Novel Genetic and Molecular Regulation of HDL Metabolism

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August 2012

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

ABSTRACT

Coronary artery disease (CAD) is the leading cause of mortality and morbidity worldwide. Low levels of high-density lipoprotein cholesterol (HDL-C) constitute a major independent risk factor for CAD, influenced by a combination of genetic and environmental factors. In this thesis, we aimed to advance our understanding of the genetic regulation of HDL-C, through the identification and characterization of novel candidate genes, and to delineate the molecular mechanisms underlying HDL biogenesis, in the hopes of better elucidating the complexities of HDL metabolism.

First, we examined whether variation at the *PCSK5* gene locus influences HDL-C levels. Through familial segregation analyses and two-stage genetic association studies in unrelated subjects of French Canadian descent and low HDL-C Finnish families (n_{total}=883), we reported a region-wide significance of *PCSK5* SNPs with HDL-C, suggesting that genetic variability at the *PCSK5* locus regulates HDL-C levels, possibly through the inactivation of endothelial lipase. Second, to search for rare-low frequency variants responsible for low HDL-C, we used whole exome sequencing in a multigenerational French Canadian family (n=75) with HDL-C<5^{th} age-sex specific percentile. Through this approach, we identified a complex combination of two non-synonymous variants, in the ATP-binding cassette transporter (*ABCA1*) and the lipoprotein lipase genes, predicted to be damaging and causing low HDL-C. These results emphasize the need for exome sequencing of complex lipid traits in unexplained familial cases. Third, we sought to characterize a novel genetic determinant involved in HDL-C regulation. Recent studies from our group identified the WW domain-containing oxidoreductase (*WWOX*) gene locus to be associated with low serum HDL-C levels in 9,798 subjects. In this study, we examined the role of WWOX in lipoprotein and HDL metabolism using a combination of *in vivo* functional studies, by means of total Wwox knock-out and Wwox liver-specific mouse models, gene microarray and next generation resequencing analyses in HDL-deficient French Canadian families. We demonstrated that the effects of WWOX

on lipoprotein metabolism involve multiple mechanisms, including cholesterol homeostasis, apoA-I and ABCA1-mediated pathways and fatty acid/triglyceride metabolism. Fourth, with novel insight into genetic regulations of HDL metabolism, we focused on elucidating the mechanistic basis of nascent HDL formation. Specifically, we investigated the lipidation of ApoA-I and its interaction with an ABCA1/phosphatidylcholine(PC)-rich microdomain, the high-capacity binding site (HCBS). Using sucrose gradient fractionation, we also demonstrated that the ABCA1/ HCBS partitions to nonraft domains, thus playing a pivotal role in the selective desorption of PC molecules by apoA-I, creating an optimal environment for nascent HDL formation and cholesterol release.

In summary, this work has collectively advanced our understanding of the molecular and genetic basis of HDL metabolism. It has brought to light novel genes governing plasma HDL-C levels in humans, highlighting the need to use multiple integrative genetic approaches to identify causal common and rare variants conferring susceptibility to low HDL-C. These findings have also helped elucidate the mechanistic basis of the nascent HDL genesis pathway. Together, this thesis has combined genetic and biochemical strategies to provide a more comprehensive understanding of the complexities of the HDL metabolism and cellular cholesterol transport, which may lead to the characterization of novel therapeutic targets for CAD.

RESUMÉ

La maladie coronarienne athérosclérotique (MCAS) est la principale cause de mortalité et de morbidité à l'échelle mondiale. Un niveau bas des lipoprotéines de cholestérol de haute densité (HDL-C) représente un facteur de risque majeur pour la MCAS et est influencé par une combinaison des facteurs génétiques et environnementaux. Dans cette thèse, nous avons abordé la régulation génétique des HDL-C grâce à l'identification et la caractérisation de nouveaux gènes candidats, et de mécanismes moléculaires liés à la biogenèse des HDL, dans l'espoir de mieux élucider la complexité du métabolisme des HDL.

En premier, nous avons examiné si la variation au niveau du locus du gène proprotéine convertase *PCSK5* peut affecter les niveaux de HDL-C. Grâce aux analyses de ségrégation familiale et aux études d'association génétique dans une population canadienne-française et chez les familles finlandaises (n_{Total}=883) avec un niveau de HDL-C bas, nous avons constaté une large corrélation régionale entre les variations polymorphiques (SNPs) de *PCSK5* et HDL-C. Ceci suggère que la variabilité génétique au niveau du locus *PCSK5* réglemente le HDL-C, éventuellement par le biais de l'inactivation de la lipase endothéliale, une enzyme clé dans la modulation des niveaux plasmatiques de HDL-C. Deuxièmement, pour rechercher des variants de fréquence rare qui sont responsables d'un bas niveau de HDL-C, nous avons utilisé la technique du séquençage de l'exome dans une famille multi-générationnelle canadienne-française (n=75) avec un HDL-C<5e percentile spécifique (âge-sexe). Grâce à cette approche, nous avons identifié une combinaison complexe de deux variants non-synonymes dans le transporteur ABCA1 et dans le gène de la lipoprotéine lipase provoquant un taux faible de HDL-C. Ces résultats soulignent la nécessité du séquençage de l'exome des traits lipidiques complexes dans les cas familiaux inexpliqués. Troisièmement, nous avons caractérisé un nouveau gène impliqué dans la régulation du HDL-C. Nous avons examiné le rôle de WWOX dans le métabolisme du HDL en utilisant des études fonctionnelles *in vivo* avec des souris Wwox total knock-out (KO) et des souris Wwox KO spécifiques au foie, une

technologie des puces à ADN et du reséquençage dans des familles canadiennes-françaises déficientes en HDL. Nous avons démontré que l'effet de WWOX sur le métabolisme des lipoprotéines implique plusieurs mécanismes, y compris l'homéostasie du cholestérol, les voies de régulation à travers l'ApoA-I et l'ABCA1 et le métabolisme d'acides gras/triglycérides. Quatrièmement, nous voulions élucider le mécanisme de formation du HDL naissant. Plus précisément, nous avons étudié la lipidation de l'ApoA-I et son interaction avec des sites constitués de microdomaines ABCA1/phosphatidylcholine (PtdC) que nous avons appelés « site de liaison de grande capacité (HCBS) ». En utilisant un gradient de densité de sucrose, nous avons observé que l'ABCA1 et le HCBS sont localisés dans des domaines membranaires solubles aux détergents et que l'ApoA-I désorbe sélectivement la PtdC de ces domaines ainsi créant un environnement optimal pour la formation des molécules naissantes de HDL et la libération de cholestérol.

Collectivement, ce travail a élargi notre compréhension des sciences moléculaires et génétiques du métabolisme du HDL. Il a mis en lumière de nouveaux gènes impliqués dans la régulation des niveaux de HDL-C, soulignant la nécessité d'utiliser plusieurs approches intégratives génétiques pour identifier les causes des variants communs et rares conférant une susceptibilité à un faible taux de HDL-C. Ces résultats ont également contribué à élucider le mécanisme de biogenèse du HDL naissant. Ainsi, cette thèse a combiné des stratégies génétiques et biochimiques pour fournir une meilleure compréhension de la complexité du métabolisme des HDL et du transport du cholestérol cellulaire, qui pourrait ainsi conduire à l'élaboration des nouvelles cibles thérapeutiques pour la MCAS.

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LIST OF ABBREVIATIONS

-C	Cholesterol
22OH	22-(*R*)-hydroxycholesterol
2D-PAGGE	Two-dimensional polyacrylamide non-denaturing gel electrophoresis
9CRA	9-cis-retinoic acid
ABCA1	ATP binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ACVD	Atherosclerotic cardiovascular disease
Apo	Apolipoprotein
ANGPTL3	Angiopoetin-like protein 3
BHK	Baby hamster kidney
BSA	Bovine serum albumin
CAD	Coronary artery disease
CCTα	CTP:phosphocholine cytidylyltransferase alpha
CDP-cho	Cytidine diphosphate choline
CDX	Methyl- β-cyclodextrin
CHD	Coronary heart disease
CE	Cholesteryl esters
CETP	Cholesteryl ester transfer protein
Cho	Choline
CHO	Chinese hamster ovary
CKα	Choline kinase alpha
CPT	CDP-choline:DAG choline-phosphotransferase
CRP	C-reactive protein
CVD	Cardiovascular disease
DMEM	Dulbecco's modified Eagles medium
DRM	Detergent-resistant membrane
DSP	Dithiobis(succinimidyl propionate)
DTT	Dithiothreitol
EL	Endothelial lipase
ES	Extracellular Space
FC	Free cholesterol
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
GWAS	Genome-wide association studies
HCBS	High capacity binding site
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
HL	Hepatic lipase
HPLC	High pressure liquid chromatography
HSPG	Heparin sulfate proteoglycans
ICCs	Intracellular compartments
IHD	Ischemic heart disease

LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LDNR	Ligand-dependent nuclear receptor
LIPE	Hormone sensitive lipase
LpA-I	Nascent apoA-I containing particle
LOD	Logarithm of odd ratio
LPL	Lipoprotein lipase
LPR1	Low desnity lipoprotein receptor-related protein-1
LRP1	Lipoprotein receptor related protein-1
LXR	Liver X receptor
MAF	Minor allele frequency
MI	Myocardial infarction
miR	microRNA
NCEH1	Neutral cholesteryl ester hydrolase-1
NPC	Niemann-Pick disease type C
PBS	Phosphate buffered saline
PC	Proprotein convertases
PC-PLC	Phosphatidylcholine-specific phospholipase C
PCSK5	Proprotein convertase subtilisin/kexin type 5
PCR	Polymerase chain reaction
Pcho	Phosphocholine
PL	Phospholipids
PM	Plasma membrane
PMA	Phorbol 12-myristate 13-acetate
PtdCho	Phosphatidylcholine
RCT	Reverse cholesterol transport
RT	Room temperature
RT-qPCR	Reverse transcriptase quantitative Polymerase chain reaction
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulfate
SM	Sphingomyelin
SMCs	Smooth muscle cells
SNP	Single-nucleotide polymorphisms
Sp1	Phosphorylated specificity protein 1
SPRI	Solid Phase Reversible Immobilization paramagnetic bead
SR-BI	Scavenger receptor class B type I
StARD3	Steroidogenic acute regulator protein
TD	Tangier disease
TG	Triglycerides
TGF	Transforming growth factor
TLC	Thin layer chromatography
TR	Transcription regulator
VLDL	Very low density lipoprotein
WT	Wild type
WWOX	WW domain-containing oxidoreductase

CONTRIBUTIONS OF AUTHORS

As permitted under the "Guidelines for Thesis Preparation" provided by the Graduate and Postdoctoral Studies Office, Chapters 1- 5 of this thesis include manuscripts either published or in preparation for publication. The contributions of the authors are listed below:

Chapter 1

Sections of this chapter (where indicated) are modified from two review articles published in *Current Atherosclerosis Reports*:

Iatan I, Palmyre A, Alrasheed S, Ruel I, Genest J. *Genetics of cholesterol efflux* Current Atherosclerosis Reports. 2012 Jun;14(3):235-46.

Iatan I, Alrasadi K, Ruel I, Alwaili K, Genest J. *Effect of ABCA1 mutations on risk for myocardial infarction*. Current Atherosclerosis Reports. 2008 Oct;10(5):413-26

Chapter 2.

The contents of this chapter have been published as:

Iulia Iatan, Zari Dastani, Ron Do, Daphna Weissglas-Volkov, Isabelle Ruel, Jenny C. Lee, Adriana Huertas-Vazquez, Marja-Riitta Taskinen, Annik Prat, Nabil G. Seidah, Päivi Pajukanta, James C. Engert and Jacques Genest. *Genetic Variation at the Proprotein Convertase Subtilisin/Kexin Type 5 Gene Modulates High-Density Lipoprotein Cholesterol Levels.* Circulation Cardiovascular Genetics. 2009;2:467-475.

The contributions made by the collaborators for this Chapter are as follows: I planned the experiments with Dr. Genest. Familial segregation analyses and sequencing studies were performed by myself, and Drs. Dastani and Ruel assisted with the analysis and interpretation of the results. DNA samples were

collected by Dr. Ruel. Preparation of all DNA samples for genotyping was performed by myself. Stage 1 of the association studies in the French Canadian subjects was performed by myself with the assistance of Dr. Do. Stage 2 of the association studies in the Finnish family subjects was performed by Drs. Weissglas-Volkov, Lee, Huertas-Vazquez and Pajukanta. I drafted the manuscript and generated all figures and tables in consultation with Dr. Genest. Drs. Prat, Taskinen, Seidah, Pajukanta and Engert provided critical review of the manuscript.

Chapter 3.

The contents of this chapter have been accepted for publication as:

MV Prasad Linga Reddy[#], Iulia Iatan[#], Daphna Weissglas-Volkov, Elina Nikkola, Blake E Haas, Miina Juvonen, Isabelle Ruel, Janet S Sinsheimer, Jacques Genest, and Paivi Pajukanta. *Exome sequencing identifies two rare variants for low HDL-C in an extended family.* Circulation Cardiovascular Genetics. Accepted for publication with no revisions July 23rd, 2012. [#]Authors contributed equally to this work.

The contributions made by the collaborators for this Chapter are as follows: The work in this manuscript was shared by Dr. Reddy and myself, in consultation of our collaborating supervisors, Drs. Genest and Pajukanta. The contributions of Dr. Reddy and myself were equivalent from an experimental and conceptual perspective, and we co-wrote the manuscript. All DNA samples were collected by Dr. Ruel. Selection of samples for exome sequencing was done by myself in consultation with Dr. Genest and Dr. Pajukanta. Samples were sent to UCLA for exome sequencing. Drs. Reddy, Weissglas-Volkov, Haas, Ms. Nikkola, and Ms. Juvonen were responsible for the exome sequencing data collection. Analysis of this data was done by Drs. Reddy and myself. The genotype by sex interaction analysis was performed by Dr. Weissglad-Volkov. I performed all functional assays, including the efflux experiments and gene-gender experiments.

Dr Reddy and I drafted the manuscript and generated the figures and tables together. Drs. Sinsheimer, Genest and Pajukanta reviewed the manuscript and provided general review of the data and project. The rest of the authors provided helpful suggestions and critically reviewed the manuscript.

Chapter 4.

The contents of this chapter are in preparation to be submitted for publication as:

Iulia Iatan, Hong Choi, Isabelle Ruel, MV Prasad Linga Reddy, Kil Hyunsuk, Mohammad Abu Odeh, Dapha Weissglas-Volkov, Elina Nikkola, Zuhier Awan, Jaeho Lee, Aqeilan Rami, Päivi Pajukanta, Marcelo Aldaz, Jacques Genest. *The WW Domain-containing Oxidoreductase Gene Modulates HDL Metabolism and Lipoprotein Gene Expression*. In preparation for submission.

The contributions made by the collaborators for this Chapter are as follows: I planned all experiments in consultation with Dr. Genest and was entirely responsible for the preparation and drafting of the manuscript and its figures. I prepared serum samples for lipid measurements and lipoprotein assays, isolated serum from 12 mice and performed western blots. Drs. Choi and Ruel provided their assistance with the experiments. Mr. Odeh and Dr. Aqeilan provided total knockout samples, while Mrs. Hyunsuk, Mr. Lee and Dr. Aldaz produced the liver-specific knockout mice, and performed measurements of these mice, while also preparing and providing mice samples. I isolated, prepared and submitted microarray RNA samples, and analyzed these data, with the assistance of Dr. Choi. Dr. Ruel performed HPLC and RT-qPCR experiments, and Drs. Reddy, Weissglas-Volkov and Pajukanta performed the human resequencing study on the samples selected and prepared by Dr. Ruel and myself. The rest of the authors provided helpful suggestions and critically reviewed the manuscript.

Chapter 5.

The contents of this chapter have been published as:

Iulia Iatan[#], Dana Bailey[#], Isabelle Ruel, Anouar Hafiane, Steven Campbell, Larbi Krimbou and Jacques Genest. *Membrane microdomains modulate oligomeric ABCA1 function: impact on apoAI-mediated lipid removal and phosphatidylcholine biosynthesis*. J Lipid Res. 2011;52(11):2043-2055. [#]Authors contributed equally to this work.

The contributions made by the collaborators for this Chapter are as follows: The work in this manuscript was shared by Dr. Bailey and me under the direction of Dr Genest. The contributions of Dr. Bailey and me were equivalent from an experimental and conceptual perspective, and we co-wrote the manuscript with the aid of Mr. Krimbou, and generated all figures and tables. As such, Dr. Bailey and I designed and performed all of the experiments described in this manuscript in consultation with Dr. Genest. Mr. Krimbou assisted with data analysis and manuscript preparation, as did Dr. Genest.

ORIGINAL CONTRIBUTION TO KNOWLEDGE

Chapter 2: Genetic Variation at the Proprotein Convertase Subtilisin/Kexin Type 5 Gene Modulates High-Density Lipoprotein Cholesterol Levels

-We found 7 novel variants for the *PCSK5* gene
-Using French Canadian and Finnish low HDL-C families, we observed significant association between the rs11144766 SNP and HDL-C
-We identified for the first time that genetic variability of the *PCSK5* gene regulates HDL-C levels

Chapter 3: Exome sequencing identifies two rare variants for low HDL-C in an extended family

-We were the first to use exome sequencing to find rare variants with large effects that work together to contribute to the low HDL-C complex trait, as this approach had previously been used for monogenic disorders
-We found that two rare, nonsynonymous variants in the ABCA1 and lipoprotein lipase genes explain much of the HDL-C genetic variance in an extended family and confer susceptibility to low HDL-C

Chapter 4: The WW Domain-containing Oxidoreductase Gene Modulates HDL Metabolism and Lipoprotein Gene Expression

-We were the first to characterize WWOX as a regulator of HDL-C and lipid metabolism
-We utilized two *Wwox* knock-out mice models to confirm that WWOX influences the expression of regulators of HDL and lipid metabolism.
-We found a gender-specific effect for WWOX in regulating triglyceride levels and lipid metabolic pathways

Chapter 5: Membrane microdomains modulate oligomeric ABCA1 function: impact on apoA-I-mediated lipid removal and phosphatidylcholine biosynthesis pathway

-We determined that the high-capacity binding site (HCBS) works in tandem with ABCA1 to bind apoA-I in the nonraft microdomains

-We characterized a three-step lipidation model by which phosphatidylcholine and cholesterol are removed from peripheral cells after interactions with non-rafts and rafts domains and that apoA-I-mediated lipid efflux induces phosphatidylcholine synthesis. This provides a biochemical basis for excess cholesterol removal from lipid-laden macrophages, which may have important implications in preventing cardiovascular disease.

ACKNOWLEDGEMENTS

There are so many people to thank for their support during my thesis studies, and your help and encouragement, whether technical, intellectual, or emotional, have meant the world to me.

First and foremost, Dr. Genest, I want to give you my sincerest thanks for your constant guidance, understanding, patience, and support for all these years. Your experimental insight, your direction and endless enthusiasm for research were fundamental to the success of this work. I will certainly miss your sense of humor, which I was able to appreciate so well...! The amazing research opportunities you have provided me with are incomparable, and I am eternally grateful for what you have given me, as your contribution to my future endeavors will continue beyond completion of these studies. I could not have asked for a better supervisor, mentor and role model.

To the many colleagues and friends I have made over the years in the Cardiovascular research laboratories, I want to thank each one of you for your friendship and help. Donna, Houssein, Khalid & Khalid, Shirya, Safia, John, Mohsen, Nick, Ron, Zari, Sven, Nathalie, Cyril, Sarah, Steve, Anouar, Zuhier, Colette, Anna, Gina, Caroline, Dr. Sniderman, Dr. Kiss and Dr. Engert - your distinct personalities each hold a permanent place in my heart, and I will always cherish the memories we have shared.

Dana, my “lab sister”, you have been an integral part of my research career and I will never forget all the great times we had, whether at the lab or during conferences. I want to thank you for your friendship, help and support.

Hong, while we have not spent much time together in the lab, your good humor and extensive scientific insight has been invaluable. Katia, you have been missed and your cheerfulness is something I will always remember. Isabelle, you have been extremely supportive throughout my studies. Your help and friendship during these many years has been greatly appreciated, and I want to thank you for continually supporting me, and especially for all your advice!

A very sincere thank you goes to Larbi and Michel for their scientific guidance, friendship, humor, patience and valuable experimental insight.

To my friends who have encouraged me every step of the way: Alex, Steph, Chiara, Debbie, Andreea, Cici, Anita, thank you for your friendship and encouragement. Chris, thank you for being there by my side during these studies, for your constant optimism, advice and important scientific insight, but most of all, for your help, and your friendship I will always cherish it.

To Ben, your incredible support has always been a source of inspiration that has helped me throughout my studies. I want to thank you for your immense understanding, for your unwavering patience during my long research hours, and most of all, for always being there for me.

Mom, dad, Tudor you are so important to me... You have instilled in me to never to give up and always pursue my dreams. Words cannot express the heartfelt appreciation that I have for your never-ending support and encouragement. Without your guidance, I would not have made it this far!

CHAPTER 1. GENERAL INTRODUCTION

1.1 Cardiovascular disease

1.1.1 Cardiovascular disease worldwide and in Canada

Cardiovascular disease (CVD) is the leading cause of mortality and disability worldwide (Yusuf et al., 2004; Linsel-Nitschke and Tall, 2005; WHO, 2011). In 2008, an estimated 17.3 million people died from CVD, representing 30% of all global mortality (WHO, 2011). Of those, approximately 7.3 million were due to atherosclerosis and its major sequel, coronary artery disease (CAD) (WHO, 2011). In Canada, despite a decline of 25% in rates of CAD in the past 10 years, heart disease and stroke remain two of the three leading causes of mortality, accounting for one-third of all deaths (Genest et al., 2009; Heart and Stroke Foundation, 2012). Accordingly, while significant progress in CAD prevention and pharmacological therapies have been made, the prevalence of CAD is still expected to dramatically increase in the next two decades. The best weapon to tackle this growing burden is to gain a greater understanding of the disease; that is, to learn more about its mechanisms of action and its genetic regulations.

1.1.2 Pathophysiology of Atherosclerosis

Atherosclerosis is a progressive disease, characterized by the accumulation of fibrous elements and lipids affecting primarily the intima of large- and medium-sized elastic and muscular arteries (Lusis et al., 2004) (**Figure 1.1**). According to the theory of Ross and colleagues, put forth in 1973, atherosclerosis can be viewed as a 'response to injury' process, with lipoprotein and other risk factors being the injurious agents (Lusis, 2000; Ross and Glomset, 1976a; Ross and Glomset, 1976b; Ross, 1993).

In its earliest stages, the atherogenetic process is characterized by perturbations in endothelial function in response to turbulent flow in the setting of an unfavorable serum lipid profile. Endothelial dysfunction allows for accumulation of lipoprotein particles, mainly low-density lipoprotein (LDL), and their aggregates into the intima at preferential sites of lesion formation, shown as (step 1) in **Figure 1.1** (Lusis et al., 2004; Ross et al., 1977). Trapping of lipoproteins results in their modification and oxidation by vascular cell waste and

lipoxygenase species (Lusis, 2000). Oxidized LDL subsequently invokes a pro-inflammatory response, stimulating the secretion of adhesion molecules (VCAM-1, E selectin, and P selectin) and cytokines (i.e. M-CSF), and promotes recruitment of lymphocytes and monocytes to the endothelium lesion site (step 2). The monocytes then transmigrate across the endothelial monolayer into the intima (step 3), where they proliferate and differentiate into macrophages (step 4) (George and Lyon, 2010). Macrophages play an essential role in atherosclerosis as they rapidly uptake highly oxidized atherogenic lipoproteins via scavenger receptors SR-A and CD36, forming lipid laden foam cells (step 5) (Lusis, 2000). These 'fatty streaks' are recognized as the onset of atherosclerosis (George and Lyon, 2010). With time, apoptotic cell death of foam cell macrophages occurs, causing the formation of a lipid-rich necrotic core (Lusis et al., 2004). With further fatty streak progression, inflammatory cytokines are secreted (step 6), promoting smooth muscle cell (SMC) recruitment, which migrate from the medial layer to the intima. With the secretion of fibrous elements by the smooth muscle cells and platelet adherence, occlusive fibrous plaques develop (step 7). The lesions continue to grow by the migration of new mononuclear cells from the blood, and are further accompanied by SMC proliferation, extracellular matrix production, and accumulation of extracellular lipids (Lusis, 2000; George and Lyon, 2010). With additional growth, the atheroma progressively narrows the arterial lumen, thereby reducing blood flow which can have severe consequences, including ischemia, unstable angina, myocardial infarction or even sudden death (Aikawa and Libby, 2004).

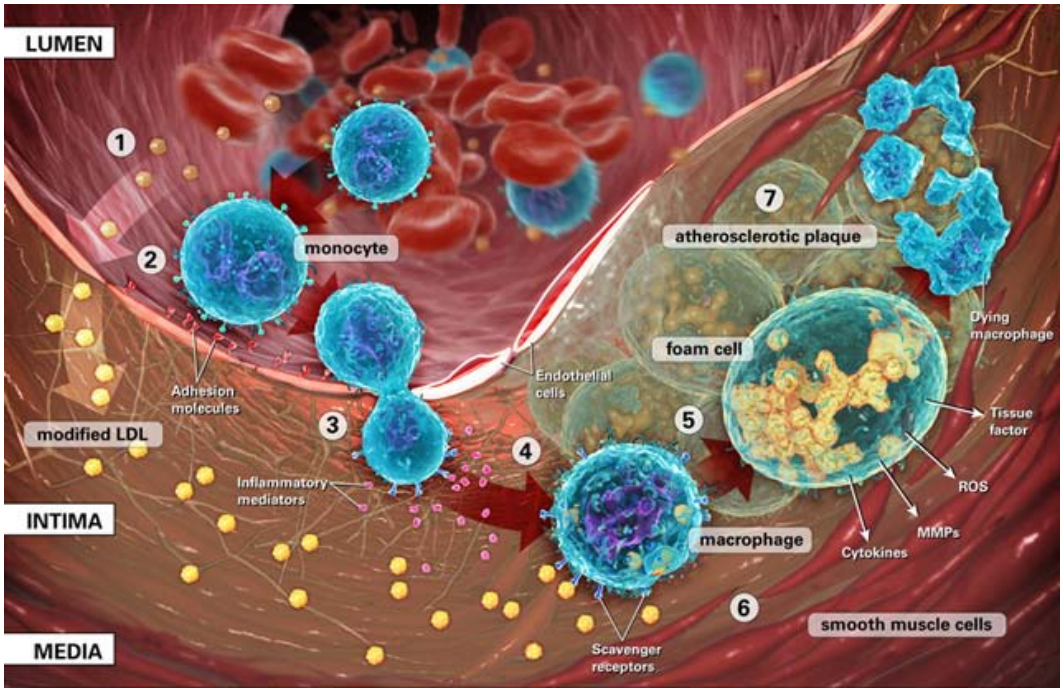

Figure 1.1. Atherosclerosis formation
The formation of an atherosclerosis plaque involves several steps. 1) Lipoprotein particles, primarily low-density lipoprotein cholesterol (LDL-C) enter the intima of the arterial wall. These lipoproteins are trapped in the intima and are modified by oxidation, triggering a pro-inflammatory response that stimulates the secretion of adhesion molecules. 2-3) This response leads to the recruitment of monocytes which transmigrate into the intima. 4-5) These monocytes differentiate into macrophages, which use scavenger receptors to uptake the oxidized, atherogenic lipoproteins, resulting in the production of foam cells. 6) Foam cells secrete cytokines to perpetuate the inflammatory response, which promotes the growth of smooth muscle. 7) As foam cells undergo cell death, their lipid-rich corpses are coated by a fibrous cap from the smooth muscle cells, producing a plaque, which may occlude the arterial lumen. Image obtained from In Vivo Communications Ltd (www.invivo.ca) (InViVo Communications™ Inc, 2012)

1.1.3 Risk factors associated with CVD

Epidemiological studies have revealed numerous risk factors associated with atherosclerosis. These can be grouped into those that are largely environmental and those with important genetic components, which may further be collectively classified as modifiable and non-modifiable. Each of these have been reviewed in depth (Yusuf et al., 2004; Genest et al., 2009; George and Lyon, 2010; Lusis et al., 2004). Environmental factors include those that relate to lifestyle, such as tobacco smoking, stress, physical inactivity, excessive alcohol consumption, and obesity. Risk factors associated with a strong genetically heritable component include dyslipoproteinemia (high serum levels of LDL-cholesterol (LDL-C; >2.0 mmol/L) and/or very low-density lipoprotein cholesterol (VLDL-C); low serum concentration of high-density lipoprotein-cholesterol (HDL-C (<1.03 mmol/L for men and <1.3 mmol/L for women)), elevated concentrations of triglycerides (TG), hypertension, lipoprotein (a), inflammatory biomarkers (e.g. C-reactive protein), homocysteine and diabetes mellitus. Despite having a genetic component, these factors are modifiable to a certain degree, while some others are not, such as age and male gender, family history of premature atherosclerosis incidents, and genetic abnormalities (e.g. familial hypercholesterolemia) (Yusuf et al., 2004; Genest et al., 2009; Lusis et al., 2004; Smith, Jr. et al., 2000). While biochemical and genetic studies strive to develop strategies to combat atherosclerosis, it is imperative that modifiable risk factors also be controlled through lifestyle changes where appropriate.

1.1.4 Epidemiology of HDL

Multiple epidemiological and clinical studies have shown a strong, continuous and graded inverse association between plasma levels of HDL-C and CAD (Yusuf et al., 2004; DiAngelantonio et al., 2009; Gordon et al., 1977). Canadian and American CAD prevention guidelines describe HDL-C as a categorical risk factor, with its absolute value used as a multivariate model to predict CAD risk and to determine the need and intensity of preventive therapies

(NCEP, 2001; Genest et al., 2009; Ridker et al., 2008). To date, HDL-C has been evaluated as a risk marker in 68 long-term population-based studies involving more than 302430 individuals (DiAngelantonio. et al., 2009) from Europe and North America. In multivariate models adjusted for both nonlipid and lipid (triglycerides and non-HDL cholesterol) risk factors, HDL cholesterol was found to be inversely correlated with CHD events. For every 0.39 mmol/L (15 mg/dL) increase in HDL cholesterol concentration, the risk of a CAD event was reduced by 22% (95% CI, 18%–26%) (Rosenson et al., 2011). Additionally, it is is estimated that approximately 40% of patients with premature CAD have low HDL-C (Despres et al., 2000; Genest, Jr. et al., 1993; Genest, Jr. et al., 1992; Weber et al., 1997), currently established as the most common lipoprotein disorder in patients with CAD. Furthermore, while low HDL-C concentrations are also associated with multiple features of the metabolic syndrome (Lorenzo et al., 2007), HDL-C plasma levels are strongly genetically determined. Altogether, this supports the traditional 'HDL hypothesis' (Vergeer et al., 2010) stating that HDL protects against CAD through multiple beneficial effects.

In order to gain a better understanding of the molecular mechanisms of HDL metabolism and function, and its relevance to cardiovascular disease prevention, an overview of the lipoprotein metabolism is needed.

1.2 Overview of lipoprotein metabolism

1.2.1 Biochemistry of lipoproteins

Cholesterol is integral to survival. It is an important constituent of cell membranes and a precursor for the biosynthesis of steroid hormones, bile acids and vitamin D. Although essential, when carried in certain lipoprotein fractions, cholesterol deposition in the arterial wall occurs and increases the risk of CAD. Therefore, a tightly regulated mechanism for the maintenance of cholesterol homeostasis is necessary.

The majority of cholesterol in human plasma is carried in the form of cholesteryl esters (CE), at the core of lipoprotein particles. Lipoproteins shuttle hydrophobic molecules between organs in the aqueous environment of plasma.

They are a heterogeneous population of macromolecules containing a core of TG and CE, surrounded by an envelope of phospholipids (glycerophospholipids and sphingolipids) and free (unesterified) cholesterol (**Figure 1.2**). Their protein components, known as apolipoproteins (apo), are arranged circumferentially, providing structural integrity to the lipoprotein, are involved in the assembly and secretion of the lipoprotein, and interact with specific cell membrane receptors (Genest and Libby, 2011).

Plasma lipoproteins vary in size, density, lipid and apolipoprotein content. The main functions of the different lipoproteins are determined by their apolipoprotein and lipid component. TG-rich lipoproteins (chylomicrons and VLDL), LDLs and HDLs are the major lipoprotein classes when classified by their density (**Table 1.1**) (Assmann and Nofer, 2003; Lewis and Rader, 2005).

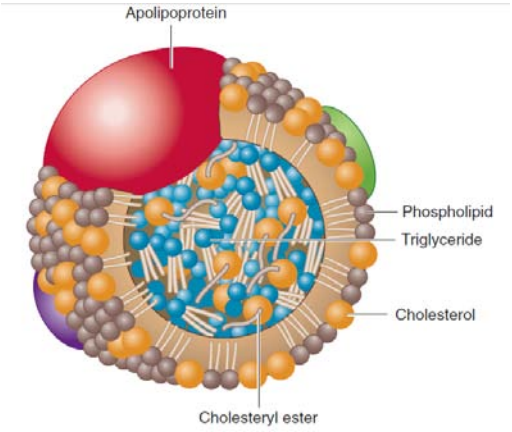

Figure 1.2 General structure of lipoproteins
Lipoprotein are spherical particles that transport lipids in the bloodstream. Triglycerides and cholesteryl esters are carried within the particle core. The external layer is hydrophilic and consists of a phospholipid monolayer in which cholesterol and apolipoproteins are embedded. Apolipoproteins are involved in the secretion of the lipoprotein, provide structural integrity, and act as cofactors for enzymes or as ligands for various receptors. Figure taken with permission from (Genest and Libby, 2011)

Table 1.1 Plasma lipoprotein composition

Component	Origin	Density (g/mL)	Size (nm)	Protein %	[Chol] in plasma (mmol/L)†	[Tg] in plasma (mmol/L)‡	Major Apo	Other Apo
Chylomicron*	Intestine	<0.95	100-1000	1-2	0.0	0.0	B48	AI, C's
Chylomicron remnants*	Chylomicron metabolism	0.95-1.006	30-80	3-5	0.0	0.0	B48, E	A-I, A-IV, Cs
VLDL	Liver	<1.006	40-50	10	0.1-0.4	0.2-1.2	B100	AI, C's
IDL	VLDL	1.006-1.019	25-30	18	0.1-0.3	0.1-0.3	B100, E	
LDL	IDL	1.019-1.063	20-25	25	1.5-3.5	0.2-0.4	B100	
HDL	Liver, intestine	1.063-1.210	6-10	40-55	0.9-1.6	0.1-0.2	AI, A-II	AIV
Lp(a)	Liver	1.051-1.082	25	30-50			B100, (a)	

* In the fasted state, serum (or plasma) should not contain chylomicrons or their remnants
†In mmol/L. For mg/dL, multiply by 38.67.
‡In mmol/L. For mg/dL, multiply by 88.5.
Apo: apolipoproteins; HDL: high-density lipoprotein; IDL: intermediate-density; LDL: low density lipoprotein; VLDL: very-low density lipoprotein; Chol: cholesterol; Tg: triglycerides. Modified from (Genest and Libby, 2011)

1.2.2 Lipoprotein transport

Lipoproteins are synthesized in the liver and intestine, mainly as VLDL and chylomicrons (**Figure 1.3**). Chylomicrons contain a single apoB48, and they transport dietary TG (~ 85% of their lipid content) that are hydrolyzed by lipoprotein lipase (LPL) and delivered primarily to adipose tissue and muscle for energy storage or production. VLDL are TG-rich particles comprising a single apoB100 (containing a domain recognized by the LDL receptor (LDLR)) as their main lipoprotein in human, and either apoB100 or apoB48 in mouse. They follow the same catabolic pathway through LPL as chylomicrons, and, during hydrolysis of TG-rich lipoproteins, an exchange takes place: VLDL particles, as well as chylomicrons acquire apoCs and apoE in part from HDL particles. VLDL also exchange TG for CE from HDL (mediated by cholesteryl ester transfer protein (CETP)). The VLDL remnant is called an intermediate-density lipoprotein, which is taken by the liver via its apoE moiety or further delipidated by hepatic lipase (HL) to form an LDL particle (**Figure 1.3**) (Genest and Libby, 2011).

