THE ROLE OF LYOSOMAL ACID LIPASE IN REGULATION OF THE ATPBINDING CASSETTE TRANSPORTER A1, HIGH DENSITY LIPOPROTEIN AND REVERSE CHOLESTEROL TRANSPORT

by

Kristin Louise Bowden

B.Sc., Queen’s University, 2005
M.Sc., Dalhousie University, 2008.

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Abstract

The key regulator of initial HDL particle formation by cells is the ATP-binding cassette transporter A1 (ABCA1). ABCA1 expression is regulated primarily by oxysterol dependent activation of the liver X receptor (LXR). We investigated the role of lysosomal cholesterol on ABCA1 regulation by studying the lysosomal disorder Cholesteryl Ester Storage Disease (CESD). CESD is caused by genetic mutations in the LIP-A gene that result in only 5% of normal activity of lysosomal acid lipase (LAL), an enzyme that hydrolyzes cholesteryl esters (CE) and triglycerides on internalized lipoproteins specifically within the lysosome. We hypothesized that the flux of unesterified cholesterol out of the lysosomes from LAL-mediated hydrolysis of LDL cholesteryl esters is a key regulator of cellular ABCA1 expression, HDL formation and reverse cholesterol transport (RCT). We found that primary skin fibroblasts derived from individuals with CESD had impaired upregulation of ABCA1 in response to LDL loading, reduced phospholipid and cholesterol efflux to apoA-I, lower production of 27-hydroxycholesterol (27-OH) production in response to LDL loading and reduced α-HDL particle formation. This defect was recapitulated in normal fibroblasts following treatment with LAL inhibitors, whereas, treatment with conditioned medium from normal fibroblasts containing secreted LAL rescued ABCA1 expression, apoA-I-mediated cholesterol efflux, HDL particle formation and production of 27-OH by CESD cells. We further investigated the role of LAL in RCT from macrophages specifically using an immortalized macrophage cell line created from LAL-deficient mouse peritoneal macrophages (LAL−/−). LAL−/− macrophages exhibited reduced basal and cholesterol-stimulated ABCA1 expression in culture, and reduced ability to support RCT in LAL−/− mice compared to wild-type (LAL+/+) macrophages injected into LAL+/+ mice. ABCA1 protein expression was reduced in lal−/− mouse liver and mRNA
expression of several LXR-dependent genes involved in reverse cholesterol transport (ABCG1, ABCG5, ABCG8, CYP7A1, SR-B1) were differentially modulated compared to LAL+/+ controls. These results indicate a critical role of LAL in promoting lysosomal flux of cholesterol for ABCA1 expression, cellular cholesterol efflux and RCT in vivo.
Preface

Chapter 2: LAL deficiency impairs regulation of ABCA1 and formation of HDL in CESD

Kristin Bowden contributed 70% towards all aspects of this work. She contributed 60% towards experiments (Figures 2.3, 2.5, 2.6 and 2.7, 2.9, 2.10, 2.11 and 2.12), completed analysis of the data, and draft the original version of this manuscript.

Contribution of co-authors:

- Nicolas Bilbey, MSc performed 40% of experiments. This includes Figures 1, 2 and 4.
- Leanne Bilawchuk, BSc and Teddy Chan, MSc provided technical assistance and performed experiments for supplementary Figure 2.11.
- Emmanuel Boadu, PhD performed the 2-dimensional gel electrophoresis of medium samples (Figures 2.6 and 2.12).
- Roshni Sidhu, BSc, performed analysis of oxysterol mass by Gas Chromatography / Mass Spectroscopy of cell and medium samples (Figure 2.8) under supervision of a collaborator Daniel Ory, PhD.
- Hong Du, PhD, provided expert advice and assisted with revision of the manuscript.
- Gordon Francis, MD, supervised this project and assisted with editing and writing of the manuscript.

All work was completed under approval of the UBC research ethics board (certificate #H07-02720 – High Density Lipoprotein Formation).

Chapter 3: LAL promotes Reverse Cholesterol Transport from Macrophages

Kristin Bowden contributed 95% towards all aspects of this work, including all experiments, analysis of data and writing of the original manuscript.

Contribution of co-authors:

- Hejin (Julia) Kong and Teddy Chan, MSc provided technical assistance with some aspects of animal experiments.
- You-Hai Xu, PhD, performed breeding and husbandry of the LAL knockout mice under supervision of our collaborator Gregory Grabowski, MD.
- Hong Du, PhD provided immortalized peritoneal macrophage cell lines used for experiments in Figures 3.1-3.4 and provided scientific advice.
- Gordon Francis, MD, supervised this work and provided assistance with editing of the manuscript.

Animal work was performed under approval by the UBC Animal Care Committee (Certificate #A12-0067).
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List of abbreviations

ABCA1: ATP-binding cassette transporter A1
ABCB4: ATP-binding cassette transporter B4
ABCB11: ATP-binding cassette transporter B11/Bile salt export pump
ABCG1: ATP-binding cassette transporter G1
ABCG4: ATP-binding cassette transporter G4
ABCG5: ATP-binding cassette transporter G5
ABCG8: ATP-binding cassette transporter G8
ACAT: acyl-CoA:cholesterol acyltransferase
acLDL: acetylated LDL
ad-hLAL: adenoviral overexpression of human lysosomal acid lipase
aggLDL: aggregated LDL
ApoA-I: apolipoprotein A1
ApoB: apolipoprotein B
ApoE: apolipoprotein E
Ca\(^{2+}\): calcium (ionic)
CVD: cardiovascular disease
CE: cholesteryl ester
CESD: cholesteryl ester storage disease
CETP: cholesteryl ester transfer protein
CFTR: cystic fibrosis transmembrane receptor
CL: cardiolipin
CQ: chloroquine
Cu\(^{2+}\): copper (ionic)
CYP7A1: cytochrome P450 type 7A1
CYP27: cytochrome P450 type 27
FPLC: fast protein liquid chromatography
EGFR: epidermal growth factor-like repeats
E-LDL: enzymatically modified low density lipoprotein
ER: endoplasmic reticulum
E8SJM: Exon 8 splice junction mutation
FH: familial hypercholesterolemia
HDL: high-density lipoprotein
HDL-C: high-density lipoprotein cholesterol
Hg\(^{2+}\): mercury (ionic)
HgCl\(_2\): Mercury chloride
Hg(NO\(_3\))\(_2\)): Mercury nitrate
HMGCR: 3-hydroxy-methylglutaryl-coenzyme A reductase
IDL: intermediate-density lipoprotein
KCl: potassium chloride
K\(_m\): Michaelis menton constant
LAL: lysosomal acid lipase
LCAT: lecithin cholesterol acyl transferase
LDL: low-density lipoprotein
LDL-C: low-density lipoprotein cholesterol
LDLR: low density lipoprotein receptor
LIPA: gene encoding lysosomal acid lipase
LPL: lipoprotein lipase
LPS: lipopolysaccharide
LXR: liver X receptor
LXRE: liver X receptor response element
Lyso-PC: lyso-phosphatidylcholine
MDR1: multi-drug resistance protein 1
NaCl: sodium chloride
NaF: sodium fluoride
nCEH: neutral CE hydrolase
NBD: nucleotide binding domain
NEFA: non-esterified fatty acids
NPC: Niemann Pick disease type C
NPC1L1: Niemann Pick disease type C1 like protein 1
oxLDL: oxidized LDL
PA: phosphatidic acid
PC: phosphatidyl choline
PCSK9: pro-protein convertase subtilisin kexin type 9
PEST: proline, glutamine, serine, threonine recognition sequence
PS: phosphatidyl serine
phLAL: LAL produced by clonal reproduction in the species *Pichia pastoris*
PI: phosphatidyl inositol
RCT: reverse cholesterol transport
RXR: retinoid X receptor
SNP: single nucleotide polymorphism
SR: scavenger receptor
SR-BI: scavenger receptor B1
SREBP: sterol regulatory element binding protein
SSD: sterol-sensing domain
TG: triglyceride
TICE: trans-intestinal cholesterol export
TM: transmembrane
UC: unesterified cholesterol
WD: Wolman disease
VLDL: very low-density lipoprotein
$V_{\text{max}}$: maximum velocity of reaction
$\text{Zn}^{2+}$: zinc (ionic)
4-MUO: 4-methylumbelliferonyl oleate
7(α)-OH: 7-hydroxycholesterol
22(R)-OH: 22-hydroxycholesterol
24(S)-OH: 24-hydroxycholesterol
25,25-OH: 24,25-hydroxycholesterol
25-OH: 25-hydroxycholesterol
27-OH: 27-hydroxycholesterol
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Dedication

I would like to dedicate this thesis to my parents Barry and Gloria and my wonderful husband James for all of their patience and loving support throughout all the ups and downs of my PhD. I couldn’t have done it without you.
CHAPTER 1: INTRODUCTION

1.1 Overview

Cardiovascular disease (CVD) is the leading cause of death worldwide (1) and accounts for 29% of all deaths in Canada (2). Heart disease and stroke exact an enormous burden on our healthcare system and our Canadian economy, accounting for 16.9% of all hospitalizations (3) and more than 20 billion dollars annually in physician and hospital costs and lost productivity (4). Atherosclerosis, or ‘hardening of the arteries’ is a common underlying cause of CVD where deposits of cholesterol accumulate within plaques that form in the artery wall (5). High levels of circulating low-density lipoprotein (LDL) cholesterol put one at higher risk of CVD. The success of statin drugs, which inhibit cholesterol synthesis and increase cellular uptake of LDL cholesterol from plasma, and significantly reduce the risk of CVD, should leave no doubt of the central role of cholesterol in atherosclerosis and CVD (6). One of the hallmarks during the development of atherosclerosis is the accumulation of foam cells within the artery wall where scavenger cells such as macrophages take up modified forms of LDL that have infiltrated the arterial endothelium and are retained in the extracellular matrix. When the balance of removal of cholesterol from cells in the artery wall is less than uptake, excess cholesterol is stored in the form of cholesteryl esters within lipid droplets of foam cells and can lead to the progression of the atherosclerotic plaque. Cellular cholesterol homeostasis is normally tightly controlled within the cells through important regulatory mechanisms discussed in the following section (Section 1.1). A process termed reverse cholesterol transport (RCT) (discussed in section 1.3) is also critical to prevent excess accumulation of cholesterol within peripheral cells. In RCT, excess cholesterol is removed from cells and carried on high density lipoprotein (HDL) particles so that they can be removed from circulation by excretion. The ATP-binding cassette transporters A1
and G1 (ABCA1 and ABCG1) (see section 1.4) perform a critical role in the RCT pathway by mediating the efflux of cholesterol from cells during HDL particle formation (7). The transcriptional regulation of ABCA1 and ABCG1 are under the control of the nuclear transcription factor liver X receptor (LXR) (8,9) which is activated by oxygenated derivatives of cholesterol known as oxysterols (10,11). It is theorized that oxysterols are formed under conditions of excess cholesterol accumulation within the cell (12) which, in turn, activate cholesterol efflux genes such as ABCA1 and ABCG1 to trigger cellular cholesterol removal and promote RCT. However, the intracellular sources of cholesterol that are used to turn on gene expression and which become substrates for efflux are currently unknown. We proposed that lysosomal cholesterol was the major pool of cholesterol responsible. Previous data from our laboratory indicated that ABCA1 regulation and cellular efflux were impaired in the lysosomal storage disorder Niemann Pick disease type C (NPC) (discussed in section 1.5), where the movement of cholesterol out of the late endosomes and lysosomes is blocked, resulting in aberrant HDL particle formation (13). Here, the role of lysosomal acid lipase (LAL - an enzyme which hydrolyzes cholesteryl esters to free cholesterol at acidic pH within lysosomes) (section 1.5) in ABCA1 regulation, HDL metabolism and whole body RCT is explored.

1.2 **Regulation of cellular cholesterol metabolism**

All eukaryotic cells require cholesterol, a vital structural component that helps maintain membrane permeability, structure and fluidity. Cells can synthesize cholesterol *de novo* or derive it by uptake of exogenous cholesterol on lipoproteins. However, aside from bile acid and steroid hormone synthesis in specialized cells, most cells cannot catabolize cholesterol. Therefore, it is essential that intracellular levels of cholesterol be tightly regulated in order to prevent cholesterol starvation or excess. The concentration of cholesterol in the cell is tightly controlled through a
feedback mechanism elucidated through the work of Goldstein and Brown and colleagues, whereby the mechanisms of regulation of sterol sensitive genes by the sterol regulatory element binding proteins (SREBPs) has been resolved.

1.2.1 SREBPs in the regulation of cholesterol homeostasis

Two proteins Scap and HMGCR are among cellular proteins that contain sterol-sensing domains (SSD), which allow them to monitor the levels of cholesterol in the endoplasmic reticulum (ER) membrane. Scap acts as an escort protein that senses cholesterol and binds to SREBP in the ER membrane and can chaperone it from ER to Golgi. The structure of SREBP contains an N-terminal domain with a basic helix-loop-helix motif DNA-binding domain and a C-terminal regulatory domain and contains two transmembrane domains connected by a central luminal loop. When SREBPs are first synthesized in the ER, they are membrane bound with the N- and C-terminal segments facing the cytoplasm to form a hairpin-like structure (14). When cholesterol levels in the cell are depleted, the cholesterol content of the ER is also low. Bound to the C-terminal regulatory domain of SREBP through its own C-terminal WD domain (15), Scap reacts to the reduced cholesterol levels in the membrane by causing a conformational change within the SSD of Scap. This conformational change allows Scap to interact with a complex of proteins (including Sar1-GTPase, Sec23 and Sec24), which triggers binding of COPII proteins that allow the Scap-SREBP complex to bud off from the ER membrane and move to the Golgi (16). Once these vesicles have fused with the Golgi, SREBP is cleaved sequentially by site 1 protease (subtilisin family serine protein), which first cleaves acidic residues within the luminal loop region leaving both halves membrane-bound. Site 2 protease (a zinc metalloprotease) then cleaves within the membrane domain adjacent to N-terminal domain, thus releasing the soluble
N-terminal transcription factor domain so that it can then enter the nucleus and regulate its target genes that work to replenish cellular cholesterol (and fatty acid) levels.

Transcriptional regulation by SREBP is achieved by binding of activated SREBP in the nucleus to target DNA sequences called sterol-regulatory elements (SREs) in the promoter region of these sterol-sensitive genes (17). There are three SREBP isoforms; SREBP1a and SREBP1c and SREBP2. SREBP1a and 1c are splice variants produced from a common gene while SREBP2 is encoded by a separate gene (17). Each of the SREBPs have different specificities for their target genes; SREBP2 is primarily involved in regulation of cholesterol synthesis genes (such as HMG-CoA reductase, HMG-CoA synthase, farnesyl pyrophosphate synthase and squalene synthase) and expression of the LDL receptor; SREBP1c activates genes primarily for fatty acid synthesis (such as fatty acid synthase and acetyl CoA carboxylase) and desaturation (such as stearoyl CoA desaturase); SREBP1a activates both fatty acid synthesis and cholesterol regulatory genes in a non-specific way (14,18).

When cellular cholesterol levels are in excess, sterols accumulate in the ER membrane. This triggers a conformational change in the sterol sensing domain (SSD) of Scap that allows it to now interact with a membrane-bound proteins called insulin sensitive genes, or Insigs (19) and sequesters the Scap-SREBP complex away from the COPII budding complex so that it remains in the ER (20). There are two isoforms of Insig: Insig 1 is an SREBP target gene (therefore Insig 1 expression is regulated by cholesterol through SREBP processing) (18), whereas Insig2 is constitutively expressed (21).

HMG CoA reductase (HMGCR), an enzyme that performs the rate-limiting step in cholesterol synthesis, is itself regulated in a sterol-sensitive manner. HMGCR also resides in the ER membrane and contains a SSD. When excess cholesterol (or lanosterol, a cholesterol
precursor) accumulates in the ER membrane, these sterols cause a conformational change in the N-terminal transmembrane SSD of HMGCR that allows binding to Insig, which leads to degradation of the HMGCR protein (22). Insig is bound to a complex of ubiquitin-conjugating enzymes \textit{(e.g.} gp78 ubiquitin ligase, E2 conjugating enzyme Ubc7, VCP ERAD ATPase\textit{)} that ubiquitinate HMGCR and accelerate its degradation in the proteasome (23). HMGCR mRNA expression is under the control of SREBP (24), which allows HMGCR to be turned on under cholesterol starved conditions, when cholesterol is needed in the cell. Oxysterols have also been shown to inhibit SREBP activation by blocking Scap-mediated transport (16), although by a different mechanism (20). Treatment with either cholesterol or oxysterols can induce a Scap-Insig complex to form, thus preventing Scap-mediated SREBP transport and cleavage (16). While cholesterol binds directly to Scap to mediate a conformational change within its SSD, the same was not shown for 25-hydroxycholesterol (25-OH) (20). Therefore, oxysterols must act by another mechanism either directly or indirectly, perhaps through an intermediary protein in order to induce Scap-Insig complex formation (16). Radhakrishnan and colleagues (25) showed that 25-OH and other oxysterols, such as 22(R)-OH, 24(S)-OH, 27-OH and 24,25-epoxycholesterol bind to Insig-2 directly. Through mutational analysis of Insig-2, five critical residues for binding of oxysterols were identified. Importantly, mutations that prevented oxysterol binding also prevented Scap binding to Insig (25). As these studies indicate, oxysterols are intimately involved in regulation of SREBP retention in the ER, and important regulators of gene expression networks in cholesterol homeostasis.

1.2.2 \textit{LDL-receptor mediated uptake of exogenous cholesterol}

SREBPs also control uptake of exogenous LDL cholesterol through regulation of the LDL receptor pathway. The LDL receptor (LDLR) is an endocytic transmembrane receptor that
is composed of a short cytoplasmic domain, a single transmembrane domain, an O-linked sugar domain, epidermal growth factor-like repeats (EGFR), Y repeats that contain a YWTD-consensus motif (together, YWTD repeats and flanking EGFR domains form a beta-propeller secondary structure) and a ligand binding domain (26). The pathway by which LDL particles are internalized via the LDLR was also elucidated through the work of Goldstein and Brown, who studied fibroblasts from patients with familial hypercholesterolemia (27). FH homozygotes have LDL cholesterol levels 6-10 times higher than normal, develop tendinous xanthomas and corneal arcus and have severe atherosclerosis, sometimes causing heart attacks in childhood (28). Incubation of normal fibroblasts with LDL caused suppression of HMGCR, but in FH fibroblasts HMGCR activity was 50-100x higher and did not go down with LDL incubation (29). By measuring the uptake of $^{125}$I-LDL, it was discovered that LDL uptake was mediated by binding to the LDL receptor, which was defective in FH fibroblasts (30). They subsequently purified the LDLR from bovine adrenal gland (31), cloned the human cDNA (32) and isolated the LDLR gene (33).

Receptor mediated uptake of LDL occurs in several stages. First LDL binds to its receptor with high affinity (this binding requires apolipoprotein B100), then is taken up by endocytosis via clathrin-coated pits (34), which anchor the NPxY sequence in the cytoplasmic domain of the LDL receptor within the internalized vesicle (35). The endosomes carrying the LDL bound to the LDLR then fuse with the lysosomes, where the LDL cholesteryl esters (CE) are broken down to unesterified cholesterol (UC) (36). In the relatively acidic environment of the endosomes, the LDLR releases its ligand and the empty receptor recycles back to the plasma membrane (37). Following receptor-mediated uptake of LDL, the UC that is released from the lysosome eventually enters the regulatory pool in the ER, which triggers not only suppression of
HMGCR, and SREBP processing but also promotes cholesterol esterification by activating the enzyme acyl-coA: cholesterol acytransferase (ACAT)(38). ACAT is an integral membrane protein in the rough ER that catalyzes the formation of CE from UC and long chain fatty acids, using coenzyme A and ATP as cofactors (39). It is an allosteric enzyme and it is hypothesized that binding of cholesterol or oxysterols to the enzyme produces a ligand-induced conformational change that brings subunits together from inactive to active form (40,41). Thus, ACAT is regulated largely by the availability of substrate rather than an SREBP-dependent mechanism.

A chaperone protein, proprotein convertase subtilisin kexin type 9 (PCSK9) has been shown to bind to the LDLR and prevent its recycling back to the PM, thereby promoting its degradation in the lysosome (42). PCSK9 reduces the level of LDLR at the cell surface of hepatocytes (43-45). Although PCSK9 is a serine protease, it functions as a chaperone protein to the LDLR independent of its catalytic activity, although, protease activity is required for PCSK9 to undergo auto-catalytic processing of its pro-domain to its mature form (46). Following its synthesis in hepatocytes, PCSK9 is secreted into the circulation where it binds to the EGFR domain of the LDLR and is internalized along with LDL (47). During endocytosis, the acidic pH of the endosomes causes PCSK9 to bind more tightly to the LDLR, which locks it into an open conformation (48). Since the LDLR must adopt a closed conformation in order to recycle back to the plasma membrane, PCSK9 prevents its sorting into recycling endosomes, thus targeting it for proteasomal degradation in the lysosome (42,49). Gain of function mutations in PCSK9 leading as a previously unknown form of FH have been identified in two French families, and subsequently in FH patients in Utah (US), Norway and the UK. Conversely, loss of function mutations in PCSK9 are associated with below normal LDL-C levels and significantly reduced global coronary heart disease risk (42). PCSK9 expression is regulated by SREBP-2, and its
transcription is activated by statins (HMGCR inhibitors) (50,51). Since PCSK9 is co-activated with the LDL receptor, this may reduce the effectiveness of statins at clearing plasma LDL-C. Altogether, this makes PCSK9 an attractive target for therapy to lower LDL-C beyond what statins can achieve, or in statin-intolerant patients. Currently, several lines of therapies targeting PCSK9 are being developed and tested in clinical trials: gene silencing using anti-sense oligonucleotides, mimetic peptides of the EGFR of the LDLR to compete for binding, or monoclonal antibodies targeting PCSK9 (42).

The SREBP-2 upregulates LDLR (52). However, cholesterol liberated from LDL via LDLR-mediated uptake in turn suppresses SREBP and down-regulates expression of the LDLR in a negative feedback loop (18,52). The cholesterol content of the membrane must be held within an appropriate concentration in the membrane that is essential for maintenance of proper membrane fluidity, permeability, as well as to support lipid rafts and integral membrane proteins. Therefore, through regulation of LDLR, HMGCR, ACAT and PCSK9, SREBP-2 keeps the levels of membrane cholesterol constant despite fluctuations in requirement of cholesterol and supply from exogenous sources.

1.2.3 Uptake of modified LDL by scavenger receptors

Fatty streaks appear during the early stages of atherosclerosis consisting of foamy lipid and cholesterol filled macrophages (53-55) and smooth muscle cells (56,57). In atherosclerosis, the damaged vascular endothelium binds and recruits monocytes to the subendothelial space, where they differentiate into macrophages and take up modified forms of LDL to become foam cells. However, the LDLR does not appear to be important for uptake of lipoprotein cholesterol that leads to foam cell formation (5,58). Scavenger receptors, thus called because of their multiple ligand binding properties, were first identified by Goldstein and Brown when they
recognized that macrophage cells took up modified lipoproteins by a different mechanism other than the LDLR (59). Scavenger receptors mediate the uptake of modified apoB-containing lipoproteins, however, unlike the LDLR, they are not subject to the same feedback regulatory mechanisms and so uptake is uncontrolled. It is now known that a subtype of smooth muscle cells within the atherosclerotic intima can also express scavenger receptors and take up modified lipoproteins to become foam cells (57). SRs can also take up non-lipoprotein ligands such as maleylated-BSA, polyribonucleotides, polysaccharides, anionic phospholipids and it is proposed that these polyanionic ligands bind to positively charged collagenous domain on SRs (60). The binding interaction between modified lipoproteins and scavenger receptors also appears to be ionic in nature. For example, positively-charged lysine residues on the apoB moiety of oxidized LDL (oxLDL) or acetylated LDL (acLDL) become derivatized by oxidation or acetylation, which neutralizes those residues and increases their net negative charge to allow recognition by the collagen-like domain on scavenger receptors (61). Some of the scavenger receptors most important for uptake of modified lipoproteins are CD36, SR-A and LOX-1 (lectin-like oxLDL receptor), while some more recently identified members include SR-PSOX (CXCL16), FEEL-1 (stabilin-1/CLEVER-1), SREC (scavenger receptor expressed by endothelial cells) and CD163 (62). The class B scavenger receptor CD36 seems to be most important for oxLDL uptake, which comes from the evidence that CD36 knockout mice have a 60-80% reduction in macrophage oxLDL uptake (63). Interestingly, apoE/CD36 double knockout mice also have a 70% reduction in atherosclerosis compared to apoE single knockout mice (64). The scavenger receptor SR-A is also responsible for the majority (~80%) of acLDL uptake and around 50% of oxLDL by macrophages based on genetically modified mouse studies. SR-A knockout mice on an apoE or LDLR deficient background also have decreased atherosclerosis (65,66). However, knockout of
SR-A, CD36 and apoE together does not abrogate foam cell formation within the lesion (67), thus calling into question the significance of these transporters for macrophage lipoprotein uptake and the pathogenesis of atherosclerosis.

