table S3. Binding constants for the binding assay of lipoprotein fractions to recombinant human biglycan.

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Appendix B

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Model of intestinal chylomicron over-production and Ezetimibe treatment: Impact on the retention of cholesterol in arterial vessels

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Abstract

The metabolic syndrome (MetS) and conditions of insulin resistance are often characterized by an increase in cardiovascular disease (CVD) risk without a concomitant increase in low-density lipoprotein cholesterol (LDL-C), suggesting that other atherogenic pathways maybe involved. Intestinally derived chylomicron remnants (CM-r) are also thought to contribute to atherogenic dyslipidemia during insulin resistance. Recent animal and human studies suggest that insulin resistance leads to an over-production of intestinal chylomicrons (CM), which can contribute to fasting and post-prandial dyslipidemia during these conditions. We and others have contributed new insights into the mucosal absorption, efflux and secretion of cholesterol and triglyceride (TG) in intestinal CM during conditions of insulin resistance. One of the pertinent discoveries has been that the intestine has the capacity to increase the secretion of CM during conditions of hyper-insulinemia (observed in the JCR:LA-*cp* rat model).

Advances to identify cholesterol-transporter targets have highlighted the contribution of the intestine to whole body cholesterol metabolism. Ezetimibe (EZ) is a novel pharmaceutical compound that reduces intestinal cholesterol absorption. We know that Ezetimibe either alone, or in combination with a HMG-CoA reductase inhibitor (such as Simvastatin [SV]) can decrease both plasma LDL-C and CM-r concentrations. However, the combined effects of these compounds (EZ + SV) on post-prandial dyslipidemia and/or impact on arterial deposition of cholesterol during MetS have not been studied. The focus of this review is to highlight studies using an animal model of MetS and CM over-production (the JCR:LA-*cp* rat), and to summarize the effects of Ezetimibe on intestinal cholesterol flux, CM metabolism and uptake of cholesterol into arterial vessels.

Keywords: Lipid metabolism; Insulin resistance; Metabolic syndrome; apoB48; Ezetimibe; Simvastatin; Cholesterol transport; Lipid absorption; JCR:LA-cp rats

Contents

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1. Increased risk of cardiovascular disease during the metabolic syndrome

The MetS is often characterized by an increase in cardiovascular disease (CVD) risk without a concomitant elevation in plasma low-density lipoprotein cholesterol concentration (LDL-C), suggesting that other pathways are contributing to atherogenic risk [1–3]. Recent studies have indicated that intestinal apolipoprotein-B48 (apo-B48) containing chylomicron remnants (CM-r) (in both fasted and post-prandial states), contribute to atherogenesis and are a significant risk factor for CVD ([4] and see other articles in this issue). The consequence of impaired clearance of small dense, cholesterol-rich CM-r contributing to atherogenesis, is proposed to be due to increased arterial exposure, permeability, retention and subsequent accumulation of these particles within the vessel wall [5,6]. Furthermore, evidence from animal and human studies have shown that insulin resistance (IR) can lead to the over-production of intestinally derived CM-r, which may contribute increased circulating concentrations of CM-r associated with post-prandial dyslipidemia [7]. Clinical studies from our own group also suggest that elevated concentrations of CM-r are prevalent during childhood obesity ([8] and abstract by Pendlebury et al., in this issue) and in type 1 diabetic subjects [9], despite the absence of a classical dyslipidemic profile.

2. Using a combination of pharmaceutical targets to inhibit cholesterol absorption and synthesis

It is well established that the total plasma cholesterol concentration is mediated via the metabolic cross-talk between endogenous (hepatic) synthesis and exogenous (intestinal) dietary and biliary reabsorption pathways. HMG-CoA reductase inhibitors (or statins) are widely used for treatment of hypercholesterolemia due to their plasma cholesterol lowering effects by blocking hepatic cholesterol synthesis. In recent years there has been a significant focus to identify cholesterol-transport (absorption) targets that have highlighted the function of the intestine as a major contributor to systemic cholesterol homeostasis. For example, Ezetimibe is a novel pharmaceutical compound that reduces intestinal cholesterol absorption [10]. Ezetimibe either alone, or in combination with statins can decrease the plasma LDL-C and CM-r concentration in humans [11,12]. However, the combined effects of Ezetimibe and statins on post-prandial dyslipidemia and/or arterial retention of CM-r, specifically under conditions of insulin resistance has remained unclear. These questions have been a focus of our group, and thus the purpose of this review is to summarize recent data in the context of known advances in intestinal cholesterol transport, CM metabolism and atherosclerosis.

3. Over-production of chylomicrons during obesity and insulin resistance in the JCR:LA-cp rat

In our laboratory we have developed an adapted lymphatic cannulation (fistulae) procedure that has enabled us to directly measure intestinal lymphatic lipid (triglyceride and cholesterol), as well as apo-B48 (CM) secretion during the basal (fasted) and post-prandial (fed) state. These methods have been invaluable in our understanding of the dynamic flux of lipids derived from the intestine, particularly during conditions of insulin resistance. Using lymph collected from JCR:LA-*cp* rats (a model of insulin resistance and MetS [13,14]), during basal (fasted state) and post-prandial (fed) conditions, we have consistently observed increased concentration of apo-B48 (or the number of CM particles secreted by the intestine) compared to lean (control) rats [4]. Increased production of CM particles in the post-prandial state has been suggested to be an adaptive response as a result of increased lipid availability within the enterocyte during insulin resistance [15,16]. Indeed, the JCR:LA-*cp* rat has been shown to have hypertrophy of the intestinal mucosal villi and an increase in the number of enterocytes per surface area [15]. It is plausible that the intestinal hypertrophy observed in the JCR:LA-*cp* rat may directly contribute to over-production of CM and increased lipid secretion during hyperphagia and/or obesity leading to increased CVD risk.

It is now also recognized that as insulin resistance progresses, the impact on the intestine, specifically enterocyte lipid metabolism is likely to be complex and multi-factorial. For example, an increase in intestinal and intracellular nutrient substrate availability during MetS may also explain enhanced *de novo* lipidogenesis and/or elevated storage capacity. Elegant mechanistic studies by Adeli et al. using a complementary fructose fed hamster model of CM over-production and insulin resistance, has provided breakthroughs in the understanding of intracellular lipidogenic dysfunction in the enterocyte [17], as well as other relevant studies conducted by Hussain et al. [18] and Zoltowska et

al. [19]. Zoltowska et al. has shown that intestinal lipogenic enzymes [such as monoacylglycerol transferase (MGAT) and diacylglycerol transferase (DGAT)] are upregulated in the insulin resistant sand rat [19]. While work by Hussain et al. has provided unique insights into the molecular regulation of microsomal transfer protein (MTP) and how this can control apo-B48 availability for particle lipidation ([18,20] and review by Hussain et al in this issue).

4. Intestinal cholesterol absorption

Cholesterol absorption is a multistep process and is dependent on the net difference between 'influx' (mucosal to serosal) and 'efflux' (serosal to mucosal) of intra-luminal cholesterol across the brush border membrane [21]. However, it is not entirely clear what proportion of exogenous dietary/biliary cholesterol is directly incorporated into CM and transported in the lymph, or if additionally, there is also a contribution from other sources. These other sources could include newly synthesized (*de-novo)* or intracellular stores of cholesterol within the enterocyte, and/or even contributions from plasma-derived cholesterol. Collectively the pathways of intestinal cholesterol metabolism are poorly understood both under normal physiological conditions and during the metabolic milieu of insulin resistance (see proposed model in Fig. 1).