While the primary role of apoB-containing lipoproteins is the delivery of lipids from the liver and intestine to peripheral tissues through a receptor mediated process (Brown and Goldstein, 1986), the later is countered by the return of excess cholesterol from peripheral tissues to the liver by HDL, in a process known as the reverse cholesterol transport (RCT).

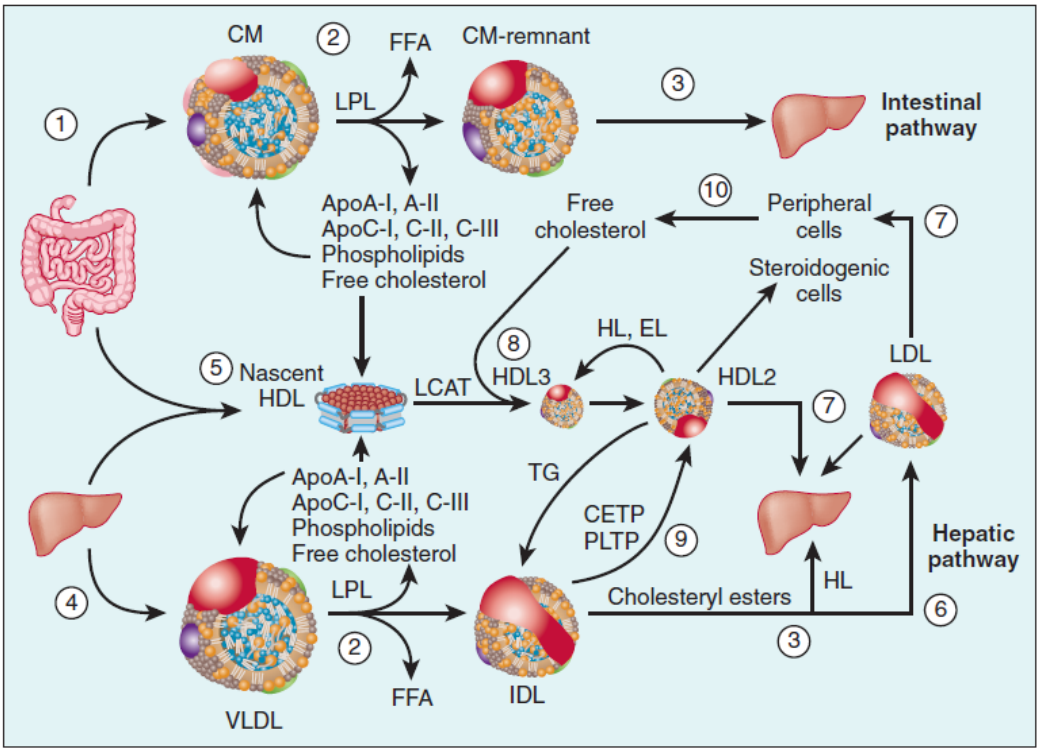

Figure 1.3 Overview of the lipoprotein transport system
Please refer to the explanation in the text. Apo: apolipoprotein; CETP: cholesteryl ester transfer protein; EL: endothelial lipase; FFA: free fatty acids; HL: hepatic lipase; HDL: high-density lipoprotein; IDL: intermediate-density lipoprotein; LCAT: lecithin cholesterol acyltransferase; LDL: low-density lipoprotein; LPL: lipoprotein lipase; PLTP: phospholipid transfer protein; VLDL: very-low density lipoprotein. Image taken with permission from (Genest and Libby, 2011).

1.3 High-density lipoproteins and the reverse cholesterol transport

1.3.1 Classification of high-density lipoproteins

High-density lipoproteins are highly heterogeneous, small (~6-10 nm) lipoproteins with a density of 1.063-1.210 g/ml, consisting of approximately 50% proteins (**Table 1.1**). Their main protein constituent is ApoA-I, (70% of the apolipoprotein content) which is synthesized in the liver and intestine. Additionally, the HDL-proteome contains other apolipoproteins such as ApoA-II, apoCs, apoD, apoE, apo M (Karlsson et al., 2005) lipolytic enzymes (CETP (Glomset, 1968)), paraoxonase, platelet activating factor acetylhydrolase (PAF-AH)), lipid transfer proteins (LCAT) (Cheung et al., 1986) and phospholipid transfer protein (PLTP) (Tall et al., 1983), as well as acute phase response proteins (e.g. serum amyloid A) (Gordon et al., 2010). Recent shotgun proteomics

analyses by our group (Alwaili et al., 2012) and others (Vaisar et al., 2007; Rezaee et al., 2006), have expanded that list. Particularly, when monitoring changes in the HDL proteome between male age-matched controls, stable CAD, and acute coronary syndrome (ACS) subjects (n=10/group), we have evaluated the HDL protein composition to 66 HDL-associated proteins, 10 of which had been previously identified (including vitronectin and complement C4B) and 14 of which were novel (Alwaili et al., 2012). Overall, we identified proteins involved in cholesterol homeostasis (~50%), with significant contributions by proteins involved in lipid binding, antioxidant, acute-phase response, immune response, and endopeptidase/protease inhibition (Alwaili et al., 2012).

HDL particles are heterogeneous in size and composition. They can be subdivided in several subspecies and isolated by a variety of techniques. Please refer to **Table 1.2** (Rosenson et al., 2011) for a recent classification of HDL subspecies. By definition, mature α-migrating particles contain Apo-I but have significant differences in lipid content and enzymes, giving rise to large light HDL_2 (1.063 g/ml - 1.110 g/ml) and small dense HDL_3 (1.110 g/ml - 1.170 g/ml) distinguished by their buoyancy with analytic ultracentrifugation (Lindgren et al., 1951; Gotto, Jr. et al., 1986; Kunitake et al., 1985). Separation by charge and size using two-dimensional polyacrylamide gradient gel electrophoresis (2D-PAGGE) identifies that the most prominent species in plasma are α-migrating particles (90%) and only a small minority migrate in the pre-β position (Kunitake et al., 1985). Of note, the term pre-β HDL describes particles migrating in the pre-β position which includes three distinct classes: lipid-free/lipid-poor apoA-I, nascent HDL (particles formed upon interaction of lipid-free ApoA-I with the ATP binding cassette cholesterol transporter (ABCA1)) and discoidal HDL particles (nascent HDL or remnant particles released after remodeling of mature HDL) (Wu et al., 2009; Silva et al., 2008).

Table 1.2 Classification of HDL subparticles

Proposed term	Very large HDL (HDL-VL)	Large HDL (HDL-L)	Medium HDL (HDL-M)	Small HDL (HDL-S)	Very small HDL (HDL-VS)
Density range, g/mL	1.063–1.087	1.088–1.110	1.110–1.129	1.129–1.154	1.154–1.21
Size range, nm	12.9–9.7	9.7–8.8	8.8–8.2	8.2–7.8	7.8–7.2
Density gradient ultracentrifugation	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
Density range, g/mL	1.063–1.087	1.088–1.110	1.110–1.129	1.129–1.154	1.154–1.170
Gradient gel electrophoresis	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
Size range, nm	12.9–9.7	9.7–8.8	8.8–8.2	8.2–7.8	7.8–7.2
2-D gel electrophoresis	α-1	α-2	α-3	α-4	Pre-β-1 HDL
Size range, nm	11.2–10.8	9.4–9.0	8.5–7.5	7.5–7.0	6.0–5.0
NMR	Large HDL-P[b]	Medium HDL-P		Small HDL-P	
Size range, nm	12.9–9.7	9.7–8.8	8.8–8.2	8.2–7.8	7.8–7.2
Ion mobility	HDL2b	HDL2a and HDL3			
Size range, nm	14.5–10.5	10.5–7.65			

Modified from (Rosenson et al., 2011). [a]One-dimensional electrophoresis was performed in nondenaturing gradient polyacrylamide gel (4%-20%). [b]HDL-P, HDL particle

1.3.2 Methods for isolating and measuring HDL subspecies

In order to study HDL, consideration must be made to the various sub-types that exist. Indeed, as described in Section 1.3.1 and **Table 1.2**, HDL particles are highly heterogeneous in size and composition (Rosenson et al., 2011). Separating HDL by their subsets provides valuable information that is of greater relevance to a patient's state than mere HDL-C levels. Several techniques exist to enable this degree of insight, which focus on the isolation and measurement of HDL particles from plasma or serum.

The "gold standard" for isolating HDL is flotational preparative ultracentrifugation. As previously mentioned (**Table 1.1**), HDL are defined as lipoprotein particles in the density range of 1.063 to 1.21 g/mL. The multi-step process of ultracentrifugation involves increasing plasma density (normally at 1.006g/ml) to 1.063 g/ml with salt, such as KBr, after which it is centrifuged to isolate VLDL, IDL, LDL and Lp(a) floating at the top, from plasma (Rosenson et al., 2011). The HDL fractions found at the bottom are collected, the density is then adjusted to 1.125 g/ml and centrifuged again to isolate HDL_2 particles, which are subsequently dialyzed to remove salts. HDL_3 particles can be isolated following another centrifugation step prior to which the density is adjusted to

1.21g/ml. The disadvantage of this approach however is the potential of shearing HDL proteins due to the strong centrifugal forces.

Another commonly used technique is the precipitation of apoB-containing lipoproteins with a charged high molecular weight compound (Khera et al., 2011). Typical examples include heparin, manganese, dextran-sulfate and polyethylene glycol (PEG). Given that apoB is not present in HDL, following precipitation, the cholesterol remaining in the supernatant is derived from HDL (Warnick and Albers, 1978). It will however also contain most plasma proteins, such as albumin, which readily binds free fatty acids. For this reason, precipitation may not be used when isolating HDL for proteomic or lipidomic studies.

As described in **Table 1.2** several other approaches exist that focus on the separation and/or analysis of HDL, including NMR spectroscopy, ion exchange chromatography, immunoaffinity chromatography, zonal ultracentrifugation and density gradient ultracentrifugation (thoroughly reviewed by (Rosenson et al., 2011)). In addition to these methods, the work described in this thesis has routinely relied on the use of two analytical techniques, size-exclusion chromatography and 2D-PAGGE, and hence additional focus will be given to describing these methods.

1.3.2.1 Size-exclusion chromatography

Size-exclusion chromatography separates the contents of a sample based on their size. The most common form of this separation is gel permeation chromatography, of which several techniques exist, including Fast Pressure Liquid Chromatography (FPLC) and High-Pressure Liquid Chromatography (HPLC) (Dernick et al., 2011). In studying HDL particles, FPLC and HPLC are largely used for analytical purposes rather than for sample preparation. The technique involves administering a serum/plasma sample into the HPLC apparatus, which is then passed through a size-exclusion column using varying pressure forces. Samples elute from the column based on their size into fractions, which represent different subspecies of particles (Chetiveaux et al., 2002; Innis-Whitehouse et al., 1998). Specifically, in our laboratory serum/plasma from individual animals or humans is separated into lipoprotein fractions using HPLC

with a Superose 6 10/300 GL column attached to a Beckman Coulter System GoldTM apparatus. A 150 mM NaCL mobile phase with a flow rate of 0.4 ml/ml is used for the separation of the specific sample into 72 HPLC fractions (400 µl) that are then collected in a 96-well plate using the ProteomeLabTM automated fraction collector. Knowing the identity of the different fractions can involve using reference samples or detection assays (Walkey et al., 1998). As such, VLDL, LDL and HDL are usually found within fractions ~15-17, ~25-35 and ~37-48, respectively. With the identity of each fraction known, total cholesterol and triglyceride concentrations can be subsequently analyzed within each fraction using the InfinityTM Cholesterol and Triglyceride Liquid Stable Reagents (Thermo Electron Corporation) following the manufacturer's instructions. This technique is not optimal as a preparative approach for isolation of samples, but is frequently used for analysis of cholesterol, triglycerides and phospholipids composition of serum/plasma samples.

1.3.2.2 Two-dimensional polyacrylamide gradient gel electrophoresis

A routinely used strategy in our laboratory for separating HDL sub-populations is the 2D-PAGGE (**Figure 1.4**). Plasma samples are separated first by charge in one dimension on a 0.75% agarose gel (100 V, 3 h, 4°C) (Krimbou et al., 1997), which separates pre-β, α and pre-α-migrating subspecies. Of note, several particles actually migrate in the pre-β position, including lipid-free apoA-I, lipid poor apoA-I and discoidal HDL (Wu et al., 2009; Silva et al., 2008). This gel is then cut into strips that are subsequently placed on a 5-23% concave polyacrylamide gradient gel, such that when run (125 V, 24 h, 4°C), samples are separated according to size, in a second dimension (Krimbou et al., 2004). This allows separation of particles by decreasing size, into preβ2, α1, α2, α3 and preβ1, which are ~12.9, 11.0, 9.2, 7.6 and 5.6 nm in diameter, respectively (Asztalos et al., 1993). This approach of resolving subspecies by two variables provides better separation of the various heterogeneous HDL subspecies. In addition, an iodinated high molecular weight protein ladder (7.1–17.0 nm; Pharmacia) is run alongside the samples as a standard on each gel, to permit size estimates to be made for the HDL particles (Krimbou et al., 1997). Electrophoretically separated samples are

then electrotransferred (30 V, 24 h, 4°C) onto nitrocellulose membranes and apoA-I-containing particles (LpA-I) are detected with immunopurified polyclonal anti-apoA-I antibody labeled with ^{125}I. This allows a visual representation of the presence or absence of specific HDL subclasses. Relative levels of HDL subspecies can also be compared by quantifying through densitometric scanning the amount of radiation for each band, thus allowing 2D-PAGGE separation to present both a quantitative and qualitative assessment of HDL subspecies.

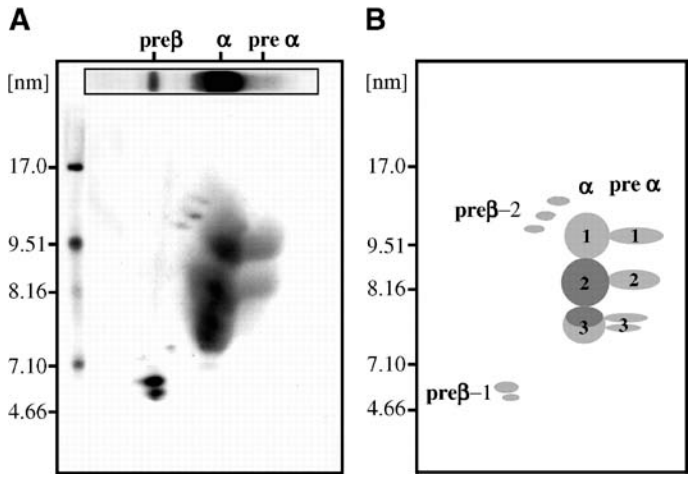

Figure 1.4.1 Separation of HDL subfractions by 2D-PAGGE
A. Separation of apolipoprotein A-I (apoA-I)-containing HDL subpopulations by 2D-PAGGE from a normolipidemic, healthy male subject. The rectangular inset represents the first-dimensional separation on an agarose gel (separation based on charge). A duplicate of that agarose strip was applied on the top of a concave gradient polyacrylamide gel (3–35%) and electrophoresed in the second dimension (separation based on size). Molecular mass standards are shown at left. B. Schema of the apoA-I-containing HDL subpopulations in human plasma. The lighter shading indicates apoA-I-containing subpopulations; the darker shading represents apoA-I- and apoA-II-containing subpopulations. Image from (Asztalos et al., 2005).

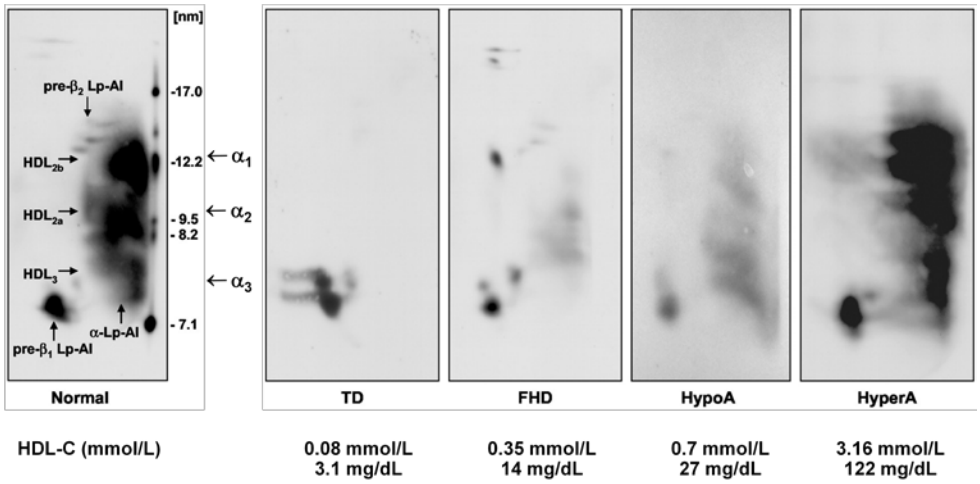

Figure 1.4.2 2D-PAGGE separation of plasma apoAI-containing lipoproteins
Plasma from normolipidemic (Normal), Tangier disease (TD, homozygous mutations at the *ABCA1* gene), familial HDL deficiency (FHD, heterozygous mutations at *ABCA1* gene), hypoalphalipoproteinemia with normal cellular lipid efflux (HypoA), and hyperalphalipoproteinemia (HyperA) subjects were separated according to charge into preβ- and α-HDL subspecies (agarose, x-axis) and size (polyacrylamide gradient, 3% to 20% gel, y-axis) into preβ2, α1, α2, α3 preβ1-HDL particles. ApoA-I was detected with ^{125}I-labeled anti-apoAI antibody. Molecular size markers are indicated on the left panel. ApoAI-containing HDL subpopulations are indicated with arrows. Modified from (Marcil et al., 2003).

1.3.4 Antiatherogenic properties of HDL

Investigation into the biochemistry of HDL has revealed not only its antiatherogenic properties, but more specifically, the mechanisms by which HDL mediates its positive effects. Experimental evidence shows that the atheroprotective effects of HDL are pleiotropic and extend far beyond removing cholesterol from lipid-laden macrophages in the atherosclerotic plaque. HDL particles have antioxidative, anti-inflammatory and antithrombotic properties, play a role in endothelial protection, modulate vasomotor tone, and improve endothelial cell survival, migration and proliferation (Iatan et al., 2008; Barter, 2005). Nonetheless, the major cardioprotective effect of HDL has been attributed to its key role in the reverse cholesterol transport (RCT), a process in which cholesterol from peripheral tissues is selectively returned to the liver for excretion in the bile (Assmann and Nofer, 2003; Alwaili et al., 2010; Iatan et al., 2008).

1.3.4.1 Antioxidant properties

As described in Section 1.1.2, the accumulation of LDL in the subendothelial matrix, and its subsequent oxidation, initiates the process of atherosclerosis. HDL antioxidative activity typically refers to its ability to inhibit LDL oxidation, through several methionine-containing apolipoproteins (such as apoA-I and apoE) and enzymes including paraoxonases (PON1 and PON3), lecithin-cholesterol acyl transferase (LCAT), lipoprotein-associated phospholipase A2 (Lp-PLA2), PAF-AH and glutathione peroxidise, which prevent the formation of highly reactive oxidized phospholipids in LDL particles (Kontush and Chapman, 2006). HDL particles also carry natural antioxidant molecules, such as vitamin E. Of interest, the apolipoproteins and enzymes possessing anti-oxidative activities are distributed nonuniformly across HDL subfractions, with many predominantly confined to the HDL_3 subfraction (Marcil et al., 2004; Van Lenten et al., 2001).

1.3.4.2 Anti-inflammatory properties

As described in Section 1.1.2, accumulation of macrophages and other immune cells in the arterial intima leads to inflammation in atherosclerosis, which involves signaling pathways and the upregulation of cytokines, mediated by activation of the transcription factor NF-κB (Navab et al., 1991; Sugano et al., 2000). HDL prevent vascular inflammation in part via suppression of NF-κB mediated expression of cell adhesion molecules on endothelial cells and by reducing neutrophil infiltration within the arterial wall (Barter, 2005; Rye et al., 2009). These adhesion molecules are generally presented on the luminal surface of endothelial cells to slow neighboring monocytes. This first occurs via loose E-selecting tethering, and then by tight association of endothelial intercellular adhesion molecular (ICAM-1) and vascular cell adhesion (VCAM-1) with leukocyte integrins. (Barter, 2005; Degoma and Rader, 2011; Rye et al., 2009). In support of HDL's anti-inflammatory properties, it has been recently demonstrated that HDL from healthy individuals, but not HDL obtained from patients with an ACS, prevents the activation of NF-kB and its movement to the cell nucleus in response to exogenous TNFα (Besler et al., 2011). Additionally, HDL also influences macrophage activation via JAK2/STAT3 interaction with apoA-I and ABCA1 (Tang and Oram, 2009). Finally, the role of HDL in removing oxidized lipids as described in 1.3.4.1 also has an effect on inflammation, as oxidized phospholipids possess proinflammatory properties (Furnkranz et al., 2005).

1.3.4.3 Endothelial cell function

It was observed that a correlation exists between an increase in HDL levels and endothelium-dependent vasodilation (Bisoendial et al., 2003). HDL-derived cholesterol has been shown to promote endothelial generation of nitric oxide (NO) via stimulation of the endothelial NO synthase (eNOS) enzyme, and as such improves endothelial function and arterial vasoreactivity (Degoma and Rader, 2011; Kuvin et al., 2002; Mineo et al., 2006).

HDL particles can also promote the proliferation and migration of endothelial cells, as well as their survival, through anti-apoptotic effects, as well

as recruitment of endothelial progenitor cells (EPC) to sites of vascular injury, dependent on NO production (Besler et al., 2011; Mineo et al., 2003; Sorrentino et al., 2010; von and Rohrer, 2009). It is via the scavenger receptor class B type I (SR-BI) that HDL influences the mobilization of endothelial progenitor cells and through Ca^{2+} that it enhances their proliferation (Bisoendial et al., 2003; Pyke and Tschakovsky, 2005). Factors such as oxidized LDL and TNF-α are thought to trigger cell death of endothelial cells, but evidence has shown that HDL deters this effect by reducing intracellular Ca^{2+} levels and caspase-3 expression (Kuvin et al., 2003; Naqvi et al., 1999).

1.3.4.4 Antithrombotic properties

Several pieces of evidence suggest that HDL may exert antithrombotic effects (Degoma et al., 2008). In addition to promoting blood flow through an increase in NO and prostacyclin production, it also attenuates thrombin generation by enhancing the activity of proteins (protein S and protein C) that inactivate coagulation factor Va (Griffin et al., 1999; Mineo et al., 2006). HDL antithrombotic actions may also involve inhibiting the release of platelet activating factor, tissue factor expression, factor X activation and thromboxane A2 synthesis (Degoma and Rader, 2011; Naqvi et al., 1999; Saku et al., 1985).

1.3.4.5 Reverse cholesterol transport

As mentioned above (1.3.4), (**Figure 1.5**), the most recognized and beneficial function of HDL in protecting against atherosclerosis is its ability to promote RCT. This refers to the process by which cholesterol and lipids from peripheral tissues, such as foam cells, are selectively returned to the liver for excretion in the bile via interaction with specific transporters, such as ABCA1, ABCG1 and/or SR-BI (Khera et al., 2011; Rader et al., 2009).

HDL originates as lipid-free or lipid-poor apoA-I, secreted by the liver (~80%, (Timmins et al., 2005)) and the intestine (~20%, (Brunham et al., 2006a)), independently of ABCA1 activity (**Figure 1.5**). It then rapidly interacts with ABCA1 to promote efflux of free cholesterol and phospholipids, forming nascent

HDL particles in hepatic and peripheral cells, including macrophages (Yancey et al., 2003). The expression and activity of the ABCA1 transporter is essential and rate limiting for nascent HDL generation (Timmins et al., 2005), as it was demonstrated by our group and others, with the identification of patients with mutations at the ABCA1 locus (Tangier disease (TD)) as having impaired HDL formation (Brooks-Wilson et al., 1999; Rust et al., 1999; Bodzioch et al., 1999; Lawn et al., 1999).Once in the circulation, nascent HDL continues to remove cellular cholesterol, the free cholesterol (FC) in the discoidal HDL is then esterified via the HDL-associated enzyme LCAT (**Figure 1.5, Figure 1.3**). The CE formed this way move to a more thermodynamically stable position in the core of the particle, assuming a spherical configuration (HDL_3) (Genest and Libby, 2011). With further cholesterol esterification, the particle increases in size to become the more buoyant, mature HDL_2. These large HDL_2 particles can efflux free cholesterol from peripheral cells via another ABC transporter, ABCG1, and by passive diffusion (Kennedy et al., 2005). Once esterified, some of the CE produced in HDL by LCAT can be transferred to apoB-containing particles (e.g. IDL, VLDL) by CETP, in exchange for other apoB-associated neutral lipids, including TG (Barter et al., 2003) (**Figure 1.5, Figure 1.3**). It is noteworthy that the modified HDL particles and the generation of a neutral lipid core converts the discoidal preβ-migrating particles to spherical α-migrating particles, a process known as HDL maturation. The TG-enriched HDL resident pool, denoted as HDL_{2b}, can be further remodeled by hepatic lipase (HL), endothelial lipase (EL) and lipoprotein lipase (LPL) (Clay et al., 1991; Olivecrona and Olivecrona, 2010). Specifically, HL hydrolyzes both phospholipids and TG associated with HDL, while EL is a phospholipase responsible for hydrolyzing HDL-associated phospholipids, converting them back to HDL_3 particles. LPL is a lipolytic enzyme known to hydrolize chylomicrons and VLDL-associated TG. The CE transferred onto Apo B-containing lipoproteins will be taken up by the liver by the superfamily of LDL-receptor (LDL-R) and LDL-R-related protein (Alwaili et al., 2010), while cholesterol from HDL is selectively taken up by the liver via the scavenger receptor BI (SR-BI) and subsequently secreted into the bile (**Figure 1.5**, **Figure 1.3**).

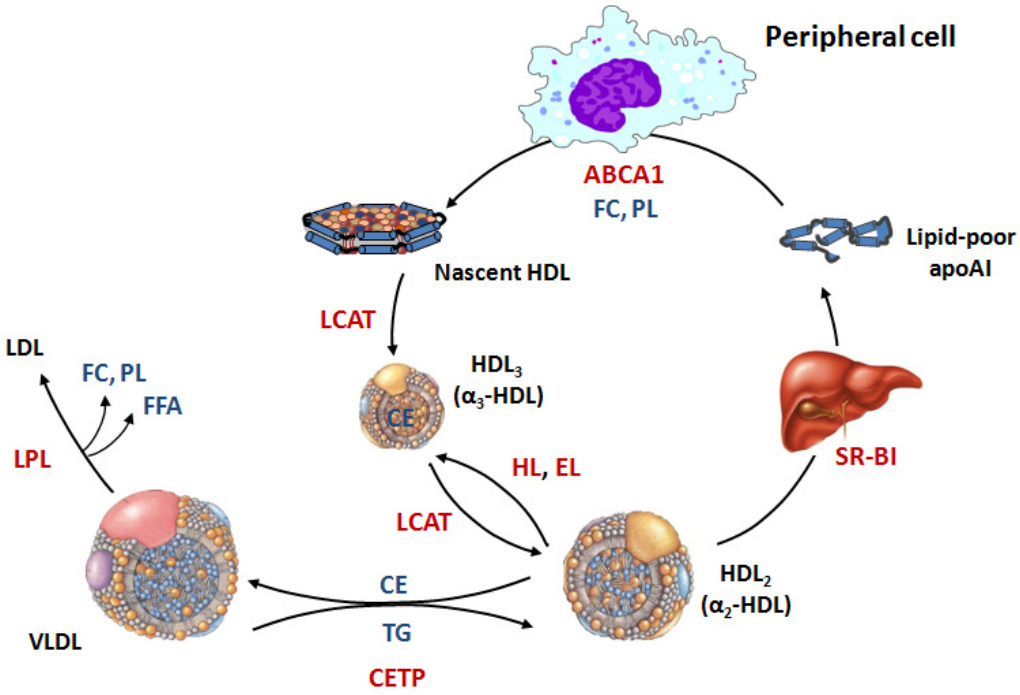

Figure 1.5. Reverse cholesterol transport pathway.
During RCT, apolipoprotein A1 (apoA-I), the main protein on HDL, is synthesized in hepatocytes and released from the liver as lipid-poor ApoA-I. It then binds to the ATP-binding cassette transporter A1 (ABCA1) located on the surface of peripheral cells, such as macrophages, and promotes efflux of free cholesterol (FC) and phospholipids (PL), resulting in the formation of nascent HDL particles. Once in circulation, these HDL particles continue to remove cellular cholesterol and are modified by the lecithin–cholesterol acyltransferase (LCAT), which esterifies FC. The hydrophobic cholesteryl esters (CE) move to the core of the lipoprotein and the HDL particle becomes a large α-HDL particle (HDL_3). With further cholesterol esterification, and exchange for triglycerides (TG) by the cholesteryl ester transfer protein (CETP), the HDL particle increases in size to become the more buoyant HDL_{2a}. Finally, the HDL-C can be taken up by the liver and cleared from the blood stream by the scavenger receptor (SR-BI) expressed in hepatocytes. The TG-rich very-low-density lipoproteins (VLDL) can be hydrolyzed by lipoprotein lipase (LPL) to become intermediate-density lipoproteins (IDL), and with further remodeling, low-density lipoproteins (LDL). During hydrolysis of TG-rich lipoproteins, redundant phospholipids can be shed.

1.3.5 Mechanisms of ABCA1-mediated lipid efflux

1.3.5.1 ATP-binding cassette transporter A1

ABCA1 belongs to the ABC transporter family consisting of 48 members in humans (Oram and Heinecke, 2005) and are characterized by the presence of three conserved Walker motifs (A, B and C) in their nucleotide binding domains (Walker et al., 1982). ABCA1 is a 250 kDA transmembrane protein, consisting of two similar halves (Oram and Heinecke, 2005) with each one of them having an ATP-binding domain, six transmembrane domains and significant intracellular and extracellular loops. Each half of the protein is associated with a large extracellular loop that is highly glycosylated (Singaraja et al., 2006).

As described in Section 1.3.4.5, the main function of ABCA1 is to efflux lipids from the cells to lipid-poor apolipoproteins, the main one being apoA-I. This is regulated by the cholesterol content of the cell, as well as by ABCA1 activity. It is the cholesterol efflux component of the lipid transport ability of ABCA1 that specifically contributes to HDL biogenesis. Phospholipids are the other major lipids transported by ABCA1 and are an essential co-factor for ABCA1-mediated cholesterol transport. The addition of phospholipids to Apo-I increases the cholesterol acceptor ability of apoA-I and promotes cholesterol efflux (Fielding et al., 2000).

In order to be functional, ABCA1 requires higher order oligomerization (Trompier et al., 2006; Denis et al., 2008). Studies from our laboratory have previously documented that human ABCA1 exists as a homotetramer and have proposed that a homotetrameric complex of ABCA1 constitutes the minimal functional unit required for apoA-I lipidation (Denis et al., 2004a) in agreement with the work by Chimini and colleagues (Trompier et al., 2006). This is also consistent with findings from our group in that apoA-I binds to tetrameric and dimeric, but not monomeric, forms of ABCA1 (Denis et al., 2004a).

It is thought that 90% of cellular cholesterol is located at the plasma membrane (PM) (Lange, 1991), thus representing a significant source available for ApoA-I. Evidence from intracellularly retained ABCA1 mutants (Singaraja et al., 2009) suggests that PM is the main site for interaction and lipidation of ApoA-I by ABCA1. Therefore, ABCA1 is predominantly located at the PM and undergoes shuttling to and from endo-lysosomal compartments (Neufeld et al., 2004). There are several regions and residues within ABCA1 that ensure correct trafficking and localization of ABCA1: the N-terminal signal anchor sequence which translocates ABCA1 so that the first hydrophilic domain is in the exoplasmic space (Fitzgerald et al., 2001) cysteines at residue positions 3, 23, 1110 and 1111 that require palmitoylation for ER exit (Singaraja et al., 2009), and the N-terminal xLxxKN Golgi exit motif that is common to ABC transporters (Beers et al., 2011).

Insights into the molecular physiology of ABCA1-mediated cellular cholesterol efflux have shown that ABCA1 is predominantly regulated by oxysterols at the transcriptional level via the liver specific receptor (LxR) pathway (Oram and Heinecke, 2005) and cyclic adenosine monophosphate (cAMP) in various cell lines (Haidar et al., 2002). In fact, the sensitivity of ABCA1 to lipids, both synthetic and natural, is largely attributable to the action of the nuclear LxR (α and β) receptors and retinoid X receptor (RxR), which play a central role in the transcriptional control of global lipid homeostatis (Costet et al., 2000; Linsel-Nitschke and Tall, 2005). Both LxR α and β isoforms associate with RxR to form heterodimers that can be activated by oxysterol and retinoid ligands. The binding of activated LxR/RxR heterodimers to the DR4 elements in the ABCA1 promoter simulates transcription (Costet et al., 2000).

Cell type speciation for HDL biogenesis has also been examined carefully (Krimbou et al., 2005). Hepatic-derived and intestinal cell lines express ABCA1 and lipidated apoA-I, providing support to the aforementioned concept that liver and intestine are the major sites producing nascent HDL particles. In addition, only hepatic cells lines produce pre-β particles, but their role is still poorly understood. Other cell types, such as fibroblasts, express ABCA1 and produce HDL as well, providing a useful model for *in vitro* studies. Interestingly, human

vascular endothelial cells, such as human umbilical vascular endothelial cells, do not express ABCA1 in significant amount and do not contribute to nascent HDL biogenesis despite the large aggregate mass constituted by endothelial cells in the body.

1.3.5.2 Tangier disease and familial hypoalphalipoproteinemia

Parts of this Section 1.3.5.2 were obtained with permission from *Current Atherosclerosis Reports* from (Iatan et al., 2008), *Effect of ABCA1 Mutations on Risk for Myocardial Infarction* and were subsequently modified for this thesis.

Tangier disease (TD) was first recognized by Frederickson et al. (Fredrickson DS et al., 1961) in 1961, but it was not until 1999 that the *ABCA1* gene associated to the disease was discovered by our groups and others (Brooks-Wilson et al., 1999; Rust et al., 1999; Bodzioch et al., 1999; Lawn et al., 1999).

A mutation at the *ABCA1* locus was identified as the cause of TD, a rare autosomal recessive disorder characterized by HDL deficiency and cholesterol deposition in reticulo-endothelial tissues. An abnormality of cellular cholesterol efflux as the major defect in TD suggested that the molecular cellular physiology of cholesterol transport was disturbed. For this reason, the interaction of ABCA1 with apoA-I is considered as the rate-limiting step for cellular cholesterol and phospholipid efflux, and is critical for nascent HDL formation and, ultimately for RCT, as previously described.

Patients with TD, described as being homozygous or compound heterozygous at the *ABCA1* gene, demonstrate hepatosplenomegaly, markedly enlarged yellow tonsils and lymph nodes, corneal clouding and a demyelinating neuropathy (Miller and Zhan, 2004). The major biochemical abnormality is an absence of HDL-C, mild to moderate increased triglyceride levels and a marked decrease in LDL-C. Pathological examination of affected tissues predominantly shows accumulation of neutral lipids within macrophages. The association of TD with premature atherosclerosis has been the subject of considerable debate (Assmann G et al., 2001). When compared to homozygous familial hypercholesterolemia, due to defects at the LDL receptor gene and CAD

encountered in the first two decades of life, the association between TD and premature CAD has been more tenuous. The low serum level of LDL-C in TD may in fact be protective.

In contrast, individuals carrying a heterozygous form of TD, identified as familial HDL deficiency (FHD) and correspondingly ~45% decrease in HDL-C serum levels, have been shown to have a three-fold greater CAD risk when compared to unaffected controls (Clee et al., 2000). The HDL-C level in these patients is markedly reduced ($<5^{th}$ percentile for age- and gender-matched patients). The prevalence of ABCA1 mutations in subjects with HDL deficiency is estimated at approximately 10-20% (Candini et al., 2010; Alrasadi et al., 2006). To date, over 100 ABCA1 mutations have been identified, associated with TD or FHD (Brunham et al., 2006b). The examination of cohorts of patients with HDL deficiency has thus shown that mutations in the *ABCA1* gene are a relatively frequent cause of severe HDL deficiency.