1.3 Reverse cholesterol transport

The concept of reverse cholesterol transport (RCT) was first introduced by Glomset in 1968 (68), in which he described the involvement of lecithin cholesterol acyl tranferase (LCAT) in a process whereby cholesterol from peripheral cells is returned via HDL to the liver for biliary excretion into feces. The observation was made that cholesterol in peripheral cells was in equilibrium with plasma lipoproteins, however, there was a deficit in the liver owing possibly to the excretion of cholesterol. Extra-hepatic cells can take up lipoprotein cholesterol from plasma or synthesize it de novo. However, most cells (with the exception of steroidogenic cells) cannot catabolize cholesterol. Therefore, the process of RCT is critical to the removal of excess cholesterol from peripheral tissues. This cycle of cholesterol is especially important for the maintenance of the homeostatic balance of cholesterol in the body and is potentially crucial to the prevention of atherosclerosis (69,70). UC is toxic to cells and can cause apoptosis by the unfolded protein response (71). Therefore, the RCT pathway is critical to removal of excess cholesterol from peripheral cells. There are 4 major steps in the RCT pathway (72): 1) cellular cholesterol efflux onto HDL particles, 2) UC on HDL particles are esterified to CE, 3) return of HDL-CE and cholesterol to the liver, either by direct uptake from HDL or by transfer to apolipoprotein B (apoB)-containing lipoprotein and uptake from LDL via the LDLR, and 4) cholesterol excretion in the bile, either directly, or following conversion to bile acids. The RCT pathway is summarized in Figure 1.1 below. The next section will discuss each of these steps in detail and the different players involved.
Figure 1.1 Reverse Cholesterol Transport.

Within the lysosomes of cells (ovals with dashed line), lysosomal acid lipase (LAL) hydrolyzes LDL cholesteryl esters (CE) following endocytosis mediated by the LDL receptor. Unesterified cholesterol (UC) is removed from the lysosomes by Niemann Pick type C proteins (NPC1). The ATP-binding cassette transporter A1 (ABCA1) mediates the efflux of cellular UC to apolipoprotein A1 (ApoA-I) to form nascent discoidal shaped HDL particles. The UC is esterified to CE by the enzyme lecithin cholesterol acyl transferase (LCAT) to form spherical HDL particles. HDL UC and CE can be taken up directly to the liver by selective uptake via the scavenger receptor B1 (SR-BI).

Alternatively, the CE on HDL can be transferred to LDL by the action of the cholesteryl ester transfer protein (CETP) and then LDL UC and CE enter the liver via LDL receptor-mediated uptake. Some of the hepatic UC is then directed towards biliary excretion either directly or indirectly following conversion to bile acids.
1.3.1 Step 1: Cellular cholesterol efflux and HDL formation

The first step in RCT is also the rate-limiting one. Apolipoprotein A-I (apoA-I), the major protein in HDL, is secreted by cells in the liver and intestine (73,74), and enters circulation in a poorly lipidated form. The ATP-binding cassette transporter A1 (ABCA1), a transmembrane protein, promotes the cellular efflux of cholesterol and phospholipids on to apoA-I to form nascent, pre-β HDL particles in the plasma. However, if ABCA1 is not functional or not present to perform this role, poorly lipidated apoA-I is rapidly catabolized in the kidney (75). ABCA1, ABCG1 and ABCG4 act in a sequential manner (76): ABCA1 first lipidates apoA-I to form nascent HDL particles, and then ABCG1 (and G4 in the brain) can further efflux cholesterol from cells onto HDL particles (77,78). ABCA1 and ABCG1 are further discussed in detail in section 1.4. Scavenger receptor B1 (SR-B1) can also promote cholesterol efflux to mature HDL particles (79), however, its relative role is unclear.

1.3.2 Step 2: HDL cholesterol esterification

Next, the enzyme lecithin cholesterol acyl transferase (LCAT) catalyzes the formation of CE from UC by obtaining fatty acids from phospholipids or TGs (72,80). Esterification of cholesterol on HDL enriches the neutral lipid core of the particle enlarging it and converting it from a discoidal to spherical shape. LCAT deficiency in mice (81,82) and humans (80) results in lipoprotein abnormalities – very low HDL and HDL that is present in discoidal form and only contains UC and phospholipid, as well as low plasma apoA-I (because poorly lipidated apoA-I is rapidly degraded) (75). Patients with LCAT deficiency or fish eye disease have corneal opacities, anemia, and proteinuria and may suffer from renal failure (68,72,80). Glomset originally proposed that LCAT was a driving force in RCT (68), however, it is now known that active transport (by ABCA1 and ABCG1) is necessary for functional RCT and that passive efflux is not
sufficient (83,84). While LCAT is crucial to the proper functioning of the RCT pathway, its importance in RCT for atherosclerosis is not well understood. Groups within the population that have high LCAT activity also had lower risk of CAD in one study (85) and certain LCAT mutations conferred higher risk of premature atherosclerosis (86,87). Interestingly, it would appear that individuals with heterozygous LCAT deficiency have a more atherogenic lipoprotein profile and more severe atherosclerosis than homozygotes (88). Overexpression of LCAT in rabbits increased HDL and reduced LDL, consistent with reduced atherosclerosis (89). However, mouse studies show conflicting results with regards to atherosclerosis and level of LCAT expression (90-93). Thus, the relative importance of LCAT for protection against atherosclerosis is not yet clear.

1.3.3 Step 3: Uptake of HDL cholesterol and cholesteryl esters by the liver

Following cholesterol esterification by LCAT, CE and remaining UC on HDL particles can be taken up by the liver directly by the scavenger receptor SR-BI. By this mechanism, SR-BI mediates the selective uptake of lipids and cholesterol (in the form of CE or UC) directly from the HDL particle independently of the apolipoprotein A-I component (94,95). With the exception of HDL containing apoE, whole HDL particles are not likely taken up via receptor-mediated uptake (96). Instead, HDL-CE uptake follows a non-endocytic mechanism that is not blocked by endocytosis or receptor-recycling inhibitors, including chloroquine (97). This indicates that CEs derived from selective uptake from HDL follow a non-lysosomal pathway and are not likely to be hydrolyzed by LAL.

An inverse relationship between SR-BI and HDL levels exists. SR-BI knockout mice have increased plasma HDL-cholesterol (HDL-C) because uptake to the liver is reduced (98-100), whereas overexpression of SR-BI reduces plasma HDL-C (101) but promotes macrophage
RCT when expressed in the liver (102). There is also strong evidence that hepatic SR-BI expression is athero-protective (103-107).

Cholesteryl esters on HDL can also be transferred to apoB-containing lipoproteins in exchange for TG by the cholesteryl ester transfer protein (CETP) (108,109). Following transfer by CETP, CEs on LDL or VLDL can then enter hepatocytes (110) by receptor-mediated uptake by the LDL receptor. In human CETP deficiency, patients have extremely high levels of HDL-C (111). Mice don’t have CETP, however, overexpression of human CETP decreases plasma HDL-C levels (112). In humans, most of the CE taken up by the liver (and converted to bile) is first transferred from HDL to apoB-containing lipoproteins (113), suggesting that CETP mediates the primary mode of hepatic uptake in RCT. However, UC on HDL particles seems to be taken up directly by the liver by SR-BI, independently of CETP (114).

### 1.3.3.1 HDL remodeling in the plasma

HDL particles in plasma can be acted upon by other proteins to alter its content and promote recycling of HDL apolipoproteins so that they can accept more lipid and cholesterol in the first step in RCT. Following CETP-mediated transfer of triglycerides (TG) onto HDL, hepatic lipase on the hepatocyte surface can hydrolyze this TG and regenerate lipid poor apoA-I, or preβ-HDL (115,116). Hydrolysis of HDL-TG by hepatic lipase further stimulates hepatic HDL-CE uptake (117), mediated by SR-BI (118). Endothelial lipase hydrolyzes phospholipids on the surface of HDL (119), generating poorly lipidated apoA-I which is rapidly catabolized (120). The phospholipid transfer protein transfers phospholipids from the surface of very low density lipoprotein (VLDL) and chylomicron remnants to HDL (121). It can remodel HDL by transferring phospholipids between different sized HDL particles (122) and plays an important
role in generation of preβ-HDL (123). All of these factors influence the size distribution and functionality of HDL particles that influence the efficiency of RCT.

1.3.4 Step 4: Hepatobiliary excretion of cholesterol and bile acids

Once taken up by the liver, CEs must first be hydrolyzed and UC can be either secreted directly into bile, or indirectly after conversion to bile acids. The classical pathway for bile acid synthesis occurs within hepatocytes, where cholesterol is converted primarily to cholic acid and chenodeoxycholic acid (in humans); the rate limiting enzyme in this conversion is cholesterol 7α-hydroxylase, or cytochrome P450 enzyme 7A1 (CYP7A1) (124). Bile acids are actively secreted from the liver into bile and then released into the intestine following ingestion of a meal, where they act as surfactants to dissolve dietary lipids and proteins for absorption. The majority of bile acids are reabsorbed and recycle in the hepatobiliary circulation, however, a small percentage (~5%) is lost to excretion (125). ABCG5 and ABCG8 work together to actively transport cholesterol across the apical membrane of hepatocytes into bile (126). The bile salt export pump, ABCB11 also actively pumps bile acids across the hepatocyte membrane into the bile canaliculus for bile production (127). There is significant evidence that the pool of HDL-derived cholesterol in the liver is preferentially shunted towards biliary excretion (113,128,129).

1.3.5 The role of the intestine in RCT

The role of the intestine in RCT is increasingly becoming apparent. In addition to hepatocytes, ABCG5 and ABCG8 are also expressed in the intestinal epithelium, where they counteract dietary cholesterol absorption (and biliary cholesterol re-absorption) by transporting cholesterol absorbed in epithelial cells back into the intestinal lumen (130,131). Also, the Niemann Pick C1-like 1 (NPC1L1) protein, the target of the drug ezetimibe (132), plays a role in
absorption of cholesterol in absorptive enterocytes, which impacts the RCT pathway, affecting whole body cholesterol homeostasis (133).

An alternative pathway for cholesterol excretion has been recently identified in rodents, whereby HDL-cholesterol may be excreted directly into the intestine, which would bypass the classical RCT pathway, by avoiding passage through the liver (134,135). Using bile duct ligation and NPC1L1 overexpression to impair hepatobiliary cholesterol transport, Temel and colleagues (135) found that fecal sterol excretion from plasma cholesterol was normal, suggesting that cholesterol may be excreted directly from the plasma into the intestine. This alternative pathway was termed trans-intestinal cholesterol excretion (TICE). In another study it was determined that HDL was not required for TICE (136). In a recent publication (137), TICE was determined to be an active process, requiring the LDLR and ABCB11, which was modulated by treatment with statin or recombinant PCSK9. Nevertheless, Nijstad and colleagues (138) challenge the relevance of the TICE pathway, maintaining that the classical RCT pathway is still required for excretion of cholesterol coming from peripheral tissues. They observed that bile duct ligation or knockout of ABCB4 in mice blocked excretion of radiolabelled cholesterol derived from peripheral cells and that very little HDL-CE is taken up from circulation into the intestine, which was confirmed by the results of Vrins et al (136). Therefore, further studies are needed to resolve this controversy and its relevance to human biology.

1.3.6 Macrophage-specific reverse cholesterol transport

The study of the RCT process has shifted in recent years. Current methods to model reverse cholesterol transport directly from macrophages have been pioneered by the laboratory of Daniel Rader, where radiolabeled cholesterol tracer is tracked through the RCT pathway ultimately to the feces (139,140). This technique is a more effective method at estimating the
flux of cholesterol through the RCT pathway, rather than measurement of HDL-C levels themselves, since it models RCT in a more dynamic sense. For example, in deficiency of apoA-I, over expression of CETP or SR-BI causes major reductions in RCT without affecting cholesterol efflux (140-142). Similarly, human subjects with natural mutations in apoA-I (ApoA-I_Milano) have very low HDL-C, but no excess risk of atherosclerosis (143). Also, the CETP inhibitor torcetrapib, while it significantly increased HDL-C levels, resulted in increased risk of cardiac and other events (144-146). Recently, much of the focus has turned toward the many known anti-atherosclerotic properties of HDL such as the anti-oxidant, anti-inflammatory, anti-thrombotic properties, as well as its dynamic role within the reverse cholesterol transport (RCT) pathway, as opposed to HDL cholesterol levels themselves (115,140,147). The ability of HDL to promote cholesterol efflux from macrophage foam cells, smooth muscle cells and endothelial cells in the arterial wall is central to its anti-atherogenic effects (140,148). Therefore, measurement of RCT from macrophages specifically is relevant to atherosclerosis, since macrophages are the primary cell type that accumulates cholesterol to become foam cells in the artery wall in mouse models of atherosclerosis (114,149).

The roles of several players in macrophage-specific RCT have been investigated using this technique. ABCA1 and ABCG1 macrophage-specific knockout mice have reduced cholesterol efflux to apoA-I and HDL, respectively and have impaired in vivo macrophage RCT (77,150). Alternatively, macrophage-specific overexpression of ABCA1 in mice increased macrophage RCT (150). Mice fed the synthetic LXR agonist GW3965 also promoted macrophage RCT by upregulation of several genes in the RCT pathway (151). Interestingly, in several instances, changes in macrophage RCT that inversely reflect changes in atherosclerosis severity did not correlate with HDL-C levels (101,152,153). For example, SR-BI overexpression
in the liver enhanced macrophage RCT, despite decreased HDL-C levels (102). In these cases, macrophage RCT may provide a more accurate reflection than HDL-C of the role of a particular gene in the RCT pathway for influencing atherosclerosis susceptibility.

1.3.7 Regulation of reverse cholesterol transport genes by the liver X receptor

The liver X receptor (LXR) belongs to a family of ligand-activated nuclear receptor transcription factors that play a crucial role in the transcriptional regulation of genes in diverse aspects of lipid metabolism, including genes that mediate RCT. Nuclear receptors such as LXRs, peroxisome proliferator activator receptors (PPARs), and farnesoid X receptors (FXRs) function as lipid and sterol sensors, which respond to cellular lipid levels and change gene expression patterns accordingly to protect cells from lipid overload and toxicity (154). The LXR works in concert with the retinoid X receptor (RXR), forming an obligate heterodimer pair (155). The LXR along with RXR binds to an LXR response element (LXRE), which consists of a direct repeat sequence (DR4) consisting of conserved bases AGGTCA spaced by 4 variable nucleotides within the promoter region of its target genes (155). There are two isoforms of LXR, LXRα and LXRβ, which are the products of two different genes, and share 78% sequence identity within their ligand binding domains (156). The LXRα isoform is most highly expressed in liver, intestine, kidney, adipose as well as in macrophages (11,155), whereas the LXRβ has a low level of constitutive expression in most tissues in the body (157). Oxysterols are the natural ligands that activate LXRs, with the strongest activators being 22-OH, 20(S)-OH, 24-OH, 25-OH, 24(S),25-epoxycholesterol and 7α-OH (158-160). Although 27-hydroxycholesterol (27-OH) is not as potent ligand of LXR, it is the most abundant oxysterol in plasma (161), making it a likely important activator of LXR in vivo (10,162). Glucose can also bind to and activate LXRs, indicating that LXR may also be involved in the regulation of glucose metabolism and insulin
signaling (163). Recently it was discovered that desmosterol, a precursor to cholesterol, also activates LXR-mediated gene expression in response to cholesterol loading of macrophages (164). This was shown to be independent of oxysterol-mediated activation of LXR, when the enzymes responsible for production of the major endogenous ligands 24-OH, 25-OH and 27-OH were knocked out in mice (164). Thus, desmosterol may be an important, previously unknown ligand for LXR.

1.3.7.1 Enzymatic and non-enzymatic synthesis of oxysterols

Oxysterols are derivatives of cholesterol that can be produced enzymatically, or non-enzymatically through free-radical lipid peroxidation such as 7-ketocholesterol, 7(α)-hydroxyl (7-OH), and 5, 6-epoxides (165,166), or by auto-oxidation (166) and can come from dietary (167) or intracellular sources in vivo. Non-enzymatically produced oxysterols are also found on oxLDL particles and are thought to contribute to atherosclerosis (58,168-170).

Oxysterols can also be synthesized enzymatically within cells. Some oxysterols such as 7-, 27-, 24- and 25-hydroxycholesterol (7-OH, 27-OH, 24-OH and 25-OH) are produced as intermediates in the initial steps in bile acid synthesis. The most abundant oxysterols 7-OH and 27-OH are produced enzymatically by the cytochrome P450 enzymes CYP7A1 and CYP27 respectively. They are involved in the two main pathways for the initial steps in bile acid synthesis. The enzyme 27-hydroxylase is ubiquitously expressed in most tissues, while 7(α)-hydroxylase expression is liver-specific (165). Mutations in the gene encoding CYP27 result in cerebrotendinous xanthomatosis (171), causing severe neuropathy as a result of accumulation of sterol around the myelin sheaths of neurons (172).

The oxysterol 24-OH is produced enzymatically by the cytochrome P450 enzyme 24-hydroxylase (CYP46) (173) and is expressed specifically in the brain (174). Since the blood
brain barrier limits the exchange of cholesterol from lipoproteins, conversion of cholesterol to 24-OH may facilitate reverse cholesterol transport out of the brain (174). 22-hydroxycholesterol (22-OH) is thought to be produced enzymatically and is present at low levels in most tissues, although the mammalian 22-hydroxylase has not yet been identified (173). It is especially abundant in steroidogenic tissues where it is used as an intermediate in the production of steroid hormones such as testosterone (175).

The human gene for 25-hydroxylase, encodes a small, polytopic membrane protein that belongs to a group of enzymes containing a non-heme di-iron core with histidine-rich regions within the active site, and is not a member of the cytochrome P450 family (176). 25-OH is produced at low amounts in most tissues, including the lung, heart and kidney (165) and functions as an intermediate for bile acid synthesis, and regulation of cholesterol sensitive genes (12,177).

1.3.7.2 The role of oxysterols in regulation of cholesterol homeostasis

The ‘oxysterol hypothesis’, first proposed by Kandutsch in 1978 (12), suggests that oxysterols can act like signaling molecules under conditions of excess cellular cholesterol. Despite their very low abundance (10^-5 to 10^-6 that of cholesterol), they potently suppress cholesterol synthesis, likely due to their hydrophilic properties which increases solubility, allows oxysterols to move freely throughout cellular compartments (12). In addition to suppression of cholesterol synthesis, during cholesterol overload, oxysterols also activate a host of transcription factors such as LXRα to promote cholesterol efflux and reverse cholesterol transport in order to alleviate cholesterol buildup within cells. Thus, LXRα act as sensors to monitor the oxysterol levels within the cell and translate this into transcriptional regulation of target genes in other lipid metabolism pathways (159). It is now understood that LXRα are involved in the control of a
number of lipid regulatory networks. The LXR target gene network includes genes involved in fatty acid metabolism (by activation of SREBP-1c and target genes fatty acid synthase, acetyl CoA carboxylase and stearoyl CoA desaturase), and phospholipid and cholesteryl ester transporters (PLTP) (178), cholesterol efflux (ABCA1 and ABCG1), lipoprotein transport and exchange (apo E, apo CI, CII, LPL and CETP), and excretion via bile synthesis (CYP7A1) (154,179).

Oxysterols can also control LDL cholesterol uptake by upregulating expression of the inducible degrader of the LDL receptor (IDOL). IDOL is an E3 ubiquitin ligase that ubiquitinates the LDL receptor, targeting it for proteasomal degradation (180). The expression of IDOL is under regulation of LXR (180). LXR induction of IDOL provides another mechanism by which oxysterols can regulate LDL uptake when cellular cholesterol levels are high (180,181). Thus oxysterols play an important role in signaling cholesterol overload and activation of cellular regulatory measures to control cellular cholesterol homeostasis and promote RCT.

1.4 ABCA1 and ABCG1 function and regulation

ABCA1 is a member of a large superfamily of ATP-binding cassette transporters, comprised of 7 subgroups (A to G) and 51 members, including the cystic fibrosis transmembrane receptor (CFTR) and multi-drug resistance protein (MDR1) (182). Generally, these membrane proteins function by using ATP to pump a variety of substrates unidirectionally across the membrane bilayer (183). The ABCA1 is a membrane protein that transports phospholipids and cholesterol in order to lipidate apolipoproteins for nascent HDL particle formation during the earliest stage of RCT.

Like other ABC proteins, ABCA1 has two transmembrane domains (TM) with 6 membrane-spanning alpha helices each, two nucleotide binding domains (NBD) composed of a
Walker motif and a signature motif, which together utilize the energy from ATP hydrolysis to catalyze conformational changes that allow translocation of the substrate (182,184). There is also a hydrophobic regulatory domain in the loop between TM domains that interacts with the membrane (185). The importance of ABCA1 in the RCT pathway became apparent with the study of Tangier disease and the discovery that mutations in ABCA1 were the underlying cause (186-189). Tangier disease is a rare autosomal recessive disorder characterized by enlarged yellow-orange tonsils, skin xanthomas, and hepato(spleno)megaly. As well, patients may have ocular manifestations and CE deposits in lymph nodes, thymus, and intestine. Deposits in Schwann cells lead to peripheral neuropathy in many cases (185,190,191). Tangier patients also have generalized hypolipidemia; homozygotes have a severe deficiency of HDL-C (<10mg/dL), nearly absent plasma apoA-I, low LDL-C (40% of normal) and may develop hypertriglyceridemia (185,192). Tangier heterozygotes have half normal HDL levels and half normal efflux activity from fibroblasts (83), implying that ABCA1 is the rate limiting step in HDL production for RCT (185). Tangier homozygotes have a 4-fold greater incidence of cardiovascular disease (CVD), although there is significant heterogeneity among patients, and heterozygotes also commonly have a higher incidence of CVD (191,193).

ABCA1 mediates efflux of PLs and UC, in a process that is dependent on the Golgi (84,194-196) and through its interaction with apolipoproteins such as apoA-I, apoA-II and Apo C-I, C-II and C-IV (197-201). Notably, Francis et al. demonstrated that Tangier fibroblasts have impaired cholesterol and phospholipid efflux to apoA-I in medium (192). Increased expression of ABCA1 at the cell surface enhances ApoA-I binding (202). ApoA-I interacts directly with the extracellular loops of ABCA1 to enhance its efflux activity (202,203). Similarly, ABCA1 activity can also be increased by phosphorylation of the NBD by casein kinase 2 (204).
Activation of Janus kinase 2 inhibits apoA-I binding to ABCA1 and reduces lipid efflux (205). However, for the purposes of HDL particle formation, apoA-I binds to high affinity binding sites, or lipid domains in the membrane created by ABCA1 (206).

1.4.1 ABCA1 transcriptional and post-transcriptional regulation

In macrophages and fibroblasts cholesterol loading upregulates ABCA1 expression (188,207,208) and activates apoA-I dependent efflux (84,196,209,210). Cyclic AMP upregulates ABCA1 in macrophages, independently of cellular sterol levels (188,199,201,207,210), which it does by activating protein kinase A that phosphorylates the NBDs of ABCA1, increasing its activity (211,212).

The ABCA1 promoter contains an LXRE, and its expression is upregulated in response to LXR and RXR ligands, oxysterols and retinoic acid respectively (8,9) in a synergistic fashion. SREBP-2 can suppress ABCA1 transcription by binding directly to an E-box motif in the ABCA1 promoter (213). Statins can also upregulate LXR-mediated ABCA1 and ABCG1 expression in macrophages by reduced mevalonate production and RhoA signaling, increasing PPAR_γ activation and its target gene LXR (214). There are a number of other transcriptional activators and repressors that regulate ABCA1 expression which are nicely reviewed by Schmitz and Langmann in (213).

Due to its rapid turnover rate (a half-life of 1-2 hours) (215), ABCA1 can also be regulated at the level of protein stability. The ABCA1 protein contains an internal sequence rich in proline, glutamic acid, serine and threonine (PEST). Phosphorylation of threonines 1286 and 1315 within the PEST motif targets calpain protease to increase ABCA1 protein degradation. However, apoA-I binding counters phosphorylation and calpain-mediated degradation, thus increasing the stability of ABCA1 at the plasma membrane (216,217). Also the proteins α1-
syntrophin and Lin7 contain PDZ-domains and can interact with the C-terminal tail region of ABCA1 to increase its stability, possibly by causing a conformational change that protects ABCA1 from calpain protease degradation (218). ApoA-I binding to ABCA1 also activates phospholipase C, which hydrolyzes PC to DAG, activating protein kinase C and phosphorylating ABCA1, increasing its stability (219).