5. Enterocyte transporters responsible for cholesterol influx

The Niemann Pick C1-Like 1 protein (NPC1L1) is considered a primary transporter involved in facilitating luminal cholesterol absorption or influx into the enterocyte [22]. The NPC1L1 transporter is abundantly expressed in the intestine, particularly in the jejunum (brush boarder membrane) and has also been proposed to act as an intracellular trafficker of cholesterol from the basolateral membrane of the enterocyte to other cytosolic compartments, such as the endoplasmic reticulum [23]. Substantial literature also supports the role of the scavenger receptor class-B type 1 (SR-B1) in cholesterol uptake (specifically in duodenal and jejunal mucosal membranes); whereas FAT/CD36 may be involved in cholesterol influx at the jejunal and ileal mucosal membranes [24] (Fig. 1).

6. Enterocyte transporters responsible for cholesterol efflux

ATP-Binding Cassette (ABC)-G5 and ABCG8 are also located at the mucosal brush border membrane of the enterocyte [25]. The expression of ABCG5/G8 is greatest in the duodenum and jejunum, and they are proposed to function in tandem (heterodimer) to efflux cholesterol (primarily, but not exclusively, plant sterols) from the enterocyte back into the lumen for fecal excretion [25].

The trans-intestinal cholesterol efflux pathway (or TICE) is intriguing in that it has been proposed to be responsible for 'reverse' transport of cholesterol directly from plasma to the intestinal lumen. During TICE, cholesterol is thought to be transported across the basolateral membrane into the enterocyte, and then shuttled to the mucosal membrane for efflux out into the lumen, contributing to cholesterol elimination in the faeces [26] (Fig. 1). In a recent study, stable isotopically labeled cholesterol was used to assess the fate of *de novo* synthesized cholesterol, and these studies revealed that TICE is impaired in mice lacking ABCG5 [27]. These studies have supported the notion that the ABCG5/8 heterodimer are intimately regulated as part of the TICE pathway (see also review by Kuipers et al., in this issue).

7. Net cholesterol absorption during insulin resistance

Intestinal cholesterol transport may be altered in subjects with diabetes and/or insulin resistance. Lally et al. revealed that the expression of ABCG5 and ABCG8 mRNA in subjects with type-2 diabetes [28] was significantly lower; and that NPC1L1 mRNA was significantly higher, compared to control subjects. Furthermore, alterations in transporter expression in subjects with type-2 diabetes have been associated with an increase in plasma cholesterol and apo-B48 concentration. Collectively, the data suggests that diabetic subjects may have an impaired capacity to efflux cholesterol while simultaneously increasing net cholesterol absorption [28].

Preliminary studies by our own group using the JCR:LA-*cp* rat are exploring whether alterations in cholesterol transport maybe involved in CM over-production and/or CM cholesterol enrichment during insulin resistance [15]. Using '*Ussing Chamber*' techniques (isolated jejunum segments mounted in a diffusion chamber under physiological conditions, in order to determine substrate transport across tissue) we have revealed that both mucosal-to-serosal (in)flux and serosal-to-mucosal (ef)flux are both elevated in the insulin resistant JCR:LA-*cp* rat. However, interestingly, the overall *net cholesterol influx* (i.e. influx–efflux) was greatest in the insulin resistant state relative to control, consistent with the clinical data from that of Lally et al. Further studies are now ongoing to delineate whether this particular observation is an adaptive response of the intestine and/or a different phenomenon of cholesterol transport in this animal model *per se*.

8. Effects of Ezetimibe on intestinal cholesterol absorption

Ezetimibe selectively inhibits intestinal cholesterol absorption through the NPC1L1 transporter [10]. Evidence

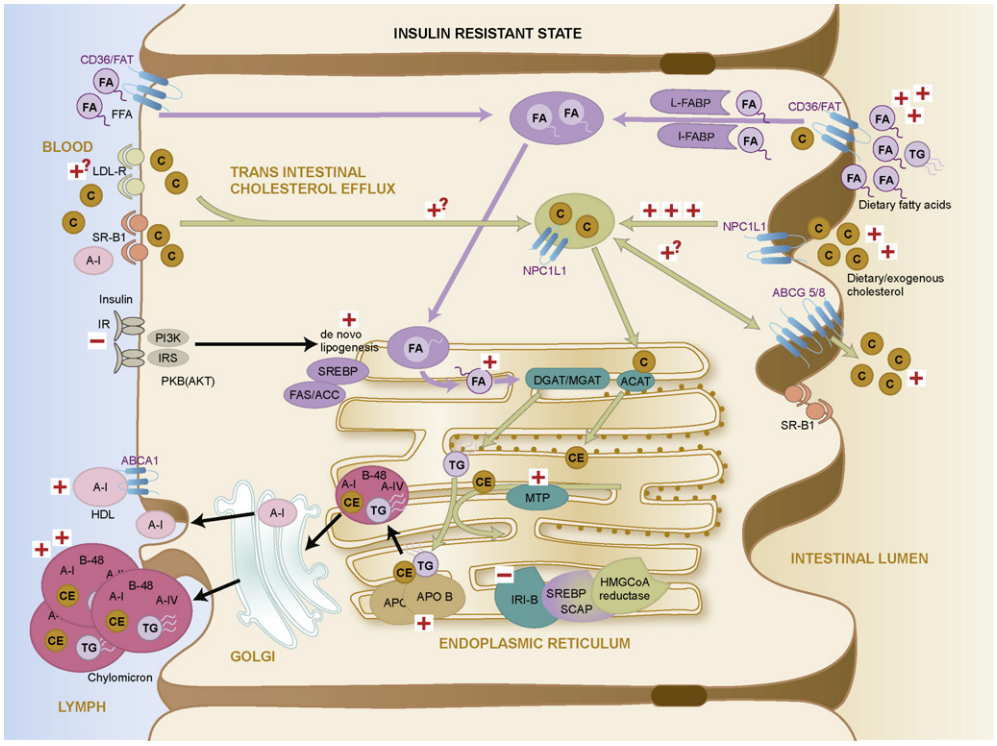

Fig. 1. Proposed schematic model of cholesterol and triglyceride flux within the intestinal epithelium (enterocyte) during dietary absorption (influx), chylomicron secretion and insulin resistance.

Under normal physiological conditions incoming cholesterol ('C') and triglyceride ('TG' and 'FA') is presented to the brush boarder membrane of the enterocyte (right side of panel) either from dietary or biliary sources to interact with the undisturbed layer and facilitated transporters (i.e. CD36/FAT, NPC1L1/SR-B1). It is likely that both TG and sterols are chaperoned intracellularly to interact with a number of trafficking proteins and/or endosomal-like compartments that regulate homeostasis between absorption, uptake, efflux and lipoprotein secretion within the cell. Efflux of sterols out of the cell via the brush boarder membrane is possible through the activity of ABCG5/G8 activity, also likely to be regulated as part of absorption and synthesis.

Trans-intestinal cholesterol efflux (TICE) has been proposed to contribute to intracellular concentration of sterols by the uptake plasma cholesterol from LDL-R and SR-B1 via the basolateral membrane (left side of panel), and able to excrete sterols out of the enterocyte via the brush boarder membrane (arrows left to right) and ABCG5/G8. It is also conceivable that the enterocyte may also uptake plasma FA from the basolateral membrane via CD36/FAT.

FAs are re-packaged into TG prior to the export as lipoproteins. There is evidence to suggest that the enterocyte can export lipoproteins via both apoB dependent (chylomicron) and apoB independent (HDL) pathways. Intracellular fatty acid and cholesterol synthesis machinery contribute substantially to the regulation of substrate availability in order to control the lipidation of apoB particle. Both fatty acid and cholesterol synthesis machinery can be influenced by insulin signaling pathways and maybe dysfunctional during conditions of insulin resistance.