In this thesis, fibroblasts from a TD ABCA1 mutant (Q597R) have been utilized. In the heterozygous form, this mutant reduces apoA-I levels to 0.82±0.10 and HDL-C levels to 60±5 relative to controls (Brunham et al., 2006b). This Q597R mutation occurs in the first extracellular domain of ABCA1 and although it does not affect plasma membrane localization of ABCA1, it does result in an absence of cholesterol efflux and in a significant reduction in phospholipid efflux and apoA-I binding (Vaughan et al., 2009).

1.3.5.3 Lipidation of apoA-I by ABCA1 at the plasma membrane

The interaction between apoA-I and ABCA1 is essential and rate-limiting for the generation of nascent HDL particles, and for the initial step in the RCT. However, the exact molecular mechanisms by which ABCA1 acts to transport lipid across the membrane and mediates apoA-I lipidation is not well-understood.

There have been different models proposed for this interaction (Krimbou et al., 2006; Rothblat et al., 1992; Yancey et al., 2003). In the 'plasma membrane' model, based on similarities between ABC transporters (P-

glycoprotein, sav1866 (Dawson and Locher, 2006; Rosenberg et al., 2003) it was proposed that ABCA1 actively transports lipids to the cell surface where they become accessible to docking apolipoproteins (Chambenoit et al., 2001; LeGoff W. et al., 2004; Lin and Oram, 2000; Vaughan and Oram, 2003; Vedhachalam et al., 2007a; Witting et al., 2003). Earlier studies by Oram et al. support this desorption model, where it was shown that ABCA1 transports phospholipids and cholesterol to generate cell-surface domains that are microsolubilized by apolipoproteins (Vaughan and Oram, 2003), and that more than 80% of apoA-I is bound to protein-deficient plasma membrane protrusions, or exovesiculated lipid domains, in cholesterol-loaded fibroblasts (Lin and Oram, 2000; Vedhachalam et al., 2007a).

This model however was met with controversy, as Fielding and colleagues described instead a two-step lipidation model (Fielding et al., 2000). The latter was compatible with ABCA1 acting as a phospholipid 'flippase', in a mechanism by which apoA-I and ABCA1 interact, phospholipid transfer occurs and subsequent membrane solubilisation transfers cholesterol to apoA-I (Fielding et al., 2000; Gillotte et al., 1998; Gillotte et al., 1999; Massey and Pownall, 2008). This two-step model thus argues that phospholipid efflux precedes cholesterol efflux. This is supported by evidence that efflux of phospholipids can be uncoupled from cholesterol efflux (Yamauchi et al., 2002; Wang et al., 2001). Central to this model is that the rearrangement of lipid domains on the plasma membrane by ABCA1 is independent of apoA-I interaction (Zarubica et al., 2009; Vaughan and Oram, 2003), but dependent on the nucleotide binding activity of ABCAI (Landry et al., 2006).

Furthermore, in light of earlier studies implying that lipid transfer to apoA-I involves direct binding to ABCA1 (Chroni et al., 2004; Wang et al., 2001) , it was also proposed that apoA-I interacts with ABCA1 and this complex is internalized for lipid loading to occur (LeGoff W. et al., 2004; Neufeld et al., 2004). In this retroendocytosis model, there is evidence that ABCA1 acts as a receptor for apolipoproteins, which are subsequently endocytosed and targeted to late endosomal or lysosomal compartments where they acquire lipids and are exocytosed (Chen et al., 2005; Neufeld et al., 2001; Neufeld et al., 2004;

Takahashi and Smith, 1999). In agreement with these findings, where apoA-I specifically mediates the continuous endocytic recycling of ABCA1, our group has identified that lipidation of apoA-I occurs in two kinetically distinguishable compartments, the PM and intracellular compartments (ICCs) (Hassan et al., 2008) and that apoA-I associated with ICCs is rapidly re-secreted. These distinct dynamics of intracellular and plasma membrane (PM) apoA-I lipidation thus suggest that the two processes co-exist (Hassan et al., 2008) and play a central role in the biogenesis of nascent HDL.

While several models of ABCA1-mediated lipid efflux have been proposed, one of the best-accepted is the model where ABCA1 acts both as a receptor and transporter in the generation of nascent HDL particles. In this two-step model, it was proposed that ABCA1, through its phospholipid translocase activity, flips phospholipids to the outer leaflet of the PM. Subsequently, apoA-I binds to these translocated phospholipids, acquires cholesterol, and solubilises the membrane (Chroni et al., 2007; Krimbou et al., 2006; Rothblat et al., 1992; Yancey et al., 2003). An earlier study from our laboratory suggested that interaction of apoA-I with ABCA1 is required for subsequent association of apoA-I with the PM and extraction of lipid (Krimbou et al., 2006). This concept is strongly supported by recent findings from our group (Hassan et al., 2007), as well as those from Phillips and colleagues (Vedhachalam et al., 2007a; Vedhachalam et al., 2007b) which have identified a second PM apoA-I binding site that works in tandem with ABCA1 to recruit and lipidate apoA-I. Using chemical crosslinking and phospholipase treatment studies, we discovered that this site does not consist of an apoA-I binding protein, as apoA-I fails to crosslink to any additional proteins other than ABCA1, but instead of a phosphatidylcholine (PC)-rich phospholipid microdomain (Hassan et al., 2007; Vedhachalam et al., 2007b). Hence, evidence supporting that the major component of this site is PC was obtained when we treated ABCA1-expressing cells with PC specific phospholipase C (PC-PLC) which significantly reduced apoA-I binding, apoA-I mediated cholesterol efflux, and nascent HDL production. ApoA-I exhibits saturable binding to both ABCA1 and this microdomain, with a B_{max} of 0.04±0.01 and 0.36±0.03 ng/μg and a K_d of 1.80±0.10 and 3.50±0.20 μg/mL, respectively

(Hassan et al., 2007). In light of this observation that the ABCA1 dependent PC-rich PM site has a low affinity and near 10-fold higher capacity to bind apoA-I (approximately 90% of cellular apoA-I) as compared to ABCA1, we characterized it as the 'high-capacity binding site (HCBS) (Hassan et al., 2007). Chapter 5 serves to further investigate the characteristics of the HCBS and the involvement of the apoA-I/ABCA1/HCBS system in nascent HDL formation.

1.4 Lessons learned from clinical trials

Multiple epidemiologic, genetic, and interventional trials have validated the central tenet of the 'lipid hypothesis', which proposes a causal relationship between dyslipidemia and atherogenesis and identifies lipid modification as a risk-reducing strategy for CAD (Genest and Libby, 2011). Confirming this theory, multiple clinical studies have shown that lowering serum levels of total cholesterol and LDL-C is associated with a proportional decrease in the risk of CAD events and strokes (Baigent et al., 2005; Delahoy et al., 2009; Genest and Libby, 2011). The 'HDL hypothesis' however, has not yet received the same level of scientific support, as there still lacks unequivocal evidence of a beneficial effect of HDL-raising treatments on cardiovascular end-points (Awan et al., 2008; Alrasadi et al., 2008; Greenland et al., 2010; Vergeer et al., 2010). This was demonstrated in a meta-analysis of 95 clinical trials involving 300 000 individuals, the results of which suggested that on-trial HDL-C concentrations were not significantly related to CAD events (Briel et al., 2009). Recent trials using statins also showed that by aggressively lowering LDL-C (<2.0 mmol/L or 80 mg/dL)), HDL-C levels no longer predict residual cardiovascular risk (Ridker et al., 2010; Genest and Libby, 2011). These findings, combined with Mendelian randomization data on genetic HDL deficiency states, stirs controversy on the validity of HDL-C as a therapeutic target (Frikke-Schmidt et al., 2008b). Mendelian randomization principles assume that the existence of a causal relationship between HDL-C and CAD would imply that association between a gene variant and HDL-C levels will translate into the CAD risk expected from the effect on HDL-C. Using this approach however, recent genetic studies by (Frikke-Schmidt et al., 2008b; Voight et al., 2012) casts doubt on a direct protective effect

of HDL-C against atherosclerotic risk, as gene variants affecting HDL-C levels do not necessarily correlate with a corresponding effect on heart disease. For example, subjects with genetic forms of HDL deficiency, such as apoA-I Milano, or some mutations at ABCA1(Frikke-Schmidt et al., 2008b) and LCAT, are not necessarily associated with premature CAD, whereas mutations in the HL or the CETP genes – leading to high HDL-C – may not be protective (Weissglas-Volkov and Pajukanta, 2010). These observations indicate that the relationship between CAD and HDL-C is more complex than originally suggested and further investigations are needed. Nevertheless, aggregate evidence from clinical trials does however suggest that raising HDL by pharmacological means is associated with a beneficial effect on atherosclerosis (Barter et al., 2007a; Brown et al., 2006; DiAngelantonio et al., 2009; Grover et al., 2009; Jafri et al., 2010; Nissen et al., 2003; Singh et al., 2007; Tardif et al., 2007).

Various strategies have been employed in an attempt to raise HDL-C levels, with varying results. Statins are inhibitors of the hydroxyl-methyl-glutaryl coenzyme-A reductase (HMG-CoA reductase), the rate-limiting enzyme in sterol synthesis (Genest and Libby, 2011). They are known to indirectly cause a decrease in hepatic VLDL and LDL production, with a modest increase in HDL-C levels (Barter et al., 2007b; Genest and Libby, 2011). There have been more than 28 large-scale secondary or high-risk prevention trials investigating the effect of statins on major cardiovascular events and mortality (Genest and Libby, 2011) revealing only a marginal increase in HDL-C (between 0 to 10%).

Another well-known pharmacological means of raising HDL-C is the use of niacin (vitamin B3), observed to increase HDL-C levels as early as of 1955 (Altshuler et al., 2010). Additionally, niacin lowers LDL-C by 15%-25% and decreases TG serum concentrations (Genest and Libby, 2011). Although it was found to increase HDL-C by 10-30% (Chapman et al., 2010; Singh et al., 2007) and several clinical trials have suggested its potent anti-atherosclerotic effects (Singh et al., 2007; Brown et al., 2006), niancin's side-effects, associated with severe skin flushing and hepatotoxicity, hamper its widespread use. Furthermore, studies using a combination of statin and niacin therapy were initially promising (Kashyap et al., 2002), but more recent trials, such as the large AIM-HIGH

(NCT00880178) study, was terminated in 2011 due to a lack of effect on outcomes etween treatment groups (Boden et al., 2011). Other trials such as HPS2-THRIVE (NCT00461630) will soon provide (2013) important insight into its clinical effectiveness.

Fibrates, another class of compounds, are PPARα agonists, and while exerting a primary effect by reducing TG via lipoprotein lipase upregulation, they also increase HDL-C levels through induction of hepatic apoA-I and apoA-II production, and by reducing the CETP-dependent transfer of cholesterol from HDL to VLDL. Clinical trials, such as the ACCORD (NCT00000620) study revealed that fibrates have a modest effect on HDL levels, but have an effect on total mortality (Ginsberg et al., 2010).

Recent pharamaceutical developments have involved targeting CETP, responsible for the exchange between VLDL/LDL and HDL. Torcetrapib was the first CETP inhibitor tested in humans. Although preliminary trials showed that it increased HDL-C by 60-100% (Brousseau et al., 2004), a phase III clinical trial of this compound, the ILLUMINATE trial (NCT00134264), was suddenly halted because of an increase in mortality and morbidity in subjects, despite an improved plasma lipid profile (72% increase in HDL-C levels and 25% decrease in LDL-C levels) (Barter et al., 2007b). The failure of torcetrapib prompted development of a second-generation of CETP inhibitors, with a focus on reduction of adverse off-target effects seen with torcetrapib in raising blood pressure and stimulating aldosterone synthesis (Barter, 2009). These include dalcetrapib and anacetrapib (Joy and Hegele, 2009), which are currently undergoing clinical trials. Though dalcetrapib only raises HDL-C by only 25%, it has been deemed safe and its clinical trial (DAL-Outcomes, NCT00658515) in 16,000 ACS patients will hopefully shed more light onto whether raising HDL-C by inhibiting CETP reduces cardiovascular events (Stein et al., 2010; Schwartz et al., 2009). Comparatively, anacetrapib has been shown to increase HDL-C levels by 138% over 76 weeks, without adversely affecting blood pressure (Krishna et al., 2007; Cannon et al., 2010). The REVEAL large-scale trial (NCT01252953) of 30,000 individuals is currently ongoing to assess the effects of anacetrapib in the secondary prevention of cardiovascular disease. Importantly, both dalcetrapib and

anacetrapib have been demonstrated to increase HDL-C and reduce LDL-C levels, while neither shares the off-target effects of torcetrapib.

From pharmacological standpoints however, direct augmentation of apoA-I serum concentration represents the most validated HDL-related therapeutic approach in terms of antiatherosclerotic potential. As described in (Degoma and Rader, 2011), at least four therapeutic approaches are currently being developed to increase plasma apoA-I levels. These include intravenous apoA-I therapies involving recombinant apoA-I_{Milano}/phospholipid complexes, purified native apoA-I/phospholipids complexes, and autologous delipidated HDL, as well as oral upregulators of endogenous apoA-I production, such as RVX-208. While intravenous infusion of ApoA-I has been shown to increase both HDL-C plasma concentrations and reduce atheroma plaque size (Nissen et al., 2003; Ross, 1993), the logistics of infusion therapies do not make them as attractive. In comparison, RVX-208, a compound from the quinazoline family, has been shown to increase apoA-I and HDL-C *in vitro* and *in vivo* (60% and 97% increase respectively), leading to the formation of nascent HDL particles, with an increase in pre-β-LpA-I, α-LpA-I levels, as well as enhanced cholesterol efflux (Bailey et al., 2010a). The *in vivo* functionality of HDL particles produced through the action of RVX-208 compound and the effect on CAD risk has yet to be defined, as current studies on RVX-208 (NCT01067820) are aiming to characterize its effects on apoA-I synthesis when administered to patients with ACS.

Collectively, these studies have attempted to exploit the fact that HDL-C has been suggested as a sound therapeutic target (Rader, 2006; Duffy and Rader, 2009; Singh et al., 2007; Degoma and Rader, 2011). As there have been several challenges along the way, additional investigation is required to identify novel regulators of HDL, in order to expand the roster of therapeutic candidates for improving treatment for cardiovascular disease.

1.5 Genetics of HDL-C

1.5.1 Heritability of HDL

While environmental and pharmacological agents can modify HDL-C levels, genetic factors are the most influential determinants of plasma HDL-C concentrations. Based on family and twin studies (Peacock et al., 2001), HDL-C has a strong inherited basis, with heritability estimates between 40-80%, shown across multiple populations (Bielinski et al., 2006; Lusis et al., 2004; Peacock et al., 2001; Qasim and Rader, 2006). In the French Canadian cohorts used by our laboratory, the overall heritability of the low HDL-C trait is 0.58 (n=699 subjects) (Dastani et al., 2006b), highlighting the need to better explore the genetics of HDL metabolism.

1.5.2 Approaches for identifying genes for HDL-C

For the past two decades, several studies have attempted to reveal the genetics behind low or high HDL-C, by using different approaches: families, cases and controls or unascertained general population samples. Nevertheless, the genetic inheritance of HDL-C is complex, and similarly to other complex traits, genetic determinants of HDL-C concentrations can be either monogenic, environmental, or in most cases result from interactions between polygenic and environmental factors (ie multifactorial) (Weissglas-Volkov and Pajukanta, 2010).

A systematic traditional strategy that can be used to identify novel genetic determinants includes first determining genetic heritability by performing families or twins studies. Then, using segregation analyses demonstrate the mode of inheritance and find the type and frequency of the susceptibility alleles. Third, one can perform linkage analysis to identify susceptible loci, and subsequently narrow down the candidate region to find the specific gene locus. Finally, identify the variation in the candidate gene and characterizing its effect on the trait in question, as well as its biochemical function.

With the advent of new technological advances however, several approaches can now be used to find disease susceptibility genes. The next section will summarize some traditionally used strategies to identify genetic factors

influencing HDL-C, such as familial segregation studies, candidate genes approaches, genome-wide searches by linkage analyses or association studies, as well as some novel approaches such as genome wide associations studies (GWAS), next generation resequencing and exome sequencing.

1.5.2.1 Segregation studies

Segregation analysis is a general method to evaluate the transmission of a trait within pedigrees. It is used to assess the mode of inheritance and can provide evidence for a major gene effect in a population. Different modes of inheritance have been observed in different segregation studies due to the complexity of HDL-C metabolism, the combination of multiple genes and environmental factors, as well as population differences (Friedlander and Kark, 1987; Kronenberg et al., 2002; Feitosa et al., 2007; Cupples and Myers, 1993; Mahaney et al., 1995). Familial segregation analysis is used throughout this thesis by building allele-specific haplotypes at a gene locus of interest. A haplotype is defined as a genomic locus identified by genetic markers (microsatellites or single nucleotide polymorephisms (SNPs)) determined to characterize alleles in individuals from the same family, in order to examine the transmission of this locus and its potential association with a specific phenotype (e.g. low HDL-C).

1.5.2.2 Candidate gene approach

Candidate gene studies are hypothesis-based analyses. Genes selected for study are based on either their location in a region of linkage or on other evidence, based on *a priori* knowledge of their biological function or a possible role in the etiology of the disease. These genes can be subsequently targeted for sequencing or associations analyses in cases and controls to identify causal variants enriched or depleted between the two groups (Weissglas-Volkov and Pajukanta, 2010). The approach is also used to follow-up genome-wide efforts to discover genomic regions that were previously found to associate with the trait under study. This type of approach was used in Chapter 2 and 4 of this thesis.

Using a candidate gene approach, variants in several genes have been associated with altered plasma HDL-C levels. As such, several monogenic

disorders have been described, with very low (often below 10 mg/dL or usually <5th percentile age-sex matched (HDL deficiency)) or high (>90th percentile age-sex matched) HDL-C levels (**Figure 1.6**). Please refer to **Table 1.3** for detailed characteristics of identified monogenic defects of low and high HDL-C levels. Mutations in the *CETP* gene are associated with increased HDL-C concentrations, whereas mutations in *apoA-I* or *LCAT* cause a low HDL-C (Inazu et al., 1994) (Kondo et al., 1989; Sorci-Thomas and Thomas, 2002; Kuivenhoven et al., 1997; Kuivenhoven et al., 1998). Of at least 47 mutations affecting the structure of apoA-I, not all are associated with CAD. Mutations in *LPL*, *HL* and several other genes (Boes et al., 2009) also affect HDL-C levels. The identification of ABCA1 as the cause of TD and familial HDL deficiency (Brooks-Wilson et al., 1999; Marcil et al., 1999a; Rust et al., 1999; Bodzioch et al., 1999; Remaley et al., 1999; Lawn et al., 1999) has led to a better understanding of the role of cellular cholesterol and phospholipid transport in the metabolism of nascent HDL particles. Based on a selected group of subjects in our laboratory, we have identified that approximately 20% of French Canadians with severe HDL deficiency have mutations at the *ABCA1* gene (Alrasadi et al., 2006), prevalence which was higher than that observed by the group of Cohen *et al*. in a cohort of subjects from Ottawa and Texas (Cohen et al., 2004) as well as from a German cohort (Frikke-Schmidt et al., 2004). Some of these variations at the *ABCA1* locus will be further addressed in Section 1.7. Studies from our laboratory have also reported that the *SMPD-1* gene (defective gene in Niemann-Pick disease, types A and B (Schuchman and Miranda, 1997) is associated with a low HDL-C (Lee et al., 2003). However, genetic variability at the *SMPD-1* gene locus does not appear to contribute to HDL-C levels (Dastani et al., 2007). In addition to the genes described here, there have been several others that are involved in HDL metabolism, such as *SR-B1*, *PLTP*, *EL*, *Apo CIII*, *Apo AIV*, *PON1*, and *ApoE*, (Boes et al., 2009; Mank-Seymour et al., 2004; Nishida and Nishida, 1997). Of note, besides modulating HDL levels, several of these genes have been shown to affect cellular cholesterol efflux with important biological consequences. These will be further reviewed in Section 1.6.

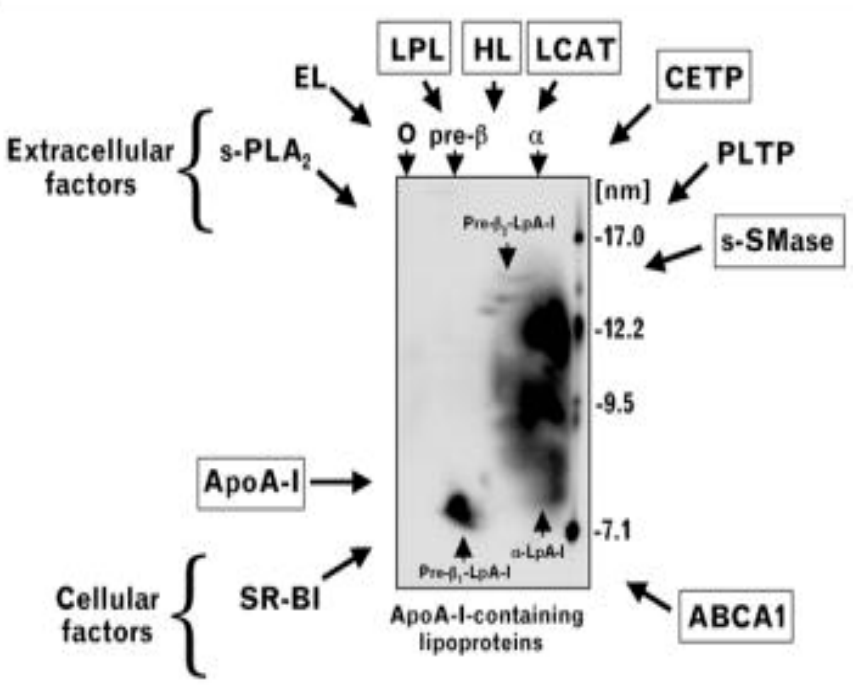

Figure 1.6. Gene defects identified in man

Separation by 2D-PAGGE of plasma apolipoprotein A-I-containing lipoproteins within the high-density lipoprotein density range. The markers on the right indicate the size of the particles. Gene products in boxes have been shown to cause monogenic HDL disorders in humans. Proteins (transporters, receptors, enzyme, transfer proteins, lipases) that modulate HDL in humans are depicted. ABCA1, ATP-binding cassette transporter A1; apoA-I, apolipoprotein A-I; SR-BI, scavenger receptor class BI; s-PLA secretory phospholipase A; EL, endothelial lipase; LPL, lipoprotein lipase; HL, hepatic lipase; LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; PLTP, phospholipid transfer protein; s-SMase, secretory sphingomyelinase; LpA-I, apoA-I-containing particles. Image fom (Dastani et al., 2006c) .

Table 1.3 Selected monogenic defects of low and high HDL-C levels

Gene	Disorder	HDL Phenotype	Lipoprotein Phenotype	Clinical Findings	Relation to CAD
APOA1 (11q23-q24)	ApoAI deficiency [MIM 604091]	HDL-C < 5 mg/dl No detectable apoAI HDL in plasma	Complete absent of apoAI, normal LDL-C, and TG levels	Xanthomas or mild-moderate corneal opacification	Increase risk and premature CAD
ABCA1 (9q31.1)	Tangier's disease [MIM 205400]	HDL-C < 5 mg/dl Only discodial preβ-1 HDL present in plasma	Extremely low levels of apoAI, reduced LDL-C and mildly elevated levels of TG	Enlarged orange tonsils, hepatosplenomegaly, peripheral neuropathy, and thrombocytopenia	Increase risk and premature CAD
LCAT (16q22.1)	LCAT deficiency [MIM 245900]	HDL-C < 5 mg/dl Only discodial preβ-1 and α-4 HDL present in plasma	Reduced levels of apoAI, and LDL-C and high TG levels Abnormal apoB-containing lipoproteins, Increased proportion of unesterified cholesterol 80–100% (normal <30%)	Striking corneal opacification, anemia, proteinuria, renal insufficiency, and organomegaly	Premature CAD has been reported in some patients, but generally uncommon
	Fish-eye disease (partial LCAT deficiency) [MIM 136120]	HDL-C < 10 mg/dl Only discodial preβ-1 and α-4 HDL present in plasma	Reduced levels of apoAI, and LDL-C and high TG levels Slightly elevated proportion of unesterified cholesterol (up to 70%)	Striking corneal opacification	Premature CAD is generally uncommon
LPL (8p21.3)	Type I hyperlipoproteinemia (familial chylomicronemia) [MIM 238600]	HDL-C < 20 mg/dl	Severe Hypertriglyceridemia, chylomicronemia, and low levels of LDL-C	Pancreatitis, xanthomas, abdominal pain, and hepatosplenomegaly	Premature CAD is generally uncommon
CETP (16q21)	CETP deficiency [MIM 607322]	HDL-C >100 mg/dl Elevation of HDL_2 particles and particles are enriched with cholesterol ester and apoE and poorer in TG	Elevated levels of apoAI, normal to decreased LDL-C and apoB	Unknown	Unclear
LIPC (15q21-23)	HL deficiency [MIM 151670]	HDL-C > ~70 mg/dl (variable, few reports available) HDL particles are enlarged (HDL_2 type particles) and rich in TG	Severe hypertriglyceridemia, modest increase in apoAI, variable elevation in LDL-C	Unknown	Some increase CAD risk reported

Table obtained and modified from (Weissglas-Volkov and Pajukanta, 2010)

1.5.2.3 Genome-wide linkage studies

Recently, genome-wide scanning techniques have been applied to the identification of genes that modulate HDL-C as a complex or quantitative trait. In linkage studies, one seeks to identify a trait locus that co-segregates with a specific genomic region, tagged by polymorphic markers and within families (Borecki and Province, 2008). The degree of linkage is measured by the logarithm of odd ratio (LOD) score, with positive LOD scores to give evidence of linkage (Lander and Schork, 1994). There are several strategies to linkage analysis developed for quantitative and complex traits: parametric and quantitative trait loci (QTL) analyses.

Parametric linkage analyses can be used to identify loci for complex traits such as HDL-C, by dichotomizing the continuous variable based on an arbitrary cut-off point (e.g. 0.9 mmol/L or 35 mg/dL to define hypoalphalipoproteinemia). This however does not take into account age or sex or the presence of concomitant metabolic disorders, and may reduce the power to detect genes (Dastani et al., 2006b). When treating HDL-C as a discrete trait, our laboratory selects patients (probands) with HDL-C <5th percentile for age and gender-matched subjects (Marcil et al., 2003; NIH Washington DC, 1980), and their families, in order to identify genes that modulate HDL-C levels. This selection criterion was routinely used in the projects described in this thesis. By utilizing linkage analysis, our group identified a locus on chromosome 4q31.21 in individuals of French Canadian descent with low HDL-C (Dastani et al., 2006c).

QTL analyses are used to identify chromosomal regions that are associated with variations in HDL-C that can underlie complex human diseases. Generally, QTL analysis is more powerful to identify loci controlling these traits than using dichotomized traits. Using this strategy, previous studies in our laboratory have performed a linkage analysis in two sets of families, a Quebec-wide study (QUE) consisting of 642 subjects from 29 multi-generational families where the proband was selected for an HDL-C <5th percentile (age and gender matched), and 410 individuals from families of the Saguenay–Lac St-Jean region (SLSJ). Significant linkage to a locus on chromosome 16q23-24 was identified

(LOD score 2.61 and 2.96 for QUE and SLSJ cohorts, respectively) (Dastani et al., 2010). We further examined the region by SNP fine mapping and family-based and case-control association analyses and observed that in the QUE cohort, four families demonstrated strong segregation of an 18.1 cM region (7.8 Mb). In addition, this locus has been implicated in previous linkage scans for HDL-C in multiple studies from different populations, making it the only reproducible region reported on chromosome 16 in different independent studies (Amos et al., 1986; Aouizerat et al., 1999; Aulchenko et al., 2009; Gagnon et al., 2003; Ishimori et al., 2004; Kathiresan et al., 2009; Mahaney et al., 2003; Mehrabian et al., 2000; Pajukanta et al., 2003; Sabatti et al., 2009; Shearman et al., 2000; Soro et al., 2002; Thompson et al., 2005; Willer et al., 2008; Yip et al., 2003; Dastani et al., 2010; Kathiresan et al., 2008b). Investigations of a novel gene at this locus are the focus of the study presented in Chapter 4.

1.5.2.4 Association studies

Associations studies seek a correlation between a specific genetic variation and trait variation in a sample of individuals, therefore implicating a causal role for the variant (Borecki and Province, 2008). This approach thus compares the prevalence of genetic markers or SNPs in a large population of affected subjects (e.g. individuals with HDL-C <5th percentile) versus controls. Statistical consideration must take into account the large number of genetic variants being investigated and thus the potential for elevated false positive results.

1.5.2.5 Genome-wide association studies

Parts of this Section 1.5.2.5 were obtained with permission from *Current Atherosclerosis Reports* from (Iatan et al., 2012) *Genetics of Cholesterol Efflux* and were subsequently modified for this thesis.

More recently, the completion of the Human Genome Project and the International Haplotype Map Project (The International HapMap Consortium, 2005; Frazer et al., 2007) have made it possible to perform genome-scale screens

for common DNA sequence variants that are associated with phenotypes of interest, an approach called genome-wide association (GWA) (Musunuru and Kathiresan, 2010a). In contrast with linkage analyses and candidate gene studies, which were successful in identifying rare disease causing variants exclusive to a few individuals, GWAS studies are designed to detect common variants, associated with a complex trait of diseases. GWAS can identify single nucleotide polymorphisms (SNPs) (and other variations) in DNA, but do not necessarily imply causality between identified genes and the biological trait under study.

As stated, GWAS compare the frequency of common DNA variants between people with the trait or the disease (cases) and similar people without (controls). DNA is extracted from various cells in a given population (with some studies surpassing 100 000 individuals total). High-throughput genechips can now access over 2,000,000 SNPs spanning the entire genome. The prevalence of genetic variants at each SNP is then examined between cases and controls and bioinformatics is subsequently used to determine the statistical strength and significance of the observation. It should be emphasized that most of the SNP variations associated with a trait or disease do not always lie in the region of DNA that codes for proteins. They are usually in the large non-coding regions (introns or intergenic regions) and may be in linkage disequilibrium with –yet unknown- genetic variants, which have functional significance.

In contrast to careful analyses of individual SNPs, GWAS are hypothesis-free and, theoretically at least, bias-free. It should be noted however that the sheer number of statistical tests performed presents an unprecedented potential for false-positive results. Despite these caveats, GWAS in the field of HDL genetics has reassuringly confirmed many of the previously documented loci (or genomic regions) that harbor genes causing Mendelian disorders in man, as well as identifying novel loci for HDL-C (Weissglas-Volkov and Pajukanta, 2010). These include, among many others, *GALNT2* (Kathiresan et al., 2009; Kathiresan et al., 2008b), *MVK/MMAB* (Willer et al., 2008), *NR1H3* (Sabatti et al., 2009), *apoB* (Aulchenko et al., 2009), *FAD1-2*, *HNF4A* and *ANGPTL4* (Kathiresan et al., 2009) for HDL-C. To date, 95 separate loci have been identified

to contribute to lipid serum concentrations, either total cholesterol, HDL-C, LDL-C or TG ($P<5x10^{-8}$) (Teslovich et al., 2010). Most of these recent findings on known and novel genes associated with HDL-C, and derived from data from (Teslovich et al., 2010), are presented in **Table 1.4**. These provide strong support for previously established known genes affecting HDL metabolism, while also offering novel avenues. While GWA studies are extremely powerful tools for the study of complex traits, studies of affected families with extremes of biological traits (in our case, HDL deficiency) can offer additional complementary insight on HDL metabolism, as will be shown in Chapter 4.

Table 1.4 Results of GWAS for HDL-C

Locus	Gene	Chrom	Functions and/or biological processes associated with gene of interest
PABPC4	PolyAdenylate-Binding Protein Cytoplasmic 4	1	Platelet activation
ZNF648	Zinc Finger Protein 648	1	Transcription factor
GALNT2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2	1	Involved in O-linked oligosaccharide biosynthesis
IRS1	Insulin Receptor Substrate 1	2	Protein phosphorylated by insulin receptor tyrosine kinase, mutations in genes associated with type 2 diabetes and insulin resistance
COBLL1	COBL-Like protein 1	2	-
SLC39A8	Solute Carrier family 39 (Zinc transporter), MEMBER 8	4	Subfamily of proteins with structural characteristics of zinc transporters
ARL15	ADP-Ribosylation Factor-Like 15	5	-
C6orf106	Chr 6 Open Reading Frame 106	6	-
CITED2	CBP/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain 2	6	Inhibits transactivation of HIF1A-induced genes, mutations in genes are cause of cardiac septal defects
LPA	Lipoprotein (a)	6	Serine proteinase that inhibits tissue-type plasminogen activator I, elevated levels are linked to atherosclerosis
KLF14	Kruppel-like factor 14	7	Kruppel-like family of transcription factors
PPP1R3B	Protein PhosPhatase 1, Regulatory subunit 3B	8	Regulation of glycogen synthase phosphatase activity; may be involved in type 2 diabetes
TRPS1	TrichoRhinoPhalangeal Syndrome I	8	Transcription factor that represses GATA-regulated genes, mutations in this genes are a cause of trichorhino-phalangeal syndrome types I-III
TTC39B	TetraTriCopeptide repeat domain 39B	9	Knockdown in mouse liver increases plasma HDL-C levels
ABCA1	**ATP binding Cassette A1**	**9**	**Cholesterol efflux**
AMPD3	Adenosine Monophosphate Deaminase 3	11	Deamination of AMP to IMP in red cells; purine nucleotide cycle
LRP4	Low density lipoprotein Receptor-related Protein 4	11	Regulator of Wnt signaling
PDE3A	Phosphodiesterase 3A	12	Mediates platelet aggregation and plays a role in cardiovascular function by regulating vascular smooth muscle contraction and relaxation
MVK	**Mevalonate kinase**	**12**	**Isoprenoid and sterol synthesis**
SBNO1 1	Strawberry notch homolog 1	12	-
ZNF664	Zinc finger protein 664	12	Transcription factor
SCARB1	**Scavenger receptor class B, member 1**	**12**	**Cholesterol uptake and efflux**
LIPC	**Hepatic lipase**	**15**	**Triglyceride hydrolase; ligand/bridging factor for receptor-mediated lipoprotein uptake**
LACTB	Lactamase, beta	15	Subunit of mitochondrial ribosome

CETP	Cholesteryl Ester Transfer Protein	16	Transfer of cholesteryl ester from HDL to other lipoproteins
LCAT	Lecithin-cholesterol acyltransferase	16	Extracellular cholesterol esterifying enzyme; mutations in gene cause fish-eye disease and LCAT deficiency
CMIP	c-Maf-inducing protein	16	T-cell signaling pathway
STARD3	**StAR-related lipid transfer (START) domain containing 3**	**17**	**Cellular cholesterol transport**
ABCA8	ATP Binding Cassette A8	17	Member of the ABC1 subfamily (transport various molecules across extra- and intracellular membranes); function of protein has not yet been determined
PGS1	Phosphatidylglycerophosphate synthase 1	17	-
LIPG	Endothelial lipase	18	Phospholipase
MC4R	Melanocortin 4 receptor	18	Interacts with adrenocorticotropic and MSH hormones
ANGPTL4	Angiopoietin-like 4	19	Glucose homeostasis regulator, lipid metabolism, and insulin sensitivity
LOC55908	Hepatocellular carcinoma-associated gene TD26	19	-
LILRA3	Leukocyte Ig-like receptor, subfamily A, member 3	19	Soluble immunoreceptor expressed on monocytes and B cells
HNF4A	Hepatocyte nuclear factor 4, alpha	20	Nuclear transcription factor, regulates expression of several hepatic genes
PLTP	Phospholipid transfer protein	20	Transfers phospholipids from triglyceride-rich lipoproteins to HDL
UBE2L3	Ubiquitin-conjugating enzyme E2L 3	22	Ubiquitination of several proteins
APOB	Apolipoprotein B	2	Major apolipoprotein of chylomicrons, VLDL and LDL
MLXIPL	MLX interacting protein-like	7	HLH leucine zipper transcription factor of the Myc/Max/Mad family
LPL	Lipoprotein Lipase	8	Triglyceride hydrolase; ligand/bridging factor
TRIB1	Tribbles homolog 1	8	-
FADS1-2-3	Fatty acid desaturase	11	Regulate unsaturation of fatty acids
APOA1	**Apolipoprotein AI**	**11**	**Major protein of HDL; involved in cholesterol efflux, LCAT cofactor**
UBASH3B	Ubiquitin associated and SH3 domain containing B	11	Inhibit endocytosis of EGFR and PDGF
LRP1	Low density lipoprotein receptor-related protein 1	12	Multiligand cell surface receptor; involved in intracellular signaling, lipid homeostasis, clearance of apoptotic cells
APOE	Apolipoprotein E	19	Chylomicron remnants and VLDL remnants clearance by the liver

Of the 95 loci identified through GWAS by (Teslovich et al., 2010), reported here are 47 loci associated with HDL-C levels as a primary trait (with exception of the 9 last genes, where HDL-C was a secondary trait in the analysis). Gene data was extracted and modified from (Teslovich et al., 2010) and gene descriptions and functions were determined by consulting the National Center for Biotechnology Information (NCBI, 2011). In bold characters are represented genes related to known efflux defects in man.