1.4.2 The ABCG1 and its role in RCT

Another member of the ATP-binding cassette transporter family, ABCG1 has also been found to play an important role in cholesterol efflux in RCT. ABCG1, homologous to the Drosophila white gene, is a half transporter (has only one TM domain and one NBD) that can form homodimers and possibly heterodimers with ABCG4 (220). ABCG1 is upregulated by acLDL cholesterol loading in macrophages, but unlike ABCA1 its protein expression is downregulated following incubation with HDL₃ (221) but not by native LDL or free cholesterol (222). ABCG1 is also upregulated by oxysterol-mediated induction of LXR (179,222,223). LXR-induced ABCG1 expression increases cholesterol efflux to exogenous HDL₃ as an acceptor (77,150,224), although it can also efflux to LDL, cyclodextrin or phospholipid vesicles (225,226), and does not require protein interaction at the plasma membrane for efflux. ABCG1 has similar tissue expression pattern to ABCA1 but is present mostly intracellularly in ER and Golgi compartments (179,221). Exactly how it functions in lipid efflux in these compartments is unknown, however, it is thought to play a role in regulating intracellular cholesterol movement (227). ABCG1 knockout mice have lipid accumulation in foamy macrophages in many tissues, especially the lung (77). Interestingly, however, neither ABCG1 knockout nor overexpression in mice has any effect on plasma HDL-C or other lipid or lipoprotein levels (228). ABCG1 is also thought to play diverse roles in cholesterol homeostasis during T cell lymphocyte proliferation,
glucose metabolism in pancreatic β-cells and surfactant secretion from type II pneumocytes (227). The relation of ABCG1 to atherosclerosis is complex. Loss of ABCG1 in the whole body or specifically in macrophages on an atherogenic mouse background (LDLR or ApoE-/−) results in either reduced or mildly increased atherosclerosis depending on the study conditions (229-234).

1.5 Regulation of cholesterol metabolism in Niemann Pick disease type C (NPC)

Niemann Pick disease type C is a neurodegenerative disease with other clinical manifestations such as hepatomegaly, neonatal liver disease, and, frequently, death in childhood or early adolescence. NPC patients typically have low plasma HDL (13,235). Biochemically, NPC is characterized by intracellular accumulation of UC, specifically within the late-endosomes and lysosomes (236). Cholesterol accumulated within this compartment is not available for normal cholesterol regulation such as for suppression of cholesterol synthesis by HMGCR and uptake by the LDLR or for upregulation of cholesterol esterification by ACAT (237). Excess lysosomal cholesterol also cannot access intracellular sites for oxysterol synthesis, resulting in reduced activation of liver X receptor (LXR) nuclear receptor and dysregulation of its target genes in NPC (238).

Previous work in our lab has demonstrated that cholesterol-dependent regulation of ABCA1 and cellular efflux of cholesterol and phospholipids to apoA-I are impaired in NPC1 deficient human fibroblasts as a result of impaired cholesterol trafficking out of the lysosomal compartment (13). However, exogenous addition of oxysterols or synthetic LXR ligand corrects ABCA1 and ABCG1 expression and cholesterol efflux in NPC1 fibroblasts (239). Larger HDL (alpha-sized) particles are nearly absent from plasma of NPC patients, however, LXR agonist treatment restores normal HDL particle formation from NPC fibroblasts (239). This work
indicates that cholesterol transport through the lysosomal compartment is important for regulation of ABCA1 for cholesterol efflux and HDL formation. However, it also shows that upregulation of ABCA1 by direct activation of the LXR pathway can overcome this defect and restore normal functioning of lipid and cholesterol efflux for HDL particle formation.

1.6 Lysosomal acid lipase (LAL)

Defects in lysosomal hydrolysis of cholesteryl esters also lead to impaired regulation of HDL formation, and are the major focus of this thesis. The enzyme lysosomal acid lipase (LAL), the sole hydrolase responsible for breakdown of cholesteryl esters within the lysosomes, is discussed in the following section.

1.6.1 The history of discovery of LAL

One of the earliest descriptions of what would later be termed Wolman Disease was made by Abramov and colleagues (240). They described a case report of a 2 month old girl with adrenal calcification and enlarged liver and spleen, which upon microscopic analysis revealed massive lipid accumulations and foamy histiocyte infiltration in the tissues of the liver, spleen, lymph, adrenals, lungs and small intestine. Histologic staining of lipids showed that much of this accumulation was cholesterol (240). This was later confirmed by Wolman and colleagues in 1961, in another case report of two affected siblings with similar symptoms and pathology (241). Upon histologic analysis of affected tissues, it was confirmed that the lipid accumulation was a mixture of triglycerides, cholesterol and fatty acids. The adrenals in particular contained 18% of their wet weight as cholesterol, 90% of which was esterified (normal is 3-12%). The authors also remarked at the superficial similarity to Niemann Pick C disease, except for the prevalence of adrenal calcification and little central nervous system involvement, for which previous cases may have been mistaken (241). Separately, Mahadevan and Tappel (242) identified a lipase enzyme
purified from rat liver and kidney that was capable of hydrolyzing triglycerides at acidic pH. Following subcellular fractionation of rat liver homogenate, they observed a 22-fold increase in lipase activity in the lysosomal fraction, suggesting that it was a lysosomal enzyme (242). Shortly after, Patrick and Lake (243) made an important discovery linking acid lipase deficiency to the pathology observed in Wolman disease. They studied a case of 3 brothers affected with Wolman disease, having accumulations of neutral lipid and cholesterol (mainly in the form of triglyceride and cholesteryl ester) in visceral organs, suggesting that it may have been an inherited disease caused by a genetic deficiency. The activity of acid lipase in liver and spleen homogenates at pH 4.2 was absent in Wolman disease samples. Thus, they concluded the cause of Wolman disease was a deficiency of acid lipase enzyme leading to the progressive accumulation of triglycerides and cholesteryl esters in affected tissues (243).

1.6.2 The molecular biology of LAL

The LIPA gene that encodes LAL has been mapped to the human chromosome locus 10q23.2-23.3 (244,245). The gene spans approximately 36 kilobases and contains 10 exons (246,247). The gene was first cloned in 1991 by Anderson and Sando (244). The resulting cDNA sequence predicts an mRNA product 26-27 kilobases long (244,248). The LIPA gene has a high level of sequence homology and shares structural similarity with human gastric lipase and rat lingual lipase, enzymes involved in the pre-duodenal breakdown of triglycerides (244). However, LAL is distinct from other neutral lipases such as neutral cholesteryl ester hydrolase (nCEH), lecithin cholesterol acyl transferase (LCAT) and lipoprotein lipase (LPL) (244). The LAL protein contains 372 amino acids preceded by a 27 amino acid signal sequence (248,249). The mature protein contains 3 cysteine residues (Cys\textsuperscript{227}, Cys\textsuperscript{236} and Cys\textsuperscript{244}) that share conservation with other lipases, suggesting their importance for catalysis (249). The polypeptide sequence also
contains conserved putative glycosylation sites (Asn-X-Ser/Thr) at three critical asparagines (244). Treatment of cells with tunicamycin produces an inactive form of LAL, suggesting that glycosylation is critical to catalysis within the folded protein (249).

1.6.2.1 Gene regulation of LIPA

The regulation of LIPA gene transcription has not been well characterized to date. Putative binding sites for Sp1 and AP2 transcription factors and a CAT-box were identified in the promoter region of LIPA (247,250). The pro-atherogenic lipoprotein(a) suppresses LAL mRNA expression in cultured and circulating monocytes (251). The bacterial endotoxin lipopolysaccharide (LPS) also downregulated LAL gene expression at concentrations above 1 ng/ml, although the mechanism is unknown. LAL expression does not, however, appear to be regulated by LDL (251) (and my own personal observations). No LXRE has been identified in the promoter region of the LIPA gene in any of the transcription factor searches (247,250) (including my own analysis), and the gene is not upregulated by LXR ligands.

1.6.3 LAL secretion and uptake

LAL is synthesized as a pre-protein containing a 27 amino acid signal peptide and a 49 amino acid prodomain (248). LAL can be found in two different isoforms that vary by cell type. The purified protein from human skin fibroblasts has an apparent molecular weight of 41 kDa for the intracellular form and 49 kDa for the form that is secreted (249,252,253). Human liver contains 41kDa and 56kDa molecular weight isoforms (248). Post-translation, LAL protein in cultured human fibroblasts has a half-life of approximately 12 to 24 hours (253).

According to the ‘secretion recapture hypothesis’, following synthesis, lysosomal enzymes are modified (tagged with a ‘recognition marker’), packaged into vesicles for secretion and can later be internalized from the extracellular fluid and targeted to their site of action in the
lysosomes (254). It is now known that uptake of lysosomal hydrolases and targeting to the lysosomes is mediated by recognition of phosphomannosyl residues on the lysosomal proteins by the mannose-6-phosphate receptor (255-257). Purified LAL enzyme added to the medium corrects hydrolysis activity and CE accumulation in cultured Wolman disease fibroblasts (258). LAL protein was taken up by endocytosis by saturable kinetics, which could be outcompeted by addition of mannose-6-phosphate to the medium or by a ‘high-uptake’ form of another lysosomal enzyme L-iduronidase, suggesting that uptake was receptor-mediated and that a common receptor is used by several hydrolases. Alkaline phosphate pre-treatment of LAL also blocked uptake, indicating that the receptor recognizes phosphomannosyl residues on the proteins that are required for uptake (258). Further evidence that glycosylation was required for LAL uptake came from the observation that treatment with endo-β-N-acetylglycosaminidase H reduced the 49kDa isoform to 41kDa (253). Endoglycosidase-treated LAL retained enzyme activity in vitro, however, this form could no longer be taken up by cells.

Zschenker and colleagues (259) identified a mutation in the signal peptide of LAL (-5 amino acid position) causing Wolman disease. When the G-5R mutant was expressed in insect cells, enzymes contained in cell lysates had normal catalytic activity, however in medium the activity was 6% of normal, suggesting a defect in secretion. Therefore, it is likely that the signal peptide is important for directing LAL trafficking towards secretory vesicles.

1.6.4 LAL enzyme properties

The enzymatic properties of lysosomal acid lipase (LAL) have been extensively studied in a number of different species and experimental conditions. This acid lipase, enzyme classification number EC 3.1.1.13, belongs to the class of serine hydrolases, with a classical GXSXG active site motif at serines 153 and 99, although site-directed mutagenesis studies have
shown that only Ser\textsuperscript{153} is critical to catalysis (249). The LAL protein has limited solubility, with high affinity for membranes. When membrane-bound, only 30% could be released into the soluble fraction (242,260). This has raised the possibility that intracellular LAL may be tightly bound to the lysosomal membrane \textit{in vivo} (242).

1.6.4.1 How LAL activity is measured

Early assays to measure lipolytic activity of LAL did not discriminate based on substrate since they quantified the release of fatty acids from hydrolysis. The substrate being tested would be dispersed in a pH adjusted citrate-phosphate buffer containing Triton X-100 detergent (242). Then, following different incubation times at 37°C in the presence of semi-purified enzyme, the free fatty acids released following hydrolysis were extracted into chloroform and quantified using a colorimetric or fluorometric assay (260,261). Later, methods for measuring hydrolysis of radioisotope labeled substrate (e.g. [\textsuperscript{14}C]Tripalmitin or cholesteryl-3-[1-\textsuperscript{14}C] palmitate) were developed. This was a more sensitive assay that allowed for a more specific analysis of enzyme activity for different substrates observed (262). However, these assays still measured the release of fatty acids since the radiolabelled moiety (\textsuperscript{3}H or \textsuperscript{14}C) was on the acyl chain (263,264). In the early 1980s, a simpler method for measuring LAL hydrolysis activity was determined, where the artificial substrate 4-methylumbelliferyl oleate (4-MUO) was incorporated into phospholipid vesicles in a citrate-taurodeoxycholic acid buffer at acidic pH (265). Upon incubation with cell lysate or tissue homogenate containing LAL, hydrolysis of 4-MUO releases the product 4-methylumbelliferate, which emits fluorescence at a wavelength of 455nm when excited at 355nm (258,265). This simplified assay allowed for an easy and accurate assessment of LAL activity in multiple samples under different enzyme purification conditions.
1.6.4.2 LAL enzyme activity parameters

The pH optimum of LAL has been studied in a range of species and under multiple different experimental conditions, making it difficult to generalize. In human tissues, LAL activity has been reported to be optimal anywhere from pH3.5 to 6.0. Rat and rabbit LAL activity is optimal at approximately 4.5 to 5.0 (242,252,260,266-268). However, the pH optimum seems to depend on the substrate used and conditions under which the assay was performed. For example, the pH optimum for CE and TG (hydrolysis of radiolabelled substrate) is approximately 3.5 to 4.5 whereas it is slightly higher towards the artificial substrate 4-methylumbelliferineryl oleate (4-MUO), between 4.0 and 5.0 (252,269,270). As well, modification of the assay conditions by addition of detergents such as TritonX-100, bile acids, acidic phospholipids or high salt concentration can cause a shift in the pH optimum (253,260,271).

The specificity of LAL towards various substrates has also been studied in detail. LAL is a carboxyl esterase that can hydrolyze the ester bond to release fatty acids from cholesteryl esters and triglycerides. LAL has 3-4 times greater affinity for TG hydrolysis than for CE (272), and hydrolyzes TG, diacylglycerols and monoacylglycerols in decreasing order of activity (260,272,273). However, cleavage of monoacylglycerols has been demonstrated for purified rat and rabbit enzyme only, and it cannot be cleaved by human LAL (272,273). Ester bond cleavage occurs most favorably at the sn1(3) position (273) and there is a preference for substrates with medium to long chain fatty acids of 10-18 carbons (242,261), for unsaturated fatty acids over saturated (261), and for cis over trans double bonds (249).

The reaction kinetics of purified LAL from different species and tissue types has been quantified by several groups in the 70s and 80s. The rate of enzymatic activity is shown to be linear from 10 minutes up to 90 minutes and is proportional to enzyme mass over a range of 100-
300 µg protein (260,261,266,274). LAL enzyme is only 30% soluble in water and is most stable when membrane bound or when integrated into lipid vesicles, where the solubility increases to 70% (242,260). The poor solubility of LAL had caused some difficulty during early attempts at purification of the enzyme. As a result, the apparent $K_m$ and $V_{max}$ of the enzyme differ greatly depending on substrate preparation and tissue type from which the enzyme was purified. A wide range of values of $K_m$ for human LAL have been reported, varying between 20 µM to 1.2 mM of substrate concentration (269,270,272). In general, the $K_m$ tends to be lower towards the 4-MUO artificial substrate than for CE or TG (252,270). Similarly, the reported $V_{max}$ of the enzyme ranges between 8 nmol/mg*min to 5.4 µmol/mg*min for TG or CE, but is much higher (up to 290 µmol/mg*min) towards 4-MUO (252,268,275,276).

1.6.4.3 Factors that affect LAL enzyme activity

1.6.4.3.1 Inhibitors

Numerous different activators and inhibitors of LAL have been identified in a number of studies in the literature, however the general properties and types of agents will be summarized here. In general, LAL enzyme activity is inhibited by high salt conditions of greater than 0.2M of salts such as NaCl, KCl, HgCl$_2$, Hg(NO$_3$)$_2$, iodoacetate or NaF (242,261,269,270), at lower concentration, by divalent cations such as Ca$^{2+}$, Cu$^{2+}$, Hg$^{2+}$ or Zn$^{2+}$ (242,252,264,266,268), or by other positively charged molecules such as stearamine (277). LAL has also been shown to be inhibited by acidic phospholipids such as lyso-Phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), cardiolipin (CL) (253,277), while other studies have found that phospholipids such as CL, PS and phosphadidylethanolamine (PE) have the opposite effect by enhancing LAL activity (263,271). Various sulfhydryl reagents have been shown to inhibit enzyme activity, which indicates the presence of a critical cysteine residue at or near the active
site of LAL (261,266,268,276). As well, lysosomotropic drugs such as chloroquine and
chlorpromazine have both direct and indirect inhibitory effects on LAL (253,275,278) as well as
the hormone progesterone (274). The fungal antibiotic esteratin also possesses potent inhibitory
properties against LAL, causing an accumulation of CE in cultured smooth muscle cells 12 times
higher than basal levels (279). More recently, a specific and potent inhibitor of LAL activity,
termed ‘lalistat’ has been developed. Lalistat contains a thiadiazole carbamate moiety and is
closely related to another drug ‘orlistat’ that had previously been shown to inhibit LAL but to a
lesser degree (280,281). In fact, although controversial, lalistat has been proposed as a potential
therapeutic strategy in the treatment of NPC disease in order to prevent the formation of UC,
which is much more cytotoxic to neurons than neutral CEs (282).

1.6.4.3.2 Activators

Among the activators of LAL, detergents such as TritonX-100 or digitonin have been
shown to enhance LAL activity in vitro (253,263,276). In accordance with this, the addition of
bile acids or salts such as deoxytaurocholate or taurocholate has also been shown increase the
activity of LAL, possibly by increasing the accessibility to its hydrophobic substrates in aqueous
solution (242,253,277). For this reason, inclusion of the substrate into phospholipid vesicles
(primarily made up of PC) enhances LAL activity, especially when negatively charged or acidic
phospholipids such as phosphatidic acid (PA), PS, CL are included (263,268,271,277,278).

1.6.5 The intracellular role of LAL in LDL cholesterol metabolism

A series of events take place during the uptake of exogenous LDL into cells (as reviewed
in (283)): 1) LDL binds to the LDLR at the cell surface (255,284), 2) LDL becomes internalized
via receptor-mediated endocytosis into clathrin-coated vesicles (285), 3) endocytic vesicles fuse
with the lysosomes and the protein and CE components of LDL are hydrolyzed (286,287), 4)
release of UC from lysosomes, 5) a regulatory response to excess intracellular UC takes place which causes the downregulation of HMGCR and LDLR expression (255,284), and stimulation of cholesteryl esterification by ACAT (38,288). This is summarized in Figure 1.2.
Figure 1.2 The role of LAL in the LDL receptor pathway.

LDL binds to the LDL receptor (LDLR) and is internalized via receptor-mediated endocytosis in clathrin-coated vesicles. LDL bound to the LDL receptor is transported to the late endosomes/lysosomes, where LDL is released from the LDL receptor and cholesteryl esters (CE) are broken down to unesterified cholesterol (UC) by lysosomal acid lipase (LAL) at acidic pH. The un-bound LDLR is recycled back to the plasma membrane for re-use. The Niemann Pick Type C protein 1 (NPC1) transports UC out of the late endosomes and lysosomes where it can be delivered to other parts of the cell such as the ER. Increased cholesterol levels in the ER lead to 1. Suppression of cholesterol synthesis by HMG CoA reductase (HMGR), 2. Decreased LDL receptor expression, and 3. Enhanced activity of acyl-CoA cholesterol acyltransferase (ACAT), which esterifies UC to CE for storage in lipid droplets. (*adapted from Brown and Goldstein (289))
The lysosomes were identified as the site of intracellular storage of CE and TG in LAL deficient fibroblasts (290). Using $[^3]$Hcholesteryl linoleate to specifically label cholesteryl esters on LDL particles, Brown and colleagues (287) were able to show that LDL-CEs were degraded intracellularly in parallel with LDL apolipoproteins and then rapidly re-esterified into $[^{14}]$Ccholesteryl oleate by ACAT. It was also demonstrated that this LDL-CE breakdown takes place in the lysosomes, because hydrolysis was blocked by the lysosomal inhibitor chloroquine and that the optimum pH of activity for this hydrolysis by cell free extracts was approximately 4. Uptake via the LDL receptor was also required, given that familial hypercholesterolemia (27) fibroblasts, which lack LDL receptor activity, had no hydrolysis of $[^3]$Hcholesteryl linoleate labeled LDL (287). It was later determined that WD and CESD fibroblasts, while having normal breakdown of LDL apolipoproteins, had defective hydrolysis of LDL-CEs, indicating that LAL was the enzyme responsible for hydrolysis of LDL-CE (237). CESD fibroblasts also had reduced suppression of HMGCR upon LDL loading and the total cholesterol content of CESD cells over time was higher than normal fibroblasts (237), which was most likely due to increased de novo cholesterol synthesis. Knowing that lysosomal enzyme deficiencies could be corrected by addition of the missing enzyme to the culture medium (see review (291)), they next tried a similar approach using co-culture of fibroblasts in order to investigate the requirement and sufficiency of LDL receptor and LAL enzyme. When either CESD or WD fibroblasts were incubated in co-culture with FH fibroblasts at varying ratios, the aberrant LDL-CE hydrolysis was rescued in a linear fashion with increasing FH/(CESD or WD) cell ratio (292). Co-incubation of CESD or WD cells in equal proportion to FH cells restored normal suppression of HMGCR activity and cholesterol re-esterification by ACAT, but was suppressed with increasing proportion of either cell line (292). Therefore, through the elegant work of the laboratory of
Goldstein and Brown, it was elucidated that the lysosome is the site of hydrolysis of CE brought in by LDLR-mediated endocytosis, and that lysosomal LDL-CE hydrolysis by LAL supplies the rest of the cell with free cholesterol, resulting in a regulatory response of the processes that regulate cholesterol synthesis and storage.

1.6.6 LAL and autophagy

Autophagy is a bulk degradative process for clearance of aged proteins or damaged organelles, provides a source of energy during starvation by breakdown of stored substrates and performs a housekeeping function for cell survival as opposed to cell death via apoptosis or necrosis (293), although it shares many of the same effectors and regulators as in apoptosis (294). Autophagy follows a conserved, multistep process whereby cytoplasmic contents are sequestered within double-membraned vesicles that then fuse with lysosomes in order to deliver their contents for degradation (293): 1) The first step involves initiation of formation of the limiting membrane or, phagophore. 2) The expansion of the limiting membrane is stimulated by production of phosphoinositol-3-phosphate by the signaling molecule phosphoinositol-3 kinase, which helps to recruit autophagy proteins for nucleation of the membrane and creates curvature in the phagophore membrane as it expands (295). 3) The autophagosome membrane fuses with the lysosome, containing digestive hydrolases. 4) The inner membrane of the autophagosome is broken down and its contents are degraded by lysosomal hydrolases. 5) The macromolecules produced by hydrolysis are released into the rest of the cell through pores in the lysosomal membrane created by permeases.

Autophagy plays an important role in lipid and cholesterol metabolism. Singh and colleagues (296) found cytosolic lipid droplets can be taken up into lysosomes by autophagy and degraded by lysosomal hydrolases. Lipid droplets were observed within double-membraned
vesicles within lysosomes. Inhibition of autophagy in Atg5 knockout mouse hepatocytes leads to TG and cholesterol accumulation, an increased number and size of lipid droplets and reduced breakdown of free fatty acids, whereas induction of autophagy relieved hepatic storage of TG (296). This process whereby lipid droplet TG and CE are delivered by autophagy to the lysosomes was termed by the authors as “lipophagy”.

The autophagy machinery becomes activated following exposure to modified lipoproteins such as acetylated LDL (acLDL) (297) and after uptake, the acLDL CEs are rapidly hydrolyzed in the lysosomes and then re-esterified by ACAT in the ER and incorporated into lipid droplets (298-302). LAL is required for hydrolysis of lipid droplet CEs derived from acLDL loading as it was blocked by chloroquine and lalistat (297). Inhibition of LAL or autophagy also significantly reduced efflux of cholesterol from cytosolic lipid droplets to the medium and Atg5/− mice had impaired macrophage to feces RCT. In addition, a role for the LAL homologue LIPL-4 in autophagy has also been identified in C. elegans and increases life span in these organisms (303). This indicates the importance of autophagy and LAL-mediated hydrolysis of lipid droplet CEs for whole body cholesterol metabolism and longevity.

1.6.7 Human LAL deficiency: Wolman disease and cholesteryl ester storage disease

LAL deficiencies, although they contain a spectrum of clinical phenotypes, are generally divided two different diseases, termed Wolman disease (WD) and cholesteryl ester storage disease (CESD) based on the disease severity (304). Wolman disease is characterized by hepatosplenomegaly, adrenal calcification and severe malabsorption and cachexia leading to death before 1 year of life (241,304). In CESD, hepatomegaly is also common and although clinical outcome is highly variable and affected individuals can survive well beyond the 4th decade.
In Wolman disease, the complete inability to hydrolyze CE and TG leads to a buildup of these substrates in enterocytes in the small intestine, malabsorption and death by starvation within the first year of life (304-307). Cholesteryl ester storage disease is the name given to a less severe form of LAL deficiency where those affected can survive well into mid life. The clinical presentation of CESD is characterized by accumulations of primarily CE in the liver and spleen, leading to enlargement of the liver and sometimes the spleen, however adrenal calcification is rare (304). The most common manifestations include hepatomegaly and hyperlipidemia. Fatty liver may develop into hepatic fibrosis and cirrhosis in some cases (308), leading to acute or chronic liver failure (304). Hyperlipidemia is also a consistent finding in CESD patients, which is commonly diagnosed as Type IIb (or Type IIa) familial combined hyperlipidemia with elevated plasma LDL and VLDL (309). Individuals with CESD also often have low plasma HDL cholesterol levels, below the 5th percentile and may be less than 20mg/dL (~0.5 mmol/L) in some (310-312). In some cases, it was observed that the HDL$_2$ to HDL$_3$ ratio was reversed (10:1 instead of 1:10) (304,313). Hyperlipidemia is not a common finding in WD, although low HDL-C levels have been reported in some patients (314).