Insulin resistance has been shown to affect the availability of apoB by increasing MTP activity resulting in over-production and excess particle export. It is not yet clear whether the enterocyte can compensate during insulin resistance to adequately maintain intracellular fatty acid and/or cholesterol homeostasis or whether increased dietary (and/or plasma) substrate exacerbates over-production of lipoprotein particles.

LDL-R: low-density lipoprotein receptor; SR-B1: scavenger receptor class B, type I; IR: insulin receptor; IRS insulin receptor substrate; PI3K: phosphoinositide 3-kinase; PKB (AKT): protein kinase B; TG: triglyceride; SREBP: sterol regulatory element binding protein; FAS/ACC: fatty acid synthase/Acetyl-CoA carboxylase; DGAT: diacylglycerol acyltransferase; MGAT: monoacylglycerol acyltransferase; ACAT: acyl-coenzyme A:cholesterol acyltransferase; MTP: microsomal triglyceride transfer protein; IR1-B IRE1-beta Inositol-requiring 1: aka endoplasmic reticulum (ER) to nucleus signaling 1 (ERN1) isoform beta; SCAP: SREBP cleavage-activating protein; NPC1L1: Niemann-pick C1 Like 1; HMGCoA: 3-hydroxy-3-methyl-glutaryl-CoA reductase; CD36/FAT: cluster of differentiation 36/fatty acid translocase; ABCG5/G8: ATP-binding cassette sub-family G member 5/member 8; L-FABP: liver fatty acid binding protein; I-FABP: intestinal fatty acid binding protein; HDL: high-density lipoprotein; B48: apolipoprotein B 48; CE: cholesterol ester; AIV: apolipoprotein AIV; AI: apolipoprotein AI; APOB: apolipoprotein B; FFA: free fatty acid.

that NPC1L1 is the target of Ezetimibe has come from an exhaustive series of studies (also see review by Davis et al., in this issue). Briefly, a deficiency in NPC1L1 leads to a 70% reduction in cholesterol absorption [22]; and in humans, with known mutations in *NPC1L1* gene, they have a reduced response to Ezetimibe treatment [29–31]. Ezetimibe has been shown to increase cholesterol elimination via bile and to improve the efficiency of reverse cholesterol transport via interaction with hepatic NPC1L1 [32]. Although it is well documented that NPC1L1 is a major target of Ezetimibe, other studies also suggest that this drug may have additional targets (reviewed in [24]). For example, there is supporting evidence that SR-BI and/or CD36 may be potential (synergistic) targets of Ezetimibe [33–35].

The effects of Ezetimibe (either alone or in combination with a HMG-CoA reductase inhibitor [or statins]) on net cholesterol influx during insulin resistance still remain unclear. Preliminary studies from our group (also presented as part of the ISCD 2010 meeting) have shown that JCR:LA-

cp rats treated with either Ezetimibe (0.01%; w/w) alone or in combination with Simvastatin (0.01%;w/w) for 8 weeks, show reduced *net* cholesterol influx when compared to non-treated controls.

9. Effects of Ezetimibe on chylomicron production

To date, there have been only a handful of studies examining the effects of Ezetimibe on CM production and CM composition. Previous animal studies have shown that Ezetimibe treatment decreases cholesterol absorption, resulting in the secretion of cholesterol ester-poor CM and reduced cholesterol delivered to the liver via the CM-r pathway [36]. As a result, a compensatory response can occur whereby hepatic LDL-receptor expression is upregulated, enhancing LDL-C clearance and lowering plasma LDL-C levels [36,37].

It is also notable that an intriguing study by Sakono et al. has determined the effect of statin therapy on intestinal lymph cholesterol in rats. Sakono et al. has shown that Sprague–Dawley rats treated with Pravastatin for 28 days had *higher* lymph cholesterol and triglyceride concentrations during 3–6 h following lipid administration compared to control rats [38]. These data suggest that statin therapy may lead to a compensatory *increase* in intestinal cholesterol absorption and/or cholesterol synthesis. In lymph isolated from JCR:LA-*cp* rats treated with Simvastatin for 8 weeks, we have observed a similar up-regulation of cholesterol and triglyceride secretion following intralipid infusion compared to non-treated rats (unpublished findings). Indeed, we would speculate that if statin use is accompanied by a compensatory increase in intestinal cholesterol absorption, then it may be beneficial to employ a combination of Ezetimibe and statin therapies during conditions of insulin resistance.

Despite findings that Ezetimibe treatment reduces intestinal cholesterol absorption, there is some clinical evidence that as a compensatory response, total cholesterol synthesis is increased [39,40]. In a randomized, double-blind, placebo-controlled cross-over study in 18 patients with moderate hypercholesterolemia treated with 10 mg/d of Ezetimibe, cholesterol absorption was reduced but a compensatory increase in total cholesterol synthesis was also observed [40]. Ezetimibe treatment was reported to induce an 89% increase in cholesterol synthesis compared to placebo and was proposed to be a result of enhanced *de novo* hepatic cholesterol synthesis [40]. Whilst this study did not measure intestinal or hepatic cholesterol synthesis separately, it eludes to the notion that intestinal cholesterol synthesis may also be up-regulated and may contribute to increased CM cholesterol content. In fact, preliminary results in JCR:LA-*cp* rats have shown that while Ezetimibe has no significant effect on *net* lymphatic apo-B48, TG or cholesterol concentration *per se* (i.e. during conditions of over-production), there is an increase in the *ratio* of cholesterol:apo-B48 in lymph. Thus, during conditions of insulin resistance, Ezetimibe reduces net cholesterol *influx* (as measured by *Ussing Chamber* techniques discussed above) but may subsequently upregulate intestinal cholesterol *synthesis* resulting in increased cholesterol availability for incorporation into CM [40,41].

Consistent with this, a study by Tremblay et al. recruited hyperlipidemic men to examine the effects of Ezetimibe on kinetics of apo-B48 *in vivo* [11]. Subjects underwent a primed-constant infusion of a stable isotope [L-(5,5,5-D3)-leucine] in the fed state (presumed to be equivalent to constant apo-B48 secretion). They found that triglyceride rich lipoprotein [TRL] (by ultracentrifugation) apo-B48 pool size (i.e. newly secreted CM particles) was significantly decreased (33%) following Ezetimibe treatment, no difference was observed in the apo-B48 production rate [11]. While the subjects recruited by Tremblay et al. were not insulin resistant *per se,* results appear to be consistent with that found in the JCR:LA-*cp* rat whereby Ezetimibe is effective at reducing CM apo-B48 pool size (possibly mediated by enhanced CM clearance), but perhaps not production *per se*.

10. Effects of combined Ezetimibe and HMG-CoA reductase inhibition on chylomicron production

To date there has been minimal literature documenting the effects of combined Ezetimibe and statin therapy on CM production and/or composition. Tremblay et al. recently reported the effects of combined therapy on the *in vivo* kinetics of apo-B48 and apo-B100 in men with mixed hyperlipidemia [12]. Plasma concentrations of total cholesterol, LDL-C and TG were significantly reduced (−43%, −53.6% and −44% respectively) with the co-administration of Ezetimibe and Simvastatin as compared to placebo. Interestingly, with the addition of a statin as combination therapy, apo-B48 pool size (−48.9%) and production rate (−38%) were both decreased compared to placebo [12]. These results suggest that combined therapy (EZ + SV) can decrease both plasma TRL apo-B48 pool size (newly secreted CM particles), as well the production rate of CM. The clinical observations of Tremblay et al. are somewhat in contrast to our findings using combination therapy in the JCR:LA-*cp* rat. The study of Tremblay et al. did not measure cholesterol per CM particle *per se,* however results from studies in the JCR:LA-*cp* rat suggest that combination therapy may actually upregulate lymph apo-B48 production. Concomitantly we observed a decrease in the ratio of lymphatic cholesterol:apo-B48 in JCR:LA-*cp* rats supplemented with both Ezetimibe and Simvastatin. The inconsistency of our data versus that of Tremblay et al. may be a result of hyperinsulinemia and associated CM overproduction in JCR:LA-*cp* rats. Whereas subjects recruited in the study of Tremblay et al. were hyperlipidemic (as determined by elevated LDL-C) and were not necessarily obese or insulin resistant. Consequently, we would propose that under conditions of established insulin resistance, whereby CM production maybe upregulated, the current recommended dose of Ezetimibe (with or without SV) may not be sufficient to correct for the these alterations.