1.5.2.6 Novel genetic approaches: next generation resequencing and exome sequencing

The investigation of severe traits of HDL will be facilitated by two other techniques that allow even faster progress in the field of human genetics. While GWAS have the potential of finding common variants (minor allele frequency (MAF) typically >0.05) explaining a relatively small percentage of variation of a biological trait (small-modest size), the focus of the new genomics era has shifted towards the identification of rare (MAF typically <0.01), and highly penetrant variants which have a significant biological relevance (Cirulli and Goldstein, 2010).

1.5.2.6.1 Next generation sequencing

Parts of this Section 1.5.2.6.1 were obtained with permission from *Current Atherosclerosis Reports* from (Iatan et al., 2012) *Genetics of Cholesterol Efflux* and were subsequently modified for this thesis.

This shift has been partly accelerated by the availability of the next generation sequencing (NGS) platforms, which enable identification of uncommon and rare variants through whole exomes and whole genome sequencing, by processing millions of sequence reads in parallel. As such, "deep" resequencing is a relatively novel technology that allows for targeted sequencing of genomic DNA over a large chromosomal region (usually in millions of base pairs). Resequencing provides a comprehensive analysis for candidate genes and regions as both common and rare variants can be identified. As opposed to the Sanger method (Sanger et al., 1977) which was utilised in the Human Genome Project, NGS have increased DNA sequencing output by more than 3 orders of magnitude and have reduced the cost of DNA sequencing by 500000-fold (Marian and Belmont, 2011; Tucker et al., 2009). Identifying sequence variations and understanding their biological significance is therefore becoming a major focus of genetics. For this reason, this strategy among others was used in Chapter 4.

1.5.2.6.2 Exome sequencing

Furthermore, common genetic variants identified so far through GWAS account for only a small proportion of serum HDL-C levels (10-15%) (Teslovich et al., 2010) and the effects on the protein and associated-disease of interest have been functionally validated for only a limited few. Accordingly, this also emphasizes the need to identify novel rare and low frequency variants (MAF<0.05) for HDL-C that impart larger effects. Since the majority of the known disease-causing DNA sequence variants are located within exons, the current focus of human genetic studies has also shifted towards exome sequencing technologies, where every exon (approximately 180,000) is sequenced by several high-throughput technologies Using reference genome sequences, and powerful bioinformatics packages, one can identify genetic variants causing disease in humans (Musunuru et al., 2010).

The recent advent of exome capture has thus emerged as a powerful method to identify genes underlying rare monogenic disorders (Choi et al., 2009b; Kryukov et al., 2007; Ng et al., 2010) as protein coding genes harbor the majority of mutations with large effects on disease-related traits (Ng et al., 2010). At the same time, there is a need to go beyond the molecular identification of Mendelian disorders, by utilizing exome sequencing to investigate causal-variants for multifactorial traits, such as low HDL-C. This will be examined in Chapter 3.

1.6 Candidate genes regulating HDL metabolism and cholesterol efflux

Parts of this Section 1.6 were obtained with permission from *Current Atherosclerosis Reports* from (Iatan et al., 2012) *Genetics of Cholesterol Efflux* and were subsequently modified for this thesis.

As seen in the previous section, the use of different genetic approaches has enabled us to identify novel HDL genes and validate biologically significant ones involved in HDL metabolism. Furthermore, it is also important to consider the effect of some of these genes and their respective products on metabolic processes regulating HDL metabolism, such as the cellular cholesterol efflux machinery.

The following section thus focuses on describing some significant novel and well-known genes from Section 1.5.2.2, identified from family studies, linkage analyses or GWAS, to investigate their role in cellular cholesterol efflux and/or modulation of HDL-C levels.

The cholesterol efflux mechanism described in Section 1.3.4.5, by which cholesterol is removed from lipid laden macrophages in the subintima of the vessel wall by ABCA1 through the lipidation of apoA-I, is a process remarkably conserved throughout evolution. Cellular cholesterol is toxic to cells and may activate at least two important events: cellular accumulation within the endoplasmic reticulum may trigger the unfolded protein response, leading to alarm, defense, or activation of apoptosis signaling pathways (Devries-Seimon et al., 2005); and cholesterol crystals activate the NLRP3 inflammasome by first inducing lysosomal damage in turn leading to the activation of interleukins-1 and -18 (Duewell et al., 2010; Rajamaki et al., 2010). Thus, the effects of HDL-mediated cholesterol efflux on specific cells such as macrophage point towards a role in innate immunity to protect cells against damage. Therefore, understanding gene products that can alter such cellular efflux machinery is of particular biological importance, as it may provide tangible therapeutic targets to prevent atherosclerosis in man. A summary of key process involved in cellular cholesterol efflux are summarized in **Figure 1.7.**

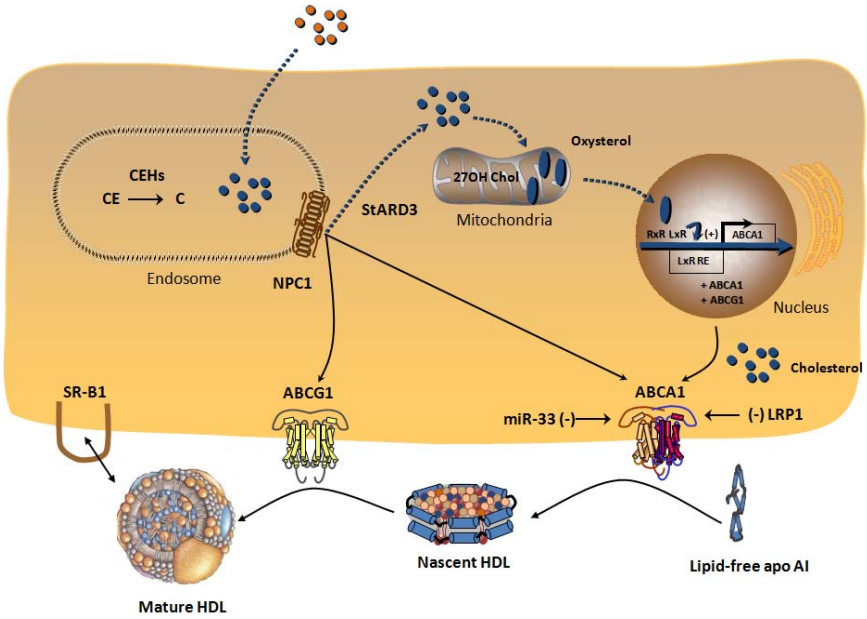

Figure 1.7 Major genes involved in genetic disorders of cellular cholesterol efflux in man

To date, genetic disorders can be associated with defects of cholesterol transport in the cytosol in Niemann-Pick disease type C (NPC), defects in cellular cholesterol efflux to apolipoprotein (apo) AI caused by defective ATP-binding cassette (ABC) transporter AI (Tangier disease), and mutations within apo AI and mutations within SR-BI. ABCG1–ATP-binding cassette transporter G1; C–free cholesterol; CE–cholesteryl esters; CEHs–cholesteryl esterases (LIPE–hormone sensitive lipase; NCEH1–neutral cholesteryl ester hydrolase-1 also called KIAA1363 and other CE hydrolases); HDL–high-density lipoproteins; LRP1–low density lipoprotein receptor-related protein-1; LXR–liver X receptor; miR–micro RNA; RXR–retinoid X receptor; SR-B1–scavenger receptor B1; StARD3–steroidogenic acute regulator protein

Table 1.5 Genes and their proteins associated with cellular cholesterol efflux

Gene Symbol	Protein	GWAS (Table 1.4)	Disease in humans
ABCA1	ATP binding cassette A1	Yes	Tangier disease
ABCG1	ATP binding cassette G1	No	-
Apo AI	Apolipoprotein AI	Yes	Apo AI deficiency
LIPE	Hormone sensitive lipase	No	-
LRP1	LDL receptor related protein-1	Yes	-
miR-33	Micro RNA -33	No	-
NCEH1	Neutral cholesteryl ester hydrolase	No	-
NPC1	Niemann Pick Type C -1	No	Niemann-Pick disease type C
SMPD1	Sphingomyelinase phosphodiesterase-1	No	Niemann-Pick disease types A&B
SR-BI	Scavenger Receptor BI	Yes	Elevated HDL-C
STARD3	Steroidogenic acute regulatory D3	Yes	

Mutations in four of these genes are associated with disorders of cellular cholesterol efflux in man. Several of these genes are also found in GWAS associated with HDL-C (refer to **Table 1.4**). GWAS, genome wide-association studies; HDL-C high-density lipoprotein cholesterol

1.6.1 Cholesteryl Ester Hydrolases

Arguably, the first step in removing cellular cholesterol involves the hydrolysis of CE by enzymes that cleave the fatty acyl chain. Neutral cholesterol ester hydrolase (nCEH1, also called KIAA1363) is the prototypical cellular enzyme involved in this process (Okazaki et al., 2008). In macrophages, where most of the cholesterol is stored in lipid droplets as CE, hormone sensitive lipase (LIPE) is considered to play a major role in the generation of free cholesterol destined for export in mouse, but not in humans. Interestingly, lysosomal CEHs also hydrolyse CE obtained from circulating lipoproteins. At least one other nCEH has recently been identified (Sekiya et al., 2011) and the CE hydrolysis system is redundant (Quiroga and Lehner, 2011). This biological redundancy likely may explain why so far, none of the genes involved in CE hydrolysis, the first steps of RCT, have been identified in GWAS (**Table 1.4**), or why targeted gene deletion of LIPE does not reduce CE hydrolysis, supporting the concept of multiple redundant systems for cellular CE hydrolysis (Buchebner et al., 2010; Kratky, 2011).

1.6.2 ABCA1

As previously described, the interaction between apoA-I and ABCA1 is essential and rate-limiting for the initial step in RCT. Recent data from our laboratory suggests that ABCA1 acts as a phospholipid translocase and contributes to the formation of a non-raft membrane domain that facilitates the lipidation of apo AI and the formation of nascent HDL particles as reviewed in Section 1.3.5.2. This will be further investigated in Chapter 5.

Genetic control of ABCA1 appears to be predominantly through the oxysterols/LxR pathway (**Figure 1.7**). Attempts to modulate the LxR pathway to increase ABCA1 and HDL have been fraught with the multiple roles of LxR on fatty acid synthesis and regulation of Sterol Regulatory Element-Binding Proteins (SREBP)-1c.

The regulation of ABCA1 differs between cell types (Denis et al., 2003). In macrophages, a novel pathway has been identified. Chen X. *et al.* (Chen et al.,

2011) examined the effect of LDL loading in macrophages and found increased levels of phosphorylated specificity protein 1 (Sp1) and protein kinase C-ζ (PKCζ), with increased amounts of Sp1 bound to the ABCA1 promoter. Inhibition of PKCζ or mutations within the Sp1 binding site on ABCA1 attenuated the LDL- induced expression of ABCA1 and the increase in cellular cholesterol efflux (Chen et al., 2011).

In a recent study of the lipoprotein receptor related protein-1 (LRP1), a cell-surface receptor of the LDL-receptor superfamily, Basford *et al.* (Basford et al., 2011) examined the effects of liver-specific LRP1 knock out ($LRP1^{-/-}$) in mice, and found lower levels of HDL-C. Primary hepatocytes from $LRP1^{-/-}$ mice displayed reduced HDL secretion and decreased cell-surface localization of ABCA1 without a change in total cellular ABCA1 content. Their results are consistent with a decreased translocation of ABCA1 to the plasma membrane in $LRP1^{-/-}$ mice hepatocytes (Basford et al., 2011) (**Figure 1.7**).

1.6.3 ApoA-I

ApoA-I is the major apolipoprotein within HDL, accounting for approximately 70% of the protein mass. Mutations within apoA-I have been previously identified, with at least 47 variants affecting apoA-I structure (Sorci-Thomas and Thomas, 2002; Dastani et al., 2006a), some leading to a marked reduction in apoA-I and HDL-C levels (Sorci-Thomas and Thomas, 2002), and concomitant CAD, while others with low HDL but no incidence of heart disease (Sirtori et al., 2001). ApoA-I was also previously identified in GWAS (**Table 1.4**) with a significant association for TG, TC, HDL (as a secondary lipid trait) and LDL lipid levels. Nevertheless, despite that apoA-I genetic variations are well characterized, the role of apoA-I structure on ABCA1-mediacted efflux has not been completely elucidated.

Considerable controversy in the past few years has emerged regarding the lipid-free and lipid-bound structure of apoA-I (Davidson and Silva, 2005; Thomas et al., 2008; Wu et al., 2009) offering different insights into the structure-function of ApoA-I domains critical for nascent HDL particle assembly. As such, Huang R

et al. have recently addressed ApoA-I structure in spherical particles by applying chemical cross-linking and mass spectrometry to HDL particles, describing the first models of authentic human plasma HDL in which ApoA-I assumes a cage-like structural framework closely resembling that in synthetic HDL (Huang et al., 2011). Specifically, ApoA-I adopts intermolecular interactions in plasma HDL similar to interactions previously described in the double-belt and trefoil models derived in reconstituted systems, suggesting that HDL particle sizes are modulated through twisting motion of the resident ApoA-I molecules (Huang et al., 2011).

Other studies have focused at better understanding the properties of the tertiary structure domains of ApoA-I and their influence on ApoA-I functionality in the RCT pathway (Alexander et al., 2011). By generating two chimeric N-terminal domain-swap variants of mouse and human ApoA-I, and expressing these hybrids in ApoA-I-null mice, Alexander *et al.* evaluated their abilities to promote macrophage RCT in vivo, in comparison to WT human and mouse ApoA-I. More cholesterol was observed to be significantly removed from the macrophages of the mouse-H ApoA-I expressing mice as compared to the other groups, as shown by a marked increase in fecal excretion via the RCT pathway. Furthermore, catalytic efficiencies (V_{max}/K_m) of the ApoA-I variants were determined and observed to be 2-fold higher in the mouse-H ApoA-I than the WT human ApoA-I and 3.5 fold higher than WT mouse ApoA-I, suggesting a more efficient ABCA1-mediated cholesterol efflux. These findings, along with a detected increase in the rate of cholesterol uptake into hepatocytes, demonstrated that substitution of the N-terminal domain of the human ApoA-I with the mouse ApoA-I counterpart, created a “gain of function” ApoA-I variant showing enhanced nascent HDL particle formation, a more efficient macrophage RCT and potentially antiatherogenic apoA-I. Additional insights into the effects of ApoA-I structure on cholesterol efflux were described by the same group through examination of ApoA-I C-terminal α-helix hydrophobicity influence on nascent HDL particle formation (Lyssenko et al., 2011). By engineering human ApoA-I variants, Lyssenko *et al.* observed that the decrease in ApoA-I hydrophobicity of

the non-polar face of the C-terminal amphipathc α-helix significantly reduced the catalytic efficacy of vesicle solubilisation and cholesterol efflux, forming large HDL particles, with reversing effects when hydrophobicity is restored (Lyssenko et al., 2011).

In addition to structural modifications, several studies have documented that oxidative modification of ApoA-I could also be a contributing factor in altering RCT levels (Bergt et al., 2004; Shao et al., 2006). As such, Heinecke and Oram (Shao et al., 2010) have demonstrated that chlorination, but not nitration, of ApoA-I through the myeloperoxidase pathway dramatically impaired the ability of ApoA-I to interact directly with ABCA1 and to activate the Janus-kinase-2 signaling pathway, preventing ApoA-I from promoting cellular cholesterol efflux in a macrophages (Shao et al., 2010).

1.6.4 ABCG1

The ATP binding cassette transporter G1 (ABCG1) is expressed in a variety of tissues. While it is predominantly located in the cytosol, ABCG1 promotes the lipidation of nascent HDL particles rather than lipid-free (or lipid-poor) apoA-I, playing a significant role in the efflux transport of excess cholesterol to HDL in macrophages, thereby reducing atherosclerosis (**Figure 1.7**). Gao X *et al.* (Gao et al., 2011) recently identified the first amino acid residue that is critical for ABCG1-mediated cholesterol efflux. Indeed, the cysteine residue located at position 514 is highly conserved and a mutation at this position, although having no effect on protein stability or trafficking, significantly decreased the efflux of cholesterol onto lipidated apoA-I.

Apart from structural changes, ABCG1 expression is mainly regulated by the cellular cholesterol content: it has been shown to be increased following treatment with acetylated LDL (cholesterol-loading condition) and downregulated after treatment with HDL_3 (Klucken et al., 2000). Nuclear receptors such as the LxR and the peroxisome proliferator-activated receptors (PPARs) have been suggested to regulate the expression of ABCG1. More recently, a significant effort has been put on the study of the various regulators of ABCG1 expression

through the LxR pathway, and their effect on the resultant cholesterol efflux. First, Rayner K.J. et al (Rayner et al., 2010) showed that ABCG1 is targeted by miRNAs (see miRNA section below), more specifically miR-33a. Hence, in mouse macrophages, miR-33 decreases ABCG1 expression, reducing cholesterol efflux to nascent HDL. Despite the presence of a miR-33a binding site in the human ABCG1 gene, the targeting of ABCG1 by miR-33a only appears to be marginal (Rayner et al., 2010; Marquart et al., 2010). The LxR pathway is also involved in the regulation of ABCG1 expression by group X secretory phospholipase A2 (GXsPLA2). In a recent study by Shridas and colleagues (Shridas et al., 2010), ABCG1 expression and associated cholesterol efflux were found to be reduced after GX $sPLA_2$ overexpression or exogenous addition in mouse macrophages, suggesting a potential role of GX $sPLA_2$ in atherosclerotic lipid accumulation.

ABCG1-mediated cholesterol translocation also plays an important role in protection against endothelial dysfunction (Terasaka et al., 2008; Terasaka et al., 2010), interaction of monocytes with endothelial cells (Whetzel et al., 2010) and regulation of insulin secretion (Sturek et al., 2010; Fryirs et al., 2010). Increase in ABCG1 expression and concomitant cholesterol efflux capacity, in the presence of erythropoietin (Lu et al., 2010) or after weight loss (Aron-Wisnewsky et al., 2011), for instance, were respectively associated with a reduction in lipid accumulation in foam cells and an improvement in the atherogenic profile in human. As for insulin, it was shown to decrease HDL-mediated cholesterol efflux from macrophages from the suppression of nCEH and ABCG1 expressions (Yamashita et al., 2010) and to be involved in the insulin secretion process from pancreatic β cells (Sturek et al., 2010). These new insights on the regulation of ABCG1 present this transporter has a novel potential target in the inhibition of the process of atherosclerosis in various human metabolic states, including obesity and diabetes.

1.6.5 Disorders of cholesterol trafficking: NPC1, NPC2, LIPA and SMPD1 genes

Niemann-Pick disease type C (NPC) is a complex lysosomal storage disorder caused by mutations in either the *NPC1* or *NPC2* genes, characterized by the accumulation of unesterfied cholesterol in the lysosomal compartment. Choi *et al.* (Choi et al., 2003) have reported that the cholesterol trafficking defect in cells from subjects with NPC disease results in a reduced activity of *ABCA1* . Low plasma HDL-C in *NPC1* disease patients occurs independent of their plasma triglyceride levels. This suggests that impaired *ABCA1* regulation is a consequence of reduced efflux of cholesterol out of the late endosome, and is the cause of the low HDL-C seen in NPC (Tangemo et al., 2011; Choi et al., 2003). Reduced plasma HDL-C was the most consistent lipoprotein abnormality found in male and female *NPC1* patients across age groups independent of changes in plasma triglycerides, representing a potential biomarker of *NPC1* disease severity (Garver et al., 2010). *NPC1* cells also develop a secondary defect in acid sphingomyelinase (SMase) activity despite a normal acid SMase gene, (*SMPD1*) (Devlin et al., 2010). Recent advances have provided potential therapeutic approaches for the treatment of NPC Disease. In one study, SMase activity defect in fibroblasts from *NPC1* patients was corrected by *SMPD1* transfection or acid SMase enzyme replacement. Both approaches resulted in a dramatic reduction in lysosomal cholesterol (Devlin et al., 2010). Recently, the use of small histone deacetylase (HDAC) led to a correction of the NPC phenotype (Pipalia et al., 2011). The HDAC inhibitor LBH589 (panobinostat) is currently undergoing clinical trials in man for several types of cancer. In cultured NPC1 fibroblasts, LBH589 restores cholesterol homeostasis, raising the possibility that this class of agents might be useful in a clinical setting (Pipalia et al., 2011).

Another form of ABCA1 impairment is seen in mutations in the lysosomal acid lipase A (*LIPA)* gene that result in less than 5% of normal lysosomal acid lipase (LAL) activity causing cholesteryl ester storage disease (CESD) (Bowden et al., 2011). *LIPA* has been identified on chromosome 10q23 as a novel CHD susceptibility locus. Elevated *LIPA* expression itself was related

to lower HDL-C levels that are attributed to reduced cholesterol efflux to apoA-I. Furthermore, treatment of fibroblasts from normal patients with chloroquine to inhibit LAL activity was observed to reduce *ABCA1* expression and activity, similar to that of CESD cells (Bowden et al., 2011). LxR agonist treatment of CESD cells corrected *ABCA1* expression, but failed to correct LDL CE hydrolysis and cholesterol efflux to apoA-I. Moreover, LDL-induced production of 27-hydroxycholesterol (27-HC) was decreased in CESD compared to normal fibroblasts. It was further determined that treatment of CESD cells with conditioned medium containing LAL from normal fibroblasts or with recombinant human LAL rescued *ABCA1* expression, apoA-I-mediated cholesterol efflux, HDL particle formation and production of 27-HC (Bowden et al., 2011).

Sphingomyelin (SM) plays an important role in the structural integrity of cellular membranes. Characterized by its high gel-to-liquid-crystalline phase transition temperature, SM often segregates with cholesterol within distinct sub-cellular compartment. As previously described, one of the genes involved in the modulation of SM levels is sphingomyelin phosphodiesterase 1, also known as acid sphingomyelinase (*SMPD1, MIM 607608*). It codes for the lysosomal and secretory SMase, a 70 kDa glycoprotein that hydrolyzes SM to ceramide and phosphorylcholine. Mutations in the *SMPD1* gene cause the autosomal recessive disorder of Niemann-Pick type I disease, which includes type A (MIM 257200) and B (MIM 607616). Deficiency of lysosomal SMase results in a lysosomal accumulation of SM and a secondary accumulation of cholesterol. We have previously reported that mutations in *SMPD1* causing Niemann-Pick disease types A and B are associated with low HDL-C levels but normal cholesterol efflux under the experimental conditions used (Lee et al., 2003; Lee et al., 2006).

Together these findings provide further evidence that the rate of release of cholesterol from late endosomes/lysosomes is a critical regulator of cellular cholesterol trafficking (**Figure 1.7**) and this, in turn may be critical in cellular cholesterol efflux pathways via the ABCA1 transporter.

1.6.6 SR-BI

The scavenger receptor BI (SR-BI) mediates the selective uptake of cholesteryl esters from HDL into hepatocytes and steroidogenic tissues (Trigatti et al., 2000; Krieger M, 1999) (Figure 1.6). The atheroprotective effects of SR-BI are therefore primarily attributable to its role in cholesterol efflux from lipid-laden macrophages to HDL (Covey et al., 2003; Ji et al., 1997; Van et al., 2004) and in the delivery of HDL CE to the liver (Zhang et al., 2005). Interestingly though, SR-BI-/- mice exhibit higher levels of HDL-C and increased atherosclerosis, a process attributed to a critical block in the RCT to the liver for biliary excretion (Leiva et al., 2011). In humans, several studies have described genetic SCARB1 variants associated with HDL-C levels (Acton et al., 1999; Hsu et al., 2003), most notably in the recent GWAS by Teslovich et al. (**Table 1.4**) where SR-BI was identified among the 95 loci significantly associated with lipid levels in more than 100,000 individuals of European ancestry (rs838880 P=3x10-14), confirming its critical role in lipid metabolism. Furthermore, Vergeer et al. (Vergeer et al., 2011) recently brought forth evidence that impaired SR-BI function can affect human physiology. Identifying a kindred with a functional missense mutation in SR-BI, P297S, they observed that carriers had significantly elevated HDL-C levels, although no considerable differences in lipid profile parameters such as atherosclerosis, or carotoid intima-media thickness. Importantly, primary murine hepatocytes expressing mutant SR-BI exhibited a marked reduction of 56% in cholesterol uptake from HDL of that of wildtype SR-BI, while P297S variants carriers showed reduced efflux capacity from monocyte-macrophages, comparatively to non-carriers (Vergeer et al., 2011).

1.6.7 StARD3 (MLN64)

The steroidogenic acute regulatory proteins comprise at least 15 proteins involved in cellular cholesterol transport and homeostasis (Soccio and Breslow, 2003). StARD3 (also called endosomal Metastatic Lymph Node protein 64**)** is involved in the key transfer of cholesterol from the late endosomal compartment a step that occurs after the NPC1 protein. Recent data from Charman *et al.*

(Charman et al., 2010) shows that StARD3 transports cholesterol from the endosome to the mitochondria and may provide an essential regulatory step between cholesterol, mitochondria-derived oxysterols and regulation of the cellular cholesterol efflux machinery (**Figure 1.7**). To date, no human disease linked to StARD3 has been identified. Moreover, targeted mutations of the StAR domain in mice do not lead to a defect in steroidogenesis or to a distinct phenotype (Rigotti et al., 2010). Yet in a recent study, Borthwick *et al.* report that the overexpression of StAD3 in macrophages induces increase in ABCA1 mRNA and protein and enhances cellular cholesterol efflux to apo AI. Interestingly, overexpression of StARD3 in macrophages also prevents the accumulation of CE in response to acetylated LDL (Borthwick et al., 2010). In GWAS (**Table 1.4**), the StARD3 locus is associated with lower HDL-C levels, suggesting an important role for this protein in cellular cholesterol homeostasis and possibly, efflux.

1.6.8 ABCA8

The *ABCA8* gene, located on chromosome 17 (17q24) and coding for another membrane-associated protein member of the ABC superfamily, has also been identified in GWAS (**Table 1.4**). The function of this protein in cellular cholesterol efflux is however still unclear. The protein has been isolated in human brain microvessels (Shawahna et al., 2011) where it could play a role in intracellular lipid trafficking rather than trans-plasma membrane transport (Kim et al., 2008). The gene could be regulated by extracellular signal-regulated kinases (ERK1 and 2) (Shukla et al., 2010).

1.6.9 Novel Genes

The study of extensive kindred with HDL deficiency has identified several chromosomal regions harboring genes related to HDL metabolism (Dastani et al., 2010; Dastani et al., 2006c; Iatan et al., 2009; Weissglas-Volkov and Pajukanta, 2010). We have recently reported a strong association between the WWOX gene locus and HDL-cholesterol levels (Lee et al., 2008a) ($P = 6.9 \times 10^{-7}$) which will be further discussed in Chapter 4. As will be discussed in Chapter 3,

we have also documented an association between single nucleotide polymorphisms (SNPs) at the proprotein convertase subtilisin/kexin type 5 (*PCSK5*) gene and HDL-C levels, which we validated in a Finnish population (Iatan et al., 2009). Similarly, it remains to be determined if these genes are related to cholesterol homeostasis of cholesterol efflux pathways.

1.6.10 miRNAs

Recently, the identification of micro RNAs (miRNAs) as key regulators of lipoprotein metabolism have been uncovered (Horie et al., 2010; Ramirez et al., 2011). Micro RNAs are short (average 22 nucleotides) strands of RNA involved in the repression of gene transcription. Single miRNAs can regulate the transcription of multiple genes by virtue of target sequences usually located with the 3'UTR of target genes. One miRNA, miR-33, located within the SREBP2 gene has been shown to repress expression of ABCA1 and to lower HDL-C levels (**Figure 1.7**). Using oligonucleotides directed against miR-33, Rayner et al. (Rayner et al., 2010; Rayner et al., 2011) showed that inhibition of miR-33 in the LDL-R-/- mouse is associated with an increase in hepatic ABCA1 and ABCG1 expression, increase in HDL-C, increase in RCT to the liver and feces and reduced atherosclerosis. This provides compelling support for a therapeutic application in man. More recently, other miRNAs (miR-106b, miR758) (Kim et al., 2011; Ramirez et al., 2011) have been shown to control a variety of aspects of lipid metabolism from HDL biogenesis to cholesterol efflux. These findings have thus highlighted the significant role that miRNA play in HDL metabolism, opening new avenues for the treatment of CAD.

1.7 *ABCA1* gene variation

Parts of this Section 1.7 were obtained with permission from *Current Atherosclerosis Reports* from (Iatan et al., 2008), *Effect of ABCA1 Mutations on Risk for Myocardial Infarction* and were subsequently modified for this thesis.

The identification of the ABCA1 transporter as the rate-limiting step in HDL biogenesis and the first step of the RCT pathway has triggered a great deal

of investigation into its role as a genetic factor in atherosclerosis and CAD, and thus as a potential therapeutic target. Accordingly, based on the necessary and essential role of ABCA1 in HDL biogenesis, it is likely that genetic variability at the ABCA1 gene locus may influence HDL-C levels and the genetic epidemiology of CAD, and is thus deserving of further descriptive detail.

1.7.1 ABCA1 variants

The ABCA1 gene is located on chromosome 9q22-q31, covers over 150 kb of genomic DNA, and is composed of 50 exons ranging in size from 33 base pairs to 249 base pairs. As previously described, the quaternary structure of ABCA1 appears to be tetrameric, requiring the assembly of four ABCA1 transporters into a functional subunit (Denis et al., 2004a). This molecular arrangement may cause the expression of a single mutation to behave as a dominant negative mutation, explaining the low HDL-C levels seen in heterozygous ABCA1 mutants. To date, more than 200 unique sequence variants have been identified in ABCA1, of which at least 145 are mutations. Recent data from the 1000 genomes project (www.1000genomes.org) has increased the reported number of coding region polymorphisms in ABCA1 to over 50. Altogether, these variants result in a broad spectrum of biochemical and clinical phenotypes.

1.7.2 ABCA1 genetic variability and HDL-C levels

Recent epidemiological studies have revealed that in the general population, genetic variations within the coding region of ABCA1 can govern susceptibility to CAD by modifying the plasma HDL levels. In fact, as previously reported in Section 1.5.1, family studies have established that the HDL-C trait is highly heritable, with estimates ranging from 40 to 80% (the most commonly cited being at 50%) (Breslow JL, 1995; Bielinski et al., 2006; Lusis et al., 2004; Peacock et al., 2001; Qasim and Rader, 2006). HDL-C levels can also be an insensitive marker of a genetic lipoprotein disorder (except in extreme values) and reflect a variety of environmental and genetic factors besides ABCA1 mutations. Nevertheless, multiple ABCA1 variants have been examined in relationship to

their association with HDL-C plasma concentrations (Singaraja et al., 2003), and functional ABCA1 mutations were found correlated with a broad range of HDL-C in humans. This genetic variability at the ABCA1 gene locus and the impact on HDL-C levels and atherosclerosis of the rare and common ABCA1 variants identified has been reviewed in detail by Brunham et al. (Brunham et al., 2006b). Nonetheless, not all association studies examining this relationship have been consistent and some frequently lack validation. To date, the largest study examining ABCA1 SNPs and HDL-C is the Copenhagen City Heart Study (Frikke-Schmidt et al., 2004), in which the relationship between six ABCA1 non-synonymous common SNPs (R219K, V771M, M825I, I883M, E1172D, and R1587K) and HDL-C was analyzed. The authors concluded that the R1587K SNP was associated with low HDL-C in both men and women.

Although the association between ABCA1 and HDL-C concentrations has been reviewed in depth (Brunham et al., 2006b), at least three groups (Cohen et al., 2004; Frikke-Schmidt et al., 2004; Alrasadi et al., 2006) reported the prevalence of ABCA1 mutations in low HDL-C. Accordingly, the molecular basis of HDL deficiency was examined in three separate cohorts of selected patients by direct exon-by-exon sequencing analysis of the ABCA1 gene (**Table 1.6**).

In the first study, carried out by Cohen *et al*. (Cohen et al., 2004), the entire coding region of ABCA1 and its consensus splice sites, were sequenced in 256 individuals representing the upper and lower 5th percentile of HDL-C levels from the Dallas Heart Study population. In 20 of the 128 (15.6%) individuals with the lowest 5th percentile distribution of HDL-C levels, rare sequence variants in ABCA1 were identified, which were not present in the high–HDL-C group. This finding was replicated in a second population, a cohort of 263 Canadians, indicating that in two separate populations, approximately 15% of individuals with low HDL-C expressed rare sequence variants at the ABCA1 gene locus.

Similar findings were reported by Frikke-Schmidt *et al*. (Frikke-Schmidt et al., 2004) in a study in which the core promoter and all 50 exons of ABCA1 were screened in individuals with the lowest 1% (n = 95) and highest 1% (n = 95) HDL-C levels from the Copenhagen City Heart Study. Six of the seven rare

variants that altered the ABCA1 amino acid sequence were only observed in individuals (*n*=9) with low HDL-C. Overall, the authors demonstrated that 10% of individuals in the general population with low HDL-C were found to harbour a mutation in ABCA1.

In our group, Alrasadi et al. (Alrasadi et al., 2006) examined 58 unrelated probands with HDL deficiency (defined as a HDL-C level $<5^{th}$ percentile for age-matched and gender-matched individuals) and reported a 20% prevalence of ABCA1 mutations in these subjects of French-Canadian descent, possibly reflecting a founder effect in some of the kindred examined.

Table 1.6 ABCA1 Variants and HDL-C

Study [ref]	Population screened	Number of individuals / Gender	ABCA1 variants	Conclusions
(Cohen et al., 2004)	Dallas Heart Study population (128 individuals: HDL-C < 5th percentile 128 individuals: HDL-C > 95th percentile)	256 (128 M and 128 F)	Rare ABCA1 variants were observed in 20 of the 128 individuals with HDL-C < 5th percentile.	15% of individuals with low HDL-C have rare sequence variants in ABCA1. None of the common ABCA1 variants (frequency > 10%) identified were associated with HDL-C levels across all gender and ethnic groups. One mutation (ABCA1 N1800H) was found in both Canadian and Dallas low– HDL-C groups.
	Canadian population (155 individuals: low HDL-C [10-34 mg/dl] 108 individuals: high HDL-C [58-116 mg/dl])	263 (179 M and 84 F)	21 (14%) of 155 Canadians in the low–HDL-C group had sequence variants that were not present in the 108 Canadians with a high plasma level of HDL-C.	
(Frikke-Schmidt et al., 2004)	Copenhagen City Heart Study (HDL-C < 1th percentile HDL-C > 1th percentile)	190 (89 M and 101 F)	6 of the 7 rare ABCA1 variants were only observed in the low–HDL-C group.	Heterozygosity for ABCA1 mutations was identified in at least 10% of individuals with low HDL-C.
(Alrasadi et al., 2006)	French-Canadian population (HDL-C < 5th percentile)	58	12/58 HDL-deficient subjects (HDL-C < 5th percentile) found to have rare ABCA1 variants.	20% prevalence of ABCA1 mutations in subjects of French-Canadian descent with HDL-C < 5th percentile.