The pathology of WD results primarily from accumulations of CE and TG in the liver, spleen, intestine, adrenal glands, lymph nodes, and other tissues. In both WD and CESD, hepatomegaly is a common finding. The liver may be enlarged greater than two-fold the normal size and, upon examination post-mortem, has a yellow-orange colour and greasy appearance. Neutral lipids such as TG and CE accumulate in the lysosomes of hepatocytes and Kupffer cells, and cholesterol/CE crystal clefts may be evident (304). The livers of WD patients have 2-10 fold higher than normal level of TG and massive CE accumulations of 5-600-fold normal levels.
(315), and 98% of the accumulation is in the form of CE (305). In CESD, CEs are similarly elevated up to 120-350 fold higher than normal, although CE accumulation is predominant with less increase in hepatic TGs as opposed to WD (304). In WD and CESD, Kupffer cells and macrophages in particular contain lipid droplets of TG and CE and free cholesterol (316). Inflammation may involve infiltration of macrophages, lymphocytes and neutrophils into the periportal spaces and can lead to destruction of tissue causing fibrosis and cirrhosis (317).

1.6.7.1 The genetics and molecular biology of CESD and WD: The genotype-phenotype relationship

Both WD and CESD are autosomal recessive disorders that arise from mutations in the LIPA gene at the chromosomal locus 10q23.2-q23.3. Evidence that WD and CESD arise from mutations in the same gene (LIPA) include: 1) low LAL activity in both WD and CESD cells (265), 2) secreted LAL corrects both WD and CESD (253) and 3) co-culture of WD with CESD cells doesn’t correct enzyme activity (292).

A large number of unique disease-causing genetic polymorphisms have been identified along the length of the LIPA gene (304). Although distinction between mutations that lead to either CESD and WD is not always clear cut, in general, genetic mutations that cause WD are commonly insertions, deletions or point mutations that lead to either a premature stop codon or incorrect splicing leading to truncation of the protein. In all cases, WD-causing mutations result in loss or dysfunction of LAL protein, and an absence of LAL activity (304). The mutations most commonly seen in CESD patients are missense mutations that may be homozygous or compound heterozygous with other known CESD or WD polymorphisms, but that result in partial residual LAL activity. Heterozygous individuals carrying only one mutated LIPA allele have approximately half normal LAL enzyme activity, but are phenotypically normal (313).
Mutations around the exon 8 splice junction (E8SJM) are common in both CESD and WD, however, the position of the mutation is critical to the outcome: If the mutation is 3 bases before (-3) or 1 base after (+1) the junction site, the protein is not properly spliced and this leads to exon skipping and a truncated, non-functional protein (304,313). However, if the mutation is at the -1 position adjacent to the splice site, there is an allowance (presumably flexibility within the spliceosome) which means that about 5% of the time, mRNA is spliced normally, leaving some ~1-3% functional protein and 5-10% residual activity (246,304,313). This effect has also been observed in other genetic disorders involving splice junction mutations that allows about 3% properly spliced mRNA and some residual enzyme activity (247,318). This mutation, termed E8SJM-1 (Exon 8 splice junction mutation at position -1 base from the splice site) is the result of a G>A transition at nucleotide 894 in the LAL gene (310) and is the most commonly detected mutation in CESD (304,313).

The reason for the primary accumulation of CE in CESD, and less TG buildup, is due to this small amount of residual enzyme activity in CESD combined with a preference by LAL for TGs (249,272). Therefore, TGs (and other glycerolipids) are hydrolyzed more rapidly than CEs in CESD cells, leading to a greater accumulation of CE and less TG. This small amount of enzyme activity accounts for the difference in cellular pathology that can mean the difference in survival of CESD patients.

1.6.8 Animal models of LAL deficiency

Due to their ease of manipulation and short life cycle, rodents provide a good model to study the biological effects of LAL deficiency and investigate potential treatment options. A naturally-occurring deficiency of LAL was identified in a colony of Donryu rats with a phenotype similar to Wolman disease (319) resulting from a truncation mutation in the rat LAL
gene (320). Yoshida and Kuriyama first observed hepato(spleno)megaly, swollen enlarged lymph nodes, thickened yellow intestine, weight loss and cachexia in these rats, leading to death within 4 months of birth (319). Lipid droplets within vacuolated hepatocytes and Kupffer cells of the liver, splenic cells and the lamina propria of the small intestine were observed. The livers of LAL deficient rats contained 13-fold higher CE levels, 3-fold higher cholesterol and 5-fold higher TG than unaffected rats and acidic hydrolysis of radiolabelled TG, CE or 4-MUO in vitro was only 10-20% of normal (321).

Similar to the rat, the murine LAL gene is 2.36 Kb, encoding 397 amino acids and has 79.4% homology to the human gene (322). The tissue and cell-specific expression of LAL mRNA and protein was first characterized in wild-type mice (322) and reflects the organs and tissues most affected in WD. Highest expression of LAL mRNA and protein was observed in the liver, spleen, brain, lung, kidney, duodenum and jejunum of the small intestine and thymus of adult mice. Lymphocytes and macrophages also had strong staining. However, most tissues had a basal level of LAL expression.

The outcome of LAL deficiency in mice has also been studied extensively. LAL deficient mice were generated by targeted disruption of the murine LIP-A gene, which results in an absence of mRNA, protein and complete ablation of LAL activity in all cells (323). Heterozygotes (LAL\(^{+/−}\)) possessed approximately 50-60% normal LAL enzyme activity. Homozygous LAL knockout mice (LAL\(^{−/−}\)) appear and behave normally at birth, with no overt symptoms and are reproductively viable. However, at ages 4-8 weeks, LAL\(^{−/−}\) livers were enlarged (2 times the normal size) and yellow-orange in colour and the spleen was also enlarged with a yellowish tinge (323). By 8 months of age, there is dramatic hepato(spleno)megaly, with enlarged livers up to 6 times the normal size, the spleen being on average 4.4X larger than wild-
type (324). Analysis of tissue lipids revealed a massive accumulation of CE and TG that progressed with age. At 6-8 weeks old, the levels of CE and TG levels in the liver were already 32- and 35-fold higher than wild-type liver, respectively (323) and CE levels were increased to 42 times the normal level by 8 months (324). Histologic analysis showed that in 4-8 week old mice most of the hepatic lipid accumulations were detected inside large vacuolated hepatocytes (323), however, with increasing age there is a shift towards storage in Kupffer cells (~40% by 5 months, ~85% by 8 months) (324). A proliferation assay using PCNA staining confirmed that these lipid-filled cells were proliferating resident Kupffer cells as opposed to monocyte-derived macrophages from circulation (324). The inner cortex of the adrenal gland was filled with lipid and cholesterol crystals, but with no evidence of calcification and the small intestine was also engulfed with lipid, giving it a yellowish, creamy colour. The curious observation was also made that LAL knockout mice have a progressive loss of white and brown adipose tissue with increasing age (324).

As in human WD, no differences in plasma CE or TG levels were observed in mice aged 4-8 weeks (323). However, at 8 months, LAL\textsuperscript{−/−} mice had increases in LDL and VLDL cholesterol and lower than normal HDL cholesterol when lipoproteins were separated by fast protein liquid chromatography (FPLC) or agarose gel electrophoresis, similar to the lipoprotein profiles observed in many CESD patients (317). There is also evidence that HDL particles of LAL\textsuperscript{−/−} mice may also be smaller than LAL\textsuperscript{+/+} mice (HDL peak on FPLC is shifted to the right and α-migrating band was lower in LAL\textsuperscript{−/−} mouse plasma) (324). Interestingly, LAL knockout mice also develop a diabetic phenotype, having increased non-esterified fatty acids (NEFA) in plasma and insulin resistance – plasma glucose levels were >6-fold higher than wild-type mice.
after glucose challenge. (324). Combined with the absence of subcutaneous adipose tissue, this suggested a syndrome comparable to lipoatrophic diabetes.

The pathological phenotype in LAL deficient mice was ameliorated either by supplementation with purified recombinant human LAL enzyme produced in the yeast strain \textit{Pichia pastoris} (phLAL) (325), or by overexpression of LAL using an adenovirus-mediated delivery system (Ad-hLAL) (326). Restoration of LAL activity in LAL-/- mice with either phLAL or Ad-hLAL relieved TG and CE storage in the liver and other tissues, and decreased the size of the liver and spleen over time. Systemic overexpression of Ad-hLAL restored adipose tissue, even though no localized staining was observed in the skin (326). Although plasma NEFAs were increased 162% over wild-type levels in LAL+/- mice, levels only increased by 33% in knockout mice supplemented with phLAL (325). Plasma CE and TG levels were also reduced in LAL+/- mice injected with Adv-hLAL compared to saline-injected controls and after 20 days post-injection, mice had a reduction in plasma LDL and IDL cholesterol and increased levels of HDL cholesterol (326). These findings thus illuminated the potential for enzyme replacement therapy in the treatment of human WD and CESD.

1.6.9  \textit{LAL and atherosclerosis}

Although the role of LAL in atherosclerosis is not yet defined, there is strong evidence to suggest that LAL plays an important role in the process of atherogenesis. In addition to the apparent hyperlipidemia observed, many cases of premature atherosclerosis have been identified in CESD patients (317,327,328). In one case study, severe lesions in the aorta and femoral arteries were identified in a 51 year old man with CESD (327). Many other similar cases of accelerated atherosclerosis have been described among CESD patients in the literature (329-332), indicating a potential relationship between LAL deficiency and atherosclerosis. In support
of this, Coates and colleagues (333) discovered low LAL activity in mononuclear leukocytes from non-LAL deficient patients with atherosclerosis. Compared to age-matched controls, 18% of patients with diagnosed atherosclerosis had LAL activity levels below the 5th percentile, most often in patients with premature atherosclerosis. For example, the lowest LAL enzyme activity was observed in a patient who died of a myocardial infarction at age 50 (333). In another study, LAL activity was 50% of normal in patients with cerebrovascular disease (334). Hagemenas and colleagues also observed reduced LAL activity levels in patients with hypercholesterolemia (335), although this differed from Coates’ findings where LAL activity did not correlate with plasma cholesterol levels (333).

A similar relationship between LAL deficiency and atherosclerosis has also been identified in mouse models of the disease. When LAL is knocked out in mice with an ApoE-deficient background (LAL/ApoE double knockout), these mice have much more severe atherosclerosis and more rapid progression than ApoE single knockout mice alone (336,337). In addition, LDLR knockout mice made deficient for LAL (LAL/LDLR double knockout mice) have such severe atherosclerosis that they die within 5 days on a high fat/high cholesterol diet (336,337). However, when LDLR<sup>−/−</sup> mice are supplemented with phLAL, which has the type of oligosaccharide glycosylation patterns that target it specifically to macrophage mannose-6-phosphate receptors, atherosclerosis was reduced (336). When mice were injected with phLAL daily for 30 days before lesions had started to develop (after only 1 month on a high fat/high cholesterol diet), the phLAL treated LDLR<sup>−/−</sup> mice had an absence of lesions compared to non-treated mice, indicating that early lesion development had been prevented. However, in a separate experiment where phLAL treatment was started after established lesions had developed (after 2 months on a high fat/high cholesterol diet), the lesions either didn’t progress any further,
or showed regression in size (i.e. decreased macrophage content, reduced necrotic core area) compared to untreated mice (336). Another study where LAL was overexpressed in transgenic mice found no difference in plasma or liver lipid metabolism or atherosclerosis after high fat feeding (338). The significance of this result is unclear however, since transgenic mice were on a non-atherogenic background and mice are not normally prone to diet-induced atherosclerosis. Taken together, studies in animal models of atherosclerosis indicate that LAL-deficiency likely plays a role in atherogenesis that can be remediated by macrophage-specific replenishment of LAL enzyme activity.

There is also in vivo evidence that LAL may be involved in atherogenesis since the enzyme has been observed to be present in human and animal atherosclerotic lesions. Rabbits can spontaneously develop arterial atherosclerosis when fed a diet that is high in cholesterol. Cellular CE and UC accumulate in the lysosomes of atherosclerotic aortic cells in cholesterol-fed rabbits (339-341) and human aortic smooth muscle cells (342). Indeed, LAL enzyme activity was found to be 2.5 times higher than normal in aorta of cholesterol-fed rabbits in one study (343) and was 3.5-fold higher in atheromatous arterial cells in another (344). LAL activity has also been detected in human aortic tissue (261). Davis et al. (345) also found LAL activity in human and rabbit atherosclerotic lesions. LAL activity was associated with macrophages in cholesterol-fed rabbit arterial lesions and macrophages within subendothelial fibrous lesions in advanced atherosclerotic plaques in human patient tissues. The LAL activity observed correlated with the amount of CE accumulation in homogenates of rabbit aortic tissue, suggesting that increased LAL activity might be implicated in foam cell formation (345). Alternatively, Takano and Imanaka (346) put forth the alternative hypothesis that an insufficiency of LAL activity in arterial cells is the cause of CE accumulation in atherosclerosis. They suggest that the increased
LAL activity in rabbit atherosclerotic lesions is actually lower than would be expected as compared to other lysosomal enzymes that are dramatically increased in atherosclerosis (such as acid phosphatase, N-acetyl-β-glucosaminidase, β-glucuronidase, β-galactosidase), whereas the relative increase in LAL activity was low. In fact, when lysosomes were specifically isolated from arterial cells, no LAL activity was detected (346), indicating that intracellular, lysosomal LAL might be insufficient despite increased overall activity in the lesion. The suggested mechanism involves lysosomal membrane destabilization caused by accumulating CE in lysosomes (and reduced UC that stabilizes lysosomal membranes, (347)), that then prevents the fusion of lysosomes with cargo-containing vesicles, thereby not allowing LAL to reach its substrates for hydrolysis (346). Although LAL activity has been associated with atherosclerotic lesions in these studies, it was unclear from these studies whether the activity was associated with cells or the extracellular environment.

There is compelling evidence to suggest that LAL may be secreted from macrophage cells in the artery wall and may enzymatically modify apoB-containing lipoproteins retained in the extracellular matrix, contributing to the atherogenic process. It is known that lipid droplets in atherosclerotic lesions have unusually high concentrations of free cholesterol (348-351). Bahkdi et al. (352) observed that enzymatically modified LDL (E-LDL), that had been treated with trypsin and cholesteryl esterase, when applied to human macrophages in culture was rapidly taken up (much faster than acLDL) and had double the increase in cholesterol esterification by ACAT. They also showed that most of the E-LDL was taken up via oxidized LDL (oxLDL) receptors and out-competed oxLDL particles for uptake. Prolonged cell-surface contact of aggregated LDL (aggLDL) retained in the extracellular matrix with macrophages in the lesion was observed by Buton and colleagues (353). They found that extracellular aggLDL maintains
extended cell-surface contact with macrophages and becomes enveloped inside invaginations in the plasma membrane. AggLDL CE hydrolysis was 3-7x higher than protein, and the enzymatically-modified lipoproteins did not require the LDL receptor or scavenger receptors for uptake into macrophages. This extracellular hydrolysis was found to be mediated by LAL enzyme, since chloroquine treatment or use of LAL knockout mouse macrophages blocked matrix-retained aggLDL CE hydrolysis (353). The work of Hakala et al. (354) revealed that LAL protein was present extracellularly in human lesions and that the enzyme was released from human macrophages and could hydrolyze LDL in vitro. Histological staining of normal and atherosclerotic human coronary arteries showed that LAL was co-localized with macrophages in the intima, but was not present in normal arteries. However, LAL proteins were mostly present extracellularly within the lesion and staining didn’t overlap with the lysosomal marker LAMP-1 in macrophages. Lysosomal enzyme secretion from macrophages was induced by zymosan A opsonization (zop treatment) and the resulting medium was found to contain large amounts of LAL enzyme in both the 41 and 49kDa forms. When conditioned medium from secretion-induced macrophages was applied to LDL in vitro, LDL-CE and TG hydrolysis resulted (creating hydrolase-modified LDL particles, H-LDL), and this also induced fusion of the modified LDL particles (354).

Oxidized LDL has also been shown to inhibit LAL activity in normal cells. Incubation of macrophages with oxLDL, but not acLDL, causes lysosomal CE accumulation (301). Prolonged oxLDL incubation impairs acidification of the lysosomal compartment due to inhibition of the lysosomal V-ATPase proton pump (299). As a result, normal upregulation of ABCA1 in macrophages (i.e. with acLDL incubation) and apoA-I dependent cholesterol efflux is blocked and oxLDL-derived cholesterol is resistant to efflux (302,355). This is relevant in the context of
native atherosclerosis, whereby it may provide a potential mechanism whereby LAL activity may be impaired within macrophages in the artery wall, consequentially leading to lysosomal CE accumulation and contributing to foam cell formation during atherogenesis.

Recent population-based genome-wide association studies (GWAS) have implicated genetic variants of the LIPA gene with risk of coronary artery disease (CAD). A replicated meta-analysis GWAS study of CAD including 21,408 cases and 19,185 controls identified 5 new loci associated with increased CAD incidence including a single nucleotide polymorphisms (SNPs) within the LIPA gene (termed rs1412444) with an odds ratio of 1.09 (P=2.76x10^{-13}) (356). This and a second SNP (rs2246833) within intronic sequences of the LIPA gene were also identified in a second study of CAD risk that included 21,428 cases and 38,361 controls (357). This new LIPA polymorphism increased susceptibility to CAD with an odds ratio of 1.1 (P=3.7x 10^{-8}). In both studies, SNPs were associated low plasma HDL cholesterol, and impaired endothelial function (as measured by flow-mediated vasodilatation), but not with LDL cholesterol or TG levels in plasma. Unexpectedly, these LIPA variants were strongly associated with increased LAL mRNA expression in plasma monocytes and in the liver (356,357). This suggested that the mechanism by which these SNPs affect CAD risk is not necessarily by altering hepatic CE and TG homeostasis and LDL uptake, but must be via some alternate mechanism. It is possible that increased LAL expression might increase UC levels (possibly by increased secretion of LAL and enzymatic modification of retained apoB-containing lipoproteins as discussed in the previous section) during early lesion development, which negatively affects endothelial function during atherogenesis.

Taken together, these studies in mouse and rabbit models of atherosclerosis as well as human data from CESD or WD patients or those with CAD strongly suggest the involvement of
LAL in the development of atherosclerosis and even link genetic variants in the LIPA gene to increased risk of cardiovascular disease. However, what is unclear is whether it is a relative deficiency of LAL or increased expression of LAL that increases atherosclerosis. Further research is clearly needed to understand this complex relationship.

1.7 Rationale, hypothesis and specific aims:

The overarching hypothesis of this dissertation is that the flux of unesterified cholesterol out of the lysosomes from LAL-mediated hydrolysis of LDL cholesteryl esters is a key regulator of cellular ABCA1 expression, HDL formation and reverse cholesterol transport in the body. In order to test this hypothesis the following specific aims were employed: 1) To elucidate the role of LAL in regulation of ABCA1 expression, cholesterol and lipid efflux and HDL particle formation using LAL-deficient human CESD patient-derived skin fibroblasts; 2) To investigate the effects of complete LAL deficiency in macrophage cells on ABCA1 regulation and cholesterol efflux and to determine the impact of macrophage LAL deficiency on macrophage RCT in vivo.

Aim 1: To elucidate the role of LAL in regulation of ABCA1 expression, cholesterol and lipid efflux and HDL particle formation using LAL-deficient human CESD patient-derived skin fibroblasts

Low HDL cholesterol has been identified as an independent risk factor for cardiovascular disease (358). The ABCA1 mitigates the accumulation of cholesterol in the atherosclerotic lesion as it performs the rate-limiting step in the RCT pathway by removing intracellular cholesterol and phospholipids to apolipoprotein acceptors during initial HDL particle formation (83,188,359,360). Previous investigations in our lab had shown for the first time that ABCA1 regulation and cholesterol efflux are impaired and that plasma HDL-C levels are low in the
lysosomal cholesterol storage disease Niemann Pick disease type C (13), a neurodegenerative disease biochemically characterized by intracellular accumulation of unesterified cholesterol, specifically within late-endosomes/lysosomes due to mutations in NPC proteins (236), suggesting that the intracellular pool of unesterified cholesterol derived from the lysosomal compartment was the most critical for ABCA1 regulation.

LAL catalyzes the hydrolysis of LDL-derived triacylglycerols (TG) and cholesteryl esters (CE) specifically in lysosomes. Due to mutations in LAL, enzyme activity in CESD is reduced to approximately 5% of normal (237), resulting in massive accumulation of cholesteryl esters particularly within tissues such as the liver, spleen and adrenal glands (317). The slowed release of UC out of lysosomes due to impaired LAL activity in CESD fibroblasts results in dysregulation of microsomal cholesterol homeostatic mechanisms downstream (237,292). The first aim is focused on cholesteryl ester storage disease (CESD) as a model to understand the impact of impaired lysosomal hydrolysis of LDL-derived CE (and thus, impaired release of UC from lysosomes) on the regulation and function of the ABCA1 pathway. **We hypothesized that reduced flux of cholesterol out of late endosomes/lysosomes, due to impaired lysosomal LDL-CE hydrolysis in CESD primary skin fibroblasts, leads to dysregulation of ABCA1, cholesterol efflux and impaired HDL formation.**

**Aim 2:** To investigate the effects of complete LAL deficiency in macrophage cells on ABCA1 regulation and cholesterol efflux and to determine the impact of macrophage LAL deficiency on macrophage RCT *in vivo.*

The second aim was to investigate the impacts of LAL deficiency in macrophages on ABCA1 regulation and to assess the impact of LAL deficiency on RCT from macrophages *in vivo.* During the initial stages of atherosclerosis, macrophages in the artery wall accumulate
cholesterol and become foam cells. ABCA1 plays a pivotal role in removal of excess cholesterol from macrophage cells, while forming HDL particles in order to promote RCT and prevent atherosclerotic lesion development (148). However, the contribution of different intracellular pools of cholesterol towards regulation of ABCA1 is poorly understood.

It is clear that a major factor in the pathology associated with LAL deficiency, both in humans and mice, involves the massive accumulations of cholesteryl esters in the lysosomes of macrophages in tissues such as the liver, spleen, adrenals and small intestine (317,361). In both CESD and LAL-deficient mice, hepatomegaly results primarily from lipid accumulation in foamy Kupffer cells, macrophage-like cells in the liver and foamy-macrophage infiltration into other tissues such as the intestine, spleen and lungs is a prominent feature (361,362). LAL deficiency has been shown to enhance atherogenesis in mouse models (337,363), however, supplementation of LDL receptor-deficient mice with macrophage-specific recombinant LAL prevents early lesion formation and drastically reduced the size of pre-existing advanced lesions (336).

We hypothesized that ABCA1 expression and cellular cholesterol efflux are impaired in LAL-deficient macrophages, leading ultimately to dysregulation of reverse cholesterol transport in vivo.
CHAPTER 2: LAL DEFICIENCY IMPAIRS REGULATION OF ABCA1 AND FORMATION OF HDL IN CHOLESTERYL ESTER STORAGE DISEASE

2.1 Summary

ATP-binding cassette transporter A1 (ABCA1) mediates the rate-limiting step in high-density lipoprotein (HDL) particle formation, and its’ expression is regulated primarily by oxysterol dependent activation of the nuclear receptor liver X receptor (LXR). We previously reported that ABCA1 expression and HDL formation are impaired in the lysosomal cholesterol storage disorder Niemann Pick disease type C (NPC), and that plasma HDL-C is low in the majority of NPC patients. Here we show that ABCA1 regulation and activity is also impaired in cholesteryl ester storage disease (CESD), caused by mutations in the LIP-A gene that result in less than 5% of normal lysosomal acid lipase (LAL) activity. Fibroblasts from patients with CESD showed impaired upregulation of ABCA1 in response to LDL loading, reduced phospholipid and cholesterol efflux to apolipoprotein A-I, and reduced α-HDL particle formation. Treatment of normal fibroblasts with chloroquine to inhibit LAL activity produced the same phenotype of impaired ABCA1 expression and activity as seen in CESD cells. LXR agonist treatment of CESD cells corrected ABCA1 expression, but failed to correct LDL cholesteryl ester hydrolysis and cholesterol efflux to apoA-I. LDL-induced production of 27-hydroxycholesterol was reduced in CESD compared to normal fibroblasts. Treatment with conditioned medium containing LAL from normal fibroblasts rescued ABCA1 expression, apoA-I-mediated cholesterol efflux, HDL particle formation and production of 27-hydroxycholesterol by CESD cells. These results provide further evidence that the rate of
release of cholesterol from late endosomes/lysosomes is a critical regulator of ABCA1 expression and activity, and an explanation for the hypoalphalipoproteinemia seen in CESD patients.