11. Effects of Ezetimibe (alone or in combination) on post-prandial chylomicron metabolism

Experiments from our laboratory have shown that Ezetimibe (either alone or in combination with Simvastatin), is effective at lowering post-prandial apo-B48, cholesterol and TG in the JCR:LA-*cp* rat. These findings are consistent with studies in hypercholesterolemic monkeys showing that Ezetimibe treatment can decrease post-prandial apo-B48 and cholesterol concentration [36]. However we have also observed that combined treatment of Ezetimibe and Simvastatin significantly increases apo-B48 secretion into lymph in JCR:LA-*cp* rats. Collectively, we would propose that combined Ezetimibe and Simvastatin treatment may enhance hepatic clearance of CM-r from the plasma compartment. A study by Gouni-Berthold et al. found that Ezetimibe and Simvastatin treatment in healthy men increased hepatic LDL-receptor gene expression [42]. Future studies assessing hepatic tissue uptake of radiolabeled CM would help determine whether Ezetimibe and Simvastatin treatment improves hepatic clearance of CM *per se* in our rat model. In addition to the potential effects of Ezetimibe and Simvastatin treatment on hepatic LDL (apoB100/E)-receptors, it is plausible that combined therapy may also improve lipolysis and/or the expression of lipoprotein lipase (LPL). Previous studies using either *in vivo* or an *in vitro* approaches have shown that LPL activity can be upregulated following statin treatment [43], however the effects of Ezetimibe on LPL activity are less clear.

It is also interesting to note that Ezetimibe treatment alone does not appear to affect fasting TG concentrations *per se* [44,45]. Recent clinical data suggests that Ezetimibe treatment coupled with weight loss through dieting exercise may decrease intra-hepatic triglyceride content following a decrease in visceral adiposity, but not circulating TG levels [45]. Our studies in the JCR:AL-*cp* rat would support the notion that Ezetimibe is not a good modulator of fasting plasma TG. The basis for this may be a preference for Ezetimibe to influence non-fasting TG and cholesterol concentrations (via the reduction in triacylglycerol-rich lipoprotein (TRL) fraction from the intestine) and not by affecting the fasting TG associated with VLDL.

12. Effects of Ezetimibe (alone or in combination) on arterial cholesterol accumulation

There is now substantial evidence implicating remnant lipoproteins in the pathogenesis of atherosclerosis [5,6]. Our group has used an *ex vivo* fluorescent arterial perfusion method to explore the interaction of pre-labeled lipoprotein particles within the vessel under different disease states [6,46]. Results from these experiments suggest that lipoprotein particles may have differential properties of accumulation (and efflux) based on their concentration, size, cholesterol enrichment, protein complement and ability to interact with the vascular matrix [5,47]. More recent studies from our group using this method in the JCR:LA-*cp* rat also indicate a greater susceptibility of arterial vessels to accumulate remnant lipoproteins (and associated mass of cholesterol) compared to vessels from lean non-insulin resistant rats. Exciting new data presented at the ISCD 2010 meeting also indicates that in the JCR:LA-*cp* rat, Ezetimibe is able to decrease the *number* of remnant particles (measured as apoB48), as well as the associated mass of cholesterol, retained in carotid vessels compared to non-treated JCR:LA-*cp* rats. Moreover, in combination with Simvastatin, Ezetimibe was able to further reduce arterial cholesterol deposition in JCR:LA-*cp* rats, compared to monotherapy alone. These new observations support a positive association of these compounds to reduce early stages of cholesterol deposition and arterial accumulation. Our interpretation from these studies is that combination therapy of Ezetimibe with a statin may add benefit to not only cholesterol absorption and metabolism, but also to other factors relating to reducing the susceptibility of the vascular matrix to lipoprotein-remnant cholesterol accumulation. Indeed, the recent VYCTOR study [48] has shown that in subjects with exacerbated coronary disease risk, when treated with a combination of Ezetimibe with Simvastatin/Pravastatin, there is a significant reduction in carotid intima medial thickness (IMT).

Collectively, emerging data from animal models of insulin resistance and MetS support the concept that the combination of Ezetimibe with statins may confer synergistic effects on cholesterol metabolism, CM production, clearance and vascular factors compared with Ezetimibe treatment alone. Clinical studies are now warranted to further address both regressive and preventative anti-atherosclerotic strategies using a combination of these lipid-lowering targets.

Conflict of interest statement

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Appendix C

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Chylomicron and apoB48 metabolism in the JCR:LA corpulent rat, a model for the metabolic syndrome

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Abstract

Postprandial (PP) lipaemia is a significant contributor to the development of dyslipidaemia and cardiovascular disease (CVD). It is also evident that PP lipaemia is prevalent during conditions of obesity and insulin resistance (IR) and may contribute to increased progression of CVD. Our group has assessed the potential of the obese JCR:LA-cp rat as a model of PP lipaemia in order to explore CM (chylomicron) metabolism during the onset and development of IR in the metabolic syndrome. Studies confirm that both fasting plasma and PP apoB48 (apolipoprotein B48) area under the curve are significantly elevated in the obese JCR:LA-cp phenotype as compared with lean controls. Mechanistic studies have also shown that the concentration of lymphatic CM apoB48 and CM size are significantly increased in this model. Furthermore, PP dyslipidaemia in the obese rat can be improved acutely with supplementation of *n* − 3 polyunsaturated fatty acids. Using a different approach, we have subsequently hypothesized that the vascular remodelling that accompanies IR may explain accelerated entrapment of apoB48-containing particles. Small leucine-rich proteoglycans (including biglycan and decorin) have been observed to co-localize with apoB in human tissue. However, the potential impact of IR on vascular remodelling, particularly in the presence of obesity, remains unclear. Preliminary observations from the JCR:LA-cp model indicate that biglycan protein core content increases with age and is exacerbated by IR, suggestive of pro-atherogenic remodelling. The focus of this review is to contribute to the perspective of PP lipaemia in CVD risk associated with the metabolic syndrome through the use of animal models.

Introduction to the clinical problem

It is evident that PP (postprandial) lipaemia is prevalent during conditions of obesity and IR (insulin resistance) and may contribute to increased progression of CVD (cardiovascular disease). However, a significant clinical dilemma still exists in diagnosing the early phases of the metabolic syndrome (i.e. pre-diabetes) and how this impacts on relative risk of CVD. In part, this has been impaired by the continued emphasis on LDL (low-density lipoprotein), which is often normal during early Type 2 diabetes, leading to undetected yet insidious progression of CVD [1,2]. Indeed it is interesting to note that the recent revision by the IDF (International Diabetes Federation) has defined the metabolic syndrome independent of LDL cholesterol concentration [3].