M: males; F: females; ABCA1—adenosine triphosphate–binding cassette A1; HDL-C—high-density lipoprotein cholesterol

1.7.3 ABCA1 mutations and risk for coronary heart disease

The relationship between ABCA1 polymorphisms, plasma lipid and lipoprotein lipid levels represents a "middle distance reality" between a genotype and an effect on clinical end points, such as the presence of CAD. This has been observed with the advent of Mendelian randomization studies. Although much emphasis has been put on the association of ABCA1 gene polymorphisms with alteration of HDL-C levels, the correlation between common or rare genetic variants in the ABCA1 gene and the risk of heart disease in the general population is currently debatable, as various studies have identified sometimes inconsistent results. This relationship between ABCA1 genotypes and CAD has therefore been investigated in several studies and while early reports identified an association between different SNPs and CAD, these were often not reproduced in large-scale prospective studies. A systematic search of association studies published in the last decade between ABCA1 genetic variability and risk of coronary heart disease (CHD), which includes ischemic heart disease (IHD), CAD and acute myocardial infarction (MI), was thus performed (**Table 1.7**).

The association between homozygous ABCA1 mutations (e.g. TD) and CHD has been subject of considerable controversy due to the paucity of worldwide documented cases of TD. Indeed, there is no firm agreement that TD is correlated with CHD. This lack of strong association is likely due to the very low LDL-C seen in such patients, which may provide a protective effect from CHD. In individuals with heterozygous ABCA1 mutations, however, the association with CHD and MI appears less ambiguous. In a study done by Clee et al. (Clee et al., 2000), the risk of CHD in individuals with heterozygous ABCA1 mutations was increased 3.5-fold compared to noncarriers (**Table 1.7**). However, this observation may reflect a selection bias of patients attending specialized cardiovascular prevention clinics.

Some other reports find minor or no association of the same ABCA1 variants with the diseased phenotype or even risk factor effects, and population differences are also significant (Harada et al., 2003; Brousseau et al., 2001; Tregouet et al., 2004). Through single-locus and haplotype-based analyses,

Tregouet et al. (Tregouet et al., 2004) investigated whether ABCA1 gene polymorphisms could be associated with the risk of MI and the variability of apoA1 levels by genotyping 26 ABCA1 SNPs in the Etude Cas-Témoin de l'Infarctus du Myocarde study, a case-control study of MI (Parra et al., 1992) (**Table 1.7**). As previously described (Clee et al., 2001; Brousseau et al., 2001; Cenarro et al., 2003), of the 16 polymorphisms located in the promoter and the 10 found in the coding region, it was concluded that only the R219K polymorphism was associated with MI, decreasing its risk (odds ratio of 0.80; 95% CI, 0.68-0.94; $P = 0.007$), whereas no haplotypes were involved in the susceptibility to CHD. Conversely, the R1587K polymorphism was discovered to decrease plasma apoA1 levels but it has no significant association with MI, although the carriers for the K1587 allele (RK and KK) were at slightly decreased risk of MI (odds ratio of 0.76; 95% CI, 0.61-0.94; $P = 0.013$) (Tregouet et al., 2004). From those findings, the authors concluded that ABCA1 polymorphisms, but not haplotypes, are involved in the variability of plasma apoA1 and the susceptibility to CHD.

Additional insights into the effects of ABCA1 on MI have been described by Frikke-Schmidt et al. (Frikke-Schmidt et al., 2008a) in a study where six nonsynonymous ABCA1 SNPs, R219K, V771M, V825I, I883M, E1172D, and R1587K (identified by resequencing ABCA1 in 190 individuals of Danish ancestry (Frikke-Schmidt et al., 2004)), were genotyped in 9259 individuals from the Copenhagen City Heart Study to assess their risk of CHD (Table 1.7). The principal finding of the study indicated that common genetic variation in ABCA1 predicts risk of CHD in the general population, but that their association was independent of plasma HDL-C levels: SNPs predicting increased MI risk were associated with either increases (V771 and V825I) or decreases (R1587K) in HDL-C, or no effect on HDL-C (R219K, I883M, E1172D). Furthermore, to retest whether the SNP genotype was associated with risk of IHD in an independent sample, a case-control study was conducted with 932 CHD cases versus 7999 controls and similar results were obtained. In addition, to determine which ABCA1 SNPs were independent predictors of CHD and not caused by linkage disequilibrium among SNPs, a stepwise Cox regression approach was performed

identifying V771M, I883M and E1172D as the most important for the final IHD prediction model. Additive effects on CHD risk were also observed for the V771M/I883M and I883M/E1172D pairs. In fact, the I883M and E1172D SNPs were previously determined by Brousseau et al. (Brousseau et al., 2001), in a study of 2028 white men, to have increased frequencies in cases compared with controls, in addition to detecting increased risk of future CHD events associated with the 1172D allele (**Table 1.7**). These data are thus supporting evidence that these two SNPs are two of the three most important ABCA1 SNPs for the IHD prediction model. Taken together, the findings of this study show that three of the six nonsynonymous SNPs (V771M, I883M and E1172D) in ABCA1 predict risk of IHD in the general population, and that ABCA1 variants may have proatherosclerosis effects independent of HDL-C levels (Frikke-Schmidt et al., 2008a). Therefore, one could argue that in most cases the risk associated with ABCA1 genetic variation was not explained by the inverse relationship between HDL-C and risk of MI, as has been observed for other important genes in RCT (ie, CETP, and HL). An alternative hypothesis is that HDL-C may be a poor biomarker of HDL function in the particles generated by the ABCA1 transporter (nascent HDL), which contain little cholesterol (Krimbou et al., 2005).

Likewise, a previous study (Frikke-Schmidt et al., 2005) performed by the same authors further provides additional experimental evidence that ABCA1 may have properties independent of plasma HDL-C. Indeed, it was documented that a completely conserved ABCA1 common mutation, K776N (frequency, 0.4%), was found to confer a twofold to threefold increase in risk of IHD in 37 heterozygous participants in the Copenhagen City Heart study (**Table 1.7**). Adjusting for HDL-C, or for smoking, diabetes, and hypertension, did not change the result, suggesting that the genotype predicted risk of IHD beyond that offered by HDL-C and by other traditional risk factors. Thus, risk of MI in individuals homozygous or heterozygous for ABCA1 mutations may not only be related to HDL-C levels, but may also depend on the local pleiotropic effects of HDL on the arterial wall (Assmann and Nofer, 2003; Barter, 2005).

Although knowledge of genetic predisposition, such as ABCA1 mutations, is important for prevention, diagnosis, and treatments, determinants of prognosis are also especially relevant. In fact, the relationship between ABCA1 polymorphisms and prognosis after MI was investigated in a study done by Martin et al. (Martin et al., 2006) (**Table 1.7**). Three ABCA1 SNPs were analyzed (-477C/T, R219K, and I883) in a cohort of 170 young male survivors of MI (mean age, 43 ± 5 years) in order to determine their influence in long-term prognosis. A significant association was found between the -477C/T ABCA1 variant (CC genotype) and significantly worse clinical outcome (56% unfavorable in comparison to 44 % favorable), without differences in HDL levels. Lack of relationship between HDL-C levels and clinical evolution supports the opinion that the increase in RCT activity could reduce the development of atheroma without altering plasma lipid levels (Cenarro et al., 2003).

Given that the ABCA1 gene is highly polymorphic, several other studies addressed the question of the potential roles of ABCA1 variants in influencing CHD and altering lipid metabolism (**Table 1.7**). Lutucuta et al. (Lutucuta et al., 2001) found no significant difference in lipid levels between the -565C>T genotype groups, whereas Zwarts et al. (Zwarts et al., 2002) found no association between lipid levels and the -191G>C, -17G>C, C69T, and 319ins polymorphisms, which were associated with coronary events or atherosclerosis severity.

Additionally, several recent studies have examined the role of the R219K SNP in lipoprotein metabolism and CHD. Most of the reports have not determined significant effects on lipid values, but some have illustrated a marginal increase in HDL-C and/or decrease in TG levels associated with the 219KK genotype. The 219K allele was shown to be associated with a decrease in angiographic CHD and thus with a protective effect (Clee et al., 2001). This was also detected in Spanish familial hypercholesterolemia patients, especially in those younger than 40, and in current and former smokers (Cenarro et al., 2003). The findings were replicated by Bertolini et al. (Bertolini et al., 2004) reporting the prevalence of the 219K carriers (42.6% in index cases and 47.7% in all

familial hypercholesterolemia patients) to be similar to that reported in other white populations (Clee et al., 2001; Singaraja et al., 2003) (**Table 1.7**).

Table 1.7 Genetic Variation in ABCA1 and Risk of myocardial infarction[a]

Study [ref *]	Population screened	HDL-C (mmol/L)	ABCA1 variants (bolded if associated with CHD)	Conclusions
(Clee et al., 2000)	Within 11 TD families: TD patients (n=5) ABCA1 heterozygotes (n=77) Unaffected individuals (n=156)	0.08±0.05 0.74±0.24 1.31±0.35	Del L693, **R2144X, Del E,D1893,94,** R909X, **M1091T, P2150L,** ivs25+1G→C, Del C6825→2145X, CTC6952-4TT→2203X, **C1477R,** Q597R, T929I	ABCA1 heterozygotes had a 40-45% decrease in HDL-C and a >3-fold increase risk of CHD versus unaffected individuals.-Age was an important phenotype modifier in ABCA1 heterozygotes.
(Brousseau et al., 2001)	VA-HIT Study, men with established CHD (n=1014) Framingham Offspring Study CHD-free control men (n=1014)	0.83±0.13 1.14±0.31	R219K, I883M, **E1172D**	Frequencies of the 3 ABCA1 variants were significantly increased in VA-HIT. None of the variants were associated with HDL-C. In the VA-HIT, E1172D was associated with an increased risk of CHD
(Clee et al., 2001)	REGRESS cohort, with established CHD (n=804)	R219K genotype: RR: 0.92±0.22 RK: 0.93±0.23 KK: 0.92±0.20	**R219K**	The R219K SNP is associated with a decreased progression of CHD, deceased TG and increased HDL-C.
(Lutucuta et al., 2001)	Lipoprotein Coronary Atherosclerosis Study patients (n=372)	C-477T genotype: CC: 1.14±0.29 CT: 1.10±0.28 TT: 1.19±0.32	**C-477T,** A-419C, G-320C	The C-477T variant was strongly associated with the severity of coronary atherosclerosis, with modest effect on HDL-C.
(Zwarts et al., 2002)	REGRESS cohort, with established CHD (*n* = 804)	G-191C genotype: GG: 0.93 ± 0.23 GC: 0.92 ± 0.22 CC: 0.94 ± 0.29 C-17G genotype: CC: 0.92 ± 0.22 CG: 0.93 ± 0.23 GG: 0.88 ± 0.21 C69T genotype: CC: 0.91 ± 0.22	**G-191C**, **C-17G, C69T,** C117G, A-362G, A-461G, G-720A, G-1027A, **A-1095G,** InsCCCT-1163, **InsG319,** G378C	G-191C and C69T are associated with a threefold and twofold increase in coronary events, respectively. C69T and A-1095G: increased progression of atherosclerosis. C-17G: decrease in coronary events and MI prior to study. InsG319: reduced atherosclerosis. Common variation in noncoding ABCA1 regions can alter risk for CHD without necessarily influencing plasma lipid levels.

Study [ref *]	Population screened	HDL-C (mmol/L)	ABCA1 variants (bolded if associated with CHD)	Conclusions
		CT: 0.91 ± 0.19 TT: 0.95 ± 0.30 A-1095G genotype: AA: 0.93 ± 0.24 AG: 0.93 ± 0.22 GG: 0.83 ± 0.20 InsG319 genotype: No ins: 0.92 ± 0.23 G: 0.93 ± 0.23 GG: 0.89 ± 0.30		
(Cenarro et al., 2003)	Spanish heterozygous FH patients with premature CHD (*n* = 216) or without (*n* = 158)	R219K genotype RR: 1.27 ± 0.36 RK + KK:1.32 ± 0.40	**R219K**	The frequency of the K219 allele was significantly higher in the FH group without premature CHD than in FH patients with premature CHD
(Evans and Beil, 2003)	Patients attending a lipid outpatient clinic (*n* = 813)	R219K genotype: RR: 1.32 ± 0.03 RK: 1.34 ± 0.03 KK: 1.27 ± 0.31	**R219K**	The K219 allele was significantly protective against CHD in patients with hyperlipidemia and elevated Lp(a), as well as decreasing TG levels.
(Harada et al., 2003)	Japanese patients (*n* = 410)	I883M genotype: II: 1.16 ± 0.30 IM: 1.26 ± 0.42 MM: 1.27 ± 0.37; P = 0.05 R219K genotype: RR: 1.26 ± 0.41 RK: 1.25 ± 0.40 KK: 1.24 ± 0.35	I883M, R219K	The I883M polymorphism was significantly associated with higher HDL-C in Japanese patients, but not with CHD
(Tan et al., 2003)	Cases: Chinese (n=512), Malay (n=110) and Indian (n=164) men with established CHD Controls: Chinese (n=271), Malay (n=179) and Indian (n=231) men	Chinese :CAD:0.89±0.28 Ctl: 1.19±0.32, P<0.0005 Malays: CAD: 0.83±0.27 Ctl: 1.15±0.27, P<0.0005 Indians: CAD:0.79±0.24	C-14T, 237indelG, **V825I, M883I,** A8994G	The V825I and M883I polymorphisms are positive markers for the CHD phenotype in Malays, but with no effect on HDL-C.

Study [ref *]	Population screened	HDL-C (mmol/L)	ABCA1 variants (bolded if associated with CHD)	Conclusions
		Ctl: 1.00±0.26, P<0.0005		
(Tregouet et al., 2004)	Subgroup of ECTIM cohort (n=452 cases and n=465 controls)	NA	C-564T, **R219K**, R1587K	ABCA1 polymorphisms, but not haplotypes are involved in variability of apoA1 and the susceptibility to CHD. C-564T and R1587K were associated with apoAI only. R219K was associated with MI (K219 allele associated with a decreased risk), but not with apoAI.
(Shioji et al., 2004)	Japanese Suita Study (n=1880) MI group (n=598 men)	Suita group: 1.44±0.02 MI group: 1.09±0.01, P<0.0001	G-273C	The G-273C polymorphism was significantly associated with HDL-C in the general Japanese population, but not on the incidence of MI.
(Bertolini et al., 2004)	FH patients: FH unrelated index cases (n=221) FH heterozygous relatives (n=349)	All FH subjects: Males: 1.18±0.02 Females: 1.39±0.02 P<0.0001	**R219K**	The K219 allele was significantly protective against CHD, and this effect was more pronounced in males than in females, and in smokers versus non-smokers (but no significant effect on lipid concentrations).
(Kyriakou et al., 2005)	Patients with CAD (n=1170)	C-565T genotype: CC: 1.29±0.64 CT: 1.28±0.32 TT: 1.26±0.35	**C-565T** (previously designated as C-477T)	In the sample as a whole, there was a trend toward greater atherosclerosis in the -565T carriers. In the non-smoker group, the TT carriers presented more diseased heart vessels than the CC carriers.
(Frikke-Schmidt et al., 2005)	Copenhagen City Heart Study: Noncarriers (n=9039) K776N carriers (n=37)	Women: Noncarriers: 1.72±0.01 Carriers: 1.82±0.11 Men: Noncarriers: 1.38±0.01 Carriers: 1.18±0.09 P=0.05	**K776N**	Heterozygosity for the K776N ABCA1 mutation conferred a 2- to 3-fold increase risk of IHD, independent of plasma HDL-C.The K776N polymorphism was a better predictor of IHD as compared with traditional cardiovascular risk factors.
(Martin et al., 2006)	Cohort of males diagnosed with MI (n=170) Controls: valvular patients with normal coronariography controls (n=100)	NA	**C-477T**, R219K, I883M	In long term MI prognosis, the C-477T ABCA1 variant was associated with an unfavourable clinical evolution.

Study [ref *]	Population screened	HDL-C (mmol/L)	ABCA1 variants (bolded if associated with CHD)	Conclusions
(Andrikovics et al., 2006)	Hungarian patients with ischemic stroke (n=244) Hungarian patients with CHD (n=150) Controls (n=193)	NA	**R219K, V771M,** I883M	A higher frequency of R219K and V771M wa observed in controls than in Hungarian strok patients, suggesting a protective role again CHD.
(Benton et al., 2007)	Subgroup of Multi-Ethnic Study of Atherosclerosis study group (n=969)	R219K genotype: RR: 1.34±0.38 RK: 1.29±0.35 KK: 1.37±0.39	C-565T, **R219K**	The R219K polymorphism was associated with 28% lower prevalence of coronary calcification a measure of subclinical atherosclerosis, and slightly higher HDL-C level.
(Tsai et al., 2007)	Taiwanese patients with CHD (n=205) and controls (n=201)	Cases: 1.04±0.25 Ctl: 1.27±0.31, P<0.0001	I823M	The M823 allele was associated with higher HDL-C.The I823M polymorphism was not a predictor of CAD, but it interacted with low HDL-C to increase the risk of CAD.
(Jensen et al., 2007)	Subgroup of the Nurses' Health Studies, with MI (n=249) or without (n=494)	Median (range) Cases: 1.70 (1.5-2.0) Ctl: 1.30 (1.1-1.6)	**C-565T, G-191C,** C-17G, I883M, R1587K	The C-565T and G-191C polymorphisms were inversely associated with the risk of CHD, independent of HDL-C.
(Pasdar et al., 2007)	Caucasien ischemic stroke patients (n=400) Caucasien controls (n=487)	Cases: 1.20±0.40 Ctl: 1.40±0.40	L158L, R219K, G316G, R1587K	The ABCA1 gene was not found to be associated with ischemic stroke, but R219K had the greates impact on lipid profile especially LDL and TG.
(Nebel et al., 2007)	German patients with CHD (n=1090) and controls (n=728)	NA	R219K, V771M, **I883M,** E1172D, R1587K	Only the I883M polymorphism was significantly associated with CHD.
(Frikke-Schmidt et al., 2008a)	Copenhagen City Heart Study: Patients with a diagnosis of IHD (n=1107) or without (n=7858)	Women: No IHD: 1.70±0.01 With IHD: 1.60±0.02 P<0.001 Men: No IHD: 1.40±0.01 With IHD: 1.30±0.02 P<0.001	R219K, **V771M, V825I, I883M, E1172D, R1587K**	Common genetic variation at the ABCA1 locus predicts IHD risk independently of plasma HDL-C levels. The V771M, I883M, and E1172D polymorphisms significantly predicted IHD risk, but their association was not related to HDL-C.
(Kathiresan et al., 2008b)	Malmö Diet and Cancer Study (n=5414)	rs3890182 genotype GG: 1.39±0.38 GA: 1.37±0.36	rs3890182	In combination with other lipoprotein risk alleles, CHD risk is increased.

Study [ref *]	Population screened	HDL-C (mmol/L)	ABCA1 variants (bolded if associated with CHD)	Conclusions
		AA: 1.31±0.31 P=0.003		

[*] Studies reporting the initial molecular and biochemical characterization of less than 5 probands with an ABCA1 gene defect and their families were not included in Table 2.

[a] Studies published from April 1st, 1999 through April 1st, 2008.

ABCA1—adenosine triphosphate–binding cassette A1; apoA-I—apolipoprotein A-I; CAD—coronary artery disease; CHD—coronary heart disease; Ctl—controls; FH—familial hypercholesterolemia; HDL-C—high-density lipoprotein cholesterol; IHD—ischemic heart disease; LDL—low-density lipoprotein; Lp(a)—lipoprotein(a); MI—myocardial infarction; NA—not available; SNP—single nucleotide polymorphism; TD—Tangier disease; TG—triglyceride

1.8 Research Rationale and Objectives

One of the principal research interests of our laboratory is to investigate the molecular genetics and cellular physiology of HDL deficiency. While recent studies from our group and others have led to important breakthroughs in the field of HDL metabolism, further investigations are needed to elucidate the complete spectrum of causes of HDL deficiency and the relationship between low HDL-C and cardiovascular risk.

The well-established inverse relationship between plasma HDL-C levels and the risk of CAD, combined with the fact that HDL-C has a strong inherited basis, has warranted an extensive search for genetic factors that modulate HDL-C levels. At the same time, gaining insight into the molecular mechanisms underlying the generation of nascent HDL particles (through the apoA-I/ABCA1/HCBS interaction) may have important implications in excess cholesterol removal from peripheral cells and understanding of the pathological mechanisms of CAD.

My research has thus focused on **gaining a better understanding of the genetic and biochemical regulation of plasma HDL-C levels, as well as the molecular mechanisms underlying HDL biogenesis**.

The work presented in this thesis consists of four inter-related studies with the central unifying theme of regulation of HDL metabolism, which can be divided into two components:

I. The identification and characterization of novel candidate genes and their corresponding genetic variants involved in regulation of plasma HDL-C levels. Our specific objectives are as follows:

1) Investigate whether genetic variability at the *PCSK5* gene locus contributes to variation in HDL-C levels through familial segregation analyses and associations studies (Chapter 2)

2) Identify novel rare-low frequency variants for low HDL-C using exome sequencing (Chapter 3)

3) Elucidate the role of the WW domain containing oxidoreoductase (*WWOX*) gene in HDL metabolism using a combination of *in vivo* and *in vitro* functional studies, as well as human genetic analyses (Chapter 4)

II. The characterization of the molecular mechanism of HDL biogenesis. The main objective of this study is:

4) Elucidate the cellular mechanism by which nascent HDL is formed though the lipidation and binding of apoA-I to the ABCA1/HCBS system within plasma membrane microdomains (Chapter 5)

CHAPTER 2. GENETIC VARIATION AT THE PROPROTEIN CONVERTASE SUBTILISIN/KEXIN TYPE 5 GENE MODULATES HIGH-DENSITY LIPOPROTEIN CHOLESTEROL LEVELS

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This research was originally published in Journal of the American Heart Association, Circulation Cardiovascular Genetics. Iulia Iatan, Zari Dastani, Ron Do, Daphna Weissglas-Volkov, Isabelle Ruel, Jenny C. Lee, Adriana Huertas-Vazquez, Marja-Riitta Taskinen, Annik Prat, Nabil G. Seidah, Päivi Pajukanta, James C. Engert, Jacques Genest. *Circ Cardiovasc. Genet* 2009; 2;467-475;

2.1 Preface and Rationale

While seeking to identify and characterize novel genetic determinants of HDL-C concentrations, we focused our attention on enzymes that modulate HDL particles. Endothelial lipase (EL) is an important phospholipid-specific phospholipase that is present on vascular endothelial cells, and has been shown to catalyze the hydrolysis of HDL phospholipids and facilitate HDL clearance from circulation (Cohen, 2003). EL can be inactivated by angiopoetin-like protein (Shimamura et al., 2007) or by a specific secretory proprotein convertase (PC) of the subtilisin/kexin type, the *proprotein convertase subtilisin/kexin type 5* (*PCSK5*) gene product, PC5/6 (Jin et al., 2007). In addition to the evidence that PC5/6 could be involved in the remodeling of HDL particles through EL inactivation, PC5/6 expression was observed to be highly upregulated in atherosclerotic plaques and during arterial restenosis. Furthermore, a genetic locus close to the *PCSK5* gene was recently implicated in lipid metabolism (Falchi et al., 2005). Given the biological plausibility for the involvement of PC5/6 as a potential proteinase in the control of circulating HDL-C levels (Falchi et al., 2005; Jin et al., 2007; Lusson et al., 1993), we undertook a candidate gene study to determine whether genetic variation within the human *PCSK5* gene contributes to inter-individual variation in HDL-C levels. The results of this work have been published in *Circulation Cardiovascular Genetics.*

2.2 Abstract

Background—A low level of plasma high-density lipoprotein cholesterol (HDL-C) is a risk factor for cardiovascular disease. HDL particles are modulated by a variety of lipases, including endothelial lipase, a phospholipase present on vascular endothelial cells. The proprotein convertase subtilisin/kexin type 5 (PCSK5) gene product is known to directly inactivate endothelial lipase and indirectly cleave and activate angiopoetin-like protein 3, a natural inhibitor of endothelial lipase. We therefore investigated the effect of human *PCSK5* genetic variants on plasma HDL-C levels.

Methods and Results—Haplotypes at the *PCSK5* locus were examined in 9 multigenerational families that included 60 individuals with HDL-C<10th percentile. Segregation with low HDL-C in 1 family was found. Sequencing of the *PCSK5* gene in 12 probands with HDL-C<5th percentile identified 7 novel variants. Using a 2-stage design, we first genotyped these single-nucleotide polymorphisms (SNPs) along with 163 tagSNPs and 12 additional SNPs (n=182 total) in 457 individuals with documented coronary artery disease. We identified 9 SNPs associated with HDL-C (P<0.05), with the strongest results for rs11144782 and rs11144766 (P=0.002 and P=0.005 respectively). The SNP rs11144782 was also associated with very low-density lipoprotein (P=0.039), TG (P=0.049) and total apolipoprotein levels (P=0.022). In stage 2, we replicated the association of rs11144766 with HDL-C (P=0.014) in an independent sample of Finnish low HDL-C families. In a combined analysis of both stages (n=883), region-wide significance of rs11144766 and low HDL-C was observed (unadjusted P=1.86x10^{-4} and Bonferroni adjusted P=0.031).

Conclusions—We conclude that variability at the *PCSK5* locus influences HDL-C levels, possibly through the inactivation of endothelial lipase activity, and, consequently, atherosclerotic cardiovascular disease risk.

2.3 Introduction

Low levels of high-density lipoprotein cholesterol (HDL-C) constitute a major risk factor for coronary artery disease (CAD) (Iatan et al., 2008). Genetic

factors account for a large component of the plasma variability of HDL-C levels with heritability being approximately 0.50 (Dastani et al., 2006b). Numerous human genetic studies have identified loci contributing to HDL-C, including both linkage analyses and association studies, explaining some these variations. Despite these, the genetic and metabolic factors that regulate HDL metabolism remain incompletely understood.

The metabolism of HDL is complex and involves a carefully orchestrated interplay between the biogenesis of nascent HDL particles (Hassan et al., 2008), the continual exchange of lipid and protein moieties of HDL in plasma and the modulation of HDL particles by a variety of enzymes, especially lipases (Lewis and Rader, 2005). Endothelial lipase (EL), discovered by Rader and colleagues (Jaye et al., 1999), is a phospholipase present on vascular endothelial cells. It can be inactivated by angiopoetin-like protein 3 (ANGPTL3) (Shimamura et al., 2007) or by secretory proprotein convertases of the subtilisin/kexin type, such as Furin, PC5/6, and PACE4 (Jin et al., 2007). The mammalian proprotein convertases comprise a family of nine members related to bacterial subtilisin and yeast kexin-like serine proteinases, critically involved in the activation/inactivation of various physiological and pathological processes, including those implicated in regulation of vascular events. These include PC1/3, PC2, Furin, PC4, PC5/6, PACE4, PC7, SKI-1/S1P and PCSK9 (Seidah et al., 2006), encoded by the genes *PCSK1* to *PCSK9*.

Although PCSK9 has been found to play a critical role in regulating lipid levels by enhancing low-density lipoprotein (LDL) receptor degradation (Maxwell and Breslow, 2004a), proof of *in vivo* functions of the proprotein convertase subtilisin/kexin type 5 (*PCSK5)* (On-line Mendelian Inheritance in Man: 600488) and its protein, PC5/6, in dyslipidemia and cardiovascular pathologies, has yet to be established. Murine *Pcsk5* is localized on chromosome 19 and encodes two alternatively spliced isoforms, soluble PC5A (915 amino acids; 21 exons) and membrane bound PC5B (1877 amino acids 38 exons) (Lusson et al., 1993). Although devoid of a transmembrane domain, PC5A can exert its proteolytic action at the cell surface, as it is retained at the plasma

membrane as a complex with tissue inhibitors of metalloproteases (TIMPs) and heparin sulfate proteoglycans (HSPG) (Nour et al., 2005). The essential role of *Pcsk5* has been highlighted by Essalmani et al. (Essalmani et al., 2008) who observed death at birth in the knock-out mice, while heterozygotes were healthy and fertile. Except in the liver where both isoforms are equally expressed, PC5A is the major isoform in most tissues (87% to 100%), and only the intestine and kidney show a predominance of PC5B (74% to 92%) (Essalmani et al., 2008; Essalmani et al., 2006). In humans, *PCSK5* is located on chromosome 9q21.13, and while 2 alternatively spliced transcripts are described for this gene (Miranda et al., 1996; Nakagawa et al., 1993), only one, generating a 21-exon isoform and typically referred to as *PCSK5*, has its full length nature known (NM_006200.3).

There is strong biological plausibility for the involvement of *PCSK5* in lipoprotein metabolism. Jin et al. (Jin et al., 2007)showed that PC5A inactivates *ex vivo* EL and lipoprotein lipase (LPL), with both lipases being present at the vascular endothelial surface. High expression of PC5/6 in enterocytes also suggests a possible role in processing protein substrates that could regulate food and/or sterol/lipid absorption (Lusson et al., 1993). Additionally, recent data from a genome-wide scan reports a region close to *PCSK5* on chromosome 9, implicated in lipid regulation (Falchi et al., 2005), but its link to HDL deficiency remains to be explored. In light of this, there is compelling evidence that suggests that PC5/6 may be a good candidate proteinase implicated in the control of circulating HDL-C levels and mutations affecting its function may influence lipoprotein metabolism and HDL subspecies more specifically. Therefore, we undertook a study to determine whether genetic variation within the *PCSK5* gene contributes to variation in HDL-C levels, which consequently, could influence atherosclerotic cardiovascular disease risk.

2.4 Results

2.4.1 Familial Segregation Analyses

We first investigated whether genetic variability at the *PCSK5* locus was associated with HDL-C in a Mendelian fashion. We examined the

segregation of *PCSK5* haplotypes with a severe HDL-C deficiency trait (HDL-C<10th percentile) in nine unrelated multigenerational families of French Canadian descent (175 subjects, mean number per kindred 17). Our study included 71 men and 104 women, of which 29 and 31 were affected (HDL-C<10th percentile), respectively. Mean age for males was 50±16 years and 48±19 years for women; the mean HDL-C level in affected men was 0.64±0.08 mmol/L, compared with 1.12±0.22 mmol/L in nonaffected men. Similarly, HDL-C levels in affected women were 0.87±0.15 mmol/L versus 1.39±0.26 mmol/L in non-affected. Four microsatellites, D9S1777, D9S1876, D9S175 and D9S1843, located upstream and downstream of the *PCSK5* locus and spanning 11.3 Mb within the 9q21.13 region, were used to construct haplotypes at the *PCSK5* gene. Only, one small (n=7 subjects) kindred was observed to display perfect segregation with HDL-C levels (data not shown). Thus, cumulatively, we did not find unambiguous segregation of this locus with the low HDL-C trait using a dominant model of inheritance, suggesting that *PCSK5* does not exert a Mendelian monogenic effect on HDL-C in these families.

2.4.2 Sequencing

We undertook a thorough examination of the *PCSK5* gene locus for coding and noncoding variants. Sequencing at the *PCSK5* locus was performed on all 21 exons and exon-intron boundaries using 22 pairs of primers for a total of 7455 bps in 12 unrelated individuals with low HDL-C levels (<5th percentile). We identified a total of 19 polymorphisms, 7 of which were novel noncoding variants (**Table 2.1**, **Figure 2.1**). Of the 12 previously characterized SNPs, we observed 4 synonymous polymorphisms (rs7040769, rs7020560, rs2297342 and rs10521468), 1 SNP in the 5'untranslated region (rs12005073), and 7 intronic. Two of the novel variants were insertions: 1 in intron 19 (IVS19-71insTAAAA) and the other in the 5'untranslated region (c.385insGAGCTGCGGCGGCCCGGGGCTGC). We also found a deletion in intron 20 (IVS20-50delTACTTTCAGGACTAAT), a variant in intron 4 (IVS4-3016T>A), and 3 polymorphisms in the 5' and 3'untranslated regions (c.125C>A; c.72C>T; c.323G>A respectively) (**Table 2.1**).

Table 2.1 PCSK5 Polymorphisms Identified by Sequencing

Functional Class		Location	Validated SNPs*	Novel Variants
Exonic	Synonymous	Exon 1	rs7040769	
		Exon 1	rs7020560	
		Exon 12	rs2297342	
		Exon 18	rs10521468	
	5'UTR		rs12005073	c.125C>A
				c.72C>T
				c.385insGAGCTGCGGCGGCCCGGGGCTGC
	3'UTR			c.323G>A
Intronic		Int.4		IVS4-3016T>A
		Int.7	rs2297344	
		Int.8	rs1416547	
		Int.11	rs3824474	
		Int.16	rs2270570	
		Int.17	rs1537183	
		Int.19	rs3830384	IVS19-71insTAAAA
		Int.20	rs10869726	IVS20-50delTACTTTCAGGACTAAT

UTR indicates untranslated region; Int, intron.

*Validated SNPs correspond to variants identifying by sequencing, but previously characterized.

Table 2.2 Association Results of Stage 1 Analysis for the HDL-C Trait

SNP	Effect (β)	*P*	MAF
rs11144782*	-0.076	0.002	0.164
rs11144766*	-0.063	0.005	0.197
rs1339246	0.056	0.018	0.176
rs1331384	0.037	0.038	0.485
rs11144688	-0.053	0.039	0.137
rs11144690	-0.093	0.040	0.040
rs1338746	-0.036	0.044	0.424
rs4745522	0.051	0.045	0.143
rs2050833	0.045	0.045	0.199

Quantitative association analysis for HDL-C in the French Canadians was performed using linear regression, after adjustments for age and sex, under an additive model. β is the linear regression coefficient corresponding to the effect size per copy of the minor allele.

*Significant variants ($P \leq 0.01$) selected for stage 2 analysis.

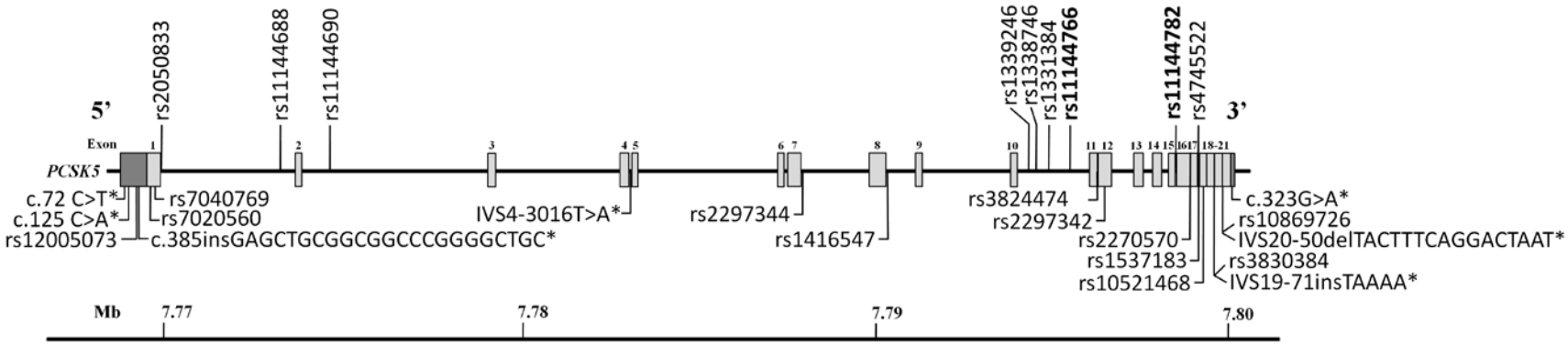

Figure 2.1 SNP locations in the PCSK5 gene.
Schematic representation of the human PCSK5 gene locus showing the exon structure and the location of the 19 polymorphisms (bottom panel) discovered through sequencing and the 9 genetic variants associated with HDL-C (upper panel) identified by genotyping. SNPs in bold are associated with HDL-C with P<0.01. Locations are based on RefSeq NM_006200.3.