2.2 Introduction

High density lipoproteins (HDL) are thought to protect against atherosclerosis for a variety of reasons, including by stimulating reverse cholesterol transport and anti-inflammatory effects (364). The rate-limiting step in HDL particle formation is the lipidation of apolipoprotein A-I (apoA-I) and other HDL apolipoproteins by the membrane lipid transporter ATP-binding cassette transporter A1 (ABCA1) (7). Expression of ABCA1 is increased at times of increased cell cholesterol content, through oxysterol-dependent activation of the nuclear receptor liver X receptor (LXR) on the promoter region of ABCA1 (8,179). However, the roles of different intracellular sources of cholesterol, including de novo synthesis in the endoplasmic reticulum, late endosomes/lysosomes, and the plasma membrane, that regulate oxysterol production and ABCA1 expression are poorly understood.

We have previously demonstrated that regulation of ABCA1 is impaired in the lysosomal cholesterol storage disorder Niemann-Pick disease type C (NPC), leading to reduced HDL particle formation by human NPC disease fibroblasts (365). The defect in the lipidation of apoA-I was overcome by addition of an exogenous non-oxysterol ligand for LXR to upregulate ABCA1, suggesting ABCA1 might be able to mobilize cholesterol from late endosomes/lysosomes even in the presence of NPC1 deficiency (239). We also determined that plasma HDL-C was low in 17/21 NPC disease patients studied (365), further confirmed in a recent report of a larger cohort of NPC1 subjects (366). Low plasma HDL-C in NPC1 disease patients occurs independent of their plasma triglyceride levels (366), further suggesting that
impaired $ABCA1$ regulation as a consequence of reduced flux of cholesterol out of lysosomes is the cause of the hypoalphalipoproteinemia in this disease.

In the current study we have extended these findings to another lysosomal cholesterol storage disorder, cholesteryl ester storage disease (CESD), caused by deficiency of lysosomal acid lipase (LAL) activity (317). CESD, and its’ more severe form Wolman disease, are caused by autosomal recessive mutations in the $LIP$-$A$ gene, which encodes for LAL, the sole enzyme responsible for acidic hydrolysis of cholesteryl esters and triglycerides delivered from lipoproteins to lysosomes (237,367). In contrast to Wolman disease, in which complete absence of LAL activity results in death usually in the first 6 months of life, splice mutations of $LIP$-$A$ that result in the 5-10% residual LAL activity in CESD allows patients to survive usually to adulthood. In addition to hepatosplenomegaly, individuals with CESD exhibit premature atherosclerosis and plasma HDL-C levels that are approximately half normal levels (317). The reason for low HDL-C in CESD has not previously been known. Here we describe impaired regulation of $ABCA1$ in human CESD fibroblasts, reduced lipid efflux to apoA-I and HDL particle formation, induction of the same defects by inhibition of LAL activity in normal fibroblasts, and correction of $ABCA1$ expression and HDL particle formation in CESD cells treated with LAL-containing conditioned medium from normal fibroblasts. We also demonstrate impaired oxysterol generation in CESD fibroblasts, as previously shown in $NPC1^{+/-}$ and $NPC2^{-/-}$ fibroblasts (238), and correction of oxysterol formation in CESD cells following incubation with LAL-containing conditioned medium. Together with our findings in NPC disease, these results provide a likely reason for the low HDL-C in CESD – impaired regulation of $ABCA1$, and further demonstrate the critical importance of the rate of flux of cholesterol out of lysosomes in the regulation of $ABCA1$ expression and HDL particle formation.
2.3 Materials and methods

2.3.1 Materials

Cholesteryl oleate, unesterified cholesterol, 1-monooleoyl-rac-glycerol, 1,2-distearoyl-rac-glycerol, triolein, oleic acid, 4-methylumbelliferyl oleate and lentivirus containing shRNA constructs were purchased from Sigma-Aldrich. Phosphatidylcholine, sphingomyelin, LXR agonist TO-901317, chloroquine diphosphate, and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Purified oxysterols were purchased from Avanti Polar Lipids. \([\text{Cholesteryl -1, 2, 6, 7-}^3\text{H}]\) cholesteryl linoleate (60-100 Ci/mmol) was purchased from American Radiolabeled Chemicals, and \([\text{methyl - }^3\text{H}]\) choline chloride (66.7 Ci/mmol) from PerkinElmer. Dulbecco’s modified Eagle’s medium (DMEM) was purchased from BioWhittaker and fetal bovine serum and lipoprotein-deficient serum (LPDS) from Cocalico Biologicals, Inc. Nitrocellulose membranes, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reagents, and pre-stained protein molecular mass markers were purchased from Bio-Rad. PE-SIL G plastic backed flexible plates used for thin-layer chromatography analysis were from Whatman. Purified recombinant human LAL (rhLAL) was from Shire Human Genetic Therapies (Lexington, MA).

2.3.2 Preparation of lipoproteins and apolipoprotein A-I

LDL was isolated from pooled plasma from healthy fasting donors by density gradient ultracentrifugation (368). Radiolabeling of LDL with \([1,2,6,7-^3\text{H}]\)-cholesteryl linoleate was performed as described (369) to a specific activity of 16-44 cpm/ng of LDL protein. Human apo A-I was purified from human plasma using Q-Sepharose Fast Flow chromatography as previously described (239).
2.3.3 Cell culture

Normal human skin fibroblasts were purchased from the American Type Culture Collection (Manassas, VA). CESD human skin fibroblasts were obtained via skin biopsy from an adult patient in the Cardiovascular Risk Reduction Clinic, University of Alberta Hospital, Edmonton, AB, following informed consent (CESD1). Genetic sequencing of CESD1 cells was performed by the laboratory of Dr. Robert Hegele, indicating a heterozygous splice mutation E8SJM (Δ254-277, 934G>A or c.894G>A), which leads to a G-to-A transition at the -1 position of exon 8 of *LIPA*. Although no previously described LAL mutations could be identified for the second allele, a second CESD mutation is assumed as the patient exhibits a classic histologic phenotype of CESD based on prior liver biopsy. The second CESD human skin fibroblast cell line from an adult patient (CESD2) was generously provided by Dr. John Parks, Wake Forest University School of Medicine). This patient is a compound heterozygote for the common E8SJM mutation (c.894G>A), which leads to excision of exon 8, and a CT deletion in exon 4, causing a frame shift and termination signal four codons downstream at codon 137 (c.397-398delCT; FS 137X) (313).

All cells were grown in monolayers and were used between the 5th and 25th passage. Cell lines were maintained in a humidified incubator at 37°C and 5% CO$_2$ in DMEM containing 10% fetal bovine serum supplemented with 1% penicillin-streptomycin (Invitrogen) (growth medium). Cells were plated at 40,000/35-mm well or 120,000 cells/60-mm dish and grown to approximately 60% confluence in growth medium. Cells were subsequently grown to 100% confluence in DMEM containing 10% LPDS. Where indicated, cells were then incubated with DMEM containing 50 μg/ml LDL for 24 h.
2.3.4 *Conditioned medium experiments*

For conditioned medium experiments, cells grown to confluence as above were incubated an additional 24 h in DMEM containing 5% LPDS. Conditioned medium was then removed from cells, centrifuged to pellet any cells and added back to the indicated cell monolayers for a further 24 h prior to incubation with 50 μg/ml LDL for 24 h, as previously described (292).

2.3.5 *Recombinant human LAL treatment*

During optimization experiments, a stock solution of 2.9 mg/ml recombinant human LAL in 20mM citrate (pH 5.3) was diluted to final concentrations of 0.15, 0.3, 0.6, 1.2, 2.4 or 6.0 μg/ml in DMEM and added to CD1 and CD2 cell monolayers for 24 hours prior to LDL loading. Further experiments were performed using a final concentration of 1.2 μg/ml rhLAL.

2.3.6 *Chloroquine treatment*

Where indicated, cells were pre-treated for 1 h prior to LDL loading with 50 μM chloroquine diphosphate by addition of 5 μl/ml of a 10mM stock solution, made up in DMEM with pH adjusted to 7, directly to the medium and then the same concentration of chloroquine was added in the presence or absence of 50μg/ml LDL for an additional 24 h as previously described (287).

2.3.7 *Lentivirus-mediated suppression of LAL*

Expression of LAL was suppressed by RNAi. Cells were transduced with lentivirus which expresses each of 5 different short hairpin RNA (shRNA) constructs (Sigma) targeting LAL mRNA (shLAL1-5), or a non-targeting, scrambled control (shNT) including a GFP tag. Prior to experiments, GFP fluorescence was detected by broad-field fluorescence microscopy in order to optimize the multiplicity of infection (MOI = #viruses/cell) and estimate the efficiency of infection and number of viruses needed for optimal expression. Briefly, fibroblasts were
grown to approximately 80% confluence in 6 well plates grown in DMEM containing 10% FBS. Cells were first treated with 8µg/ml of hexadimethine bromide and then infected with the volume of lentivirus stock calculated to reach an MOI of 5 for each of the 5 shRNA constructs.

2.3.8 Cholesterol and phospholipid efflux

Radiolabeling of LDL-derived cellular cholesterol pools was achieved by incubating cells for 24 h with 50 µg/ml [³H]-cholesteryl linoleate-labeled LDL. Cell choline-containing phospholipids were labeled in the presence of 50 µg/ml LDL by addition of 5 µCi/ml [³H]-choline chloride for 24 h (239). Cells were washed two times with PBS and then incubated in DMEM containing 10 µg/ml apoA-I for 24 hours or the indicated time points. Efflux media were collected and centrifuged at 3,000 rpm for 10 min at 4°C to remove cell debris. [³H]-cholesterol in the medium was then measured directly for cells labeled with [³H]-cholesteryl linoleate-labeled LDL by liquid scintillation counting. For phospholipid efflux, lipid extraction of medium was performed prior to separation of lipids by thin layer chromatography (370). Cell monolayers were kept on ice and rinsed on ice two times with cold PBS containing 1 mg/ml BSA, two times with cold PBS and stored at -20°C until lipid extraction. Cellular lipids were extracted and separated by thin layer chromatography and assayed for radioactivity as previously described (371). For LDL-derived cholesterol efflux assays, the radioactivity of cholesteryl esters and unesterified cholesterol spots was measured using unlabeled carrier lipids to identify spots. For phospholipid assays, radiolabeled sphingomyelin and phosphatidylcholine spots were measured. Protein content of cell monolayers was determined by standard Lowry assay using BSA as standard (372).
2.3.9 **Cholesterol mass assay**

Cells were grown in 6-well plates and loaded with LDL as described above. Following LDL incubation, cells were washed twice with PBS/1mg/ml FAFA and incubated 24 h in the presence of 10 μg/ml apoA-I. The media fraction was collected and cells were scraped in PBS, collected and homogenized by sonication. Phospholipids from media or cell homogenates were digested using phospholipase C to remove the polar head groups, and samples were vortexed for 2 h at 30°C. Total lipids were extracted by organic phase extraction with tridecanoin as the internal standard. Samples were derivatized with Sylon BFT (Supelco) and sterols were separated and analyzed by gas chromatography (Agilent Technologies, 6890 Series equipped with a Zebron capillary column (ZB-5, 15 m x 0.32 mm x 0.25 μm) and connected to a flame ionization detector (Zebron, Palo Alto, CA) (373). The separation of cholesterol and cholesteryl esters was identified and their mass calculated using the retention times and mass of the internal standard (239).

2.3.10 **Quantitative real-time PCR analysis of ABCA1 mRNA**

Total RNA was isolated from fibroblast monolayers using Trizol Reagent extraction (Invitrogen) and cDNA synthesis was performed as previously described (365). ABCA1 DNA amplification was performed by initial denaturation at 95 ºC for 3 mins. Thereafter, denaturing was at 95 ºC for 20 seconds, annealing at 58 ºC for 20 seconds, and extension at 72 ºC for 40 seconds for at total of 40 cycles (239). SYBR Green (Quanta Biosciences) was used to detect PCR products in real-time using a Realplex² Mastercycler thermocycler (Eppendorf). The human housekeeping gene cyclophilin cDNA was amplified using the same conditions. ABCA1 mRNA levels were calculated using the comparative Cₜ method relative to cyclophilin. The following primers were used: human ABCA1, 5’-GAC ATC CTG AAG CCA ATC CTG
(forward), 5’-CCT TGT GGC TGG AGT GTC AGG T (reverse); human cyclophilin, 5’- ACC CAA AGG GAA CTG CAG CGA GAG C (forward), 5’-CCG GTG CTC TTT TGA GCT GTT TGC AG (reverse).

2.3.11 Western blot analysis of ABCA1 and LAL

Cells in 60-mm dishes were harvested in 300 μL of lysis buffer containing 20 mM Tris, 5 mM EDTA, 5 mM EGTA, 0.5% Maltoside and 1X Complete Mini Protease Inhibitor (Roche) and homogenized using a glass mortar and Teflon pestle. Samples were then centrifuged at 2500 rpm for 10 mins at 4°C to pellet nucleic acids. The sample protein concentration was quantified using Biorad reagent (Biorad). Samples to be used for human LAL (hLAL) analysis were boiled for 5 min at 100°C. Samples used for ABCA1 analysis were not boiled, according to the antibody manufacturer’s guidelines (Calbiochem). Fifteen to thirty micrograms of proteins were separated by a 5% to 15% gradient SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane. Immunoblotting was performed according to standard protocols using polyclonal rabbit anti-human ABCA1 antibody (1:1000 dilution) (Calbiochem), a polyclonal rabbit anti-hLAL antibody (1:2000 dilution, Seven Hills Bioreagents) and a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution, Sigma). Chemiluminescence was detected using Super Signal West Femto Maximum Sensitivity Substrate (Pierce Protein Research Products) and the Chemigenius BioImaging System (Syngene).

2.3.12 Two-dimensional gel electrophoresis of HDL particles

In order to characterize apoA-I containing particles generated by normal and CESD fibroblasts, cell monolayers in 35-mm wells were treated as described above for conditioned medium experiments with the exception of the last hour of incubation with apoA-I, when 1 mg/ml FAFA was added to the medium to stabilize HDL particles. Fibroblast-conditioned media
were then centrifuged at 2000 X g for 5 min at 4 °C to pellet cells and the supernatant concentrated 10-fold by ultracentrifugation (Amicon Ultra-4, MWCO 10,000, Millipore). The concentrated media were stored at -80°C. HDL particles in each of the concentrated apoA-I-conditioned media were separated according to our previously described method (239), with some modifications. Briefly, 25 μl of each sample was separated in the first dimension by 0.75% agarose gel in 50 mM barbital buffer, pH 8.6, at 200 V for 5 h at 6°C. Second dimension electrophoresis was performed with a 5-23% polyacrylamide concave gradient gel at 125 V for 24 h at 6°C in 0.025 M Tris, 0.192 M glycine buffer, pH 8.3. High molecular weight protein standards (7.1-17.0 nm, Amersham Biosciences) were run on each gel. After electrophoresis, samples were transferred (35 V, 24 h, 4°C) onto nitrocellulose membranes (Trans-blot, Bio-Rad), stained with Ponceau S to locate the standard proteins and blocked for 1 h in Tris-buffered saline containing 1% Tween 20 (TBS-T) and 10% skim milk at room temperature. ApoA-I-containing particles were detected by immunoblotting with rabbit polyclonal anti-human apoA-I antibody (Calbiochem) and goat-anti-rabbit horseradish conjugated polyclonal secondary antibody (Sigma). Chemiluminescence was detected using chemiluminescent substrate (Pierce).

2.3.13 Statistics

Results were analyzed using Graph-Pad Prism version 5.0 and are presented as the mean ± the standard deviation. Significant differences between experimental groups were determined using the Student’s t test, with the exception of Figures 2.1D, and 2.5C,D,E where two-way analysis of variance was performed and differences between experimental groups were determined using a Bonferroni post-hoc comparison.
2.4 Results

2.4.1 Reduced LDL-induced expression of ABCA1 in CESD fibroblasts

We previously reported impaired regulation of ABCA1 in Niemann Pick C1 (NPC1)-deficient human fibroblasts (365), where the rate of release of unesterified cholesterol out of the late endosome/lysosome compartment is slowed (374,375) and oxysterol generation is reduced (238). The slowed hydrolysis of LDL and other lipoprotein cholesteryl esters in cholesteryl ester storage disease (CESD), resulting from mutations in lysosomal acid lipase (LAL), also results in reduced cholesterol release from this compartment and impaired cholesterol regulatory responses (237). We therefore hypothesized this would lead to impaired regulation of ABCA1 expression and activity in CESD cells. Impaired LAL activity was confirmed in skin fibroblasts obtained from two unrelated human CESD patients (CD1, CD2) using an in vitro 4-methylumbelliferyl oleate fluorescence assay (249,258), with CD1 showing $3.86 \pm 0.86\%$ and CD2 $2.17 \pm 0.85\%$ the LAL activity of normal (NL1) fibroblasts (Supplementary Figure 2.9). To determine ABCA1 expression, normal and CESD fibroblasts were grown to confluence in LPDS and then incubated in the absence or presence of 50 μg/ml LDL for 24 hours. Quantitative real-time-PCR analysis of cellular RNA extracts revealed the fold increase in ABCA1 mRNA expression of CESD fibroblasts in response to LDL loading was reduced to approximately $\frac{1}{4}$ that in two normal fibroblast cell lines (Figure 2.1A). Basal and LDL-stimulated expression of ABCG1 mRNA were also significantly reduced in both CESD cell lines compared to normal fibroblasts (Figure 2.1B). In the absence of LDL loading, levels were consistently so low as to be undetectable from CESD2 fibroblasts; LDL loading failed to upregulate ABCG1 mRNA expression to normal levels in either cell line (Figure 2.1B). The increase in ABCA1 protein levels following LDL loading was also blunted in CESD compared to normal fibroblasts.
(Figures 2.1C and 2.1D). These results suggest that, as previously seen in human NPC1 disease fibroblasts (239,365), reduced flux of cholesterol out of the late endosome/lysosome compartment in CESD cells also results in impaired transcriptional regulation of \textit{ABCA1} and \textit{ABCG1}.
Figure 2.1 Reduced expression of ABCA1 in response to LDL loading in CESD fibroblasts.

Normal (NL1, NL2) and CESD (CD1, CD2) fibroblasts were grown to confluence in DMEM containing 5% LPDS and then incubated in the presence (+) or absence (−) of DMEM containing 50 μg/ml LDL for 24 h. A,B. RNA extracts were analyzed by quantitative real-time PCR using primers targeting human ABCA1, ABCG1 and cyclophilin. Results are the mean fold increase in ABCA1 mRNA with LDL loading corrected for cyclophilin expression ± SD of three replicates, and are representative of 3 experiments with similar results. C. Cells lysates were analyzed by SDS PAGE and western blot using poly-clonal antibodies for human ABCA1 and protein disulfide isomerase (PDI) loading control. D. Western blots were analyzed by densitometry and results expressed as the mean of ABCA1:PDI ratio ± SD for 4 experiments with NL1 − LDL sample ratio set as 1. *, values less than NL1 or NL2, $p < 0.01$. 
2.4.2  

**Reduced phospholipid and LDL-derived cholesterol efflux to apoA-I from CESD fibroblasts**

Efflux of cellular phospholipids and cholesterol to HDL apolipoproteins to form viable HDL particles is critically dependent on ABCA1 activity, and is not substituted by any other known cellular mechanism or transporter (7). To determine the functional activity of ABCA1 in CESD fibroblasts, efflux of radiolabeled phospholipids and LDL-derived cholesterol as well as total cholesterol mass to apoA-I-containing medium was measured. Efflux to apoA-I of cell phosphatidylcholine and sphingomyelin radiolabeled by addition of \[^3\text{H}\]-choline during the LDL-loading phase was significantly reduced in both CESD fibroblast lines when compared to two normal fibroblast lines (Figures 2.2A and B). To specifically measure efflux of cholesterol derived from LDL, cells were loaded with LDL containing \[^3\text{H}\]cholesteryl linoleate prior to incubation with apoA-I. Efflux of LDL-derived cholesterol as a percentage of total medium plus cell radiolabel to 10 μg/ml apoA-I over a 48 h period was significantly lower from the two CESD cell lines (7.4 ± 0.67% and 10.3 ± 1.04% from CESD1 and CESD2 cell lines respectively) when compared to the two normal cells lines (23.6 ± 2.70% and 18.8 ± 0.69% from NL1 and NL2 respectively) (Figure 2.2C). The percentage of total medium plus cellular radiolabel in cell cholesteryl esters was persistently higher in CESD cells, consistent with reduced LAL activity and reduced hydrolysis of lysosomal cholesteryl esters over the 48 h time course of the experiment (Figure 2.2D). Radiolabeled unesterified cholesterol was much lower in CESD cells at the start of the apoA-I incubation, but, in contrast to normal cells, rose during the apoA-I incubation as cholesteryl esters were gradually hydrolyzed (Figure 2.2E). The increase in unesterified cholesterol counts is consistent with reduced ABCA1 activity in these cells and reduced efflux to apoA-I compared to normal cells. Differences in cholesterol mass in the
medium and cell cholesteryl ester pools after 24 h incubation with 10 μg/ml apoA-I mirrored changes seen in the radiolabel experiments, with much lower efflux of cholesterol mass to apoA-I-containing medium and much higher cholesteryl ester levels in CESD compared to normal cells (Figures 2.2F and 2.2G). Normal and CESD cells had unesterified cholesterol mass ranging between 33.0 ± 3.03 μg/mg (NL1) and 63.4 ± 6.62 μg/mg (CD1) cell protein; taken together, no significant difference could be observed between normal and CESD cells in this parameter (Figure 2.2H). This result was not unexpected since, as opposed to radiolabeling experiments where the fate of [3H] LDL-cholesteryl esters was measured specifically, the total mass of unesterified cholesterol also includes that from de novo cholesterol synthesis by HMGCR, known to be upregulated in CESD cells (237), and neutral hydrolysis of cytosolic cholesteryl esters (284).
Figure 2.2 Reduced phospholipid and cholesterol efflux to apoA-I from CESD fibroblasts.

Normal (NL1, NL2) and CESD (CD1, CD2) fibroblasts were grown to confluence in DMEM containing 5% LPDS and then in DMEM containing 5 μCi/ml [3H]choline-chloride and 50 μg/ml LDL for 24 h (A,B), 50 μg/ml [3H]cholesteryl-linoleate LDL (C,D,E), or 50 μg/ml LDL (F,G,H), followed by further incubation with DMEM in the presence (+) or absence (-) of 10 μg/ml apoA-I for 24 h or the indicated time points. The radioactivity of the medium was counted directly by liquid scintillation counting (C) or following lipid extraction (A,B). Medium and cellular lipid extracts were separated by thin layer chromatography and radioactivity was measured in phosphatidylcholine (A) and sphingomyelin (B) spots, or cholesteryl ester (CE, D) and unesterified cholesterol (UC, E) spots. Total lipids were extracted from medium (F) and cells (G,H) and UC (F,H) and CE mass (G) were determined by gas chromatography and mass spectrometry. Error bars are the standard error of the mean and are
representative of 3 experiments with similar results. *, values less than (A,B,C,E,F) or greater than (D,G) NL1 or NL2 controls, \( p < 0.05 \).
2.4.3 Chloroquine blocks LDL-dependent upregulation of ABCA1 expression and activity in normal fibroblasts

To test whether inhibition of LAL activity impairs ABCA1 expression and activity in normal fibroblasts, cells were exposed to 50 μM chloroquine for 1 h prior to and during LDL loading (237). Chloroquine treatment of normal fibroblasts reduced basal ABCA1 mRNA to nearly zero, and completely blocked the increase in ABCA1 mRNA seen in response to LDL-loading, which was reduced to a greater extent than seen in untreated CESD fibroblasts (Figure 2.3A). The increase in ABCA1 protein in response to LDL loading was also blocked by chloroquine treatment in normal fibroblasts to a similar degree as non-chloroquine treated CESD cells, indicating the importance of lysosomally-derived cholesterol as a regulator of ABCA1 expression (Figure 2.3B). Interestingly, LAL protein levels were also decreased in normal and CESD fibroblasts following chloroquine treatment (Figure 2.3B), which has been attributed to enhanced secretion of newly synthesized enzyme and/or decreased re-uptake of secreted LAL (376). Therefore, chloroquine blocks LDL cholesteryl ester hydrolysis not only by inhibition of LAL activity but also by reduction of overall LAL enzyme level within the cell. Efflux of LDL-derived cholesterol to apoA-I was abolished in normal cells following chloroquine treatment before and during delivery of [3H]cholesteryl linoleate-labeled LDL to cells (Figure 2.3C). Chloroquine treatment also resulted in accumulation of [3H]cholesteryl esters and reduction of cellular [3H]-unesterified cholesterol in normal fibroblasts, to similar levels as those seen in non-chloroquine-treated CESD cells, confirming the inhibition of LDL-cholesteryl ester hydrolysis by chloroquine (data not shown). In addition, LAL expression was suppressed using RNAi by lentivirus-mediated delivery of short hairpin RNA (shRNA) directed against the LAL transcript. We were able to achieve significant knockdown of LAL protein expression (~80% reduction) in
normal cells transduced with lentivirus expressing shRNA against LAL (shLAL3 and 4) compared to non-targeting shRNA (shNT) (Supplementary Figure 2.10A). However, LAL activity was only reduced to ~30-40% of normal (compared to only 5-10% residual activity in CESD fibroblasts) (Supplementary Figure 2.10B) and there was no apparent effect on ABCA1 expression (Supplementary Figure 2.10A). Therefore, the metabolic phenotype of impaired ABCA1 regulation and cellular cholesterol efflux to apoA-I observed in CESD fibroblasts could be recapitulated by pharmacological inhibition of LAL in normal fibroblasts, indicating that LAL deficiency is the reason for the impaired ABCA1 regulation observed in CESD fibroblasts.
Figure 2.3 Chloroquine blocks upregulation of ABCA1 in response to LDL loading and apoA-I-mediated cholesterol efflux in normal fibroblasts.