In the clinic, we continue to recognize the positive effects of LDL-lowering therapy on atherosclerosis and CVD. While these efforts are well documented, increasing evidence supports a causal role between the metabolism of intestinally derived CMRs [CM (chylomicron) remnants] and the development of atherosclerosis [4]. CMs are TAG (triacylglycerol) rich when initially secreted by the enterocyte. Once in circulation, CM particles rapidly undergo hydrolysis to produce cholesterol-dense remnants [5,6]. These TAG-depleted remnants have been shown to be atherogenic as they are able to penetrate arterial tissue and become entrapped within the subendothelial space [7]. It has also been demonstrated that CMRs can induce macrophage lipid loading, which is a hallmark feature of early atherogenesis [8,9]. Moreover, raised fasting concentrations of apo (apolipoprotein) B48, a specific marker for CMs and their remnants [10], have been shown to be elevated in obese, insulin-resistant and Type 2 diabetic subjects [11–14]. However, clinical studies have so far failed to provide a definitive association between impaired PP metabolism and the very early phases of IR and corresponding risk indices [11–14]. Thus animal models offer the potential for further characterization of the metabolic syndrome in order to understand the metabolic and PP profile of this condition. Despite a greater emphasis on the study of CVD risk in the metabolic syndrome, there remains a dearth of well-characterized pre-diabetic models in order to investigate the role of PP lipoprotein metabolism in the development of atherosclerosis.

Key words: animal model, arterial proteoglycan, atherosclerosis, chylomicron remnant, insulin resistance, obesity, postprandial lipaemia.

Abbreviations used: apo, apolipoprotein; CVD, cardiovascular disease; CM, chylomicron; CMR, CM remnant; ERK, extracellular-signal-regulated kinase; IR, insulin resistance; LDL, low-density lipoprotein; LPL, lipoprotein lipase; PCSK-9, proprotein convertase subtilisin kexin type 9; PUFA, polyunsaturated fatty acid; PP, postprandial; SREBP, sterol regulatory element-binding protein; TAG, triacylglycerol; TGF, transforming growth factor; VLDL, very-low-density lipoprotein.

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JCR:LA-cp rat as a model of PP lipaemia

Animal models of obesity and the metabolic syndrome usually have defects in the metabolism of leptin and its receptor. Some of these models include the *ob*/*ob* and *db*/*db* mice, the Zucker (*fa*/*fa*) and the JCR:LA-cp rat [15,16]. Phenotypically, these rodent models develop symptoms

associated with the metabolic syndrome including obesity, IR, hypertriglyceridaemia and/or hyperleptinaemia [15,16]. Of these animal models, only the JCR:LA-cp rat model has been reported to spontaneously develop pathological complications, such as atherogenesis and myocardial ischaemia that are consistent with CVD complications seen in men [15].

The plasma lipid profile of the JCR:LA-cp (*cp*/*cp*) rat has been characterized extensively over recent years [15]. The *cp*/*cp* phenotype has mildly higher total plasma cholesterol levels compared with lean (+/?) counterparts [15]. The observed increase in total cholesterol has been associated with the VLDL (very-low–density lipoprotein), and not the LDL fraction, similar to that observed in the pre-diabetic state in humans. As a result, the JCR:LA-cp model provides a unique opportunity to study dyslipidaemia in the pre-diabetic state.

Recently, we assessed the metabolism of PP CMs in the JCR:LA-cp rat. Our approach for these studies has been to develop a novel, oral fat challenge test for our rodents analogous to the existing approach used in clinical studies [17,18]. After an overnight fast (16 h), animals are offered a 5 g food pellet supplemented with dairy fat (approx. 30% carbohydrate, 60% fat, 10% protein w/w). Our methodology uses a well-established standardized conscious, non-restraint protocol [19]. Given that the PP phase (0–4 h) predominantly represents plasma apoB48 derived from the intestine, we utilized this as a guide to assess CM metabolism in the JCR:LA-cp rat [19]. In addition, our group has developed techniques to accurately detect the presence of apoB48 in plasma using a highly sensitive Western blotting/enhanced chemiluminescent procedure [19–21].

Using our approach in the JCRL:LA-cp rat, we have observed significant lipaemia associated with apoB48-containing particles, particularly in the early PP phase. Importantly, we have determined that fasting concentrations of apoB48 in this model, despite having contributions from both intestinal and hepatic sources [19,22,23], can predict the corresponding change in PP response, as measured by the incremental area under the curve [19]. We have also observed that the kinetic profile of PP apoB48 demonstrates a significant and exacerbated delay following an oral fat challenge. These results are consistent with reports in human subjects that also show elevated levels of fasting apoB48 in individuals at risk of CVD [11,14]. Thus an oral fat load (and/or challenge) in these animals is likely to contribute to pro-atherogenic processes by either facilitating the saturation of lipolytic pathways, reducing the clearance capacity of cholesterol-rich lipoproteins, or exacerbating the permeability and retention of cholesterol in arterial vessels [11,14]. Consequently, evidence from our studies supports the hypothesis that the JCR:LA-cp model provides a unique means to clarify further the pathological role of PP lipaemia during the pre-diabetic state.

Hypertriglyceridaemia and VLDL overproduction: a major feature of IR

An important characteristic of patients with IR is the overproduction of VLDL, with increased secretion of both apoB100 and TAG [24]. Physiologically, it is also essential to consider the PP phase and the often hyperphagic-like behaviour observed during clinical obesity. Humans (and animals) are potentially in a PP state continuously for up to 16–20 h per day. This in turn contributes to a sustained secretion of CM-associated TAG from the intestine, which contributes to circulating TAG levels. Increases in the dietary carbohydrate substrate can also facilitate increased synthesis of lipid at the site of the liver. Thus hyperphagia and IR collectively generate significant biochemical modulations in the liver including the up-regulation and hypersecretion of VLDL [15]. Consistent with observation in humans, the JCR:LA-cp model also presents with classic hypertriglyceridaemia and VLDL oversecretion which appears to develop in response to several lipidogenic factors, including SREBP-1c (sterol regulatory element-binding protein-1c) regulation [15,19].

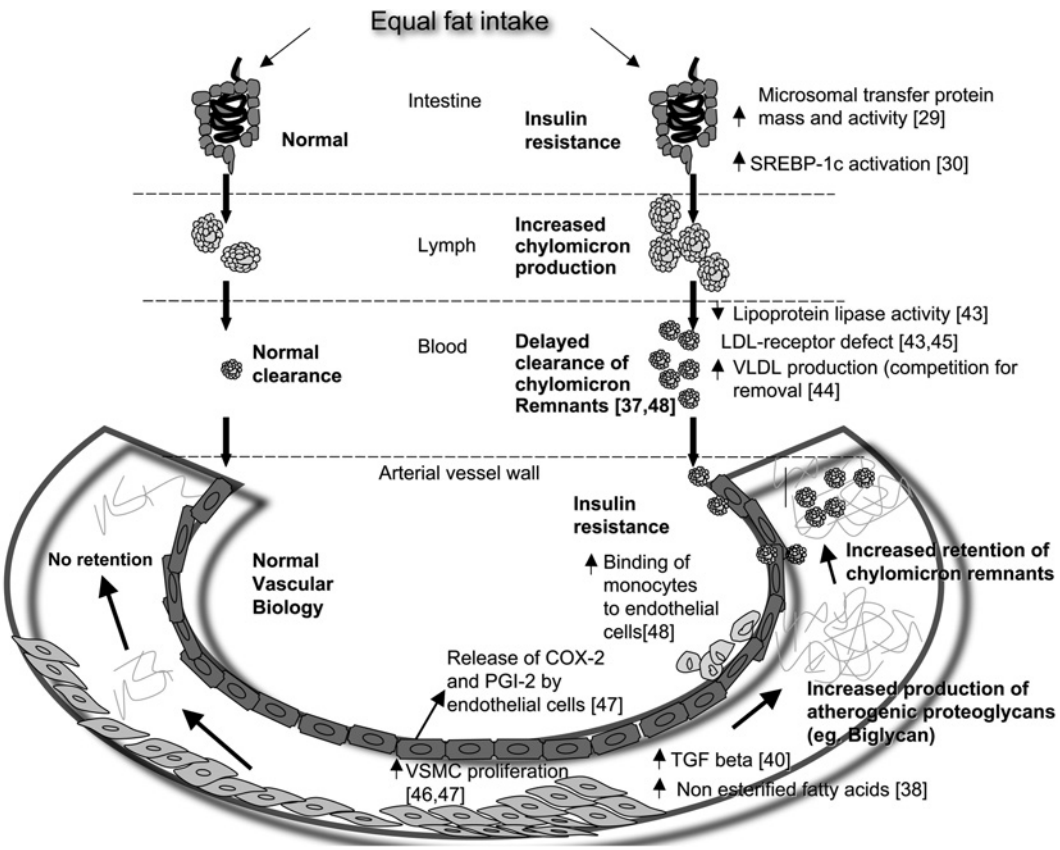
SREBPs are a family of transcription factors that regulate cholesterogenesis and lipogenesis. *In vivo* studies have shown that the SREBP-1c isoform is primarily regulated by insulin [25]. SREBP-1c levels are increased in the liver of obese, insulin-resistant and hyperinsulinaemic *ob*/*ob* mice [26,27], and the oversecretion of VLDL in JCR:LA-cp animals has been attributed to the dysregulation of endogenous fatty acid synthesis via increased expression of SREBP-1c [15]. Further contributions to increased plasma TAG concentrations may be due to down-regulation in muscle LPL (lipoprotein lipase) activity and increased adipose LPL activity observed in the JCR:LA-cp rat [15]. Very recent evidence also implicates the overexpression of PCSK-9 (proprotein convertase subtilisin kexin type 9) in VLDL oversecretion [28]. However, the regulatory aspect of PSCK-9 during IR in influencing LDL (apoB100/E) receptor expression and impact on apoE-containing lipoproteins remains largely unknown.