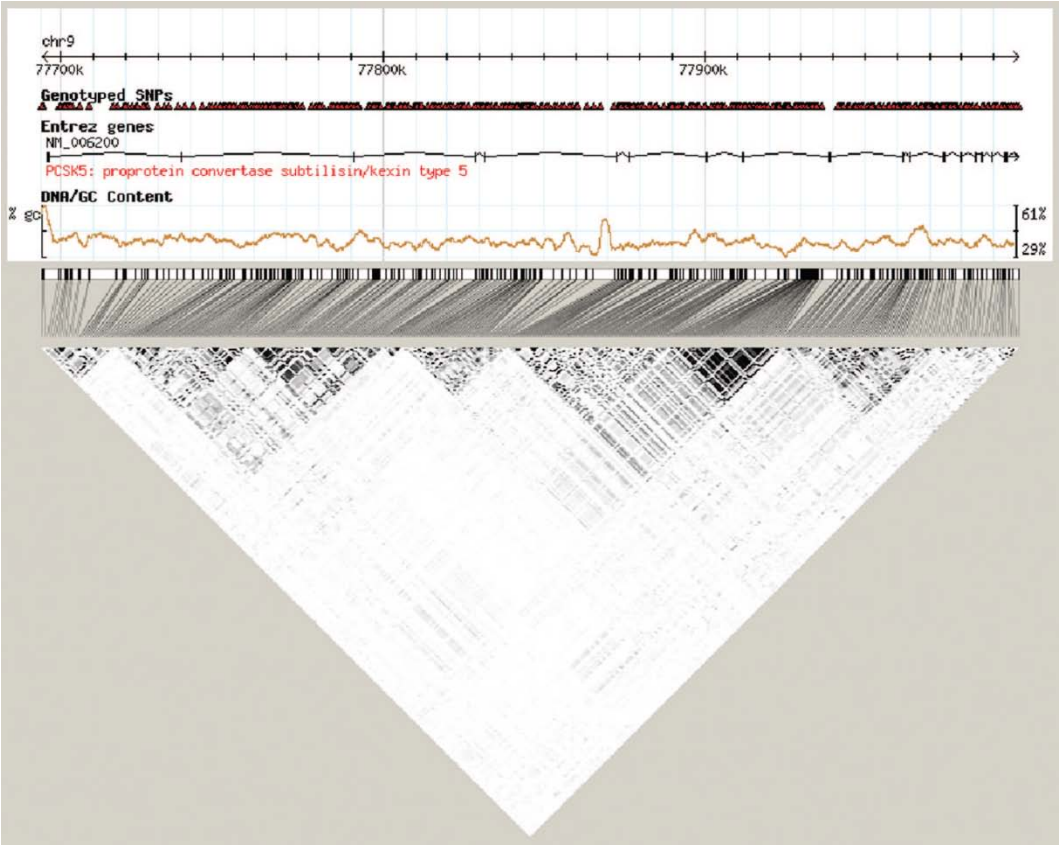

Figure 2.2 LD map of SNPs investigated in the PCSK5 gene
The LD map was generated using Haploview (Barrett et al., 2005). Numbers and white to black shading indicate r^2 values (black, high; white, low).

2.4.3 Association studies

To investigate whether common genetic variants at the *PCSK5* locus influence HDL-C levels and therefore explain some of the interindividual variation of HDL-C plasma concentrations, we conducted a quantitative association analysis utilizing a two-stage approach. In stage 1, we genotyped 169 tagSNPs in 457 unrelated subjects of French Canadian descent to screen for associations. In stage 2, we genotyped significant signals (P<0.01) in Finnish low HDL families, and subsequently performed a combined analysis of the two stages to identify variants of region-wide significance. (Skol et al., 2006) originally introduced this approach to reduce the cost of genotyping in stage 1 while maintaining the overall power of the study.

In stage 1, a total of 169 SNPs (**Figure 2.2**) were tested for association in 457 French Canadian individuals (Weber et al., 1997). Using an additive model and adjusting for age and sex as covariates, we identified 9 SNPs significantly associated with HDL-C (P<0.05), with the strongest results being rs11144782 and rs11144766 (MAF 0.164, β=-0.076 mmol/L, P=0.002; MAF 0.197, β=-0.063 mmol/L, P=0.005 respectively; **Table 2.2, Figure 2.1)**.

The rare G-allele of rs11144782 decreased HDL-C levels by 0.076 mmol/L on average per allele, while the A risk allele of rs11144766 decreased plasma HDL-C levels by 0.063 mmol/L. The effect of these minor alleles on HDL-C are displayed in **Table 2.3**. In addition, of the 9 polymorphisms identified, three other SNPs were shown to be significantly associated with decreased plasma HDL-C levels (rs11144688, rs11144690, and rs1338746) while four others were associated with an increase in HDL-C (rs1339246, rs1331384, rs4745522, rs2050833) (**Table 2.2**). These 9 variants were all found in non-coding regions and were not in linkage disequilibrium (LD).

We next tested our two most significant polymorphisms (P<0.01), rs11144782 and rs11144766, for association with other lipoprotein traits and, adjusting for age and sex under an additive model, observed a significant positive effect on plasma TG (P=0.049), very low-density lipoprotein cholesterol (VLDL-C; P=0.039), and apolipoprotein B (P=0.022) levels (**Table 2.4**) for rs11144782, suggesting that it modulates several aspects of lipid metabolism. We also conducted a stepwise conditional regression analysis in the presence of rs11144782 and age and sex as covariates. Using this approach, we re-identified rs11144766 (P<0.001) and two other nonredundant SNPs (rs2050833, P<0.036; rs4745488, P<0.038) that contributed to the variability of HDL-C (**Table 2.5**) independently of one another, providing further evidence for the role of *PCSK5* in HDL-C metabolism.

In stage 2, we followed up rs11144782 and rs11144766 which provided the most significant associations ($P\leq$0.01) in the French Canadian cohort, for replication in an independent sample of 39 low HDL-C Finnish dyslipidemic families (n=426) (Pajukanta et al., 2003). We tested for association between these

variants and low HDL-C using the quantitative transmission disequilibrium test with age and sex as covariates. While we did not observe an association with rs11144782, rs11144766 was found to be significantly associated with HDL-C in the same direction as in the French Canadians (P=0.014).

Next, we performed a combined analysis of the stage 1 and 2 unrelated and family samples (n=883) for the two SNPs by combining the Z statistics, as described in Materials and Methods. We observed a strong association between rs11144766 and low HDL-C (P=1.86x10^{-4}) for the additive model and the same A risk allele. This result is region-wide significant: it surpasses the Bonferroni correction for all 171 tests performed [169 SNPs tested in stage 1 and 2 SNPs in stage 2 (Bonferroni adjusted P=0.031)]. Thus, the association between rs11144766 and HDL-C in these Finnish dyslipidemic families is consistent with the results from the French Canadian unrelated individuals, and provides strong evidence for the influence of rs11144766 on HDL-C levels.

Table 2.3 Distribution of Genotypic Classes for rs11144782 and rs11144766 in the Stage 1 French Canadian Subjects

	rs11144782			rs11144766		
Genotypes (n)	CC (n=301)	GC (n=123)	GG (n=10)	GG (n=275)	AG (n=128)	AA (n=19)
Frequency	0.69	0.28	0.02	0.65	0.30	0.04
Mean HDL-C* (mmol/L)	0.98±0.29	0.89±0.24	0.8±0.20	0.97±0.30	0.91±0.23	0.85±0.18

Analyses were performed using PLINK version 1.04 with age and sex as covariates, under an additive model.

*The mean±SD of HDL-C are stratified by genotype, and weighted by the frequencies of their genotypic class.

Table 2.4 Lipid Traits Associated with rs11144782

SNP	Trait	Effect (β)	*P*
rs11144782	HDL (mmol/L)	-0.076	0.002
	TG (mmol/L)	0.502	0.049
	VLDL (mmol/L)	0.169	0.039
	apoBtot (mg/dL)	10.720	0.022

Analyses were performed using PLINK version 1.04 with age and sex as covariates, under an additive model. β is the linear regression coefficient, corresponding to the effect size per copy of the minor allele. apoBtot indicates total apolipoprotein B.

Table 2.5 Independent Signals Found at the *PCSK5* Locus for the HDL-C Trait

SNP	Effect (β)	*P*
rs11144782	-0.076	0.002
rs11144766	-0.072	0.001
rs2050833	0.047	0.036
rs4745488	-0.039	0.038

Conditional analyses were performed using stepwise linear regression adjusting for age, sex, rs11144782, rs11144766 and rs2050833, respectively. β is the linear regression coefficient, corresponding to the effect size per copy of the minor allele.

2.5 Discussion

The investigation of the molecular genetics and pathophysiology of HDL-C deficiency has been an area of fertile research. Despite a large body of information identifying HDL-C as a potent antiatherosclerosis lipoprotein, the fundamental mechanisms underlying the genetic regulation of the HDL-C metabolic pathway remain complex and poorly understood.

In this study, we have demonstrated that genetic variability at the *PCSK5* gene modulates HDL-C levels. By sequencing the gene, we identified seven novel non-coding variants in patients with HDL-C deficiency (**Figure 2.1**). Although none of these newly identified variants represent a missense, frameshift or non-sense polymorphism with obvious functional consequences, the possibility of their regulatory role cannot be excluded and further studies to delineate their mechanistic effects are needed. We next performed an association study of *PCSK5* SNPs with HDL-C through a two-stage study design. This approach was an efficient way to optimize the power to detect true associations, while minimizing the overall amount of genotypes required for sufficient regional coverage (Skol et al., 2006). In stage 1, we investigated the association of *PCSK5* SNPs with HDL-C levels in 457 unrelated subjects of French Canadian descent, using quantitative analyses. We identified nine significant SNPs ($P<0.05$, **Figure 2.1, Table 2.1**), five of them being associated with a decrease and four with an increase in HDL-C plasma concentration. The strongest signals, rs11144782 and rs11144766 (MAF 0.164, $\beta=-0.076$, $P=0.002$; MAF 0.197, $\beta=-0.63$, $P=0.005$ respectively), were found to be negative modulators of HDL-C levels, displaying an allele dosage-effect (**Table 2.3**): the minor allele (G) of rs11144782 was observed to contribute to a decrease of 8% in plasma HDL-C levels, while the minor allele (A) for rs11144766 lowered HDL-C serum concentration by 6% (**Table 2.2** and **2.3**). Interestingly, the MAF of rs11144782 and rs11144766 in the HapMap-CEU samples (MAF 0.156 and 0.142) was in concordance with both the French Canadian (MAF 0.164 and 0.197) and the Finnish study cohorts (MAF 0.168 and 0.197). In stage 2, we followed up significant markers in the previously mentioned independent study sample consisting of low HDL Finnish

families to confirm the observed associations. By means of a combined analysis of both stages (n=883), region-wide significance between rs11144766 and low HDL-C was observed (unadjusted $P=1.86x10^{-4}$). Using the Bonferroni correction, rs11144766 remained significant ($P=0.031$) after adjusting for multiple testing (n=171), providing sound and consistent evidence for its role in HDL-C metabolism.

Furthermore, in conditional regression analysis, we observed two additional SNPs putatively contributing to HDL-C ($P<0.05$), independent of the effects of rs11144782 and rs11144766 (**Table 2.5**). As a result, in the present study, we identified four signals at the *PCSK5* locus, independent of one another. Interestingly, in stage 1 analysis, the rs11144782 variant was also associated with other lipid traits including VLDL-C ($P=0.039$), TG ($P=0.049$) and total apoB ($P=0.022$) levels (**Table 2.4**), translating in an absolute increase in VLDL-C (0.169 mmol/L), TG (0.502 mmol/L), and apoB (10.72 g/L) per allele respectively, suggesting that *PCSK5* genetic variability may influence other aspects of lipoprotein metabolism and not solely HDL-C.

The 2 polymorphisms identified in this study, rs11144766 and rs11144782, are both located in introns of *PCSK5*. rs11144766 is found in the 10^{th} intron, between exons coding for a region of the catalytic domain of PC5/6, while rs11144782 occurs in the 15^{th} intron, between exons encoding the cysteine-rich domain (CRM) of PC5/6 (**Figure 2.1**). Despite their intronic localization, we show here that both of these SNPs are important regulators of HDL-C levels, potentially mediating PC5/6 activity.

There are several possible explanations for such effects. These SNPs may be involved in regulating splicing of the *PCSK5* transcript, to either enhance or suppress proper intron-removal. To explore this possibility, we used the ACESCAN2 Web Server (http://genes.mit.edu/acescan2/index.html) to scan for possible sites that may affect splicing. We identified a specific GTGTGG sequence present in the rare A-allele of rs11144766 as an Intronic Splicing Enhancer (ISE). This suggests that individuals carrying this variant may have modified splicing of the *PCSK5* gene which could impact PC5/6 activity. The

catalytic domain of *PCSK5* is crucial for its proteolytic convertase function, necessary for EL cleavage. Indeed, deleting exon 4 resulted in embryonic lethality (Essalmani et al., 2008; Essalmani et al., 2006), stressing the importance of this domain. In contrast, while no ISEs were found to be associated with rs11144782, it may have other effects on splicing which could alter PC5/6 function. The cysteine-rich domain of the latter is involved in regulating PC5/6A localization, which, in combination with TIMPs, binds to HSPG at the cell surface (Seidah and Prat, 2007). Data reveals that the cysteine-rich domain confers protein-protein interactions, cell surface tethering and is essential for the efficacious processing of the human proEL precursor, likely due to a proximity effect resulting from close juxtaposition of the convertase and EL through interactions with cell-surface HSPGs (Seidah et al., 2006; Nour et al., 2005). Therefore, altering either the cysteine-rich domain or the catalytic domain would likely impair PC5/6 activity and, indirectly, overexpress EL. Subsequently, this might explain the observed decrease in HDL-C levels in individuals carrying these SNPs. Beyond the possibility of modulating splicing, these variants may be located in important binding sites for unknown factors, such as microRNAs. Furthermore, it is also possible that they are in LD with other un-genotyped SNPs.

Though we have highlighted the importance of rs11144782 and rs11144766 in this study, much work remains to be done to better elucidate their functional mechanisms and effects on PC5/6 activity. Despite this, our findings suggest an *in vivo* conceptual mechanism for how rs11144766 and rs11144782 could potentially affect HDL-C metabolism (**Figure 2.3**). We propose that by altering PC5/6 function, these variants could prevent the internal cleavage of the HSPG-bound EL by PC5/6 or its inhibition by activated ANGPTL3. As a result, EL will be fully active and its effect on HDL unopposed, resulting in a pronounced phospholipase activity and consequently, hydrolysis of HDL phospholipids that will produce smaller HDL particles. This will reduce plasma HDL-C levels, in concordance with studies in which mice overexpressing human EL revealed a marked depletion in HDL-C levels (Jaye et al., 1999). The EL-mediated reduction of HDL-phospholipids can also alter the lipid composition and

physical properties of HDL, resulting in a diminished ability of HDL to mediate scavenger receptor class B type I-dependent cholesterol efflux (Yancey et al., 2004). Additionally, association of rs11144782 with increased VLDL-C, TG and apoB levels implicates the effects of other lipases, such as LPL and hepatic lipase. Further studies are needed, however, to elucidate their exact physiologic role on plasma lipoprotein metabolism in the presence of *PCSK5* variants affecting PC5/6 function. Indeed, a PC5/6 loss of activity could allow the activation of the heparin-like glycosaminoglycans-bound LPL and its triglyceride hydrolase function on chylomicrons and VLDL, subsequently decreasing them. Similarly, ANGPTL3, a liver-derived member of the vascular endothelial growth factor family, shown to be an endogenous inhibitor of EL (Shimamura et al., 2007) in cell-free systems, plays a potential role in regulating HDL levels (Koishi et al., 2002). In line with the study done by Shimizugawa et al. (Shimizugawa et al., 2002) in which overexpression of ANGPTL3 in KK/San mice resulted in a marked increase of TG-enriched VLDL levels through the inhibition of LPL activity, loss of hepatic PC5/6 activity under pathophysiological, genetic or diseased conditions could increase EL and LPL activities, resulting in reduction of plasma HDL-C, VLDL-C and TG concentrations. Thus, there are several venues by which *PCSK5* variants could impact lipoprotein metabolism. The relative contribution of these pathways upon PC5/6 inactivation is still unknown, and further work is required to determine the overall importance of each component in the system.

Given the significance of our results, a clearer understanding of the molecular interactions between the PC5/6-EL system and HDL structure, as well as the direct impact of HDL remodeling by PC5/6-EL on the reverse cholesterol transport process and endothelial function, should be the focus of future scientific studies. It would also be essential to replicate our findings in larger and more diverse study samples (Kathiresan et al., 2008a) and functionally characterize these variants. Accordingly, unravelling the *in vivo* and *in vitro* effects of the PC5/6-EL system could refine our comprehension of the complex HDL metabolic

pathway and provide novel insights into the human atheroprotective system in health and disease.

In conclusion, we observed an association of region-wide significance between rs11144766 and HDL-C under an additive model in an unrelated and family-based sample (n=883). These results support the contribution of *PCSK5* to HDL-C levels and its pivotal role in HDL-C metabolism. While previous work by Cao et al. (Cao et al., 2001) identified 2 silent SNPs in *PCSK5* varying in frequency among ethnic groups, no other studies thus far have analyzed the genetic variability at the *PCSK5* gene locus and its contribution to HDL-C levels. This report is therefore the first comprehensive examination of such genetic variation, implicating *PCSK5* as an important and influential modulator of HDL-C serum levels in humans. These findings can thus firmly place *PCSK5* on the list of genes associated with HDL-C and emphasize the need to investigate PC5/6 and its related substrates for identification of specific therapeutic targets for treatment of cardiovascular disease.

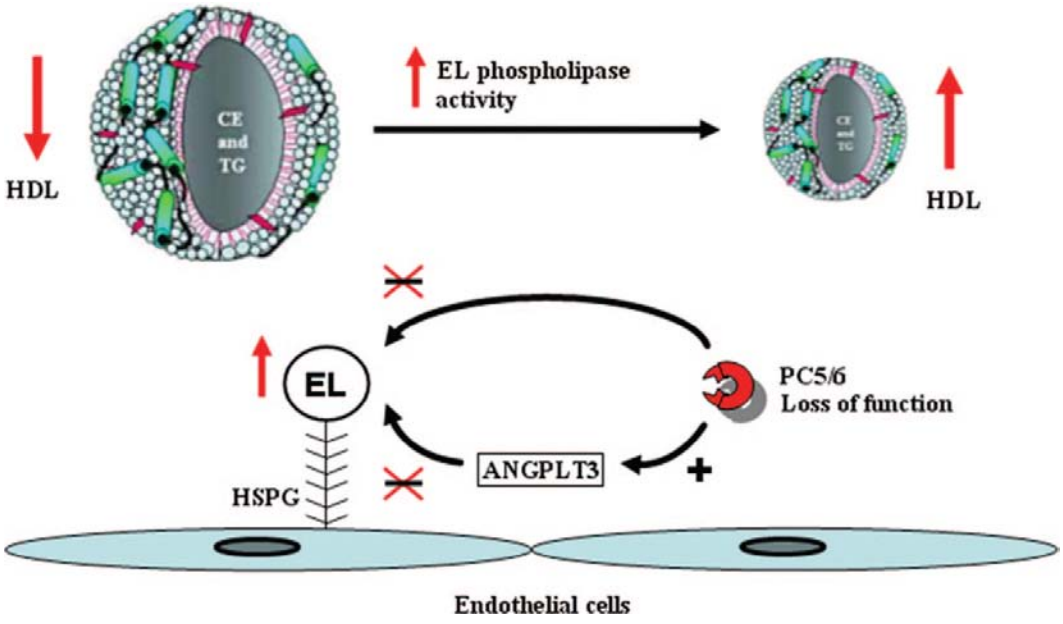

Figure 2.3 Conceptual model of PCSK5 variants' effect on EL activity
Genetic variation at PCSK5 resulting in complete or partial PC5/6 loss of function, may be responsible for the inability of PC5/6 to activate ANGPLT3 or directly inactivate EL. This will overexpress EL, increasing phospholipase activity on HDL particles resulting in smaller HDL with decreased cholesterol content.

2.6 Acknowledgements

We thank the French Canadian and Finnish families who participated in this study. Leena Peltonen is thanked for sample collection, as well as E. Nikkola and M. Lupsakko for their laboratory technical assistance. We gratefully acknowledge the Montreal Genome Centre staff for their sequencing and genotyping services.

This work was supported by the Canadian Institutes of Health Research (MOP 62834, MOP 44363, and CTP 82946) and the Heart and Stroke Foundation of Quebec. Dr Genest holds the McGill Novartis Chair in Cardiology at McGill University. I. Iatan is a recipient of a Doctoral Research Award from the Fonds de la Recherche en Sante du Quebec and a Frederick Banting and Charles Best Canada Graduate Scholarships Doctoral Award from the Canadian Institutes of Health Research. Dr Dastani is a recipient of a Doctoral Research Award from the Heart and Stroke Foundation of Canada, and Dr Do holds a Canada Graduate Scholarship Doctoral Award from Canadian Institutes of Health Research. This study was also funded by the National Institutes of Health (HL-28481 and HL082762 to P.P.); the Clinical Research Institute Helsinki University Central Hospital Ltd (to M.-R.T.); and the Finnish Heart Foundation (to M.-R.T.). Drs Weissglas-Volkov and Huertas- Vazquez are supported by the National Human Genome Research Institute (T32 HG02536) and the American Heart Association (0725232Y), respectively.

2.7 Materials and Methods

2.7.1 French Canadian family subjects

A total of nine multigenerational French Canadian families consisting of 175 genotyped members were examined and sampled in the Preventive Cardiology/Lipid Clinic of the McGill University Health Centre (MUHC). The selection criterion for probands was HDL-C<5th percentile (age and gender-matched), based on the Lipid Research Clinics Population Studies Data Book (NIH Washington DC, 1980). All subjects provided informed consent for plasma

and DNA sampling, isolation, and storage. The research protocol was reviewed and approved by the Research Ethics Board of the MUHC.

2.7.2 Stage 1 and 2 association study samples

2.7.2.1 French Canadian subjects

For stage 1 analysis, unrelated patients (n=457) of French Canadian descent were selected from the Cardiology Clinic of the Clinical Research Institute of Montreal that were <60 years of age and had angiographically documented CAD (Weber et al., 1997). Individuals had HDL-C values ranging from HDL-C<5^{th} percentile to HDL-C>95^{th} percentile (age and gender-matched) (NIH Washington DC, 1980). We excluded patients with known causes of HDL deficiency (severe hypertriglyceridemia defined as plasma triglycerides (TG)>10 mmol/L, cellular phospholipid or cholesterol efflux defect or previously identified mutations in genes associated with HDL deficiency) (Marcil et al., 1999b; Alrasadi et al., 2006). Demographic and clinical information, medications, and lipoprotein profiles were determined on all participating subjects as previously described (Marcil et al., 1999b). The research protocol was approved by the Research Ethics Board of the MUHC.

2.7.2.2 Finnish family subjects

The stage 2 study sample consisted of 39 Finnish low HDL-C families (426 genotyped individuals) recruited at the Helsinki and Turku central hospitals as previously described (Pajukanta et al., 2003). Each subject involved in this study provided written informed consent. The study design was approved by the ethics committees of the participating institutions. Inclusion criteria for the probands were age 30-60 years, at least 50% stenosis in one or more coronary arteries, HDL-C level below the Finnish age- and sex-specific 10^{th} population percentile (subjects coded as affected), total cholesterol (TC)<6.3 mmol/L for males and <6.0 mmol/L for females, and TG<2.3 mmol/L for both genders (Pajukanta et al., 2003). Serum lipid and glucose parameters were measured as previously (Pajukanta et al., 1998). Exclusion criteria for the probands were type

1 and 2 diabetes mellitus, severe hepatic or renal disease, or body mass index >30 (Pajukanta et al., 2003)

2.7.3 Haplotyping

Microsatellite genotypes were determined by deCODE Genetics (Reykjavik, Iceland) at markers D9S1777, D91876, D9S175, D9S1843 spanning 11.3 Mb and flanking the *PCSK5* gene on chromosome 9q21.13. Haplotypes were constructed using Cyrillic version 2.1.3 (Cherwell Scientific Publishing Ltd, Oxford, United Kingdom) to examine the segregation of the *PCSK5* locus with the low HDL-C trait in nine families with HDL-C deficiency.

2.7.4 Sequencing

Sequencing of the 21 exons and exon-intron boundries of the *PCSK5* gene was performed in 12 unrelated individuals with HDL-C<5^{th} percentile. Exon-specific oligonucleotides were synthesized (Integrated DNA Technologies, Coralville, Iowa) and designed using the Primer3 software (http://frodo.wi.mit.edu/) (Rozen and Skaletsky, 2000) to include at least 22 bp of intronic sequence at each intro-exon boundary. Polymerase chain reaction (PCR)-amplified fragments were purified using the Millipore purification plate (Multiscreen PCR) and directly sequenced at the McGill University and Genome Québec Innovation Centre Sequencing Platform using the Applied Biosystems 3730-xl DNA Analyzer system. The data were analyzed by Sequencing Analysis version 5.2 and Mutation Surveyor version 2.41 (SoftGenetics, State College, Pa).

2.7.5 Single nucleotide polymorphisms (SNPs) selection

To select the most informative SNPs for the first-stage genotyping of *PCSK5*, we utilized a tagSNP strategy using HapMap Utah Residents with Northern and Western European Ancestry (CEU) spanning 304 714 bp of genomic DNA and including 2kb upstream of *PCSK5* (HapMap Rel27 PhaseII+III; Haploview version.4.0 (Barrett J.C.)). We used a minor allele frequency (MAF)>0.05 and r^2 threshold of 0.80. In addition, we included novel

variants identified through sequencing (n=7), as well as SNPs selected from public genetic databases [(NCBI, UCSC, SeattleSNPs, and Human SNP (Broad Institute)] (n=12), for a total of 182 SNPs to be genotyped in stage 1.

In the second stage, out of 9 SNPs that provided significant evidence of association with $P<0.05$, we selected 2 SNPs with $P\leq 0.0\ 1$ for stage 2 genotyping.

2.7.6 Genotyping

Stage 1 genotyping of 182 SNPs was performed using the Sequenom iPLEX Gold Assay (Sequenom, Cambridge, Mass). Locus-specific PCR primers and allele-specific detection primers were designed using MassARRAY Assay Design 3.1 software. DNA was amplified in a multiplex PCR and labeled using a single base extension reaction. The products were desalted and transferred to a 384-element SpectroCHIP array. Allele detection was performed using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Mass spectrograms and clusters were analyzed by the TYPER 3.4 software. Ehrich et al. (Ehrich et al., 2005) have previously provided details of the procedure. Prior to association analysis, quality control-check was performed by assessing integrity of genotypic data. We obtained a 96% success rate for the SNPs and 99% of subjects (451 individuals) were successfully genotyped. For the remaining SNPs, a genotyping call rate >98% was obtained. After frequency and genotype pruning, there were 169 SNPs that were analyzed.

Genotyping of the 2 second-stage replication SNPs was performed in the Finnish low HDL families using the pyrosequencing technique on the PSQHS96A platform with a >94% genotyping call rate. Both SNPs were in Hardy-Weinberg Equilibrium in the unrelated founders ($P>0.5$). The Pedcheck program was used to detect Mendelian errors in the families (O'Connell and Weeks, 1998).

2.7.7 Statistical analyses

Statistical analyses for the French Canadian association study were performed with PLINK version 1.04 software (http://pngu.mgh.harvard. edu/ purcell/plink/) (Purcell et al., 2007) and the SAS package version 9.1.3 (SAS Institute Inc., Cary, NC). Quantitative association analysis for HDL-C was performed using linear regression, after adjustments for age and sex. The additive model was tested in the stage 1 analysis as it has been shown to be robust for detecting association even when the true genetic model is not additive (Tu et al., 2000). We estimated the effect of significant SNPs on the basis of the linear regression coefficient (β). Conditional analyses were performed using step-wise linear regression. Significance was set at $P<0.05$.

Association analysis in the Finnish family cohort was performed using the quantitative transmission disequilibrium test (QTDT) (Abecasis et al., 2000) implemented in the genetic analysis package SOLAR (Havill et al., 2005). The quantitative transmission disequilibrium test approach approach is robust to population stratification (Havill et al., 2005) and has been recognized as a powerful method that utilizes data from all available relatives. The orthogonal model of association within a variance component framework that included age and sex as covariates (Abecasis et al., 2000) was used in our analyses, where the total association was partitioned into orthogonal within and between family components.

We also performed a combined analysis of both stages for rs11144766 and rs11144782, using the Z-method to combine statistics. Test statistics from the French Canadian cohort and the Finish family-based study were weighted by the square-root of the sample size to calculate the corresponding combined *P* value (Skol et al., 2006). To correct for multiple testing, we adjusted for 169 SNPs tested in stage 1 as well as for the 2 SNPs tested in stage 2, resulting in a Bonferroni correction for 171 independent tests in the overall combined analysis.

2.7.8 Statement of responsibility

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

CHAPTER 3. EXOME SEQUENCING IDENTIFIES TWO RARE VARIANTS FOR LOW HDL-C IN AN EXTENDED FAMILY

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This research was accepted on July 23rd 2012 in the Journal of the American Heart Association, Circulation Cardiovascular Genetics. MV Prasad Linga Reddy[1#], PhD; Iulia Iatan[#2], PhD student; Daphna Weissglas-Volkov, PhD;[1] Elina Nikkola, Graduate Student;[1] Blake E. Haas, PhD;[1] Miina Juvonen, Student Volunteer;[1] Isabelle Ruel, PhD;[2] Janet S. Sinsheimer, PhD;[1,3] Jacques Genest, MD, PhD;[2] Päivi Pajukanta, MD, PhD[1]*.*Circ Cardiovasc. Genet*. accepted with no revisions on July 23rd 2012.

3.1 Preface and Rationale

In our search for novel genetic determinants of HDL-C, we identified in Chapter 2 that genetic variability at the *PCSK5* gene locus modulates HDL-C levels using familial segregation analyses and associations studies. This involved *a priori* biological evidence and knowledge of the target gene locus of interest, leading to subsequent investigation of its effect on HDL-C levels. In addition, the identified genetic variants are relatively common and their effects on the functionality of the PC5/6 protein require further validation. Similarly, multiple common genetic variants identified so far through GWAS account for a small proportion of serum HDL-C levels (10-15%) (Teslovich et al., 2010) and their functional effects on the protein and associated-disease of interest have not been all validated. Accordingly, this emphasizes the need to identify novel, highly penetrant-low frequency variants (MAF<0.05) for HDL-C that impart larger effects. As previously described, exome capture is an emerging technology that allows the identification of rare monogenic disorders that occur in protein coding genes. At the same time, there is a need to go beyond the molecular identification of Mendelian disorders, by utilizing exome sequencing to investigate causal-variants for multifactorial traits, such as low HDL-C. In this study, we therefore used whole exome sequencing to search for rare-low DNA sequence variants for the complex low HDL-C trait in a multigenerational French Canadian family. The results of this work have been accepted to *Circulation Cardiovascular Genetics.*

3.2 Abstract

Background- Whole exome sequencing is a recently implemented method to discover rare mutations for Mendelian disorders. Less is known about its feasibility to identify genes for complex multifactorial traits. We used exome sequencing to search for rare variants responsible for a complex trait, low levels of serum high-density lipoprotein cholesterol (HDL-C).

Methods and results- We conducted exome sequencing in a large multigenerational French Canadian family with 75 subjects available for study of which 27 had HDL-C values less than the 5^{th} age-sex specific population percentile. We captured ~50 Mb of exonic and transcribed sequences of three closely related family members with HDL-C levels $<5^{th}$ age-sex percentiles and sequenced the captured DNA using the Illumina Hiseq2000 platform. Approximately 82,000 variants were detected in each individual of which 41 rare non-synonymous variants were shared by the sequenced affected individuals after filtering steps. Two rare nonsynonymous variants in the ATP-binding cassette, sub-family A (ABC1), member 1 (ABCA1) and lipoprotein lipase (LPL) genes predicted to be damaging were investigated for co-segregation with the low HDL-C trait in the entire extended family. The carriers of either variant had low HDL-C levels and the individuals carrying both variants had the lowest HDL-C values. Interestingly, the ABCA1 variant exhibited a sex effect which was first functionally identified, and subsequently, statistically demonstrated using additional French Canadian families with ABCA1 mutations.

Conclusions- This complex combination of two rare variants causing low HDL-C in the extended family would not have been identified using traditional linkage analysis, emphasizing the need for exome sequencing of complex lipid traits in unexplained familial cases.

3.3 Introduction

Low HDL-C is the most common lipoprotein abnormality and established risk factor of coronary heart disease (CHD). Low HDL-C is caused by multiple genetic factors, common and rare, interacting with one another and with the environment and behavior. In the last two decades, significant effort has been

devoted to the identification of low HDL-C susceptibility genes. This was initially done using the genome-wide linkage analysis (Pajukanta et al., 2003; Soro et al., 2002). However, progress in identification of the actual disease genes was very slow despite the discovery of many linked intervals. More recently, genome-wide association studies (GWAS) have successfully identified multiple common variants associated with decreased levels of HDL-C (Teslovich et al., 2010).

However, the sum of common variants identified so far through GWAS explains only a small fraction (10-15%) of the variance in the HDL-C levels (Teslovich et al., 2010). Hence, it has become evident that other types of DNA variants must contribute substantially to HDL-C levels as well. To identify new rare and low-frequency variants underlying low HDL-C, massive parallel sequencing technologies can be utilized. The whole-genome sequencing is the most complete approach, but it remains significantly more expensive than exome sequencing that only analyzes coding and transcribed regions which constitute less than 5% of the whole genome sequence (Ng et al., 2010). It is estimated that the protein coding regions of the human genome constitute about 85% of the disease-causing mutations (Ng et al., 2010).

We used whole exome sequencing to search for rare variants conferring susceptibility to low HDL-C. We sequenced the exomes of closely related family members with low HDL-C from a large multigenerational French Canadian family with 75 subjects available for study and followed up the candidate variants by examining the co-occurrence patterns in the entire extended family.

3.4 Results

3.4.1 Exome sequencing

To identify rare genetic variants underlying low HDL-C, we sequenced the entire exomes (~50 Mb) of 3 family members with HDL-C less than the 5th age-sex specific population percentile from a large multigenerational French Canadian family. The three sequenced family members were closely related, as one affected, his sibling and child were sequenced. Exome capture and sequencing were performed using the Agilent Sure Select in-solution method and

Illumina Hiseq2000 platform as described in the Methods. We obtained an average of 90 million reads per person and successfully mapped ~90% of these reads to the reference sequence (**Table 3.1**). After quality control the mean coverage was 50X.

3.4.2 Filtering of identified variants

On average 82,000 SNVs were detected in each individual. We focused on variants shared by all three exome sequenced subjects and filtered the variants based on their type, frequency, and functional predictions. Filtering for missense and stop gain or stop loss variants that were shared by all three affected individuals resulted in 3,428 non-synonymous variants and 31 stop gain or loss variants (**Table 3.2**). The transition/transversion (Ti/Tv) ratio of the coding variants was 3.4, whereas the Ti/Tv ratio of the non-coding was 2.5, in good agreement with the expected ratios (Clark et al., 2011).

The identified variants were further filtered against variants present in the HapMap (Altshuler et al., 2010), 1000 Genomes Project (The 1000 Genomes Project Consortium, 2010), and dbSNP132 (The 1000 Genomes Project Consortium, 2010) databases, resulting in 332 novel variants and known variants with MAF<5%. These variants were further filtered by selecting variants predicted to affect protein function using PolyPhen (Ramensky et al., 2002) and SIFT (Ng and Henikoff, 2003) and expressed in a relevant tissue including liver, adipose, and heart, resulting in 41 shared potentially functional variants that were either novel or known but relatively rare (**Table 3.2**). Among the shared variants there were two rare functional variants in the ABCA1 and LPL genes that are excellent susceptibility candidates as their key role in HDL-C metabolism is well established. (Weissglas-Volkov and Pajukanta, 2010). We confirmed their presence by both Sanger sequencing and genotyping.