Normal (NL1) and CESD (CD1, CD2) fibroblasts were grown to confluence in LPDS. Cells were treated ± 50 μM chloroquine (72) for 1 h prior to and during a 24 h incubation ± 50 μg/ml LDL (A, B) or 50 μg/ml [3H]cholesteryl-linoleate LDL (C). A, cellular RNA extracts were analyzed by quantitative real time-PCR as described in Figure 2.1. B, cell lysates were collected and analyzed by western blot as described in Figure 2.1. C, radioactivity of the medium and cell lipid extracts was determined as in Figure 2 and results are expressed as the percent of total medium and cell [3H]UC plus cell [3H]CE. Values are the mean ± SD of triplicates and are representative of 3 experiments with similar results. *, values less than non-chloroquine-treated and LDL-loaded NL1 controls, p < 0.05.
2.4.4  **LXR agonist corrects ABCA1 expression but fails to correct apoA-I-mediated cholesterol efflux in CESD fibroblasts**

We and others have previously reported that treatment with the LXR agonist TO901317 corrects *ABCA1* expression and apoA-I-mediated cholesterol efflux (239,377), and reduces lysosomal cholesterol accumulation (239) in NPC1-deficient human fibroblasts. As found in NPC1-deficient cells, LXR agonist treatment corrected *ABCA1* expression in CESD cells to similar (mRNA) or greater (protein) levels than seen in non-LXR agonist-treated normal fibroblasts (Figures 2.4A and 2.4B). Efflux of radiolabeled LDL-derived cholesterol to apoA-I, however, while increased in normal fibroblasts, was not corrected to the same level in CESD fibroblasts compared to untreated normal fibroblasts (Figure 2.4C). These results suggest that although *ABCA1* expression can be corrected in CESD fibroblasts, the residual deficiency of LAL activity is not overcome with this treatment, resulting in a persistent reduction of substrate unesterified cholesterol from late endosomes and lysosomes for ABCA1-mediated cholesterol efflux.
Figure 2.4 LXR agonist upregulates ABCA1 expression but fails to correct apoA-I-specific cholesterol efflux in CESD fibroblasts.

Normal (NL1) and CESD (CD1, CD2) fibroblasts were grown to confluence in DMEM containing 5% LPDS and then incubated with DMEM containing 50 μg/ml LDL (A,B) or 50 μg/ml [3H]cholesterol-linoleate LDL (C) in the presence (+) or absence (-) of 5 μM TO901317 for 24h. ABCA1 mRNA expression was quantified (A) and cell lysates were analyzed by western blot (B) as described in Figure 2.1. C, following LDL and LXR agonist incubation cells were further incubated with DMEM containing 10 μg/ml apoA-I for 24 hours and cell and radioactivity of the medium and cell lipid extracts was determined as in Figure 2. Results are expressed as the percent of total (medium and cell [3H]UC plus cell [3H]CE); mean ± SD of triplicates and representative of 3 experiments with similar results. *, values less than non-LXR agonist-treated NL1 cells, p <0.05.
2.4.5  Rescue of ABCA1 expression and apoA-I-dependent cholesterol efflux in CESD fibroblasts treated with LAL-containing medium

Our data indicate that hydrolysis of endocytosed LDL cholesteryl esters by LAL is required for normal regulation of ABCA1 expression and activity, and that the impaired ABCA1 expression and lipid efflux phenotype of CESD cells can be reproduced by inhibiting LAL activity in normal fibroblasts. Restoration of LAL activity would therefore be expected to correct the impaired ABCA1 expression and lipid efflux defects in CESD cells. Functional LAL enzyme is known to be secreted from human fibroblasts, and can be taken up again via the mannose-6 phosphate receptor pathway for specific targeting to late endosomes/lysosomes (253,258). The Goldstein and Brown laboratory have previously shown that conditioned medium from normal human fibroblasts can rescue the hydrolysis of LDL cholesteryl esters and restore normal cholesterol regulatory responses including suppression of HMG-CoA reductase and stimulation of cholesterol re-esterification in CESD fibroblasts (292). We adapted this method to determine whether we could also rescue ABCA1 expression and lipid efflux in these cells. Normal and CESD fibroblasts were grown to confluence in LPDS, followed by an additional 24 h incubation in medium obtained from a 24 h incubation of normal fibroblasts (conditioned medium). Cells were then loaded with unlabeled or radiolabeled LDL for 24 h prior to determination of ABCA1 expression and cholesterol efflux to apoA-I. Conditioned medium from normal cells restored the LDL-induced increase in ABCA1 mRNA in CD1 and CD2 fibroblasts up to the same level seen in conditioned medium-treated normal fibroblasts (Figure 2.5A). ABCA1 protein levels were also increased in both CESD cell lines in response to LDL-loading following treatment with conditioned medium (Figure 2.5B). No change was observed in ABCA1 mRNA or protein when CD1 or CD2 fibroblasts were treated with
conditioned medium from either CESD cell line (data not shown). Conditioned-medium treatment also corrected LDL-derived cholesterol efflux to apoA-I in both CESD cell lines by ~3.5-fold when compared to non-treated cells, to levels similar to those seen in normal fibroblasts untreated or treated with conditioned medium (Figure 2.5C). In addition, conditioned medium reduced cell cholesteryl esters and raised cell unesterified cholesterol radiolabel in CESD cells to levels similar to those found in normal cells with or without conditioned medium treatment (Figures 2.5D and 2.5E). These results indicate LAL from the conditioned medium was internalized and targeted correctly to late endosomes/lysosomes, to restore LDL cholesteryl ester hydrolysis and cholesterol homeostasis as determined by ABCA1 expression and activity.
Normal (NL1) and CESD (CD1, CD2) fibroblasts were grown to confluence in DMEM containing 5% LPDS. Fresh medium was applied for 24 h and the resulting conditioned medium from normal cells or non-conditioned medium was applied as indicated for 24 h. Cell monolayers were then incubated in DMEM with (+) or without (-) 50 μg/ml LDL (A,B) or 50 μg/ml [3H]cholesteryl-linoleate LDL (C,D,E) for 24 h. ABCA1 mRNA expression was quantified (A) and cell lysates were analyzed by western blot (B) as described in Figure 2.1. Cells were incubated with 10 μg/ml apoA-I for 24 h and [3H]cholesterol in the medium and cell lipid extracts was determined as in Figure 2.2. Results indicate percent of total [3H]cholesterol in the medium (C), cell CE (D) or cell UC (E), mean ± SD of triplicates, and are representative of 3 experiments with similar results. *, values significantly greater (A,C,E) or lower (D) than non-conditioned medium-treated CESD cells, p < 0.05.
2.4.6  *Impaired HDL particle formation by CESD fibroblasts and correction by treatment with conditioned medium from normal cells*

We previously reported a reduction in generation of α-migrating HDL species in apoA-I efflux medium of NPC-deficient cells, and in the plasma of a patient with NPC disease, using 2-dimensional (2-D) gel electrophoresis of HDL particles (239). We also found a correction of α-HDL formation following LXR agonist treatment in NPC disease cells (239). In order to correlate our findings of reduced ABCA1 expression and apoA-I-dependent phospholipid and cholesterol efflux from CESD fibroblasts with the reported low plasma HDL-cholesterol concentrations in CESD patients (317), we performed 2D gel analysis of HDL formed in the efflux medium containing 10 μg/ml apoA-I for 24 hours from normal and CESD cells. As seen in NPC1-deficient fibroblasts (239), medium from both CESD cell lines showed normal levels of pre-β HDL but a reduction in formation of α-HDL particle species following incubation with apoA-I (Figure 2.6, CD1 and CD2 –CM). Incubation of CESD cells with conditioned medium from normal fibroblasts to increase LAL activity restored the level of α-HDL produced by CESD cells up to levels similar to those seen in normal cells without CM treatment (Figure 2.6, CD1 and CD2 +CM).
Figure 2.6 Reduced α-HDL particle formation by CESD fibroblasts is rescued following treatment with normal fibroblast conditioned medium.

Fibroblasts were treated as in Figure 2.5 with or without conditioned medium from normal fibroblasts (CM). Efflux medium following a subsequent 24 h incubation with 10 μg/ml apoA-I was concentrated to 1/10 volume and 20 μl of each sample was run in the first dimension on 0.75 % agarose, separated in the second dimension on a 5-23% non-denaturing polyacrylamide gradient gel, and analyzed by western blot using a rabbit polyclonal antibody to human apoA-I. Boxed areas represent α-HDL particles. Blots are representative of 3 experiments with similar results.
2.4.7 Impaired LDL-induced oxysterol formation in CESD cells and correction by treatment with conditioned medium from normal cells

Production of endogenous oxysterols in response to LDL loading is impaired in NPC1- and NPC1-deficient human fibroblasts (238), providing a reason for the impaired regulation of ABCA1 in NPC disease (365). The reduced flux of unesterified cholesterol out of lysosomes in CESD, plus the ability of an exogenous LXR agonist to correct ABCA1 expression in these cells (Figures 2.4A and 2.4B), suggest that endogenous oxysterol generation required for LXR activation and ABCA1 expression is also impaired in CESD. To test this hypothesis we measured the increase in production of 27-hydroxycholesterol, the predominant oxysterol generated (162,378) and key regulator of HMG-CoA reductase (378) and ABCA1 (162) expression in response to LDL loading of fibroblasts, following incubation of CESD cells with LDL. The increase in combined medium and cellular 27-hydroxycholesterol mass in response to LDL loading was less in both CESD cell lines when compared to normal fibroblasts (Figure 2.7). Treatment with conditioned medium from normal cells to provide LAL activity significantly increased production of 27-hydroxycholesterol in both CESD cell lines when compared to non-treated cells, to levels similar to or higher than LDL-loaded normal fibroblasts (Figure 2.7). These results provide the first demonstration of reduced oxysterol production in CESD fibroblasts, and further evidence that the impaired flux of cholesterol out of lysosomes and the consequent reduction in oxysterol formation is responsible for the impaired regulation of ABCA1 and HDL formation in CESD.
Figure 2.7 Reduced 27-hydroxycholesterol mass in CESD cells and correction following treatment with normal fibroblast conditioned medium.

Normal (NL1) and CESD (CD1,CD2) fibroblasts were treated as in Figure 2.5, either with (+) or without (-) conditioned media (CM). Lipids from cells and media were extracted and the pooled lipid fractions were separated by gas chromatography and the mass of 27-HC quantified as described in Experimental Procedures. Results represent the difference between LDL loading (+LDL) and non-LDL-loaded controls, mean ± SD of triplicates, and are representative of two experiments with similar results. *, values significantly greater than non-CM-treated cells, \( p < 0.05 \).
2.5 Discussion

The studies presented here provide several additional lines of evidence that the rate of flux of cholesterol out of lysosomes is a key regulator of ABCA1 expression and activity and therefore HDL particle formation. Human CESD fibroblasts with very low residual activity of LAL and therefore rate of hydrolysis of cholesteryl esters in late endosome/lysosomes exhibited a marked decrease in LDL-stimulated expression of ABCA1 at the mRNA and protein level, resulting in impaired ABCA1-dependent phospholipid and cholesterol efflux to apoA-I, and reduced production of larger α-HDL particles. Treatment of normal fibroblasts with chloroquine to inhibit LAL activity induced the same defects in LDL-stimulated increases in ABCA1 expression and cholesterol efflux to apoA-I as seen in the CESD cells. Treatment of CESD cells with LAL-containing conditioned medium from normal cells corrected cholesteryl ester hydrolysis, ABCA1 expression, cholesterol efflux to apoA-I, and formation of α-HDL particles to levels similar to those seen in normal fibroblasts. Formation of the key oxysterol regulating ABCA1 expression in response to LDL loading, 27-hydroxycholesterol, was reduced in CESD fibroblasts and restored upon addition of LAL-containing medium to correct cholesteryl ester hydrolysis. These findings are summarized in Figure 2.8 below. Together with our previous findings using cells from patients with the lysosomal cholesterol storage disorder NPC disease (239,365), these results demonstrate further the critical role of the rate of flux of cholesterol out of late endosomes/lysosomes in regulating ABCA1 expression and HDL particle formation. They also provide the first likely explanation for the low plasma HDL-C seen in CESD patients, impaired regulation of ABCA1.
Figure 2.8 Summary of results and proposed mechanism.

Partial lysosomal acid lipase (LAL) deficiency in CESD fibroblasts leads to LDL cholesteryl ester (CE) accumulation in the lysosomes and reduced release of cellular unesterified cholesterol (UC) from this compartment, leading to reduced upregulation of oxysterol production, reduced ABCA1 expression via the LXR pathway. Ultimately this leads to impairment of ABCA1-mediated cholesterol efflux and HDL alpha particle formation.
In both NPC disease and CESD, previous studies using cultured fibroblasts showed that the reduced rate of release of unesterified cholesterol from late endosomes/lysosomes leads to impaired downregulation of HMG-CoA reductase and LDL receptor activity, and, therefore, inappropriately high levels of *de novo* cholesterol synthesis and LDL uptake (237,379). Reduced trafficking of unesterified cholesterol to the endoplasmic reticulum also results in reduced levels of cholesterol esterification by acyl-CoA:cholesterol acyltransferase in both diseases (236,237). At the same time, our previous (239,365) and current work suggests the low rate of cholesterol egress from lysosomes and oxysterol generation in both these diseases results in reduced ABCA1 expression and HDL particle formation. Fibroblasts from NPC and CESD patients, therefore, fail to sense the accumulation of excess cholesterol in late endosomes/lysosomes and to upregulate ABCA1 appropriately in response to cholesterol loading, thereby resulting in impaired HDL formation.

Using the drug chloroquine to inhibit LAL activity, ABCA1 expression and ABCA1-dependent cholesterol efflux were significantly decreased in normal cells (*Figure 2.3*). Interestingly however, despite successful knockdown of LAL expression using RNAi by a lentivirus delivery method, which caused significant reduction of LAL protein (to ~20% of normal) in normal fibroblasts, LAL activity was not suppressed enough to cause measurable changes in ABCA1 expression (*Supplementary Figure 2.10*). This indicates that a threshold of LAL activity must exist, (between the 5-10% residual activity in CESD fibroblasts and the 35-40% remaining activity following LAL knockdown by RNAi) in which a sufficient amount of CE hydrolysis takes place to allow enough lysosomal UC release in order to modulate ABCA1 regulation.
27-hydroxycholesterol has been shown to be the predominant oxysterol formed and primary regulator of both HMGCoA reductase and ABCA1 expression in response to LDL or acetylated LDL loading in human fibroblasts and other cell types (162,378). The reduced generation of 27-hydroxycholesterol in NPC disease (238) and in CESD cells in response to LDL loading found in the current studies is striking also in demonstrating the key role of lysosomally-derived cholesterol in the formation of this key regulatory oxysterol required for ABCA1 expression. Despite increased HMG-CoA reductase activity and de novo cholesterol synthesis in both CESD and NPC disease cells (237,379), newly-synthesized cholesterol is apparently not contributing a significant pool of cholesterol or regulatory oxysterols affecting ABCA1 expression. While synthesis of 24(S),25-epoxycholesterol has been shown to increase coordinately with de novo cholesterol synthesis via HMG-CoA reductase (380), synthesis of this oxysterol is apparently not enough to impact ABCA1 expression in the face of reduced flux of cholesterol out of lysosomes. Further demonstration of the specific and critical role of lysosomally-derived cholesterol in regulating ABCA1 expression was seen in our experiments using chloroquine, where levels of ABCA1 mRNA were reduced to near zero in cells pretreated with lipoprotein-deficient serum or following LDL loading (Figure 2.3A).

Treatment of CESD cells with the non-oxysterol LXR agonist TO-901317 increased ABCA1 mRNA and protein levels up to either normal or higher levels than those seen in untreated normal fibroblasts (Figures 2.4A,B). In contrast to our previous findings of complete correction of apoA-I-mediated cholesterol efflux and HDL particle formation in NPC1-deficient fibroblasts treated with this LXR agonist (239), however, CESD cells showed a persistent reduction in apoA-I-mediated cholesterol efflux (Figure 2.4C). These results suggest that treatment with this agonist and increased expression of ABCA1 can bypass a deficiency in NPC1
activity to induce mobilization of lysosomal (unesterified) cholesterol, but cannot overcome the
deficiency in LAL activity and reduced LDL cholesteryl ester hydrolysis in CESD cells. These
results also support the conclusion of previous studies (381,382) that lysosomally-derived
cholesterol forms a significant fraction of the substrate pool of cholesterol mobilized by ABCA1
for HDL particle formation.

Our findings that addition of LAL-containing conditioned medium from normal
fibroblasts rescued ABCA1 expression and activity (Figure 2.5) are consistent with previous
results showing correction of LDL cholesteryl ester hydrolysis and new cholesteryl ester
formation as well as suppression of HMG-CoA reductase in similarly-treated human CESD
fibroblasts (292). Correction of LDL-derived radiolabeled cholesteryl ester and unesterified
cholesterol levels in CESD cells to the same levels as seen in normal fibroblasts (Figures 2.5D,E)
indicated LAL in the conditioned medium was targeted to and active in late
endosomes/lysosomes of the CESD cells. Correction of LAL activity also resulted in
normalization of apoA-I-mediated cholesterol efflux (Figure 2.5C), HDL particle formation
(Figure 2.6), and 27-hydroxycholesterol formation (Figure 2.7) in the CESD cells. While we
cannot rule out the possibility that 27-hydroxycholesterol present in the conditioned medium
from normal cells had a partial role in this correction, the normalization of radiolabeled
cholesteryl ester levels by LAL, which is not known to be an LXR-responsive gene, suggests the
primary effect of the conditioned medium was indeed the correction of LAL activity in the
CESD cells. This correction of the regulatory defect in ABCA1 expression and activity by
adding back LAL to CESD cells provides further evidence for reduced lysosomal cholesteryl
ester hydrolysis and flux of unesterified cholesterol out of the late endosome/lysosome
compartment as the reason for impaired ABCA1 regulation and HDL formation in CESD.
In summary, the results presented provide further evidence that the rate of flux of cholesterol out of late endosomes/lysosomes is a critical regulator of the expression of ABCA1 and HDL particle formation, and is not corrected by the increased de novo cholesterol synthesis seen in cells from patients with two different diseases of lysosomal cholesterol storage, cholesteryl ester storage disease and Niemann Pick disease type C. These results also provide the first plausible explanation for the hypoalphalipoproteinemia seen in CESD, impaired regulation of ABCA1. We therefore propose a model for CESD cells where hydrolysis of cholesteryl esters from endocytosed LDL in late endosomes and lysosomes is impaired, there is a reduced rate of unesterified cholesterol release from these compartments to other intracellular sites for regulatory effects including production of 27-hydroxycholesterol, and therefore reduced LXR-dependent regulation of ABCA1. In addition less unesterified cholesterol is available to join the substrate pool of ABCA1 for new HDL particle formation. Although we did not find an upregulation of ABCA1 expression in normal cells in response to addition of conditioned medium containing LAL, further studies are required to assess the potential role of LAL as a target to increase ABCA1 expression and HDL formation at the cellular and clinical level.
2.6 Supplementary data

2.6.1 Supplemental figures

![Bar graph showing LAL activity of CESD fibroblasts.](image)

**Figure 2.9 Average LAL activity of CESD fibroblasts.**

Normal (NL1) and CESD fibroblasts (CD1 and CD2) were grown to confluence in DMEM containing 5% LPDS. Cells were harvested from dishes by trypsinization, washed extensively in PBS and redissolved in sterile distilled water. Cells were lysed by sonication and enzyme activity was determined by quantification of fluorescence following hydrolysis of the substrate 4-MUO incorporated into phospholipid vesicles at pH5. LAL activity was calculated as the rate of increase of fluorescence over time and calculated as a percentage of normal controls (NL1). Error bars are the standard deviation of the mean from 6 separate experiments.
Figure 2.10 LAL knockdown by lentivirus delivery of short hairpin RNA reduces LAL expression and activity but does not alter ABCA1 protein expression.

Normal (NL1) and CESD (CD1 and CD2) were grown to 80% confluence in culture and NL1 cells were infected with lentivirus constructs expressing each of 5 different short hairpin RNA sequences targeting LAL (shLAL #1-5) or a non-targeting scrambled short hairpin sequence (shNT) for 18 hours. Cell samples were collected and analyzed by (A) Western blotting or (B) LAL activity following methods described for Figures 2.1 and 2.8. Once the efficiency of knockdown of LAL was determined (A), the lentivirus shRNA constructs that reduced LAL expression by the most (shLAL3 and shLAL4) were used for further experiments. Results in (A) are representative of 4 separate experiments with similar results. Results in (B) represent the standard deviation of the mean.*, values significantly lower than shNT controls, One-way ANOVA, n=6, p < 0.05.
Figure 2.11 Recombinant human LAL rescues ABCA1 expression and apoA-I-mediated cholesterol efflux in CESD fibroblasts.

Normal (NL1) and CESD (CD1, CD2) fibroblasts were grown to confluence in DMEM containing 5% LPDS. Recombinant human LAL (rhLAL) was added to CD1 and CD2 fibroblasts at the concentrations indicated (μg/ml medium) (A) or 1.2 μg/ml rhLAL (B,C,D) for 24 hours. Cell monolayers were then incubated in DMEM with (+) or without (-) 50 μg/ml LDL (A) or 50 μg/ml [3H]cholesterol-linoleate LDL (B,C,D) for 24 h. ABCA1 protein in cell lysates was analyzed by western blot (A) as described in Figure 2.1. Cells were incubated with 10 μg/ml apoA-I for 24 h and [3H]cholesterol in the medium and cell lipid extracts was determined as in Figure 2.2. Results indicate the percent of total [3H]cholesterol in the medium (B), cell CE (C) or cell UC (D), mean ± SD of triplicates, and are representative of 3 experiments with similar results. *, values significantly greater (B,C) or lower (D) than non-conditioned medium-treated CESD cells, p < 0.05.
Figure 2.12 Reduced α-HDL particle formation by CESD fibroblasts is rescued following treatment with recombinant human LAL.

Fibroblasts were treated as in Supplemental figure 2.11 with or without 1.2 μg/ml recombinant human LAL (rhLAL or no addition, NA). Efflux medium following a subsequent 24 h incubation with 10 μg/ml apoA-I was concentrated to 1/10 volume and 20 μl of each sample was run in the first dimension on 0.75 % agarose and, separated in the second dimension on a 5-23% gradient gel, and analyzed by western blot using a rabbit polyclonal antibody to human apoA-I. Boxed areas represent α-HDL particles. Blots are representative of 2 experiments with similar results.
Figure 2.13 27-hydroxycholesterol formation following treatment with recombinant human LAL.

Normal (NL1) and CESD (CD1,CD2) fibroblasts were treated as in Figure 2.5, either with (+rhLAL) or without treatment with recombinant human LAL. Lipids from cells and media were extracted and the pooled lipid fractions were separated by gas chromatography and the mass of 27-OHcholesterol was quantified as described in Experimental Procedures. Results represent the difference between LDL-loaded and non-LDL-loaded cells, mean ± SD of triplicates, and are representative of four experiments with similar results.
CHAPTER 3: LAL PROMOTES REVERSE CHOLESTEROL TRANSPORT FROM MACROPHAGES

3.1 Introduction

The ATP-binding cassette transporter A1 performs the rate-limiting step in HDL synthesis by removing intracellular cholesterol and phospholipids to apolipoprotein acceptors during initial HDL particle formation (83,188,359,360). ABCA1 is therefore a critical component of the reverse cholesterol transport pathway whereby excess intracellular cholesterol is removed from the cell and transported on HDL to the liver for removal via biliary excretion (383).