The role of insulin in intestinal CM production and metabolism

CMs are synthesized in the enterocyte and deliver endogenous and dietary lipids to the circulation via the lymphatic system. Insulin has been shown to have an acute inhibitory effect on apoB48 production in enterocytes isolated from chow-fed animals, but this effect is not seen in animals that have hyperinsulinaemia [29,30]. Studies in humans have shown that modest delays in PP apoB48 peak response may be associated with insulin levels in both individuals with fasting hyperinsulinaemia and normal controls [31]. Collectively, it appears that the enterocyte is sensitive to circulating insulin levels, but in conditions of chronic hyperinsulinaemia, the enterocyte may become resistant to these effects [30,31]. However, very little is known regarding the effect of the amount and type of dietary lipid on CM synthesis and clearance in the circulation, particularly in IR. Today, few laboratories have the expertise to undertake *in vivo* lymphatic isolation of CMs in order to measure CM intestinal synthesis and secretion directly. Preliminary data from our laboratory have shown that in the hyperinsulinaemic JCR:LA-cp phenotype, the lymphatic apoB48 concentration

Figure 1 | Proposed mechanism of increased atherogenicity due to accumulation of CMRs during metabolic syndrome

There is evidence from several studies (using both animal models and humans) that an equivalent fat load (challenge) will result in very divergent response of the intestine when comparing normal and insulin-resistant conditions. The exaggerated secretion of CMs from an 'insulin-resistant' intestine is now thought to contribute to the accumulating presence of TAG-rich particles in plasma. Furthermore, delayed clearance of apoE-containing CMR particles via the LDL-R (LDL receptor) and LRP-R (LDL receptor-related protein receptor) pathways ensures a continued exposure of atherogenic particles to the arterial wall. This in turn provides a platform for modified vascular function and exacerbated atherogenesis both during pre-diabetes and the metabolic syndrome.



is doubled following a lipid meal (1188 μg/ml) compared with normo-insulinaemic animals (555 μg/ml, $P < 0.05$, $n = 5$). This is associated further with a reduced TAG/apoB48 ratio (indicative of the size of particles) in the hyperinsulinaemic phenotype, suggesting that dietary lipids may play a key role in the regulation of PP CM synthesis. Indeed high levels of insulin may exacerbate an overproduction of apoB48 in the PP phase following a lipid-containing meal.

In addition, evidence indicates that intestinal overproduction of apoB48 may be associated with alterations in insulin signalling pathways in the enterocyte [30]. Specifically, acute studies in primary cultured enterocytes isolated from fructose-fed hyperinsulinaemic hamsters have shown alterations in phosphorylated ERK (extracellular-signal-regulated kinase) and the associated MEK1/2 [MAPK (mitogen-activated protein kinase)/ERK kinase 1/2] [30]. This represents a possible mechanism linking the dysregulation of insulin-signalling in the hyperinsulinaemic state and elevated intestinal apoB48 production. Thus these recent findings provide the impetus to extend the research focus onto studying the chronic regulation of intestinal apoB48 production in IR using the JCR:LA-cp model of pre-diabetes.

Effect of $n-3$ PUFA (polyunsaturated fatty acid) supplementation on PP lipaemia in the JCR:LA-cp rat

The literature demonstrates that consumption of $n-3$ PUFAs decreases total plasma TAG and lipogenesis, potentially by inhibiting the activation of SREBP-1c [32]. We have assessed the effect of $n-3$ PUFA supplementation on PP lipid, adipokine and inflammatory markers in the JCR:LA-cp rodent model. We have discovered that relative to obese control animals, treatment of JCL:LA-cp rats with $n-3$ PUFA significantly reduces concentrations of fasting plasma TAG,

total cholesterol, leptin and apoB48. Furthermore, $n-3$ PUFAs can significantly improve the PP response (area under the curve) for both TAG and apoB48 in this animal model. In this meeting, we report evidence supporting the view that modest dietary $n-3$ PUFA supplementation can potentially reduce both PP dyslipidaemia and the pro-inflammatory status associated with IR [33].

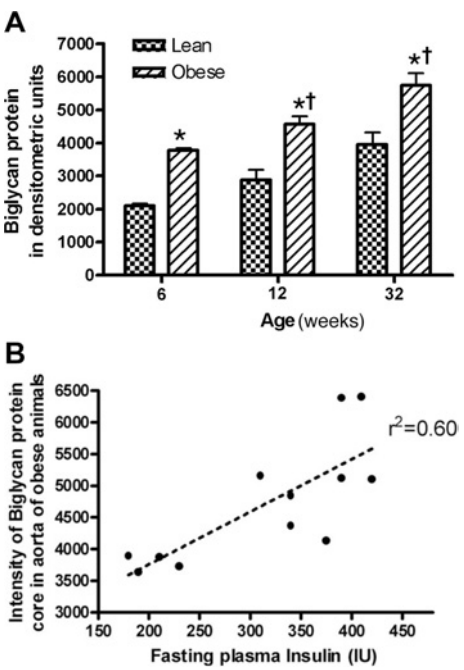
CMRs: increased atherogenicity for the metabolic syndrome

Evidence now suggests that remnant lipoproteins (including intestinal apoB48-containing CMRs) can contribute to atherogenesis and are a significant risk factor for CVD [4–7,34–36]. We have compared the delivery and efflux of both CMR lipoproteins and LDL in the vessel wall in order to further understand the factors that regulate cholesterol accumulation in early atherogenesis [37]. Our results indicate that, whereas LDL particles have a higher rate of delivery, they efflux more readily from arterial tissue compared with the larger CMRs. Collectively, our findings have highlighted that lipoproteins permeate through arterial tissue differently, and the flux of particles may be dependent on the phenotype and potential interactions with extracellular matrix components such as arterial proteoglycans. In disease states, such as diabetes, the arterial wall demonstrates an exacerbated uptake and retention of lipoprotein-derived cholesterol, including apoB48-containing CMR particles [34–36]. Thus we have hypothesized that the response-to-retention aetiology of atherosclerosis should extend beyond circulating concentrations of cholesterol to include the rate of lipoprotein particle accumulation and retention in the sub-endothelial space within the arterial wall (see the model proposed in Figure 1).