Table 3.1 Summary of reads mapped to the human reference genome (hg19)

Samples	L range*	H range†	Average
Total reads	87816621	103315979	95566300
Mapped reads	80911740(82.45%)	85185325(92.14%)	83048533(87.29%)
Duplicate reads	24513472(27.91%)	34633913(33.52%)	29573693(30.72%)
Unmapped reads	6904881(7.86%)	18130654(17.55%)	12517768(12.70%)
Uniquely mapped reads	50551412(48.93%)	56398268(64.22%)	53474840(56.58%)

*The lowest range limit of the total reads.
†The highest range limit of the total reads

Table 3.2 Number of variants shared by the 3 sequenced affected family members after a series of filtering steps

Variant Filter	Variants	Nonsynonymous	Stop gain or loss	Total
Shared by all 3*	Known†	3389	30	3419
	Novel	39	1	40
	Total	**3428**	**31**	**3459**
MAF<5% and new‡	Known	293	3	296
	Novel	39	1	40
	Total	**332**	**4**	**335**
Damaging§	Known	52	3	55
	Novel	24	1	25
	Total	**76**	**4**	**80**
Functional#	Known	18	3	21
	Novel	19	1	20
	Total	**37**	**4**	**41**

*****Nonsynonymous and stop gain or loss variants shared by all three sequenced individuals.
†Present in the HapMap, The 1000 Genomes Project and/or dbSNP132 databases.
‡Novel variants (not present in the HapMap, The 1000 Genomes Project and/or dbSNP132 databases) and known rare variants with a minor allele frequency (MAF) < 5%.
§Novel and known rare (MAF<5%) variants predicted to be damaging either by PolyPhen and/or SIFT.
#The novel and known rare (MAF<5%) variants located in genes functionally relevant to lipids; or in genes expressed in relevant tissue; and/or present within 500 kb of the 95 known lipid GWAS loci (Marcil et al., 1999b).

3.4.3 Rare missense variant in ABCA1

The ABCA1 (S1731C) variant is not present in dbSNP132 (The 1000 Genomes Project Consortium, 2010) or The 1000 Genomes Project data and is located in exon 38. This previously reported rare variant is changing a conserved amino acid from serine to cysteine and is known to result in decreased cholesterol efflux (Alrasadi et al., 2006; Clee et al., 2001; Brunham et al., 2005; Cohen et al., 2004).

In order to further determine the effect of the S1731C variant on cholesterol efflux, we used human fibroblasts from the affected proband homozygous for the S1731C variant, and compared these cells to a normal control. Assays were performed in 22OH/9CRA stimulated fibroblasts (to induce ABCA1 expression), and unstimulated cells, in the presence or absence of lipid free ApoA-I (**Figure 3.2A** (**Figure 3.1 is first referred to in Material and Methods**). We observed a significant decrease (~40%) in apoA-I-mediated cellular cholesterol efflux in the proband, as compared to the control without the ABCA1 variant ($P=1.23x10^{-4}$ using Student's *t*-test and P=0.045 using a non-parametric two-sample Wilcoxon rank sum test). These results are in agreement with previously documented findings (Alrasadi et al., 2006; Brunham et al., 2005; Cohen et al., 2004). Low efflux levels were also observed in unstimulated cells, presumably due to basal levels of ABCA1 expression and the presence of other apoA-I binding sites at the cell surface. Also, as expected, background basal conditions of passive diffusion of cellular cholesterol were not affected by mutations at the ABCA1 gene locus.

As the lipid levels of the ABCA1 S1731C variant carriers suggested a possible gene-gender effect (**Tables 3.3**, **3.4**), we further investigated whether exposure to 17β-estradiol steroid hormone endogenously expressed in females, possibly corrects the cholesterol efflux defect in fibroblasts from the S1731C male ABCA1 carrier during the 22OH/9CRA ABCA1 stimulation phase of 17 hours (**Figure 3.2B**). Interestingly, after adjusting for basal cholesterol diffusion, we observed that upon treatment with elevated doses of estradiol (>20 nM), efflux in the S1731C proband significantly increased ($P =7.2x10^{-6}$, r=0.78 using a non-

parametric Spearman trend test), while that in the wildtype control remained constant (P=0.2, r=0.25) (**Figure 3.2B**). Taken together, these results support a genotype-sex interaction effect, as hormonal regulation with 17β-estradiol partially restored the low efflux observed in the S1731C male proband but had no significant effect on the efflux of a wild-type control.

3.4.4 Rare missense variant in LPL

The identified LPL variant rs118204060 is present in the dbSNP132 (The 1000 Genomes Project Consortium, 2010) and The 1000 Genomes Project data with an unknown frequency. The rs118204060 located in exon 5 changes a conserved amino acid from proline to leucine (P234L). This variant was initially identified in familial chylomicronemia and was reported as P207L (Brisson et al., 2002; Normand et al., 1992; Ma et al., 1991) due to differences in genome builds. Upon sequence comparisons, we confirmed that they are indeed the same variant.

3.4.5 Investigation of the ABCA1 and LPL variants in the entire family

We examined the pedigree members for co-occurrence of non-synonymous ABCA1 and LPL variants with low HDL-C. By stratifying individuals by their HDL percentiles, we can see that all the affected family members with HDL-C<5th percentile carry a risk allele for either one or both of the variants (3 P234L, 11 S1731C, and 8 P234L/S1731C), except in one separate branch of the extended family in which the low HDL-C traits appears to be inherited from the affected spouse's side (**Figure 3.1**). No family member with an HDL-C value greater than the 5th percentile had both LPL and ABCA1 variants. Furthermore no family member with HDL-C values greater than the 15th percentile had the LPL variant. Seven subjects (1 male and 6 females) had the ABCA1 variant with the HDL-C percentile of 22% for the male and with an average HDL-percentile of 35% for the six females (**Figure 3.1**). Two of the three exome sequenced subjects were heterozygous for both variants and one was homozygous for the ABCA1 variant and heterozygous for the LPL variant (**Figure 3.1**). There were four homozygous subjects for the ABCA1 variant two

of which were also heterozygous for the LPL variant, whereas the LPL variant was heterozygous in all 14 family members it was observed (**Figure 3.1**). Thus, a heterozygous, milder form of LPL deficiency exists in this family. Accordingly, the LPL variant P234L is also associated with elevated levels of triglycerides (1.65±0.27 mmol/l, $P=6.14x10^{-3}$). In addition, we observed that the subjects with both variants have a lower HDL-C than the subjects with only one variant, and that the subjects heterozygous or homozygous for the ABCA1 variant do not differ in the HDL-C levels (**Table 3.4**).

3.4.6 Explained variance and heritability

We estimated that the effect of the ABCA1 and LPL variants on continuous HDL-C measurements in the extended family is -0.17±0.08 mmol/l (P=0.025) and -0.27±0.09 mmol/l (P=0.006), respectively. Together these two variants explain 60% of the genetic variance in this family and 26% of the total (genetic + environment) variance in this family, which amounts to 46% of the heritability explained as assessed in a measured genotype analysis (Lange et al., 2005). We also repeated the analysis while excluding the three affected subjects that were exome sequenced to reduce the potential for ascertainment bias. In this analysis, the effect sizes of ABCA1 and LPL remained the same (-0.18±0.08 and -0.27±0.10 mmol/l, respectively) and the additive and total variance explained were 50% and 24%, respectively, with 34% of the heritably explained. Importantly, if the subfamily with the bilineal introduction of the low HDL-C trait through the affected spouse is excluded from these analyses, virtually all of the additive variance of HDL-C and virtually all of the heritability of HDL-C is explained by the ABCA1 and LPL variants, suggesting that the ABCA1 and LPL variants can explain the low HDL-C in the ‘non-bilineal’ part of the extended family.

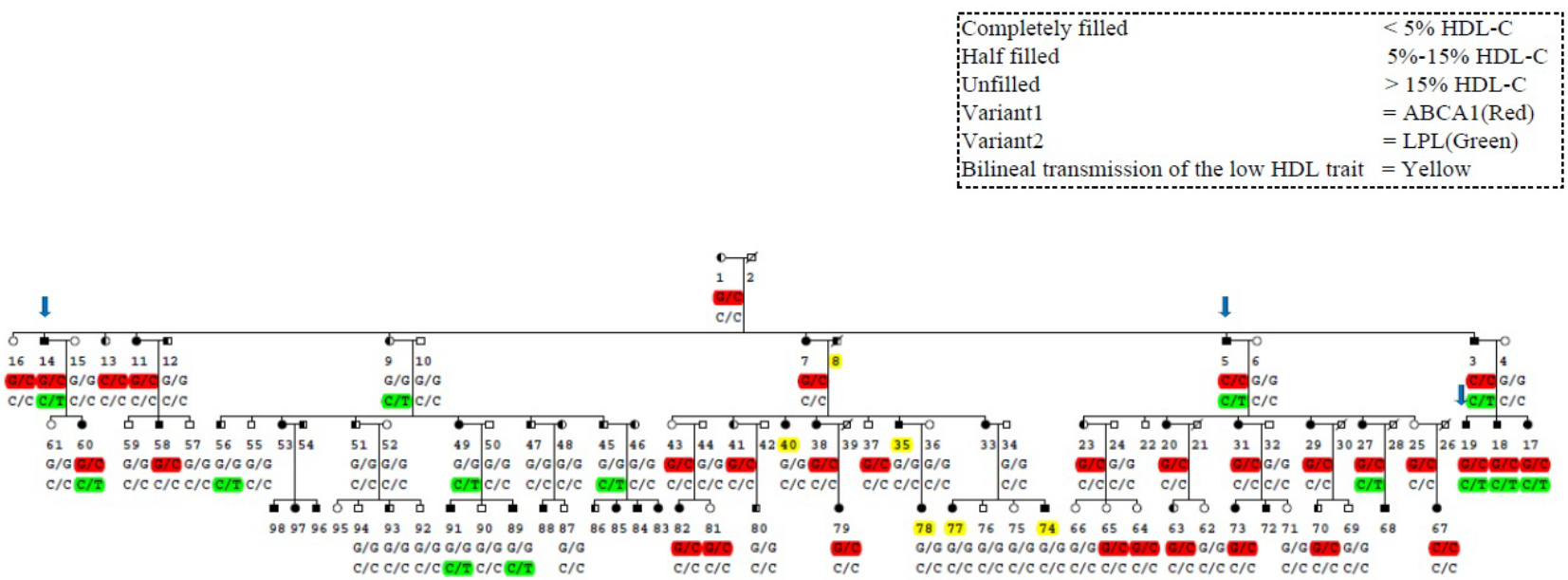

Figure 3.1 ABCA1 and LPL variants co-segregate with low HDL-C in the multigenerational French Canadian low HDL-C family with 75 (35 males and 40 females) genotyped family members

ABCA1 and LPL variants co-segregate with low HDL-C in the multigenerational French Canadian low HDL-C family with 75 (35 males and 40 females) genotyped family members. All of the affected subjects who have HDL-C<the 5th age-sex specific population percentile comprise risk alleles for either one of the two variants or both, except in one separate branch the low HDL-C traits appears to be inherited from the affected spouse's side (indicated in yellow). The subjects with both variants have a lower HDL-C than the subjects with only one variant. The subjects whose samples were exome sequenced are indicated by an arrow.

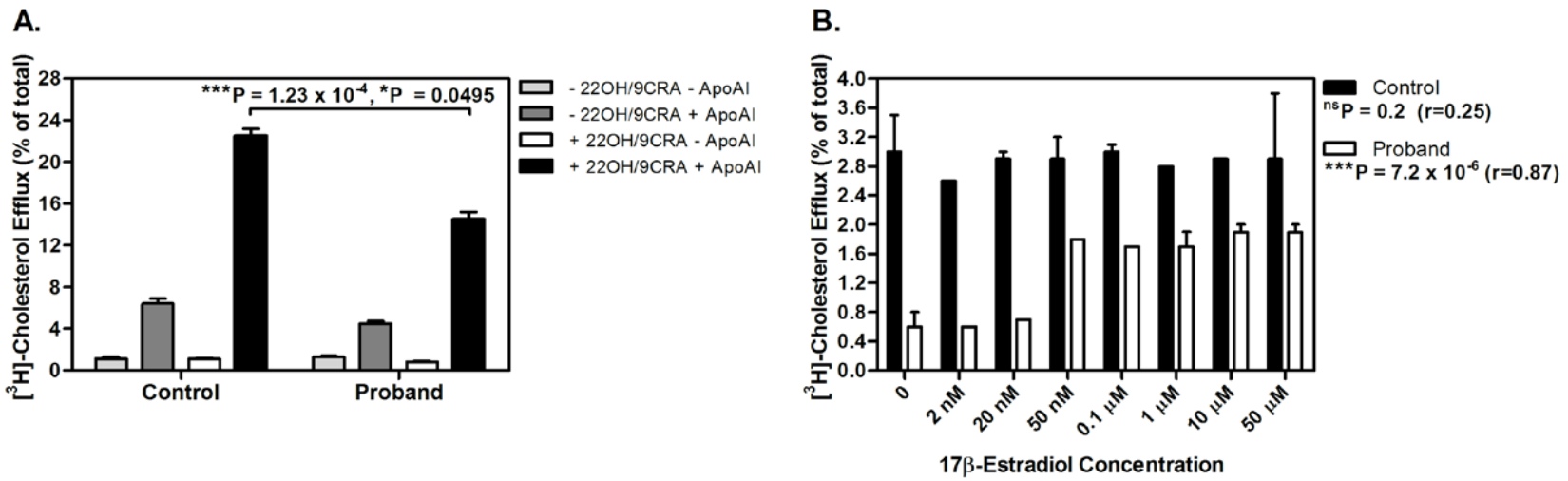

Figure 3.2A-B A) Effect of the ABCA1 variant on cholesterol efflux in fibroblasts from a proband homozygous for S1731C and a healthy control. B) Elevated concentrations of 17β-estradiol improve cholesterol efflux in the male proband with the ABCA1 S1731C variant

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Figure 3.2A-B A) Effect of the ABCA1 variant on cholesterol efflux in fibroblasts from a proband homozygous for S1731C and a healthy control. B) Elevated concentrations of 17β-estradiol improve cholesterol efflux in the male proband with the ABCA1 S1731C variant

A) Effect of the ABCA1 variant on cholesterol efflux in fibroblasts from a proband homozygous for S1731C and a healthy control. Fibroblasts were isolated by taking a biopsy from the forearm of the proband and a healthy individual, plated in 12-well plates and radiolabeled with [^{3}H]-cholesterol for 48 hours. Cholesterol efflux was performed as described in Methods under background diffusion conditions (-22OH -ApoA-I), unstimulated (-22OH +ApoA-I) and stimulated (+22OH -ApoA-I, +22OH +ApoA-I) conditions, with or without ApoA-I. The proband had a significantly reduced ApoA-I-mediated efflux as compared to the control without the variant. Values represent the mean ±S.D. from triplicate wells. Results shown are a representative of three independent experiments. ***P=1.23x10^{-4} by Student's *t*-test; and *P=0.0495 using a non-parametric two-sample Wilcoxon rank sum test. 22OH indicates 22(R)-hydroxycholesterol; 9CRA, 9-cis-retinoic acid; and ApoA-I, apolipoprotein A-I

B) Fibroblasts from a male proband with the ABCA1 S1731C variant and a healthy male control were isolated by taking a biopsy from the forearm of plated in 24-well plates and radiolabeled with [3H]-cholesterol for 24 hours. Cholesterol efflux was performed as described in Methods, with addition of increasing concentrations (2 nM, 20 nM, 50 nM, 0.1μM, 1 μM, 10 μM and 50 μM) of 17β-estradiol while stimulating ABCA1 expression with 22OH/9CR for 17 hours. As in Figure 3.2A, experiments were done under four conditions (-22OH -ApoA-I, -22OH +ApoA, +22OH -ApoA-I, +22OH +ApoA-I). Efflux results were subsequently adjusted for background basal conditions of passive diffusion. The final stimulated ApoA-I mediated efflux condition is shown. Upon exposure to increasing estradiol concentrations (>20 nM), cholesterol efflux in the S1731C proband significantly increases. Of note, the overall [3H]-cholesterol efflux counts were lesser in magnitude than those observed in Figure 3.2A given the shorter labelling time period (24 hours). In addition, the difference in efflux between the control and proband was greater than in Figure 3.2A, given the different basal diffusion of the selected conditions (data not shown), which was now removed from the net ApoAI-mediated efflux. Values represent the mean ±S.D. from triplicate wells. Results shown are representative of three independent experiments. 22OH indicates 22(R)-hydroxycholesterol; 9CRA, 9-cis-retinoic acid; and ApoA-I, apolipoprotein A-I. ***P=7.2x10^{-6} (r=0.78) using a non-parametric Spearman trend test for the efflux in the S1731C proband; and ^{ns} P=0.2 (r=0.25) using a non-parametric Spearman trend test for the efflux in the wildtype control

Table 3.3 The lipid levels and other clinical characteristics of the three individuals that were exome sequenced

IND ID	ABCA1	LPL	TC	TG	HDL-C	HDL %	BMI	LDL-C	AGE	SEX
Ind5	C/C	C/T	4.61	1.3	0.67	<5	22.02	3.36	66	Male
Ind14	G/C	C/T	4.60	6.1	0.62	<5	25.81	NA	55	Male
Ind19	G/C	C/T	2.43	2.4	0.62	<5	27.22	0.70	21	Male

The lipid levels are shown in millimoles per liter.

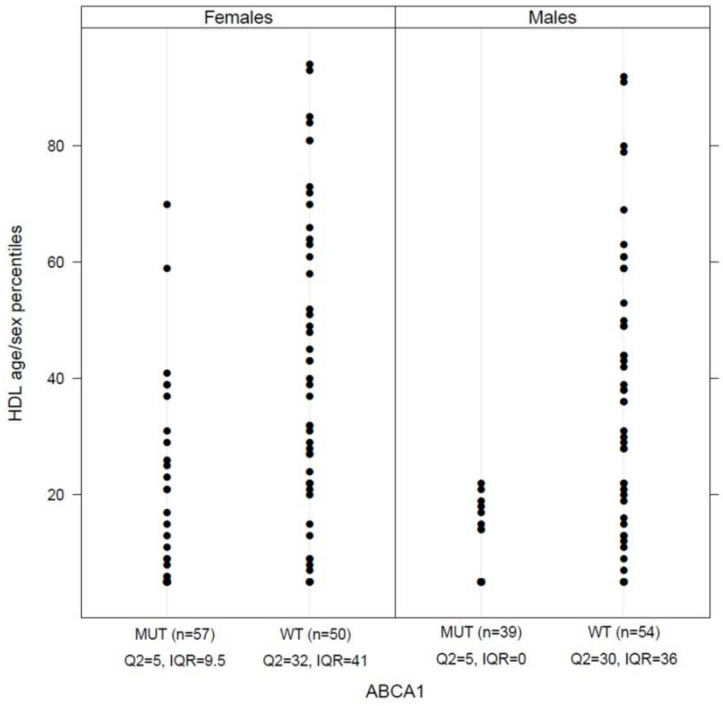

Figure 3.3 Sex-dependent effect of ABCA1 variants

Sex-dependent effect of ABCA1 variants. Figure 3.3 shows the age-sex specific population HDL-C percentiles by ABCA1 genotypes and sex in 200 French Canadian family members from 11 French Canadian families with different ABCA1 mutations (DelED1893, G616V, K776N, N1800H, Q2210H, R1851X, R2084X, R909X and S1731C). MUT indicates carriers of a mutation in the ABCA1 gene and WT stands for wild-type genotype (i.e. non-carriers). Q2 stands for the median quartile and IQR for interquartile range (Q3-Q1) of HDL-C percentiles. The distributions of the age-sex percentiles are similar between genders in the WT genotype group whereas in the MUT genotype group the distribution is more restricted to the lower tail in males than in females. This difference describes the significant result of the genotype-sex interaction analysis. It should be noted that the displayed age-sex percentiles are not adjusted for relatedness whereas the family relation was taken into account in the genotype-sex interaction analysis performed using SOLAR.

Table 3.4 The mean lipid levels and other clinical characteristics of individuals with different ABCA1 (S1731C) and LPL (P234L) genotypes

ABCA1/LPL	All Individuals	TC	TG	HDL-C	AGE	BMI	LDL-C
CC/CC	2	3.76(1.29)	0.72(0.01)	0.94(0.30)	35(33.94)	19.45(7.35)	1.67(0.71)
CC/CT	2	3.60(1.44)	3.81(3.61)	0.52(0.22)	61(7.07)	23.17(1.63)	2.19(1.65)
GC/CC	22	5.11(1.84)	1.22(0.69)	0.95(0.21)	37(20.04)	20.65(3.27)	3.53(1.92)
GC/CT	6	4.13(1.25)	2.97(1.99)	0.68(0.07)	34.3(11.36)	24.36(3.18)	2.03(1.90)
GG/CC	37	4.88(1.24)	1.60(0.90)	1.16(0.35)	34.6(17.03)	22.64(5.67)	2.94(1.13)
GG/CT	6	3.91(1.21)	2.18(1.37)	0.78(0.12)	35(17.37)	22.07(3.38)	2.06(0.87)
Total	**75**	**4.74(1.47)**	**1.68(1.21)**	**1.01(0.33)**	**36(17.86)**	**22.12(4.75)**	**2.95(1.51)**
ABCA1/LPL	**All Males**	**TC**	**TG**	**HDL-C**	**AGE**	**BMI**	**LDL-C**
CC/CC	0						
CC/CT	2	3.59(1.44)	3.81(3.61)	0.52(0.22)	61(7.07)	23.17(1.63)	2.19(1.65)
GC/CC	3	4.59(1.59)	0.94(0.45)	0.89(0.21)	30(17.44)	22.08(3.03)	3.28(1.56)
GC/CT	3	3.59(1.09)	4.43(1.84)	0.65(0.06)	35(17.78)	25.28(2.25)	0.35(0.49)
GG/CC	22	4.73(1.35)	1.45(0.89)	1.06(0.26)	34.3(17.44)	22.88(3.62)	2.97(1.31)
GG/CT	4	4.02(1.54)	2.62(1.51)	0.73(0.08)	26.2(11.67)	23.39(3.30)	2.04(1.08)
Total	**34**	**4.47(1.36)**	**1.94(1.55)**	**0.94(0.29)**	**34.6(17.25)**	**23.12(3.30)**	**2.68(1.41)**
ABCA1/LPL	**All Females**	**TC**	**TG**	**HDL-C**	**AGE**	**BMI**	**LDL-C**
CC/CC	2	3.76(1.29)	0.72(0.01)	0.94(0.30)	35(33.94)	19.46(7.36)	1.67(0.00)
CC/CT	0						
GC/CC	19	5.19(1.91)	1.27(0.72	0.96(0.22)	38.1(20.63)	20.49(3.33)	3.57(2.01)
GC/CT	3	4.67(1.36)	1.51(0.33)	0.71(0.09)	33.7(2.31)	23.46(4.22)	3.15(1.54)
GG/CC	15	5.09(1.06)	1.83(0.89)	1.30(0.43)	35.1(17.00)	22.27(7.92)	2.89(0.85)
GG/CT	2	3.69(0.23)	1.32(0.52)	0.88(0.16)	52.5(13.44)	19.46(1.92)	2.09(0.49)
Total	**41**	**4.97(1.53)**	**1.47(0.78)**	**1.06(0.36)**	**37.2(18.47)**	**21.29(5.58)**	**3.17(1.57)**

*The value in the parenthesis indicates standard deviation.

3.4.7 Genome-wide linkage analyses

To further investigate that we did not miss a major susceptibility variant, we performed a whole-genome two-point linkage analysis for low HDL-C using a dominant mode of inheritance. We first estimated using the SLINK simulation program (Weeks et al., 1990) that under the assumption of homogeneity the maximum lod score this family can provide is 4.34. However, none of the actual 553 microsatellite markers reached this lod score, most probably due to the existence of multiple low HDL-C variants in the family (i.e. heterogeneity). In more detail, no lod scores >3 were observed anywhere in the genome. The only lod score >2.0 was observed on chromosome 21 for marker D21S1255. However, we noticed that this signal on chr 21seems to arise from the bilineal branch (**Figure 3.1**) since the signal diminishes to lod score of 0.6 when we excluded this subfamily from the analysis and increases to 2.5 when we analyzed this bilineal branch of the family alone. Hence the genome-wide linkage data suggest that there might be another susceptibility variant on chr 21 that accounts for the low HDL-C in the bilineal subfamily branch of the extended family. However, since we did not sequence any family members from this branch, none of the 3,459 filtered-out variants would be good candidates. Importantly, we observed lod scores >1 near the LPL and ABCA1 genes (lod scores of 1.62 and 1.28 10.3 Mb and 5.8 Mb from LPL and ABCA1, respectively). Without the bilineal branch these lod scores increased to 2.14 and 1.45, respectively.

3.4.8 Genotype by sex interaction

The effect of the ABCA1 S1731C variant on low HDL-C levels appears more profound in the males than in females in the extended family (**Table 3.4**). Furthermore, our efflux study also suggested a gene x sex interaction (**Figure 3.2B**). Although the frequency of the S1731C variant may be individually too rare for testing genetic interactions (as large sample sizes are necessary), rare variants with large phenotypic effects are collectively common in low HDL-C families (Cohen et al., 2004). We hypothesized that the apparent sex effect may not be

restricted to the S1731C allele, but rather it may generally extend to ABCA1 alleles with major phenotypic effects. Thus to further investigate this intriguing relationship between ABCA1 and sex, we examined the collective effect of multiple rare variants in ABCA1 by sex on HDL-C affection. All in all, 10 additional low HDL-C French Canadian families with known mutations in ABCA1 (Alrasadi et al., 2006; Brooks-Wilson et al., 1999; Marcil et al., 1999a) were included in the sex interaction analysis using the SOLAR program (Almasy and Blangero, 1998), comprising a total of 93 males and 107 females. The percentage of mutation carriers was 42% and 53% in males and females, respectively (**Figure 3.3**). The S1731C variant was present in 3 of these additional low HDL-C pedigrees (Alrasadi et al., 2006) and together with the exome sequenced family the association signal for the main effect of S1731C on low HDL-C status resulted in a p-value of 0.008. In all 11 families, we observed, as expected, a highly significant main effect for ABCA1 genotypes ($P=1x10^{-09}$), as well as a significant ABCA1 genotype x sex interaction on the qualitative HDL-C affection (P=0.03) (**Figure 3.3**). Furthermore, the interaction effect appeared to be more pronounced when comparing pre-menopausal women (age<50 years) to men and post-menopausal women (P=0.003).

3.5 Discussion

By using exome sequencing, we identified two functional rare variants in the ABCA1 and LPL genes, co-segregating with low HDL-C and explaining a major proportion of the HDL-C variance and heritability in an extended family. We also observed a sex effect for ABCA1 variants, male carriers exhibiting significantly lower HDL-C levels than females. Furthermore, none of the unaffected family members had the LPL variant or both variants. Our study exemplifies how utilization of exome sequencing was critical to reveal the complex combination of two variants of which one is less severe in females. Traditional linkage analysis was unable to elucidate this type of complex pattern of variants in this extended family (Dastani et al., 2010), suggesting that many

such combinations have been missed in previous linkage analyses of complex traits.

ABCA1 and LPL are major players of lipid metabolism. The ABCA1 is a key protein involved in reverse cholesterol transport (RCT) that transports cellular cholesterol to lipid-poor acceptor apolipoproteins, such as apolipoproteinA-I (Oram and Lawn, 2001; Marcil et al., 2003). As a result, the apolipoprotein is released with the extracted phospholipid and cholesterol, forming nascent HDL particles. Mutations disrupting the normal function of ABCA1 result in little or no circulating HDL (Albrecht et al., 2004). Previous studies have shown that cell lines with the identified ABCA1 variant S1731C exhibit low levels of protein expression (Brunham et al., 2005), and that cells transfected with the S1731C allele express abundant ABCA1 mRNA but fail to generate significant amounts of ABCA1 protein (Brunham et al., 2005). Furthermore, the cholesterol efflux of S1731C has been shown to be reduced to 12.3-68.0% of the wildtype (Alrasadi et al., 2006; Brunham et al., 2005; Cohen et al., 2004). Here, we observed a ~40% cholesterol efflux reduction in the proband homozygous for the S1731C variant as compared to a normal control, in line with the earlier findings (Alrasadi et al., 2006; Brunham et al., 2005; Cohen et al., 2004). In our previous paper (Alrasadi et al., 2006), we showed that three heterozygous subjects with the S1731C variant have cholesterol efflux values of 63%, 66%, and 68% of the wildtype. Thus, about the same 40% decrease is observed in the heterozygous subjects as in the homozygous subject with the S173C variant, in line with their similar HDL-C values (**Table 3.4**). Although the phenotype data suggest that the S1731C variant does not have a gene dose effect, this conclusion warrants additional functional studies in future, as there are only 4 homozygotes in the family two of which are also heterozygous for the LPL variant, and furthermore the variant has a large range (12.3-68.0% of the wildtype) in its effect on the cholesterol efflux (Alrasadi et al., 2006; Brunham et al., 2005; Cohen et al., 2004).

The main function of LPL is to hydrolyze triglycerides in order to deliver fatty acids to the tissue. LPL also hydrolyzes very-low-density

lipoproteins (VLDL). Sequence variation in LPL has been reported to be associated with the risk of CHD, TGs and HDL-C (Teslovich et al., 2010). An efficient LPL function is associated with lower TG and low-density lipoproteins (LDL) and higher HDL. Regarding the identified P207L variant, individuals with this mutation have reduced HDL particles compared with the control subjects (Ruel et al., 2002) Previous studies have also shown that missense mutations in exon 5 of the LPL gene where the P207L variant resides are the most common cause of LPL deficiency (Garenc et al., 2005; Peterson et al., 2002). Importantly, Ma et al. reported that upon site-directed in vitro mutagenesis this variant produces a catalytically inactive lipoprotein lipase protein which is the cause of the lipoprotein lipase deficiency in the patients (Ma et al., 1991). Taking together these previous data, along with our PolyPhen (Ramensky et al., 2002) and SIFT (Ng and Henikoff, 2003) predictions, it is highly likely that both identified variants S1731C and P207L affect protein function.

The two identified variants, S1731C and P207L have been reported previously in French Canadian dyslipidemic individuals but not in normal controls (Alrasadi et al., 2006; Brisson et al., 2002; Brunham et al., 2005; Clee et al., 2001; Cohen et al., 2004; Ma et al., 1991; Normand et al., 1992). The S1731C ABCA1 variant was present in three French Canadian dyslipidemic families with low HDL-C levels (Alrasadi et al., 2006) but not in 528 chromosomes from French Canadian subjects with normal HDL-C levels (Clee et al., 2001). It was also absent in 108 French Canadian subjects with high HDL-C (Cohen et al., 2004). The P207L LPL variant was previously observed in 37 unrelated French Canadian patients with lipoprotein lipase deficiency with 54 mutant alleles present in that study sample (Ma et al., 1991). In the same study, the variant was also genotyped in 34 unrelated patients with LPL deficiency from ancestries other than French Canadian. Only one German patient was found to be heterozygous for the risk allele. Furthermore, 11 out of 180 French Canadian hyperlipidemic cases were heterozygous for the P207L variant, whereas none of the 170 normolipidemic controls had the P207L variant (Ma et al., 1991).

It is important to study the effect of sex on lipid traits to better understand the sex-specific differences in incidence of dyslipidemia and cardiovascular disease. The results of an earlier study demonstrated that ABCA1 has a sex-specific effect as elevated levels of ABCA1 were observed in females (Zhang et al., 2011), which is in line with the higher HDL-C levels and the lower risk of females for coronary artery disease. In this study, we observed that functional mutations in ABCA1 affecting the cholesterol efflux (Alrasadi et al., 2006; Brunham et al., 2005; Cohen et al., 2004) have a larger effect on HDL-C levels in male than female carriers of these variants. It is possible that the observed genotype-sex interaction results from the previously observed gender differences in ABCA1 expression levels (Zhang et al., 2011), because if males have lower baseline levels of ABCA1, the effect of the mutations could be even more profound in males. These interesting sex-specific mechanisms of ABCA1 may involve hormonal regulation of ABCA1, a hypothesis supported by our efflux experiment (**Figure 3.2B**), demonstrating that exposure of fibroblasts of a male proband with the ABCA1 S1731C variant to increasing concentrations of 17β-estradiol led to a significantly increased efflux in the male proband with the ABCA1 variant. These intriguing findings warrant further investigation in future studies.

Our results demonstrate that two relatively rare functional ABCA1 and LPL variants contribute to the risk of low HDL-C in a unique combination involving a sex-effect in an extended family. We first identified a set of variants by filtering the variants shared by the exome sequenced affected family members for variant type, frequency, and functional predictions. Because filtering has limitations caused by heterogeneity of complex traits (Ng et al., 2010), we then utilized the extended family structure for statistical analysis exploring how much of the trait variance and heritability the two key ABCA1 and LPL variants explain. Thus, our study highlights the fact that the filtering strategy used in exome studies of Mendelian disorders (Ng et al., 2010) is not directly applicable for complex disorders and that new methodologies that incorporate multiple susceptibility variants within a family are warranted. As the two variants explain a

major part of the variance in HDL-C and are shown to be functional (Alrasadi et al., 2006; Brunham et al., 2005; Cohen et al., 2004; Ma et al., 1991) they represent the key underlying HDL-C variants in this family, though other rare and common variants are likely to explain the remaining portion of the variance. As both ABCA1 and LPL are known to affect HDL-C, our study did not reveal a novel HDL gene. However, our study does highlight the importance of exome sequencing of dyslipidemic families, because traditional linkage or haplotype analysis cannot detect complex segregation of several functional rare variants due to the inherent parametric restrictions of linkage analysis. This type of underlying biological complexity must have contributed to the low lod scores and weak success of linkage analysis in gene identification of complex lipid traits. In this study, we demonstrate for how family-based exome sequencing can successfully identify multiple rare variants to be followed up utilizing the effective co-segregation information available in extended dyslipidemic families. To the best of our knowledge, our study is the first described example of two functional rare variants conferring the susceptibility to low HDL-C in an extended family.

3.6 Acknowledgements

We thank the family members who participated in this study. We also thank Cindy Montes and UCLA core facilities for laboratory technical assistance.

This research was supported by the grants HL095056 and HL-28481 from the National Institutes of Health (PP, JSS); by grants MOP 97752 from the Canadian Institutes of Health Research (CIHR) and from the Heart and Stroke Foundation of Canada (JG). MVPLR is supported by the American Heart Association grant 11POST7380028.

3.7 Materials and Methods

3.7.1 Study samples

The study sample consists of a large multigenerational French Canadian family collected in the Cardiovascular Genetics Laboratory, McGill University Health Centre, Royal Victoria Hospital, Montreal, Canada, as described

previously (Dastani et al., 2006c). There are 75 family members (35 males and 40 females) with both DNA and extensive demographic and clinical phenotype information available for study in this family. We selected three closely related family members with HDL-C levels ≤5th age-sex percentile from the uppermost generations (**Figure 3.1**) for exome sequencing to focus on most severe cases and avoid genetic heterogeneity typical for complex lipid traits.

For a gene-sex interaction analysis, 10 additional French Canadian families with previously identified mutations in ABCA1 (Marcil et al., 1999a; Brooks-Wilson et al., 1999; Alrasadi et al., 2006) comprising 125 individuals were also included in the study. The affection status in all families was determined using the 5th age-sex specific population percentile of HDL-C (Dastani et al., 2006c). Family members were sampled (blood collection for lipoprotein analyses, DNA isolation for genetic studies and skin biopsy for culture of skin fibroblasts used in cellular cholesterol efflux assays) after a 12-h fast and discontinuation of lipid modifying medications for >4 weeks. Lipids and lipoproteins were measured using standardized techniques as described previously (Brooks-Wilson et al., 1999; Marcil et al., 1999b). The research protocol was approved by the Research Ethics Board of the McGill University Health Center, and all subjects gave informed consent.