Previous work in our lab has shown that ABCA1 regulation and cholesterol efflux are impaired in two lysosomal cholesterol storage disorders: Niemann Pick disease type C (NPC)(368) and Cholesteryl Ester Storage Disease (CESD) (13,384), both characterized by low levels of plasma HDL cholesterol. NPC is a neurodegenerative disease with other clinical manifestations such as hepatomegaly, neonatal liver disease, and, frequently, death in childhood or early adolescence, and is the result of mutations in either NPC1 or NPC2 proteins which help to remove cholesterol from the lysosomes intracellularly (385). CESD is an autosomal recessive disorder resulting from mutations in the lysosomal acid lipase (LAL) gene. LAL catalyzes the hydrolysis of LDL-derived triacylglycerols (TG) and cholesteryl esters (CE) in lysosomes. Due to mutations in LAL, enzyme activity in CESD is reduced to approximately 5% of normal (237), resulting in massive accumulation of cholesteryl esters particularly within tissues such as the liver, spleen and adrenal glands (317), resulting in hepatosplenomegaly and sometimes leading to premature atherosclerosis (367). In both NPC and CESD, patients typically have low plasma HDL (13,366), which we’ve demonstrated is most likely due to decreased ABCA1 expression
and activity (13,384). In both diseases, it is thought that excess lysosomal cholesterol also cannot access intracellular sites for oxysterol synthesis, resulting in reduced activation of liver X receptor (LXR) nuclear receptor and dysregulation of its target genes (238).

It is clear that a major factor in the pathology associated with LAL deficiency, both in humans and mice, involves the massive accumulations of cholesteryl esters in the lysosomes of macrophages in tissues such as the liver, spleen, adrenals and small intestine (317,361). In both CESD and LAL-deficient mice, hepatomegaly results primarily from lipid accumulation in foamy Kupffer cells, macrophage-like cells in the liver. Foamy-macrophage infiltration into other tissues such as the intestine, spleen and lungs is a prominent feature (361,362). LAL deficiency has been shown to enhance atherogenesis in mouse models (337,363), while supplementation of LDL receptor-deficient mice with macrophage-specific recombinant LAL prevents early lesion formation and drastically reduced the size of pre-existing lesions (336).

Epidemiological evidence suggests an inverse relationship between plasma HDL cholesterol and atherosclerosis risk (358), however steady-state plasma HDL-C levels themselves aren’t necessarily indicative of the efficiency of HDL in this pathway (144-146). Recent focus has turned toward the many known anti-atherosclerotic properties of HDL, including its dynamic role within the reverse cholesterol transport (RCT) pathway, as opposed to HDL-C levels themselves (115,140,147). Current methods to model reverse cholesterol transport directly from macrophages in vivo have been pioneered by the laboratory of Daniel Rader, where radioisotope-labeled cholesterol in macrophages is traced through the RCT pathway ultimately to the feces (139), an effective approach to assess dynamic changes in the whole RCT pathway in vivo.
Here we show that ABCA1 expression and cellular cholesterol efflux are impaired in LAL-deficient macrophages, leading to dysregulation of reverse cholesterol transport in vitro and in vivo.

3.2 Materials and methods

3.2.1 Materials

Chloroquine diphosphate and 4-methylumbelliferyl oleate were from Sigma. [Cholesteryl –1,2-\(^{3}\)H (N)] cholesteryl oleate was from Perkin Elmer (Waltham, Massachusetts). DMEM and RPMI 1640 media were from HyClone (Logan, Utah). Fetal bovine serum was from Cocalico Biologicals, Inc. Nitrocellulose membranes, SDS-PAGE reagents, and pre-stained protein molecular mass markers were purchased from Bio-Rad. Pre-SIL G plastic-backed flexible plates used for thin layer chromatography analysis were from Whatman. Purified recombinant human LAL enzyme (rhLAL) was from Shire (Lexington, MA).

3.2.2 Preparation of \(^{3}\)H cholesteryl-oleate labeled acetylated LDL and isolation of apolipoprotein A-I from plasma

LDL was isolated from pooled plasma from fasting, healthy donors by gradient density ultracentrifugation (368). Radiolabeling with \(^{3}\)Hcholesteryl oleate was performed as previously described (369), then acetylated according to the methods of Basu et al. (386). Briefly, an equal volume of \(^{3}\)Hcholesteryl-oleate labeled LDL in saline solution (typically 4-5 ml) was mixed with an equal volume of a saturated sodium acetate solution and 1.5\(\mu\)l/mg LDL protein was added in 1\(\mu\)l aliquots over 1 hour, while stirring on ice and then dialyzed extensively in a saline-EDTA solution. Apolipoprotein A-1 (apoA-I) was purified from human plasma using Q-sepharose Fast Flow chromatography as described (359).
3.2.3  **LAL knockout mice**

LAL null mice (*lat*+/−) were generated on a (CF-1, 129Sv genetic background) as previously described (323). Mice were group housed in microisolation under a 12-hour light cycle and fed a chow diet *ad libitum* with free access to water. Mice of ages between 8 and 12 weeks were shipped from Cincinnati Children’s Hospital, (Cincinnati, OH) to the University of British Columbia (Vancouver, BC) and allowed to acclimatize for 1 week prior to the commencement of experiments. Animals were cared for in accordance with the guidelines of the US National Institutes of Health and the Canadian Council on Animal Care and all procedures were carried out under protocols approved by the UBC Animal Care Committees.

3.2.4  **Cell culture**

Mouse immortalized peritoneal macrophage cell lines were created by isolation of cells following peritoneal lavage of mice that were generated by crossing wild-type (*lat*+/+) or LAL knockout mice (*lat*−/−) with ImmortoMice (Charles River laboratories), engineered to have temperature-sensitive expression of SV40 large T-antigen under an IFN-γ inducible promoter, using methods described in Castoreno *et al.* (387). The isolated cells were then grown in monolayer in continuous culture at 33°C and 8% CO₂ in RPMI 1640 medium supplemented with 10% fetal bovine serum, antibiotics and 5 units/ml IFN-γ (growth medium) for the first 10 passages only.

Primary dermal fibroblasts were isolated from skin sections from *lat*+/+ and *lat*−/− mice grown in DMEM medium supplemented with 20%FBS, antibiotics and anti-fungal reagents during clonal cell growth. The individual colonies of cells isolated were then propagated in
DMEM with 10% FBS and antibiotic, incubated at 37°C and 5% CO₂. Cells were used for experiments between passages 3 and 10.

3.2.5 **Tissue homogenization and Western blotting**

Mouse tissues were collected immediately following euthanasia, flash-frozen in liquid nitrogen and stored until use. Tissues were crushed to a frozen powder using a mortar and pestle cooled in liquid nitrogen and 30-50mg of powdered tissue was homogenized on ice (388) in 0.5 ml lysis buffer containing 20mM Tris, 5mM EDTA, 5mM EGTA, 0.5% maltoside and 1X complete Mini Protease Inhibitor (Roche Applied Science) (384). Macrophages were harvested on ice in 0.25ml lysis buffer and then homogenized using a glass mortar and Teflon pestle. Cell proteins were separated by 7.5% (top half) and 12% (bottom half) SDS-PAGE and transferred to nitrocellulose overnight at 35V. Immunoblotting was performed using polyclonal antibodies against ABCA1 (1:1000 dilution, Novus Biologicals), ABCG1 (1:500 dilution, Novus Biologicals), LAL (1:1000 dilution, Abcam) and protein disulfide isomerase (PDI) (1:1000 dilution, Enzo Life Sciences) or β-actin (1:1000, Abcam) and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution, Sigma), detected using Super Signal West Femto chemiluminescence substrate (Pierce).

3.2.6 **Measurement of acid cholesteryl ester hydrolase activity**

Acid CE hydrolase enzyme activity from macrophage cell lysates was quantified using methods previously described (249,265). Cell lysates were prepared by removing cells from dishes in a 0.05% trypsin solution and cells were washed 5 times in PBS and reconstituted in sterile distilled water and sonicated in an ice-water bath for 30 seconds. Cell lysates were centrifuged at 14,000 rpm for 30 seconds in a microcentrifuge to precipitate cell debris, and
supernatants were collected and stored at -80°C until the assay was performed. Protein concentrations were estimated by Lowry assay (372). The substrate mixture was formed by first combining 25 μM (0.011g) of the artificial substrate 4-methylumbelliferyl oleate (4-MUO) with 40 μM (0.031g) L-α-phosphatidylcholine and evaporating carrier solvents under nitrogen gas. The dried lipids were then reconstituted in 10 ml of 2.4 mM sodium taurodeoxycholate and sonicated at 50 watts for 3-5 minutes to form a dispersion of lipid vesicles. The enzymatic reaction was carried out by combining in a glass test tube 50 µl of the substrate mixture with 400 µl of 0.2 M sodium acetate (pH 5.5) and 5-10 µg of cell lysate in 96 well black-bottom plates and incubated at 37°C. The fluorescence generated by the reaction was quantified (excitation wavelength 335 nm, emission wavelength 455 nm) using a Tecan Safire II plate reader (Mannedorf, Switzerland) detected at 5 minute intervals over 30 minutes. The rate of the reaction was calculated as the slope of the linear curve of the fluorescence units over time (min).

3.2.7 Quantitative real-time PCR analysis of mRNA

Total RNA was extracted from cell monolayers or frozen powdered frozen liver tissue (see above) using 1ml TRIzol extraction reagent (Invitrogen), and cDNA libraries were constructed by reverse transcription as previously described (384). DNA amplification was performed following initial denaturation at 95°C for 3 min, 40 cycles of [dentaturation at 95°C for 20 s, annealing at the temperature indicated in Table 1. for 20 s, extension at 72°C for 40 s] using SYBR Green (Quanta Biosciences) used to detect PCR products in real-time using a Realplex² Mastercycler thermocycler (Eppendorf). The sequences of primers used are listed in Table 1. The mRNA levels for genes listed were the mean cycle times, \( C_T \), corrected relative to the housekeeping gene m-cyclophilin (m-cyc) and expressed as a ratio relative to untreated controls. For semi-quantitative PCR, amplification of LAL and m-cyc mRNA was performed as
in Choi et al. (13) using primers and annealing temperatures described in Table 1. Products were run on a 1.1% agarose gel and detected by GelRed (Biotium, Inc.) nucleic acid stain fluorescence using a GeneFlash (Syngene) detection unit.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’→3’)</th>
<th>Reverse (5’→3’)</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>m-cyc</td>
<td>ACCCAAGGGGAACTGCAGCGAGAGC</td>
<td>CCGGTTCCTCTTTTGAGCTGTTCGAGC</td>
<td>58°C</td>
</tr>
<tr>
<td>ABCA1</td>
<td>GACATCTGAAGCCAATTCTGT</td>
<td>CCTTGTGGCTCACTGTGAAGGT</td>
<td>58°C</td>
</tr>
<tr>
<td>ABCG1</td>
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<td>GCCAGGTAGTAGGCTCTCCAG</td>
<td>60.9°C</td>
</tr>
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<td>CAAGCATCTCTCTCTGGGGGT</td>
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</tr>
<tr>
<td>ABCG8</td>
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<td>ACGTCGAGTAGTAGGCTCTTCT</td>
<td>54°C</td>
</tr>
<tr>
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</tr>
<tr>
<td>ApoE</td>
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<td>ACTGCGGCTGCACTGCTCC</td>
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</tr>
<tr>
<td>SR-B1</td>
<td>AACAGGGGAAGATCGAGCCAG</td>
<td>CTCAAGTAGGCTGAGTGGGC</td>
<td>54°C</td>
</tr>
<tr>
<td>LAL</td>
<td>GCTGCCAGATTCTAGTAGACTGGG</td>
<td>TGTGGGTACTCTCTAGGAGG</td>
<td>58°C</td>
</tr>
</tbody>
</table>

Table 3.1 PCR Primers and Annealing Temperatures Used

3.2.8 *Cholesterol efflux assay*

A preparation of [³H]cholesteryl oleate – labeled acetylated LDL ([³H]CE – acLDL) was generated by incubation of native LDL with [³H]cholesteryl oleate in the presence of CETP-containing plasma and then acetylated by incubation with concentrated sodium acetate with intermittent addition of acetic anhydride. Macrophage cell monolayers were grown to confluence and incubated with 50 µg/ml [³H]CE – acLDL for 24 hours followed by incubation with 10µg/ml apoA-I in RPMI 1640 medium for a further 24 hours. Medium was removed and centrifuged for 10 minutes at 3000 rpm to precipitate cell debris and [³H]sterol in medium was quantified by liquid scintillation counting. Meanwhile, cell monolayers were washed extensively with cold
PBS, 1mg/ml BSA and PBS on ice and cellular lipids were extracted by Folch extraction as previously described (370). Cellular lipids were separated by thin layer chromatography. Unesterified cholesterol (UC) and cholesteryl ester (CE) spots were located on plates using unlabeled carrier lipids stained with iodine gas, and radioactivity was quantified by liquid scintillation counting (389) as previously described (371,384). Medium UC and cell UC and CE were calculated as percent of total $[^3]$Hsterol (cell + medium).

3.2.9 **Macrophage reverse cholesterol transport**

Methods to measure macrophage to feces RCT were adapted from (139,151,390). Mice were housed individually in cages with wire mesh floor inserts and were fed *ad libitum* with free access to water for the duration of the experiment. Three days prior to injection, lal+/+ and lal-/- peritoneal macrophages were seeded in 100mm dishes at 3x10$^6$ cells/dish in growth medium and incubated for 2 days. Cells were then incubated in 6ml/well RPMI 1640 medium containing 50µg/ml $[^3]$HCE – acLDL for 24 hours. Prior to injection, cells were gently lifted from dishes using 0.05% trypsin, suspended cells were washed several times in warm PBS. Cells were counted using a hemocytometer, resuspended in warm PBS to 8-12x10$^6$ cells/ml and a small aliquot (~50µl) of cells was removed to quantify the specific activity of $[^3]$H by liquid scintillation counting. Cells were drawn into individual 1ml syringes (500 µl cells/syringe) and injected intraperitoneally into mice using 26 gauge needles within 1 hour after collection. Typically, 6x10$^6$ cells containing 7-10x10$^5$ counts per minute (CPM) in 0.5ml PBS per mouse was injected.

A blood sample was collected after 24 hours via the facial vein into tubes containing EDTA and placed immediately on ice. Blood was centrifuged at 1500 x g for 30 min at 4°C and
the plasma layer removed (391). Plasma volumes were estimated (~50-100 µl/mouse) and $[^3]H$ radioactivity was quantified by LSC. Mice were fasted for 4 hours and at 48 hours post-injection, mice were deeply anesthetized by inhaled isofluorane gas and blood was drawn by cardiac puncture. Plasma was collected as above and an aliquot of 200µl was counted directly by LSC. A second aliquot of 200µl plasma was removed and apoB-containing lipoproteins were precipitated by addition of 400µl precipitation buffer (555µM phosphotungstic acid, 25mM magnesium chloride, pH 2.5) and centrifuged at 14,000 x g for 2 min at 10°C (392) and the supernatant containing HDL was collected for quantification of $[^3]H$sterol by LSC.

Following anesthesia and removal of blood by cardiac puncture, the vasculature of mice were perfused with 20ml of ice-cold saline via the left ventricle of the heart and tissues (liver, spleen, kidney, adrenal gland, small intestine, heart, brain, lung, adipose, testes) were dissected, frozen in liquid nitrogen and stored at -80°C.

Feces were continuously collected from cages over 48 hours and stored at -20°C until assayed. Feces were weighed and soaked at 4°C overnight in distilled water at a ratio of 1ml per 100mg feces. The following day, an equal volume of ethanol was added and feces were homogenized by vortexing vigorously. Triplicate aliquots of 200µl of feces homogenate were quantified for $[^3]H$sterol and $[^3]H$bile acid radioactivity by LSC.

3.2.10 Statistical analysis

Results were analyzed using GraphPad Prism version 5.0 and standard errors of the mean are indicated. A one-way analysis of variance was performed, using Bonferroni post-hoc comparisons to analyze statistical differences between treatment groups.
3.3 Results

3.3.1 ABCA1 expression and cholesterol efflux are impaired in immortalized peritoneal macrophages that lack LAL

Macrophage lipid accumulation and foam cell formation in the artery wall is central to the dogma of the process of atherogenesis and, at least in mice, make up the majority of cells in the plaque (393). Alternatively, cholesterol efflux from macrophages, mediated by ABCA1, ABCG1 and others, is an important process to counter foam cell formation through removal of excess lipids and cholesterol. In order to investigate the role of LAL in ABCA1 regulation in macrophages, immortalized mouse peritoneal macrophage cell lines were created following isolation from wild-type (LAL\textsuperscript{+/+}) and LAL knockout mice (LAL\textsuperscript{-/-}) (387).

We observed significantly lower basal levels of ABCA1 mRNA and protein expression in lal\textsuperscript{-/-} macrophages and little increase after loading with 50 µg/ml acetylated LDL (acLDL) for 24 hours as compared to wild-type macrophages (Figure 3.1). However, when LAL enzyme was replaced by treatment with 5-20 µg of recombinant human LAL (rhLAL), ABCA1 expression was increased (Figure 3.1). The mRNA transcript levels of three other cholesterol efflux genes were also quantified in macrophage cell lines (Figure 3.2). The levels of ABCG1 were significantly reduced in lal\textsuperscript{-/-} macrophages compared to lal\textsuperscript{+/+}, even after acLDL loading, but no significant differences were observed in apolipoprotein E (apoE) or scavenger receptor B1 (SR-B1) in either cell line.
Figure 3.1 ABCA1 (A) mRNA and (B) protein are reduced in lat<sup>−/−</sup> mouse peritoneal macrophages but are partially rescued by rhLAL.

Wild-type (<i>lal<sup>+/+</sup></i>) and LAL knockout (<i>lal<sup>−/−</sup></i>) mouse immortalized peritoneal macrophage cell lines were grown in culture to confluence and supplemented with (+) or without (-) 50 µg/ml acetylated LDL (acLDL) for 24 hours. Some dishes of <i>lal<sup>−/−</sup></i> cells were also treated with 5, 10 or 20 µg of purified recombinant human LAL protein (from Shire) during, and 1 hour prior to acLDL loading. (A) ABCA1 mRNA expression was measured by quantitative realtime PCR, corrected for the housekeeping gene m-cyclophilin (m-cyc) and protein levels were resolved by SDS-PAGE and detected by Western blotting using polyclonal antibodies against ABCA1, LAL or PDI loading control. Error bars are standard error of the mean from (A) 7 or (C) 5 separate experiments (*p<0.05, One-way ANOVA, significantly different from LAL<sup>+/+</sup>, unless indicated).
Figure 3. ABCG1 but not ApoE or SR-B1 mRNA expression is reduced in LAL-/- macrophages.

Macrophages were grown in culture and incubated with or without 50µg/ml acetylated LDL (acLDL) for 24 hours. Cell lysates were collected and mRNA levels were analyzed by quantitative real-time PCR as in Figure 1A, using primers against ATP-binding cassette transporter G1 (ABCG1), Apolipoprotein E (apoE) or scavenger receptor B1 (SR-B1). Error bars are the standard error of the mean from 5 separate experiments (*p<0.05, significantly different compared to LAL+/+, One-way ANOVA with Bonferroni comparisons).
For cholesterol efflux studies, macrophages were loaded with 50 μg/ml acetylated LDL or [$^3$H]cholesteryl oleate-labeled acetylated LDL ([$^3$H]CE-acLDL) for 24 hours and [$^3$H]cholesteryl ester (CE) and [$^3$H]unesterified cholesterol (UC) counts were quantified in cells and medium following 24 hours incubation with (+) or without (-) 10 μg/ml apolipoprotein A1 (apoA-I). Both non-specific and ABCA1-dependent cholesterol efflux to apoA-I in the medium were significantly impaired and reduced to almost half of wild-type levels in lal-/—cells (Figure 3.3). Alternatively, treatment of lal-/—cells with 10 µg rhLAL was sufficient to restore ABCA1-dependent cholesterol efflux (Figure 3.3A), reflective of the upregulation of ABCA1 in these cells (Figure 3.1). As expected (237), there was an accumulation of [$^3$H]CE and reduced hydrolysis to [$^3$H]UC within the cell (Figure 3.3B and C). However, we were surprised to observe a significant proportion (~40%) of normal CE hydrolysis was still taking place in lal-/—cells. The source of this residual hydrolysis was investigated (Supplemental Figure 3.7). Briefly, when cells were treated with the lysosomal inhibitor chloroquine, although it caused some inhibition of CE hydrolysis in wild-type cells, it did not further reduce [$^3$H]UC production or increase [$^3$H]CE accumulation in lal-/—macrophages (Supplemental Figure 3.7A). When CE hydrolase activity of macrophage cell lysates was assessed using the artificial substrate 4-methylumbelliferoyl oleate (4-MUO) under acidic conditions (pH 5), approximately 50% normal activity was detected in lal-/—macrophages (Supplemental Figure 3.7B). The absence of LAL transcript was also confirmed in the lal-/—cells (Supplemental Figure 3.7C).
Figure 3.3 Rescue of impaired apoA-I-dependent cholesterol efflux in lal<sup>-/-</sup> macrophages by treatment with rhLAL.

Immortalized mouse peritoneal macrophages were grown to confluence and loaded with 50 µg/ml [³H]cholesteryl oleate-labeled acetylated LDL ([³H]CE-acLDL) for 24 hours and then washed and incubated in the presence (+ apoA-I) or absence (- apoA-I) of 10µg/ml purified apolipoprotein A-I for 24 hours. A. Medium was removed and [³H] cholesterol was quantified by LSC. B, C. From cells that were not incubated with apoA-I, cellular lipids were extracted, cellular [³H]cholesteryl esters and [³H]unesterified cholesterol were separated by TLC and counted by LSC. Measurements are expressed as the percent of total [³H] counts (medium + cell). Error bars are the standard error of the mean from 7 separate experiments (*p<0.05, One-way ANOVA, significantly different from LAL+/+, unless indicated).
3.3.2 Differential tissue-specific expression of RCT genes in LAL knockout mice

The liver is an important player in the RCT pathway, both for nascent HDL particle production and for lipoprotein cholesterol uptake, and production of bile for sterol excretion (72). Expression of several hepatic genes that are involved in RCT was analyzed by quantitative real-time PCR. ABCA1 mRNA expression in the liver was not significantly different between cell lines, nor was SR-B1 (Figure 3.4). Strikingly, ABCG1 transcript levels were approximately 17-fold higher in livers of lal-/ mice compared to wild-type mice. Alternatively, the other LXR-dependent genes ATP-binding cassette transporter G5 and G8 (ABCG5/G8) and cholesterol 7α-hydroxylase (CYP7A1) were significantly reduced in LAL knockout mouse liver (Fig 3.4).
Figure 3.4 Differential RCT gene mRNA expression in LAL+/+ and LAL-/ mouse liver.

Differential gene expression in LAL+/+ and LAL-/ mouse liver. Livers were dissected from mice, homogenized, and RNA was extracted. Quantitative real-time PCR was performed using primers against ATP-binding cassette transporters A1, G1, G5, G8, cholesterol 7 alpha hydroxylase (CYP7A1) and scavenger receptor B1 (SR-B1) as described in methods. Error bars are the standard error of the mean (*p<0.05, student T-test, significantly different compared to LAL+/+, n (+/+, +/-) = 6,6.)
When the tissue-specific expression of ABCA1 was analyzed in wild-type and LAL knockout mice, we observed a significant reduction in ABCA1 membrane protein levels in the liver, spleen and kidney of lal-/- mice (394), whereas there was little difference in tissues such as the heart, lung or brain (results not shown). The levels of ABCG1 protein were also reduced in lal-/- mice (Figure 3.5C). This disconnect between ABCA1 and ABCG1 mRNA and protein levels in the liver suggests that the differences between lal+/+ and lal-/- mice occur at a post-transcriptional level. We’ve previously reported differences in ABCA1 regulation in LAL deficiency in human cholesteryl ester storage disease skin fibroblasts (384). In order to compare these results to our mouse model, we isolated primary skin fibroblasts from wild-type and LAL knockout mice. Similar to human CESD fibroblasts, we also observe impaired upregulation of ABCA1 protein levels upon in mouse lal-/- skin fibroblasts with LDL cholesterol loading (Figure 3.5B).
Figure 3.5 ABCA1 and ABCG1 tissue-specific protein expression in wild-type and LAL knockout mice.