Among the series of pathogenic mechanisms that contribute to atherosclerosis during IR is the remodelling of extracellular proteoglycans [37,38]. The proliferation of vascular smooth muscle cells can stimulate the secretion of arterial proteoglycans, which in turn can increase the capacity of lipoprotein binding (*in vitro*) [39,40]. More specifically, transforming growth factor (TGF) β-1 has been identified in atherosclerotic vessels and has been shown to stimulate the synthesis of chondroitin sulfate and dermatan sulfate-containing proteoglycans by arterial smooth muscle cells [40]. JCR:LA-cp rats also have increased circulating concentrations of TGFβ-1 [41]. Recent data from our group suggest that relative to lean controls, there is a significant increase in biglycan protein core content in the obese JCR:LA-cp rats, which increases with both age and progressive stages of IR (Figure 2). Moreover, there is also a significant positive correlation between aortic biglycan protein core content and fasting insulin levels at 6-, 12- and 32-week time points in obese rats (Figure 2). Chait and colleagues have demonstrated that TGFβ-1 can increase proteoglycan–lipoprotein binding due to the increased length of the glycosaminoglycan chain [40]. Furthermore, Scott et al. [42] have shown (using a comparable cell culture model of fibroblasts) that TGF β-1 can have profound effects on the regulation of both biglycan and decorin.

Figure 2 | Content of biglycan (protein core) from aorta of lean and obese (JCR:LA-cp) rats with increasing age

(**A**) Aorta from at least five animals for both phenotype and each age 6, 12 and >32 weeks (representative of pre-, during and developed IR in this model) was subjected to proteoglycan extraction and purification, as well as GAG digestion, separation by SDS/PAGE and Western blotting and incubation with an antibody cocktail for biglycan. Bars represent pooled animal samples ($n \geqslant 5$) that were repeatedly blotted ($n=3$) and measured by densitometry (means ± S.E.M.). $^{*}P<0.01$ between phenotype at each age; †$P<0.01$ for increasing content of biglycan with increasing age in obese animals. (**B**) Correlation of aortic biglycan protein (μg/μl) with increasing fasting plasma insulin concentrations.



Conclusion

More than 25 years after Zilversmit put forth his alternative hypothesis (reviewed in [34]), endogenous CM cholesterol metabolism by the arterial wall has been shown to be a significant contributor to dyslipidaemia and CVD. Evidence from our studies supports the hypothesis that the JCR:LA-cp model can provide a unique means to clarify further the pathological role of PP lipaemia during the onset and development of IR in the metabolic syndrome. Preliminary observations from the JCR:LA-cp model indicate that biglycan protein core content increases with age and is exacerbated by IR, supporting the theory that vascular dysfunction associated with IR can remodel extracellular proteoglycans (Figure 1). The insights gained from these studies will help elucidate our understanding of atherosclerosis, obesity and Type 2 diabetes and may help to identify new forms of intervention or direct strategies to reduce further the mortality and morbidity associated with CVD.

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Appendix D

Protocol for Extraction of Proteoglycans from Tissue

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Protocol for Extraction of Proteoglycans from Tissue

1.Extraction of tissue

1. Aorta is extracted and cleaned of adventitia and blood using foreceps. To get blood out let it clot first and then shake and swirl the aorta a little. Use cold PBS while cleaning and place the petridish on ice while handling tissue.
2. Collect blood for insulin and glucose studies later.
3. Once cleaned snap freeze in liquid nitrogen and store in -70 freezer.

2. Weighing the tissue

Thaw the aorta sample and weigh it. Note the animal ID, age, weight etc. Keep the sample back in freezer.

3. Preparation of Extraction Solution (ES)

Make ES approximately 20 times the weight of tissue for eg for 0.453 gms of tissue need 8ml of ES, for 0.384 gms of tissue need ~6ml of ES.

The following recipe is for 20 ml of ES. Use the required amount store the rest in -20 freezer. Stays good for a long time. To change the recipe, change 0.02 (for 20 ml) to as desired.

In a beaker weigh the following. Use different or clean spatulas each time.

Formula = Molarity x volume in Litre x molecular or formula weight

4M GnHCl (4x0.02x95.53) = 7.64gm

0.15M NaCl (0.15x0.02x58.44) = 0.175gm

0.05M Trizma base = (0.05x0.02x157.60) = 0.158gm

0.01M EDTA = (0.01x0.02x372.2) = 0.074gm

0.1M AHA = (0.1x0.02x131.18) = 0.26gm

0.005M Benzamidine = 0.005x0.02x156.61=0.0156gm

0.005M NEM = 0.005x0.02x125.13= 0.0125gm

5μg/ml leupeptin for 20ml = 100μg or 0.1mg or 0.0001gm

Try to make leupeptin as 1 mg/ml conc in water. Make in a small glass vial then add the required amount of water and store at -70. So weigh 0.001gm in 1ml water or 0.002gm in 2ml water and use 100 μL for 20ml of extraction buffer.

5μg/ml pepstatin for 20 ml= 100μg or 0.1mg or 0.0001gm

Make and store pepstatin the same way as leupeptin but it dissolves only in ethanol or methanol.

0.001M PMSF = (0.001x0.02x174.20) = 0.00348 gm

In a small glass vial weigh PMSF 0.003gm in 1ml or 0.006gm in 2ml of ethanol and use 1ml of this for 20 ml extraction buffer. Save the rest at -70.

4. Cutting aortic tissue

1. Thaw aorta sample and place it in a petridish kept on ice. Use a scalpel and a forecep to cut the tissue in the smallest possible pieces while still on ice. (if in Carole's lab use scalpel and blade no 11). Use a fresh blade.
2. Transfer it into a small glass bottle that can be capped. Pour half the amount of ES required. (for eg for 0.453 gms of tissue need 8ml of ES, so pour 4ml)
3. Place a micro stir bar.
4. Place bottle in a small beaker and place the beaker in the cold room on a stirrer. Let it rotate for 24 hrs. tape the beaker to the stirrer to prevent from moving or falling.

5. Adding fresh Extraction Solution

Next day remove solution from cold room and transfer into centrifuge tubes using plastic transfer pipette (don't use glass pipettes as the solution is viscous and

might stick to it. Cut the tip of the transfer pipette. Keep everything cold that way it doesn't stick much and sample won't degrade).

Weigh the tubes and centrifuge them at around 9000 to 9500 rpm for 30 mts at 4 degrees Celsius.

For weighing tubes and centrifuge instructions in Carole's lab see additional information.

After centrifugation remove the tubes and transfer the supernatant to a clear plastic tube (15 ml culture tube). Label supernatant as extract No 1. And keep in -20 fridge.

Remove the residue with the same or fresh (tip cut) plastic transfer pipette and put in the same glass bottle again and pour fresh ES. Try to get all the residue out from the centrifuge tubes so pour 500μl ES and try to remove all residue Pour the same amount as poured earlier (In this eg 4ml).

Again leave it rotating in cold room overnight.

Next day again centrifuge samples as before and collect supernatant in the same tube with Extract no I and store at -20 fridge.

Remove residue in a small 2ml plastic bottle with cap. Try to put all residue in it and label it as Extraction resistant tissue and keep in -70 freezer.

6. Making buffer for dialyzing the sample.

The sample is dialyzed using 7 M urea. But first we make 9 M urea, deionise it *and then* use it to make 7 M urea and add inhibitors to it.