A, C. Sections of mouse liver, spleen and kidney were dissected and homogenized. B. Primary dermal fibroblasts were isolated from mouse skin and grown in culture and then treated with (+) or without (-) 50 µg/ml LDL for 24 hours. Membrane and soluble proteins were extracted and resolved by SDS-PAGE and probed by Western blot using antibodies against ABCA1, ABCG1 and β-actin loading control. Results (A and C) are representative of samples taken from 6 LAL+/+ and 6 LAL-/- mice or (B) 3 separate experiments showing similar results.
3.3.3 Macrophage to feces RCT is impaired in LAL deficiency

Since these results in macrophages suggested a dysregulation of ABCA1, ABCG1 and other RCT genes in LAL knockout mice, we next set out to investigate the impact LAL deficiency on the RCT pathway in vivo. Using a technique originally developed by the laboratory of Daniel Rader (139), the flux of radio-labeled cholesterol specifically from macrophages can be traced through the RCT pathway to the feces. There is strong evidence that ABCA1 plays an important role in promotion of macrophage to feces RCT using this method (150,395). In order to measure macrophage reverse cholesterol transport in our system, wild-type and LAL knockout mice were injected with immortalized peritoneal macrophages of the same genotype. As has been reported (323,324), the LAL knockout mice had developed significant hepatomegaly (i.e. liver enlargement) and their spleens, kidneys and intestines were also enlarged and had noticeable lipid accumulation as indicated by their yellowish colour. However, the knockout mice and wild-type controls were used between 8 and 12 weeks of age for the study before the age where this pathology typically progresses to illness and death (Gregory Grabowski personal observations). Macrophages grown in monoculture (lal+/+ or lal−/−) that had been loaded with radiolabeled with [3H]CE-acLDL for 24 hours were suspended in saline and injected intraperitoneally into mice of the same genotype. We observed a significant reduction in [3H]cholesterol in plasma at 24 and 48 hour time points when lal−/− mice were injected with lal−/− macrophages with a similar total level of radioactivity (1.36 ± 0.08% of total [3H] counts injected at 48 hours, n= 30 mice), compared to wild-type mice injected with lal+/+ macrophages (2.43 ± 0.26% of injected, n= 27) (Figure 3.6A). This was reflective of the impaired cholesterol efflux to medium observed in these macrophages in vitro (Figure 3.3A). Over 48 hours, lal−/− mice injected with lal−/− macrophages also had a reduced ability to support RCT to feces (1.55 ±
0.35% of total injected) compared to the lal+/+ into lal+/+ group (5.38 ± 0.92% of injected) (Figure 3.6B). In a third experimental group, lal/-/- macrophages were injected into lal+/+ mice to test whether endogenously secreted LAL (253,258,396) in wild-type mice could correct LAL activity in deficient macrophages and whether this would affect macrophage RCT. Indeed, macrophage RCT of [$^3$H]sterol to plasma and feces (2.60 ± 0.46%, n=19) was partially corrected to intermediate levels between LAL+/+ into +/+ and LAL -/- into -/- experimental groups (Figure 3.6A,B). Just as treatment with purified recombinant rhLAL enzyme could correct acLDL [$^3$H]cholesterol ester hydrolysis and [$^3$H]cholesterol efflux from macrophages in culture (Figure 3.3), these results suggested that some endogenous LAL had been taken up, resulting in an improvement of CE hydrolysis, cellular cholesterol (UC) efflux and RCT to feces.
Figure 3.6 Impaired macrophage reverse cholesterol transport in LAL knockout mice.

Macrophage reverse cholesterol transport to efflux to plasma (total) (A), plasma lipoproteins (B) and feces (C) is impaired in LAL knockout mice injected with \textit{lal}^{-/-} macrophages (LAL-/- into -/-) compared to wild-type (LAL+/- into +/-), but is only partially reduced in LAL+/+ mice injected with \textit{lal}^{-/-} macrophages (LAL-/- into +/-).

Macrophage cells (\textit{lal}^{+/+} or \textit{lal}^{-/-}) were loaded with 50 µg/ml $[^3]$H]-acetate into LAL+/- mice as indicated. Plasma and feces were collected at 24 and 48 hours post-injection and $[^3]$H]sterols were extracted and quantified by LSC. (B) From aliquots of plasma taken at 48 hours. ApoB-containing lipoproteins (LDL, VLDL) were precipitated using a sodium phosphotungstate solution and $[^3]$H]sterols were quantified in the HDL-containing supernatant fraction by LSC. The LDL and VLDL counts were calculated by subtracting from the total plasma $[^3]$H]sterols. Error bars are the standard error of the mean (*p<0.05, One-way ANOVA, significantly different compared to LAL+/- into LAL+/-, n(+/- into +/-, -/- into +/-, -/- into -/-) =27, 19, 30, respectively).
3.4 Discussion

The removal of excess cholesterol by macrophages in the artery wall by efflux is thought to be an important protective mechanism against atherosclerosis through the reverse cholesterol transport pathway (148). However, the contribution of different pools of intracellular cholesterol that the cell uses to regulate gene expression and promote cholesterol efflux and thus RCT are poorly understood. Here we show that when lysosomal cholesterol egress is blocked in LAL deficient macrophages, there is a dysregulation of the cholesterol transporters ABCA1 and ABCG1 and reduced cholesterol efflux (Figures 3.1 and 3.3). These data also demonstrate that this impaired ability of LAL-/- macrophages to release excess intracellular cholesterol significantly diminishes the flux of cholesterol through the RCT pathway to the feces in vivo (Figure 3.6).

Reduced LAL activity results in reduced flux of cholesterol from the late endosomes and lysosomes (LE/LY), reduced trafficking of cholesterol to intracellular sites necessary for generation of oxysterols necessary to activate LXR, and upregulate genes involved in RCT. We’ve previously shown reduced regulation of ABCA1 and formation of HDL in fibroblasts from patients with CESD, providing a reason for the low HDL-C seen in CESD (384). This was also confirmed in LAL knockout mouse primary dermal fibroblasts, which also exhibit impaired LDL-dependent upregulation of ABCA1 (Figure 3.5). Therefore, it is likely that expression of efflux cholesterol genes such as ABCA1, ABCG1 and cholesterol efflux observed in macrophage LAL deficiency (Figures 3.1, 2, 3) are reduced by a similar mechanism.

We unexpectedly observed a significant residual level of acLDL-CE hydrolysis and apparent LAL activity (Supplemental Figure 3.7B) was taking place, despite the confirmed
absence of LAL transcript in LAL-/- macrophages (Supplemental Figure 3.7C), which could not be further inhibited using chloroquine (Supplemental Figure 3.7A). Like LDL, acetylated LDL particles are taken up by endocytosis and cholesteryl esters are broken down in the lysosomes (59,298). However, Wang and colleagues have shown that LDL and acLDL are trafficked to different endosomal compartments and the cholesterol pools from either LDL or acLDL are functionally distinct (397). It is possible that some acetylated LDL-CE may escape the LAL-containing lysosomes or may be hydrolyzed by neutral hydrolases in other parts of the cell. Alternatively, although LAL is the only known lipase to hydrolyze cholesteryl esters at acidic pH within the cell (258,323), the acidic hydrolysis (at pH 5) of the artificial substrate 4-MUO by macrophage cell lysates (Supplemental Figure 3.7B) raises the possibility of the existence of another acid CE hydrolase in mouse macrophages.

Despite this residual hydrolysis, however, LAL deficiency has a major impact on ABCA1 and ABCG1 protein expression and significantly reduces cholesterol efflux, meaning that the source of this LAL-independent hydrolysis does not channel cholesterol to the key sites of oxysterol generation to upregulate LXR-dependent genes in the absence of LAL. This is reinforced by the findings of Ouimet et al. (398), indicating that LAL is also essential for hydrolysis of cytoplasmic lipid droplet cholesteryl esters. Therefore, even in the case where CE hydrolysis, and re-esterification by ACAT may occur (292), these CEs would still be returned to the lysosome via autophagy ultimately to be hydrolyzed by LAL (398), which our data suggests is necessary for UC to reach the regulatory pool for LXR activation of ABCA1 (and ABCG1).

The expression of several genes important in the RCT pathway were analyzed in liver homogenates of wild-type and LAL knockout mice. The mRNA levels of the membrane
transporters ABCG5 and G8, which play crucial roles in biliary cholesterol export (130), were
significantly reduced in LAL/-/- liver, as was cholesterol 7-α hydroxylase (CYP7A1), which
performs the rate limiting step in bile acid synthesis (Figure 3.4). These genes are known to be
LXR-responsive in normal mouse liver (110,399,400). Therefore, in LAL/-/- mice, the reduced
release of cholesterol from lysosomal CE hydrolysis may reduce the pool of unesterified
cholesterol available to stimulate LXR-dependent expression of these genes in the liver.
Interestingly, ABCG5/G8 and CYP7A1 expression are also reduced in the liver in a mouse
model of inflammation where macrophage to feces RCT is shown to be impaired (153). In this
regard, inflammation in the LAL knockout mouse (401,402) might indirectly alter gene
expression in the liver and negatively impact RCT. The levels of SR-BI transcript were not
significantly altered in LAL/-/- mouse liver (Figure 3.4). SR-BI has an LXR-response element in
its promoter (403) and its expression is increased with LXR agonists in HepG2 cells in vitro
(404). However, SR-BI expression in the liver of mice was not increased by cholesterol feeding
(405) or with the LXR agonist TO901317 (27), suggesting that it is not functionally LXR-
responsive in vivo. The transcription factor SREBP also has a dominant negative effect on LXR
activation (406) and SREBP activation would be expected to be increased in LAL-deficient cells
(292), therefore SR-BI upregulation by LXR might be suppressed.

Curiously, the mRNA and protein levels of ABCA1 and ABCG1 in the liver don’t seem
to correspond. While liver ABCA1 mRNA transcripts are equal between genotypes, and ABCG1
is significantly increased in LAL/-/- mice, the levels of ABCA1 and ABCG1 protein are much
decreased in LAL knockout mice (Figures 3.4 and 3.5). ABCA1 expression is differentially
regulated in different tissues (283) and is not thought to be LXR-dependent in the mouse liver as
hepatic ABCA1 levels are not increased in the liver of mice treated with LXR agonists
Wang and colleagues (390) showed that ABCA1 and ABCG1 mRNA and protein, and cholesterol efflux are low in NPC null mouse macrophages, but increased in primary hepatocytes. This indicates that, unlike in macrophages or fibroblasts, the flux of cholesterol out of the lysosomes does not regulate gene expression of these two transporters in the liver. However, ABCA1 and ABCG1 protein levels seem to be negatively affected at the post-transcriptional level since their levels are markedly decreased in LAL deficient mouse liver. Interestingly, the tissues where ABCA1 was lower in LAL-/- mice (liver, spleen, kidney) (Figure 3.5) also happen to be the tissues where LAL is more highly expressed, and where CE and TG accumulation is most prominent (323,324). Our data (Supplemental Figure 3.8), using a lysis buffer other than maltoside, which is more effective at extracting membrane proteins specifically, shows ABCA1 total protein levels are the same. Therefore it is possible that, although transcriptional regulation seems to be unaffected, the proteins are being degraded at the plasma membrane more rapidly in LAL-/- hepatic cells. LAL knockout mice have insulin resistance and high plasma free fatty acids compared to wild-type mice (324) and unsaturated free fatty acids are known to destabilize ABCA1 protein at the plasma membrane (409) and in HepG2 cells (410). Also, since whole liver homogenates were used, this would represent a mixed population of cells, including the macrophage-like Kupffer cells. The levels of ABCA1 and ABCG1 were significantly lower in peripheral macrophages (Figure 3.1 and 3.2). Although Kupffer cells, normally only make up approximately 10% of the resting population of liver cells (411,412), there is evidence that the Kupffer cell population is expanded in LAL knockout mice (324). Therefore, although Kupffer cells normally express ABCA1 and ABCG1 highly (413,414), it is tempting to speculate that that Kupffer cells of LAL-/- mice would have reduced expression, as in peritoneal macrophages, which might be confounding the results.
Ultimately, the absence of LAL in macrophages resulted in reduced macrophage RCT to feces in knockout mice injected with macrophages of the same genotype (Figure 3.6), indicating that lysosomal cholesterol from LAL-mediated cholesteryl ester hydrolysis is not only important for regulating gene expression and cholesterol efflux from macrophages, but is also plays a critical role in the RCT pathway. Similarly, other studies have shown that ABCA1 (and ABCG1) deficiency impairs macrophage RCT (150,395). Therefore, the differences in macrophage RCT observed in LAL knockout mice could be directly related to differences in macrophage ABCA1 and ABCG1 expression and reduced UC available for efflux. LAL-/- mice are reported to have low plasma HDL cholesterol levels and the HDL peaks of FPLC profiles from LAL knockout mice are shifted to the right compared to wild-type mice (324), suggesting smaller particle sizes. This might be explained by the reduced ABCA1 and ABCG1 levels in several tissues of LAL knockout mice, including the liver (Figure 3.5), since these transporters are important for cholesterol efflux and nascent HDL formation (77,83,185). Indeed, our data that LAL-/- macrophages had reduced cholesterol efflux to plasma of LAL-/- mice (Figure 3.6A) supports this. Adenovirus-mediated expression of LAL in LAL-/- mice increases plasma HDL-C levels compared to uninfected mice (326), providing further evidence that HDL cholesterol levels are related to LAL activity. Similarly, when LAL-/- macrophages were injected into LAL+/+ mice, there was a correction of macrophage RCT (Figure 3.6), consistent with the uptake of endogenous LAL in the peritoneal cavity of wild-type mice by LAL-/- macrophages, suggesting that LAL is sufficient to restore macrophage RCT.

These findings implicate LAL as an important player in the RCT pathway and may have a protective role in prevention of atherogenesis. The association of LAL deficiency with the development and progression of atherosclerosis has been studied both in mice and humans. LAL
and apoE double knockout mice have rapidly progressing and severe atherosclerotic lesion
development compared to apoE-deficient mice alone and LAL-deficient mice on an LDLR-/-
background die of cardiac thrombotic events after only 5 days on a high fat/high cholesterol diet
(337). Conversely, supplementation of LDL receptor-deficient mice with a macrophage-specific
form of recombinant human LAL produced in *Pitchia pastoris* prevented early lesion formation
and drastically reduced the size of pre-existing advanced lesions (336). NPC/LDLR double
knockout mice have accelerated atherosclerosis on a high fat diet, despite having lower plasma
cholesterol levels (415). It is suggested that NPC deficiency impacts macrophage intracellular
cholesterol trafficking and homeostasis through LXR since 27-OH production was low, as it is in
CESD fibroblasts (384). Human CESD patients are typically hypercholesterolemic, have low
plasma HDL-C (310,312) and increased risk for premature atherosclerosis (317,327). In addition,
CESD is thought to be under-diagnosed in the population and LAL deficiency may be more
prevalent among people with low HDL-C and premature cardiovascular disease than previously
thought (333,416). Together with the current data, this suggests that lysosomal cholesterol plays
a critical role in regulation of lipid and cholesterol transporters for removal of excess cholesterol
from peripheral cells, including macrophages, but that this also has a significantly impact on the
whole body flux of cholesterol through the RCT pathway.
3.5 Supplementary data

3.5.1 Supplemental figures

![Graph A: Cell [3H] CE](image)

**A** Cell [3H] CE

![Graph B: Cell [3H] UC](image)

**B** Cell [3H] UC

![Graph C: LAL activity of MPMs grown in serum-free medium](image)

**C** LAL activity of MPMs grown in serum-free medium

![Graph D: Confirmation of absence of LAL mRNA in LAL-/- MPMs](image)

**D** Confirmation of absence of LAL mRNA in LAL-/- MPMs

Figure 3.7 Investigation of residual CE hydrolysis in LAL-/- macrophages.

Macrophages were labeled with [3H]CE-acLDL for 24 hours with cells treated with 50 µM chloroquine for one hour prior to and during [3H]CE-acLDL labelling, and cellular [3H]CE (A) and [3H]UC (B) were analyzed as in figure 2.3. C) Acid CE-hydrolase activity from macrophage cell lysates was measured by fluorescence of cleaved 4-MUO substrate in vitro at pH 5. D) RNA was extracted from macrophages and reverse transcribed to cDNA as in Figure 1. LAL and m-cyc transcripts were amplified by PCR and separated on 1.1% agarose gels. (A, B) Results are the standard error of the mean from 5 separate experiments (*p<0.05, Two-way ANOVA, significantly different from LAL+/+). (C) Results are the standard error of the mean (*p<0.05, one-tailed t-test, significantly different from LAL+/+, n = 4). (D) Results are representative of 2 separate experiments with similar results.
Figure 3.8 A comparison of ABCA1 protein expression in mouse liver using different homogenization buffers.

Livers were dissected from LAL+/+ and LAL-/− mice and tissue sections were homogenized in either buffer A (20mM Tris, 5mM EDTA, 5mM EGTA, 0.5% maltoside and protease inhibitor) or buffer B (20mM Hepes, 1mM EDTA, 250mM sucrose, 100mM sodium pyrophosphate, 10mM sodium orthovanadate, 100mM sodium fluoride). Results are representative of multiple Western blots using tissue homogenates from 6 LAL+/+ mice and 6 LAL-/− mice.
CHAPTER 4: CONCLUDING REMARKS

4.1 Summary and discussion

Cardiovascular disease (CVD) is the leading cause of death in adults worldwide (1). While current therapies such as statin drugs and cholesterol absorption inhibitors are effective at lowering LDL-C and reducing the risk of CAD by about 30%, there is still clearly a need for strategies to further reduce morbidity and mortality from this devastating disease. Low levels of high density lipoproteins (HDL) are one of the strongest predictors of risk for CVD (358), however effective methods of increasing HDL formation clinically – shown to markedly reduce atherosclerosis in animal models – are not currently available. An enhanced understanding of the pathways cells use to initiate HDL formation is required to design novel therapies to increase HDL formation clinically. Among the anti-atherogenic properties of HDL is its ability to reduce cellular cholesterol burden by promoting reverse cholesterol transport from cells within the arterial intima to promote excretion of this essential but potentially toxic molecule (68,148). The membrane transporter ABCA1 plays an essential role in this pathway by mediating the efflux of phospholipids and cholesterol to apolipoprotein acceptors during initial HDL particle formation (7). It is known that ABCA1 is upregulated in response to cellular cholesterol loading by generation of oxysterols that activate ABCA1 gene transcription through a pathway mediated by LXR (8,9). However, the intracellular sources of cholesterol that promote ABCA1 regulation and become a substrate for efflux are poorly understood.

In this thesis, we proposed that the flux of cholesterol from the lysosomal compartment was the most important for regulation of ABCA1 expression and cholesterol efflux for HDL particle generation and RCT. Specifically, we set out to understand the role of the enzyme responsible
for acidic hydrolysis of cholesteryl esters from LDL taken up via endocytosis on the ABCA1 pathway.

In chapter 2 it was shown that patient-derived fibroblasts from individuals with CESD had significantly reduced basal ABCA1 expression and blunted upregulation with LDL cholesterol loading. This results in impaired ABCA1-mediated phospholipid and cholesterol efflux to apoA-I, reduced formation of alpha-migrating HDL particles in the medium, which we demonstrated is likely due to impaired generation of oxysterols in response to cholesterol loading which would impact ABCA1 expression via the LXR pathway. We’ve shown that lysosomal acid lipase is both necessary and sufficient for regulation of ABCA1 expression and function by inhibiting LAL activity in normal cells and re-introducing exogenous LAL using either conditioned medium from normal fibroblasts containing secreted LAL or by addition of purified recombinant human LAL. The effect of LAL deficiency in these studies indicates that lysosomal cholesterol derived from LAL-mediated hydrolysis of LDL-CE is the most critical for regulation of ABCA1 expression and cellular efflux, despite increased endocytosis via the LDL receptor and impaired suppression of de novo cholesterol synthesis reported in CESD fibroblasts (237,287), indicating that the regulatory pool of cholesterol within the cell is not sufficient to promote ABCA1 regulation in conditions of slowed release of lysosomal cholesterol. Significantly, we also demonstrated for the first time that the likely cause of low HDL in CESD patients was due to impaired ABCA1 expression and activity due to impaired intracellular lysosomal LDL-CE hydrolysis.

In chapter 3, the importance of LAL in macrophages and its influence on whole body RCT was investigated. Macrophage foam cells are a prominent feature during atherogenesis and neutral lipid and CE accumulation in Kupffer cells within the liver and foamy macrophage
infiltration into tissues is a prominent feature in LAL deficiency in both humans and animal models (361,362). LAL deficiency enhances lesion development in mouse models of atherosclerosis (337,363), however, supplementation of LDL receptor-deficient mice with macrophage-specific recombinant LAL prevents early lesion formation and drastically reduced the size of pre-existing advanced lesions (336). Therefore, we proposed that complete deficiency of LAL in macrophages causing a buildup of modified LDL-derived CE within lysosomes would have a negative impact on the ABCA1 pathway for cholesterol efflux, thus contributing to foam cell formation and ultimately leading to impairment of RCT in the body.

In addition to skin fibroblasts, it was shown that peritoneal macrophages derived from LAL knockout mice had a significant impairment of ABCA1 expression and cholesterol efflux. Modern techniques to trace the movement of cholesterol from macrophages through the RCT pathway to the feces were employed by injecting normal and LAL deficient macrophages into wild-type and LAL knockout mice, indicating that lysosomal CE hydrolysis by LAL is also critical for RCT from macrophages. This has significance in the context of atherosclerosis, where macrophage foam cells accumulate in the earlier stages of the developing plaque and excess cholesterol from modified LDL uptake must be removed via the RCT pathway in order to maintain cellular cholesterol homeostasis and prevent plaque progression (114).

Ouimet and colleagues (297) importantly showed that LAL is required not only for initial hydrolysis of LDL-CEs but is essential for hydrolysis of cytoplasmic CE stored in lipid droplets, which must return to the lysosomes via autophagy to be hydrolyzed by LAL before cholesterol is available for efflux. This reinforces our current findings and emphasizes the role of LAL and lysosomal cholesterol in ABCA1 regulation and RCT.
In summary, with these studies we’ve shown that impaired LAL activity affects HDL cholesterol metabolism in detail at the cellular level and followed up on the effects on whole body RCT in vivo and these principles have been demonstrated in multiple cell types in both human disease and mouse models of LAL deficiency which we believe provides strength to the current research.

4.2 Future directions

Novel screening and treatment options for LAL deficiency syndromes such as enzyme replacement therapy or improved screening methods to detect LAL deficiency in the population are currently being developed and tested clinically. Since CESD is under diagnosed in the general population (416), this is also important as LAL deficiency may be the underlying cause for many individuals currently diagnosed with similar presentation such as non-alcoholic hepatic steatosis, metabolic syndrome or hypoalphalipoproteinemia (416). Given our current findings that LAL deficiency leads to impaired HDL formation and RCT, enhancement of LAL activity or LAL enzyme therapy may help to increase the movement of cholesterol through the lysosomes, which is critical for ABCA1 regulation and cholesterol efflux to prevent buildup of cholesterol in the arteries which causes atherosclerosis and potentially cause premature CVD.

Some limitations of these studies, however, include the use of the species Mus musculus as a model for atherosclerosis and the focus placed on macrophages for the study of RCT in the context of atherosclerosis. Lipoprotein metabolism in mice differs from humans in several ways. For example, mice carry most of their cholesterol in plasma on HDL particles, while they do not have CETP to mediate transfer of cholesterol to LDL. While macrophages appear to be the major cholesterol-accumulating cell type within lesions of mice made genetically susceptible to atherosclerosis (393), this dogma of macrophage foam cell formation recently being called into
question in the context of human atherogenesis. Studies suggest that smooth muscle cells (57) and dendritic cells (417,418) may also play an important role in lesion development and progression in human cardiovascular disease. Therefore, it is important to consider the relevance of applying such animal models to the study of human atherosclerosis and future studies would benefit from study of RCT from other cell types and other animal models as well. While deficiency of LAL has a negative impact on plasma lipoprotein metabolism including HDL formation, RCT and increased atherosclerosis in human CESD and WD and in mouse models, as presented here and in (317,327,328,336,337), it appears that increased LAL activity may also contribute to atherosclerosis. Recent GWAS studies suggest that increased LAL expression is also associated with higher risk of CVD. Evidence that uptake of oxidized or aggregated LDL impairs ABCA1 expression in macrophages (355) suggests that this might be a mechanism for impairment of cellular LAL activity in normal individuals affected by CVD. Also, secreted LAL has been shown to hydrolyze LDL extracellularly (352,354) and enhanced LAL activity (261) as well as enzymatically modified LDL has been detected in human atherosclerotic lesions (261,354), which may contribute to the buildup of cholesterol in the artery wall, thus contributing to atherogenesis. It is possible that in this context, an increase in LAL activity may become pathogenic. Therefore, it is clear that further investigation into the role of LAL in human atherosclerosis is needed in order to determine the optimal level of LAL activity; the minimum activity to promote lysosomal cholesterol flux for regulation of HDL formation and RCT, or the maximum level of activity that becomes pathogenic in the context of atherosclerosis. Our research suggests that reduction of LAL activity is detrimental as it leads to reduced expression of ABCA1 and reduced HDL-C formation. These findings are consistent with, along with increased LDL-C levels, the increased risk of premature atherosclerosis in CESD (317). While
LAL deficiency in the forms of Wolman Disease or CESD may still be relatively rare, it is known that LAL activity in macrophages is reduced in response to exposure to oxidized LDL (299,301). This suggests that there may be a relative deficiency of LAL activity in the atherosclerotic plaque that may benefit in terms of increasing ABCA1 expression by supplementing LAL or activating its expression as a novel treatment for atherosclerosis. While we did not see further enhancement of ABCA1 expression in LAL+/+ mouse macrophages by adding additional LAL enzyme to the medium, this does not rule out the possibility that such supplementation may benefit cells in the plaque where LAL activity is reduced, and thereby assist with enhancing ABCA1 expression and increasing efflux of cholesterol from these cells. Future research will be needed to understand the complexity of the role of intracellular LAL vs. extracellular secreted LAL on lipoprotein and cholesterol metabolism in order that therapies may be targeted towards LAL in the interest of promoting RCT and reducing the risk of cardiovascular disease.
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