How much 9 M urea has to be made depends on mow much 7 M urea is needed.

Calculation for 7 M urea :

In this example 8ml of ES was used, and it had 4M Guanidine Hydrochloride which is what we need to get rid off.

So if we make 300 ml then 300/amount of total ES used

= 300/8 = 37.5

= 4M/ 37.5 = 0.1066 M GnHCl

If we put another 200 ml then 200/8 = 25

And then 0.1066/25 = 0.00426M

The aim is to take the molarity of GnHCl down to three decimal places, though the lower the better.

So in this eg we need 500 ml but another 100 ml for washes so total ~ 600 ml of 7M urea.

How much of 9M urea will we use to make 600 ml of 7M urea.

M1 V1= M2 V2

9y = 7x600

y= 600x7/9= 466.66ml.

So we will make 500 or 600ml of 9M urea and use 466.66 ml of it to make 600ml of 7M urea.

To make 600ml of 9M urea

9x60.06x0.6= 324.324

Weigh 324.324 gms of urea in a beaker add ~500 ml water stir and when it becomes clear pour into a graduated cylinder and make volume up to 600ml.

Deionizing the urea.

Take Biorad resin 5gm /100ml to be deionized so in this case 0.05x600=30gms resin

Put the resin in the beaker with urea solution. Keep on a stirrer. Stir for 1hr.

After an hour filter this using a 0.22 μm Millipore vaccum filter.

Now of this deionised 600ml 9M urea, take 466.66 ml. (don't make up the volume up to 600 ml here since the inhibitors have to be added.)

Inhibitors to be added to 600ml of 7M urea:

0.05M trisHCl (0.05x0.6x157.60) = 4.73gm

0.01M EDTA (0.01x0.6x372.2) = 2.23gm

0.005M benzamidine (0.005x0.6x156.61) = 0.469gm

0.15M NaCl (0.15x0.6x58.44) = 5.259gm

0.001M PMSF (0.001x0.6x174.20) = 0.1045gm (dissolve in MeOH)

0.5μg/μl Leupeptin 0.5μg x600ml = 300μg (dissolve in water)

0.5μg/μl Pepstatin 0.5μg x600ml = 300μg (dissolve in MeOH)

(Make leupeptin and pepstatin as described before …)

0.005M N-ethyl maleamide (NEM) = 0.005x0.6x125.13 = 0.375gm

Add these inhibitors to the 466.66 ml 9M urea above. Let it stir for about an hour. When everything turns clear set pH to 6.6 and make up the volume up to 600 ml with DDW. So now 600ml of 7M urea is ready with the inhibitors.

7. **Prepare dialysis tubing and keep sample for dialysis;**

Take out some dialysis tubing. Approximately 1cm/ml and then quite a bit extra for tying the knots. So since 8 ml ES used so 8cm + about 10cm more to tie knots. Put water in a glass beaker, add 3-4 pinches of Na_2CO_3 and keep it for boiling for about 5 minutes.

Then take the sample out from the cold room that has been rotating for a while. Transfer it into centrifuge vials using a Pasteur pipette that has been cut. Weigh the tubes and centrifuge as before ..for 30 minutes at 4 deg C.

Keep tubes on ice all the time.

Clean dialysis tubing with DDW from inside as well as outside. Tie knot on one end. Pull on the knot, never on the knot in a way such that the dialysis surface

gets pulled so the surface that gets pulled is out of the dialyzing surface. Pour the supernatant from the centrifuge and the saved sample which was collected after the first round of extraction. Tie both ends tightly. Put orange clips on both ends. In a beaker put a stir bar and pour 300ml of 7M urea + inhibitors and let it stir for 48 hrs and then dump off the urea solution and add 200ml more. Let it stir for another 24 hrs.

After the dialysis is done open the tubing very carefully over a beaker to prevent spilling. And collect sample in a small beaker.

8. Purification-running DEAE column:

First of all wash a small column with 7M urea (called buffer A). In a small beaker mix one and a half pasteur pipette full of DEAE and buffer A. Pour into the column till it packs to 1.5 cm or else add more resin. It has to be washed with 10 times the resin volume. So wash the packed resin with 15ml 7M urea.

***Make buffer B**: 0.85M NaCl in buffer A*

X = 0.85x0.02x58.44

= 0.993gms NaCl in 20 ml of buffer A.

Put the sample on column and collect flowthrough in a microfuge tube. Mark it as unbound extract.

Mark eppendorf microfuge tubes as $Wash_1$ to $Wash_6$.

6 times add **500µl of buffer A** and keep collecting the washes in the marked tubes.

Next mark 20 tubes as $Extract_1$ to $Extract_{16}$.

Add **250µl of buffer B** 16 times, and keep collecting the extract. These are bound proteins.

Put water in resin, take it out and save it for using again.

9. **DMMB assay to determine which fractions have proteoglycans:**

Stock dye solution:

1.6 mg SERVA DMB MWt. 347.9 Cat # 20335

0.2 gm sodium formate

0.2 ml formic acid (88% pure)

-make up to 100ml with Milli Q water

-store in brown glass bottle.

Working Dye solution:

Mix 9 parts of stock dye plus 6 parts of stock buffer (ratio 3:2)

One 96 well plate needs approximately 15ml working dye solution.

Dermatan sulphate standards:

Weigh out approximately 50μg Miles DS (Chondroitin Sulphate B)

Dissolve at a concentration of 1 μg per μl in water.

Dilute again 1:10 in water.

Store standards at -22 deg C.

Can be thawed and refrozen several times.

Std. No.	*ng/well*	*μg/ml*	*μl DS sol used*	*μl water*
1.	100	2	20	980
2.	200	4	40	960
3.	300	6	60	940
4.	400	8	80	920
5.	500	10	100	900
6.	1000	20	200	800

Take out Elisa well strips and fix them in the frame. In the first two wells add 50μl of water. Then add 50μl of stds in duplicates and then 15-25 μl ofv sample.(depending on the yield of the sample). In the last two wells add 25μl of the before column extract and 25μl of the first unbound fraction before washes.

Make active dye solution in a 50 ml grad cylinder in a 3:2 ratio as described before.

Then use multichannel pipetter and add 200 μl of the active dye solution in each well.

Read immediately. Plate reader gives results as ng/well.

Collect all the tubes with +ve values and pool their sample into one tube. Wash the tubes with a little PBS and pool with the sample.

10. Concentration and desalting of sample:

Take a concentrator and an old one to balance. Add PBS in the unused one to the top ~ 3ml and let it spin down in cold room for 5 mts. This step is to wet the filter. Doesn't matter if it doesn't get all down. Dump it and add sample and let it run down till it goes to 300 μl. Then top it up to cap with PBS and let it spin another 5 mts. Don't forget to remove the waste from the bottom. Pool it in one tube and save this flow thru just in case .

Do 4 times 5 minute runs, keep adding PBS and once 15' run and then concentrate the sample up to 100 μl. Don't try to concentrate too much. If the solution is concentrated then the proteins precipitate out.

Collect this sample using a gel-loading tip in a preweighed microfuge tube.

Note the wet weight of the sample now.

Dermatan sulphate standards:

Weigh out approximately 50μg Miles DS (Chondroitin Sulphate B)

Dissolve at a concentration of 1 μg per μl in water.

Dilute again 1:10 in water.

Store standards at -22 deg C.

Can be thawed and refrozen several times.

Std. No.	***ng/well***	***μg/ml***	***μl DS sol used***	***μl water***
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4.	400	8	80	920
5.	500	10	100	900
6.	1000	20	200	800

Present standards were made and tested.

Weighed 328 μgm in 328μl water.

Took 170μl of this and added 1530 μl water .i.e total 1700 μl. From this made standards as in recipe above.