3.7.2 Library construction and sequencing

Library construction was performed using 3 μg of genomic DNA and Agilent SureSelect All Exon Kit (50-Mb design) according to the manufacturer's instructions. Further details of library construction and sequencing are given in the online-only Data Supplemental methods.

3.7.3 Data analysis

3.7.3.1 Exome sequencing

We converted the qseq files into a Sanger-formatted FASTQ files that were aligned to a reference sequence (hg19) using the default options of the Burroughs-Wheeler Aligner (BWA) (Langmead et al., 2009). Duplicates were

removed and a pileup file was generated using SAMtools (Li et al., 2009). The pileup file was used to run the quality control metrics including: a minimum read depth of 4, a maximum read depth of 600, a maximum of two SNPs per a window size of 10 bases, a minimum indel score of 25 for filtering nearby SNPs and Phred quality >40. The BED file supplied by Agilent was used to filter only those reads corresponding to the 50 Mb targets.

Annovar was used for functional annotation, dividing the variants into coding and non-coding variants (Wang et al., 2010). The coding variants were further divided into synonymous, nonsynonymous (missense), and stop gain or stop loss variants. The synonymous variants were subsequently discarded because they are less likely to be causal. The variants were filtered against the variants present in the HapMap (Altshuler et al., 2010), The 1000 Genomes Project (The 1000 Genomes Project Consortium, 2010) and dbSNP132 (The 1000 Genomes Project Consortium, 2010) databases. Along with novel variants, we selected known rare variants with a minor allele frequency (MAF) <5%. These variants were classified into damaging and benign based on their predicted protein effect using PolyPhen (Ramensky et al., 2002) and SIFT (Ng and Henikoff, 2003).

3.7.3.2 Parametric linkage and association analysis

Two-point parametric linkage analysis was performed in the extended family using the 'Location-Score' option of the Mendel software (Lange et al., 2001) as described in detail in the online-only Data Supplemental methods.

Association analysis was performed using a measured genotype approach utilizing the 'Polygenic-QTL' option of Mendel (Lange et al., 2005), using continuous HDL-C levels with age and sex as covariates and allele counts of either the ABCA1 variant, LPL variant, both variants or none (i.e. null model). The heritability and variance explained were calculated as the percent change in total and genetic variance between the null model and the models including the genotypes as covariates. The LPL variant was further tested for association with log transformed TG values in a similar fashion.

3.7.3.3 Genotype by sex interaction

We included the extended family together with 10 additional families with previously identified mutations in ABCA1 (Alrasadi et al., 2006; Brooks-Wilson et al., 1999; Marcil et al., 1999a) in a gene-sex interaction analysis, comprising 200 individuals and 9 different mutations in ABCA1 (DelED1893, G616V, K776N, N1800H, Q2210H, R1851X, R2084X, R909X and S1731C). Genotype by sex interaction was tested by the SOLAR program (Almasy and Blangero, 1998) using variance-component analysis for discrete traits. We compared models with and without the gene-sex interaction term while keeping the ABCA1 genotypes in both the null and interaction model. We assumed a dominant genetic inheritance, classifying carriers of a mutation as 1 and 0 otherwise, and a multiplicative interaction term, multiplying the genotype score by sex (men=1 and women=0). We also coded a sex-interaction term in which men and post-menopausal women (≥50 years) were coded as 1 and pre - menopausal women (<50 years) were coded as 0. Subjects with HDL-C levels < the age-sex specific 10th percentiles were classified as affected and subjects with HDL-C levels > the age-sex specific 20th percentiles as unaffected. P-values were generated by comparing the two models using a likelihood ratio statistic with one degree of freedom. Since the affection status is adjusted for gender, the inclusion of the main effect of sex in the model was no longer necessary. The binary HDL-C affection was tested because the variance of HDL-C levels in these ascertained families is reduced and thus limited for effective quantitative analysis (Pajukanta et al., 2003).

3.7.4 Cell culture

Human skin fibroblasts were obtained from 3.0-mm punch biopsies of the forearm of a healthy control subject and the affected proband homozygous for the ABCA1 S1731C variant. The fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% nonessential amino acids, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum.

3.7.5 Cellular cholesterol efflux assays

Cholesterol efflux was performed as described previously (Denis et al., 2004b; Marcil et al., 2003) with minor modifications. Further details of cellular cholesterol efflux assay are given in the online-only Data Supplemental methods.

3.7.6 Supplementary Methods

3.7.6.1 Library construction and sequencing

The genomic DNA was sheared (sonication) with Covaris S2 to achieve a uniform distribution of fragments with a mean size of 200 bp. The sheared DNA was purified using Agencourt AMPure XP Solid Phase Reversible Immobilization paramagnetic bead (SPRI) and the quality of DNA was tested with the Agilent 2100 Bioanalyzer. The end repair was done by removing the 3′ overhangs followed by the addition of a single "A" base to the 3′ end of the DNA fragments using Klenow fragment (3′ to 5′ exo minus). Specialized adaptors that have a T - base overhang at their 3′ ends were ligated. Following ligation, the samples were purified (using SPRI beads), PCR amplified and the quality was checked by the Agilent 2100 Bioanalyzer.

After hybridization the captured DNA was purified and amplified. The quality of the library was evaluated using the Agilent Bioanalyzer. Finally the 100-bp single end sequencing was performed on the Illumina Hiseq2000 platform with one sample per lane.

3.7.6.2 Mutation validation

Sanger sequencing was used to confirm the presence and genotype of variants in the candidate genes identified via exome sequencing and to screen the variants in additional family members.

3.7.6.3 Cellular cholesterol efflux assays

Human skin fibroblasts were seeded in 12-well plates and at mid-confluence labelled with 2 μCi/ml [^{3}H]-cholesterol (Perkin-Elmer Life Sciences)

for 48 hours. Cells were subsequently stimulated, or not, with 2.5 μg/ml 22(R)-hydroxycholesterol (22OH) and 10μM 9-cis-retinoic acid (9CRA) for 17 hours and then incubated, or not, with 15 μg/ml lipid-free apolipoproteinA-I (ApoA-I) (Meridian Life Sciences) for 5 hours. Radioactivity was counted in both the medium and the cells. Cellular cholesterol efflux was determined as follows: ^{3}H cpm in medium / (^{3}H cpm in medium + ^{3}H cpm in cells); the results were expressed as percentage of total radiolabeled cholesterol. For the cholesterol efflux assays in the presence of 17β-estradiol (Sigma-Aldrich), fibroblasts were labeled with 2 μCi/ml [^{3}H]-cholesterol (Perkin-Elmer Life Sciences) for 24 hours, stimulated, or not, with 2.5 μg/ml 22OH and 10μM 9CRA for 17 hours and subsequently incubated, or not, with 15 μg/ml lipid-free apolipoproteinA-I (ApoA-I) (Meridian Life Sciences) for 4 hours. During the 17 hours incubation with 22OH/9CRA, cells were simultaneously treated with increasing concentrations of 17β-estradiol. As above, assays were performed in 22OH/9CRA stimulated fibroblasts (to induce ABCA1 expression), as well as in unstimulated cells, in the presence or absence of lipid free ApoA-I. Cellular cholesterol efflux was determined as described above, but in order to specifically assess the effect of estradiol on the ABCA1 variant, we adjusted for background basal conditions of passive diffusion of cellular cholesterol. Student t-test and non-parametric two sample Wilcoxon rank sum test were used to assess differences between cholesterol efflux of a S1731C male proband and a healthy male control. A non-parametric Spearman trend test in R was used in-order to test whether increasing concentrations of 17β-estradiol has a significant influence on the cholesterol efflux of the carrier proband and wild-type control. The triplicate data for each concentration was utilized by setting the number of observations per unit equal to 3. All functional experiments were performed three times independently, involving triplicate sample measurements from individual wells for each experiment. Figure 3.2A-B presents one such replicate, representative of all three experiments performed where values represent the mean ± S.D. from triplicate wells.

3.7.6.4 Parametric linkage analysis

Two-point parametric linkage analysis of the low HDL-C status was performed in the extended family using the 'Location-Score' option of the Mendel software (Lange et al., 2001). We utilized an affecteds-only strategy, coding the family members as either "affected" or "unknown" based on the age- and sex-specific population 10^{th} percentiles for HDL-C (Soro et al., 2002) to avoid problems of incomplete penetrance and ambiguity of the "unaffected" disease status. We used a dominant mode of inheritance, with gene frequencies set to 0.4% as described previously (Soro et al., 2002). The genome scan was executed using 553 genome-wide microsatellite markers with an average density of 6 cM (Dastani et al., 2006c). Genotyping and quality control procedures of the microsatellite markers were explained in detailed previously (Dastani et al., 2006c). The SLINK program (Weeks et al., 1990) was utilized to approximate the maximum possible lod score of the extended family under the assumption of homogeneity within the pedigree. We used linkage parameters as given above and a marker with 4 alleles with equal frequencies. Based on 100 replicates the maximum lod score at θ=0.05 was 4.34.

3.7.6.5 Genotype by sex interaction

We included the extended family together with 10 additional families with previously identified mutations in ABCA1 (Alrasadi et al., 2006; Brooks-Wilson et al., 1999; Marcil et al., 1999a) in a gene-sex interaction analysis, comprising 200 individuals and 9 different mutations in ABCA1 (DelED1893, G616V, K776N, N1800H, Q2210H, R1851X, R2084X, R909X and S1731C). Genotype by sex interaction was tested by the SOLAR program (Almasy and Blangero, 1998) using variance-component analysis for discrete traits. We compared models with and without the gene-sex interaction term while keeping the ABCA1 genotypes in both the null and interaction model. We assumed a dominant genetic inheritance, classifying carriers of a mutation as 1 and 0 otherwise, and a multiplicative interaction term, multiplying the genotype score by sex (men=1 and women=0). We also coded a sex-interaction term in which

men and post-menopausal women (≥50 years) were coded as 1 and pre - menopausal women (<50 years) were coded as 0. Subjects with HDL-C levels < the age-sex specific 10th percentiles were classified as affected and subjects with HDL-C levels > the age-sex specific 20th percentiles as unaffected. P-values were generated by comparing the two models using a likelihood ratio statistic with one degree of freedom. Since the affection status is adjusted for gender, the inclusion of the main effect of sex in the model was no longer necessary. The binary HDL-C affection was tested because the variance of HDL-C levels in these ascertained families is reduced and thus limited for effective quantitative analysis (Pajukanta et al., 2003). SOLAR uses a liability threshold model in the variance-component analysis to handle discrete traits, assuming that the logarithm of the odds of being affected is a linear function of a major gene, additive genetic component, covariates, and random environment.

3.8 Supplementary Tables

Supplementary Table 3.1 Lipid levels and other clinical characteristics of the 75 genotyped family members.

IND ID	ABCA1	LPL	TC	TG	HDL-C	% HDL	BMI	LDL-C	AGE	SEX
1	G/C	C/C	3.68	1.06	0.95	9	NA	2.24	88	F
3	C/C	C/T	2.58	6.36	0.36	<5	24.3	1.03	56	M
4	G/G	C/C	5.44	1.61	1.75	66	30.5	2.95	59	F
5	C/C	C/T	4.61	1.26	0.67	<5	22.0	3.36	66	M
6	G/G	C/C	6.04	1.04	2.04	84	30.7	3.53	68	F
7	G/C	C/C	3.57	1.80	0.85	<5	24.1	1.90	66	F
9	G/G	C/T	3.86	0.95	0.99	5	18.1	2.44	62	F
10	G/G	C/C	6.17	2.61	1.06	30	26.6	3.92	69	M
11	G/C	C/C	4.71	3.78	0.90	<5	23.0	1.92	64	F
12	G/G	C/C	3.65	2.11	0.87	11	23.4	1.82	75	M
13	C/C	C/C	4.68	0.73	1.16	15	24.7	NA	59	F
14	G/C	C/T	4.60	6.05	0.62	<5	25.8	NA	55	M
15	G/G	C/C	6.84	3.27	1.45	40	23.1	3.90	55	F
16	G/C	C/C	5.56	1.32	1.26	23	23.0	3.70	57	F
17	G/C	C/T	3.60	1.89	0.79	<5	21.9	1.94	31	F
18	G/C	C/T	3.74	4.80	0.72	<5	22.8	0.00	29	M
19	G/C	C/T	2.43	2.43	0.62	<5	27.2	0.70	21	M
20	G/C	C/C	8.70	1.62	0.77	<5	23.9	7.19	43	F
23	G/C	C/C	4.01	1.40	1.04	11	18.1	2.33	42	F
24	G/G	C/C	5.13	0.61	1.03	31	22.9	3.82	45	M
25	G/C	C/C	3.35	0.88	1.06	17	17.6	1.89	38	F
27	G/C	C/T	6.20	1.29	0.73	<5	20.2	4.88	35	F
29	G/C	C/C	4.95	1.16	0.80	<5	20.1	3.40	39	F
31	G/C	C/C	7.58	1.31	0.59	<5	21.1	5.97	45	F
32	G/G	C/C	4.68	1.94	1.22	59	22.7	2.58	48	M
34	G/G	C/C	6.61	0.84	1.60	92	26.9	4.62	45	M
35	G/G	C/C	4.45	3.29	0.54	<5	29.7	2.04	47	M
36	G/G	C/C	5.26	1.41	1.62	63	23.7	3.00	48	F
37	G/C	C/C	6.40	0.98	0.91	22	19.9	5.06	38	M
38	G/C	C/C	4.56	1.69	0.79	<5	26.4	2.83	45	F
40	G/G	C/C	4.54	1.90	0.64	<5	20.2	2.86	33	F
41	G/C	C/C	9.66	0.79	0.88	5	23.9	8.42	39	F
43	G/C	C/C	6.67	0.57	1.19	31	21.1	5.22	36	F
44	G/G	C/C	5.06	2.52	0.91	22	26.1	3.00	37	M
45	G/G	C/T	6.26	4.45	0.72	5	24.6	3.50	43	M
46	G/G	C/C	4.63	2.64	0.98	8	32.9	2.45	44	F
47	G/G	C/C	5.10	2.68	0.79	9	21.5	3.09	44	M
48	G/G	C/C	5.85	2.04	1.08	13	29.8	3.84	40	F

IND ID	ABCA1	LPL	TC	TG	HDL-C	% HDL	BMI	LDL-C	AGE	SEX
49	G/G	C/T	3.53	1.69	0.76	<5	20.8	1.75	43	F
50	G/G	C/C	5.58	1.41	1.19	61	28.1	3.75	43	M
51	G/G	C/C	5.37	2.31	0.82	13	24.2	3.49	37	M
52	G/G	C/C	4.79	0.80	1.82	85	18.8	2.61	37	F
55	G/G	C/C	3.24	2.46	1.07	39	19.3	1.04	27	M
56	G/G	C/T	2.84	0.83	0.85	12	18.8	1.61	24	M
57	G/G	C/C	2.72	1.03	1.10	49	24.4	1.15	43	M
58	G/C	C/C	3.92	1.37	0.67	<5	24.2	2.63	42	M
59	G/G	C/C	4.83	1.20	1.23	69	23.0	3.05	36	M
60	G/C	C/T	4.22	1.36	0.62	<5	28.2	2.62	35	F
61	G/G	C/C	4.56	3.44	1.35	52	20.4	1.65	29	F
62	G/G	C/C	6.11	3.02	1.46	64	25.2	3.28	24	F
63	G/C	C/C	6.51	0.70	0.98	6	17.0	5.21	13	F
64	G/C	C/C	4.57	1.66	1.24	39	19.5	2.58	21	F
65	G/C	C/C	5.16	1.00	1.41	59	17.6	3.30	19	F
66	G/G	C/C	3.22	0.74	1.26	43	17.1	1.62	16	F
67	C/C	C/C	2.85	0.71	0.73	<5	14.3	1.67	11	F
69	G/G	C/C	3.63	0.58	1.15	20	17.8	2.22	12	M
70	G/C	C/C	3.44	0.47	1.08	15	NA	2.15	10	M
71	G/G	C/C	3.51	1.23	1.31	48	18.4	1.64	21	F
73	G/C	C/C	2.71	1.01	0.75	<5	17.4	1.16	15	F
74	G/G	C/C	9.08	1.69	0.61	<5	28.1	7.29	25	M
75	G/G	C/C	6.18	1.59	1.40	58	21.5	4.06	23	F
76	G/G	C/C	4.23	0.77	1.36	80	20.3	2.52	20	M
77	G/G	C/C	5.57	1.66	0.68	<5	0.0	3.82	14	F
78	G/G	C/C	3.80	1.03	0.61	<5	22.0	2.28	15	F
79	G/C	C/C	3.18	0.72	0.83	<5	23.9	1.83	27	F
80	G/G	C/C	4.87	0.51	0.90	12	18.2	3.74	17	M
81	G/C	C/C	3.77	0.63	1.24	41	14.8	2.24	16	F
82	G/C	C/C	5.77	0.98	0.80	<5	16.5	4.52	10	F
87	G/G	C/C	3.86	0.91	1.07	36	23.1	2.38	21	M
89	G/G	C/T	3.81	2.19	0.70	<5	26.6	2.11	22	M
90	G/G	C/C	3.59	0.51	1.11	42	21.2	2.25	20	M
91	G/G	C/T	3.18	2.99	0.66	<5	23.5	0.94	16	M
92	G/G	C/C	4.04	0.55	1.39	79	18.4	2.40	16	M
93	G/G	C/C	3.98	0.87	0.93	15	19.7	2.65	15	M
94	G/G	C/C	4.21	0.42	1.43	53	17.6	2.59	13	M

The lipid levels are shown in millimoles per liter.

Supplementary Table 3.2 List of 41 variants shared by the three exome sequenced individuals after filtering.

Chr no	Position no	rs number	Gene name	PolyPhen*	SIFT†
2	42990225	New	OXER1	Probably	Damaging
2	160993949	New	ITGB6	Stop	Stop
3	15477933	New	EAF1	Probably	Damaging
3	47047500	New	NBEAL2	Probably	Damaging
3	196529902	New	PAK2	Probably	Damaging
4	1388675	New	CRIPAK	Probably	Damaging
5	140784743	New	PCDHGA9	Probably	Tolerated
8	145094836	New	SPATC1	Possibly	Tolerated
8	145112971	New	OPLAH	Probably	Tolerated
9‡	**107558635**	**New**	**ABCA1**	**Probably**	**Damaging**
10	34606158	New	PARD3	Possibly	Tolerated
11	57076419	New	TNKS1BP1	Probably	Tolerated
12	124362332	New	DNAH10	Probably	Damaging
15	59139625	New	FAM63B	Benign	Damaging
16	2003016	New	RPL3L	Benign	Damaging
17	45234303	New	CDC27	Possibly	Tolerated
17	44144993	New	KIAA1267	Benign	Damaging
19	14675764	New	TECR	Probably	Damaging
22	45821982	New	RIBC2	Probably	Damaging
22	39069227	New	CBY1	Benign	Damaging
1	115537367	rs61730058	SYCP1	Probably	Tolerated
1	144852390	rs61804988	PDE4DIP	Stop	Stop
2	11943082	rs4669781	LPIN1	Possibly	Tolerated
3	49162583	rs35713889	LAMB2	Probably	Tolerated
5	35753763	rs79487218	SPEF2	Benign	Damaging
5	140255119	rs114654172	PCDHA12	Possibly	Tolerated
8‡	**19811790**	**rs118204060**	**LPL**	**Probably**	**Damaging**
8	144995494	rs76803079	PLEC	Probably	Damaging
10	43871158	rs41307500	FXYD4	Probably	Tolerated
10	127697954	rs1666	FANK1	Possibly	Damaging
11	68174189	rs4988321	LRP5	Probably	Tolerated
11	56310356	rs17547284	OR5M11	Stop	Stop
11	36458997	rs62621409	PRR5L	Probably	Damaging
15	45491082	rs80131405	SHF	Benign	Damaging
16	28488943	rs77595156	CLN3	Probably	Tolerated
16	1537693	rs61734779	PTX4	Possibly	Tolerated
17	37224211	rs75117355	PLXDC1	Probably	Tolerated

19	42341407	rs35476281	LYPD4	Probably	Tolerated
19	23545516	rs112713994	ZNF91	Probably	Tolerated
19	49445774	rs10423255	DHDH	Stop	Stop
19	41235167	rs112628847	ITPKC	Benign	Damaging

* PolyPhen-2 was used to predict the possible impact of an amino acid substitution on the structure and function of the protein. A score larger than 0.85 is considered as probably damaging, a score smaller than 0.15 as benign, and a score between 0.85 and 0.15 as possibly damaging, respectively.

† SIFT predicts the amino acid substitution to be damaging if the score is less than 0.05, and tolerated if the score is greater than 0.05.

‡ The ABCA1 (S1731C) and LPL (P234L) variants are highlighted in bold.

CHAPTER 4. THE WW DOMAIN-CONTAINING OXIDOREDUCTASE GENE MODULATES HDL METABOLISM AND LIPOPROTEIN GENE EXPRESSION

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The contents of this chapter are in preparation to be submitted for publication.

4.1 Preface and Rationale

The findings in Chapter 3 highlight the utility of exome sequencing for identifying a set of causative rare functional variants for complex cardiovascular traits using large families. In line with our objectives to search for novel genes associated with HDL, recent studies from our group have identified a significant linkage peak for low HDL-C on chr16q23-24 in two independent French Canadian cohorts of 29 multi-generational Quebec families with HDL-C<5th percentile (LOD 2.61) and 61 families of the Saguenay-Lac St-Jean region (LOD 2.96) (Dastani et al., 2010). In addition, the chromosome 16 locus has been implicated in previous linkage scans for HDL-C with LOD scores >1 in at least 15 other independent genome-wide studies from different populations (Amos et al., 1986; Aouizerat et al., 1999; Aulchenko et al., 2009; Gagnon et al., 2003; Ishimori et al., 2004; Kathiresan et al., 2009; Mahaney et al., 2003; Mehrabian et al., 2000; Pajukanta et al., 2003; Sabatti et al., 2009; Shearman et al., 2000; Soro et al., 2002; Thompson et al., 2005; Willer et al., 2008; Yip et al., 2003; Kathiresan et al., 2008b). In collaboration with Pajukanta *et al*., we narrowed down this region to the rs2548861 SNP within the WW domain containing oxidoreductase (*WWOX*) gene where we observed a region-wide significance for low HDL-C in dyslipidemic families of Mexican and European descent, and in low HDL-C cases and controls of European descent, comprising 10 000 subjects ($P=6.9x10^{-7}$) (Lee et al., 2008b). Interrogation of several online databases further demonstrated that WWOX is strongly associated with low HDL-C (P=0.0000225) (Willer et al., 2008), which is supported by recent evidence that Wwox knockout mice exhibit marked reduction in serum levels (Aqeilan et al., 2007). Based on these observations, there is a biological plausibility for WWOX in modulating HDL-C levels, and therefore a strong rationale for investigating it as a novel HDL determinant. In this study, we thus examined the role of WWOX in lipoprotein and HDL metabolism using a combination of both *in vivo* functional studies, and human genetic analyses.

4.2 Abstract

Low plasma HDL cholesterol (HDL-C) constitute a major risk factor for coronary artery disease. We previously identified a region-wide association between low HDL-C and the WW-domain containing oxidoreductase (*WWOX*) gene in dyslipidemic families and low HDL-C cases and controls (rs2548861, $P=6.9x10^{-7}$). Here, through a combination of *in vivo* functional studies, gene microarray and next generation resequencing, we investigated the role of WWOX in lipoprotein and HDL metabolism. Using total *Wwox*$^{-/-}$ and *Wwox* liver-specific (*hep*$^{-/-}$) mice, we examined key regulators of HDL metabolism. We found decreased apoA-I and ABCA1 protein levels in hepatic tissues in both mice models. Analyses of lipoprotein profiles in *Wwox*$^{-/-}$ littermates showed marked reductions in serum HDL-C and apoA-I levels, with no effect in *Wwox hep*$^{-/-}$ mice. A sex-specific difference was however observed in female *Wwox hep*$^{-/-}$ mice, showing a significant increase in plasma TG levels (P=0.0025). We further obtained evidence of a gender-specific effect by gene microarray analyses in *Wwox hep*$^{-/-}$ hepatocytes, where it was identified that *WWOX* disruption alters primarily lipid metabolic pathways in females. We observed a significant ($P<0.05$, $|FC|>1.5$) reduction in mRNAs of apoA-I, LPL, INSIG and SCAP and upregulation in FAS, ANGPTL4 and LIPG, among others. These results suggest that the effects of WWOX on lipoprotein metabolism activate multiple pathways, including cholesterol homeostasis, apoA-I/ABCA1 pathway, and fatty acid biosynthesis/triglyceride metabolism. A significant increase in body mass and fat percentage (45% fat increase, P=0.009) in *Wwox hep*$^{-/-}$ mice fed a Western diet further validated the role of WWOX in regulating lipid metabolism. Finally, we used next-generation resequencing on the Wwox region and identified 8 variants (MAF<5%) significantly associated with low HDL (P=0.0013-0.0005; beta= -0.3544 - -0.3333) segregating in two French Canadian families with HDL-C$\leq 5^{th}$ age-sex percentile. Taken together, our data indicate that WWOX disruption alters lipoprotein metabolism through several mechanisms and contributes to the genetics of HDL-C.

4.3 Introduction

Low plasma high-density lipoprotein cholesterol (HDL-C) constitute a major cardiovascular risk factor that is strong, coherent and seen across all populations (Yusuf et al., 2004). HDL-C are levels are regulated by a combination of multiple environmental and genetic factors, with heritability estimates of 40-60% (Heller et al., 1993), emphasizing the need to characterize novel genetic regulators of HDL metabolism.

We have recently identified a region-wide significant association between low HDL-C and the WW-domain containing oxidoreductase (*WWOX)* gene on chromosome 16q23-24 (rs2548861, $P = 6.9 \times 10^{-7}$), in families of Mexican and European descent and in low HDL-C cases and controls, as well as in population-based cross sectional and prospective cohorts, comprising 10 000 individuals (Lee et al., 2008b). Interrogation of published genome-wide association studies (Leduc et al., 2011) and online databases (Willer et al., 2008) show that WWOX is also strongly associated with HDL-C ($P=2.25 \times 10^{-5}$). In addition to affecting HDL-C, it was further demonstrated that SNPs at the *WWOX* gene are associated with triglyceride (TG) levels (P=0.003) (Saez et al., 2010) and left ventricular thickness ($P=1.89 \times 10^{-7}$) (Vasan et al., 2009).

The *WWOX* gene spans 1.1 Mb and is located at the common fragile site FRA16D (chr16q24). It encodes a 46-kDa tumor suppressor (Aqeilan et al., 2007; Ludes-Meyers et al., 2009), the expression of which is altered in several types of human malignancies (Aqeilan et al., 2007; Aqeilan et al., 2008; Bednarek et al., 2000; Del Mare S. et al., 2011; Ludes-Meyers et al., 2009). *Wwox* disruption in mice results in metabolic abnormalities, impaired growth, and postnatal lethality, implying that WWOX serves a nonredundant metabolic role that cannot be compensated by other proteins (Aqeilan et al., 2007; Aqeilan et al., 2008). WWOX contains two WW domains and a central short-chain dehydrogenase/reductase (SRD) domain, the presence of which indicates a role in protein-protein interactions, but most importantly, suggest a role in steroid metabolism. WWOX interactions are believed to be largely driven by binding to proline-rich PPxY motifs found within an array of potential ligands, such as p73,

RUNX, c-Jun, AP2, and NF-κB transcription factors, as well as several other cellular proteins including SIMPLE, ErbB4 and Ezrin (Del Mare S. et al., 2009; Sudol, 1996). WWOX is expressed in several distinct isoforms across various tissues, regulating a wide variety of cellular functions such as protein degradation, transcription, apoptosis, cellular trafficking and metabolic reactions, supporting its many roles in cellular signaling networks central to health and disease (Del Mare S. et al., 2009). Interestingly, the highest normal WWOX expression was detected in hormonally regulated tissues such as testis, ovary, and prostate, as well as liver. This expression pattern, coupled with the presence of an SRD domain, and specific amino acid features, suggests a role for WWOX in steroid metabolism. Furthermore, it was recently observed that Wwox knock-out (KO) mice exhibit marked reductions in serum lipid levels and display impaired gene expression of key stereoidogenic enzymes (8;35). Its role in cellular lipid homeostasis and lipoprotein metabolism remains however unknown.

Based on these genetic and physiological observations, we investigated the potential role of WWOX in lipoprotein and HDL metabolism using a combination of in *vivo* functional studies and human genetic analyses. We examined Wwox total KO mice, and then generated a Wwox liver-specific KO (hep KO) model. We specifically examined the expression of key regulators of HDL biogenesis, apolipoproteinA-I (apoA-I) and the ATP-binding cassette transporter A1 (ABCA1), established as critical components in nascent HDL formation and reverse cholesterol transport (Iatan et al., 2011). Using gene micorarray in Wwox hep KO mice and next generation sequencing in French Canadian families with low HDL, we further characterized the implications of WWOX in metabolic pathways. Our findings are a first evidence of the functional role of the WWOX gene in HDL and lipoprotein metabolism.

4.4 Results

4.4.1 Impaired expression of key nascent HDL players in Wwox null mice

In order to investigate the role of WWOX in HDL metabolism, we first examined circulating lipid levels of *Wwox*-deficient (*Wwox*$^{-/-}$) mice, previously

described as hypoglycemic, with impaired expression of key steroidogenic enzymes, as compared to wild-type (WT) littermates (Aqeilan et al., 2007; Ludes-Meyers et al., 2009). HPLC serum characterization of Wwox null mice revealed lipoprotein cholesterol profiles with marked reduction in HDL-C levels (**Supplementary Figure 4.1A**). We next examined circulating apoA-I levels in serum from $Wwox^{-/-}$ mice, and observed that the decrease in HDL-associated cholesterol correlates with a reduction in ApoA-I, as assessed by SDS-PAGE (**Supplementary Figures 4.1B-C**). Additionally, 2D-PAGGE analysis of HDL sub-species in Wwox KO mice revealed a significant absence of larger α-LpAI subpopulations, as well as pre-β migrating particles (**Supplementary Figure 4.1D**).

Given the majority of HDL is produced in the liver, we further assessed the effect of Wwox deletion in hepatic tissues from Wwox null mice. We examined expression of key regulators of HDL biogenesis, apoAI and ABCA1. mRNA levels of both ABCA1 and apoAI were significantly decreased (ABCA1 P=0.00058) in Wwox KO mice, as assessed by real-time PCR (**Figure 4.1A**). In line with these findings, protein levels of ABCA1 and ApoAI were reduced by 40% and 80%, respectively, suggesting that WWOX ablation may alter endogenous production of apoAI and subsequent lipidation through the ABCA1 transporter (**Figures 4.1B-E**). Together, these data are consistent with the observed reduction in HDL-C levels and nascent HDL subspecies in serum from $Wwox^{-/-}$ mice.

Figure 4.1. Impaired expression of key nascent HDL players in Wwox null mice.

(**A**) *Effect of WWOX knock-out (KO) on ABCA1 and ApoAI mRNA levels*. Total RNA was isolated from liver samples from WT and Wwox total KO mice, as described in Methods. Analysis of mRNA expression of WWOX, ApoA1, ABCA1 was carried by RT-qPCR from 200 ng RNA/sample. Values shown are means ± SEM of three independent experiments, each performed in triplicates. Expression of each gene was normalized to GAPDH expression, and mRNA fold changes relative to controls were determined. ***P < 0.00058 by Student's *t*-test. (**B**-**E**) *Effect of WWOX KO on ABCA1 and ApoAI protein levels*. Total protein extracts were isolated from liver samples from WT and Wwox total KO mice as described in Methods. Equivalent quantities of lysate were separated by SDS-PAGE and analyzed by western blot, using antibodies against ABCA1, WWOX, GAPDH (loading control) in (**B**) and ApoAI, WWOX and α-tubulin (loading control) in (**C**). Band intensities of ABCA1, ApoAI and loading controls were quantified, and relative levels of ABCA1 (**D**) and ApoAI (**E**) were graphed. Graphs represent the average values of 4 (**D**) and 3 (**E**) different mice samples, with error bars representing the SEM. Statistical analysis was performed as described (**P=0.0015 and ***P=0.007 by Student's *t*-test). Blots shown are representative of four independent experiments.

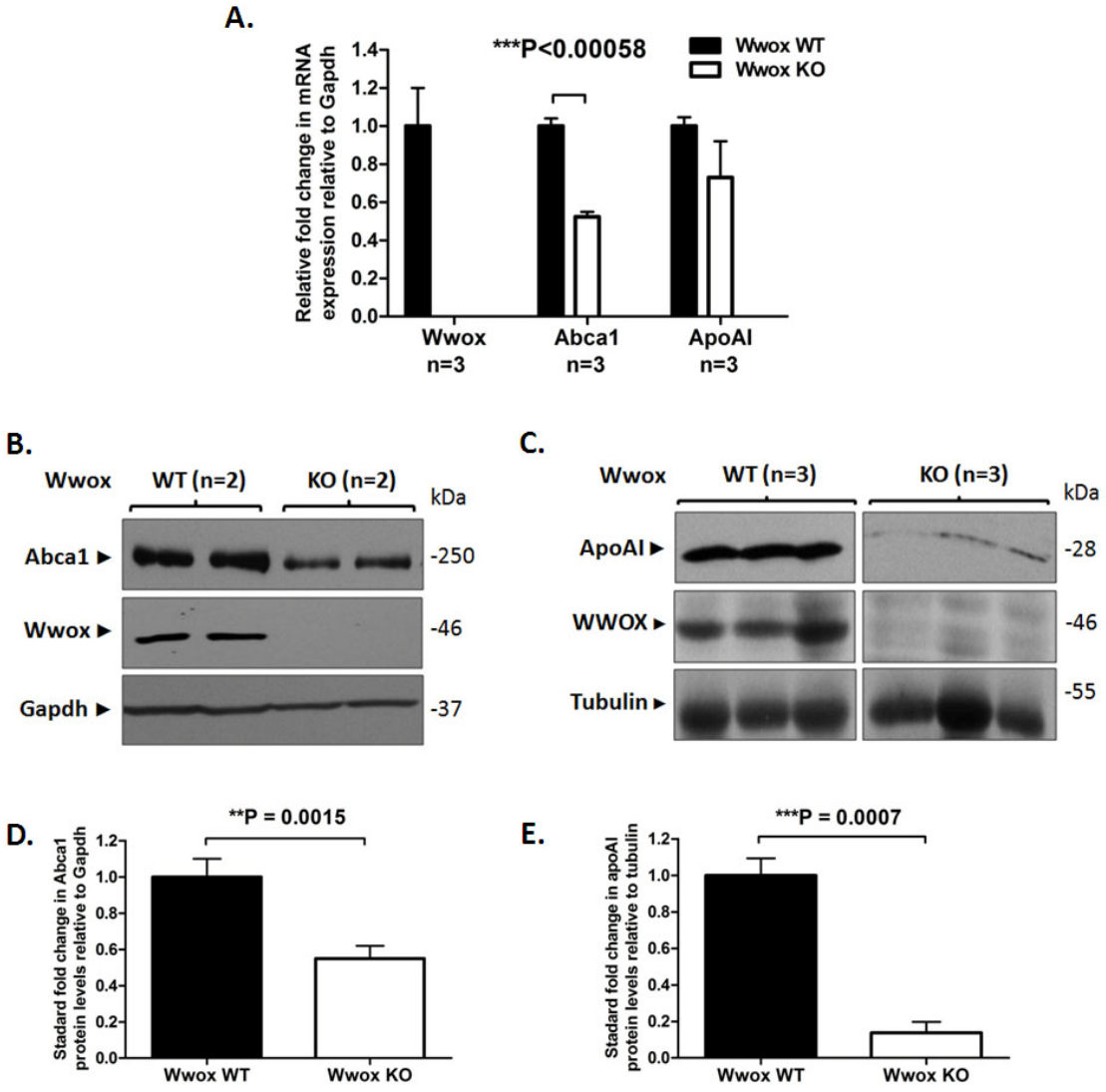


Figure 4.1. Impaired expression of key nascent HDL players in Wwox null mice

4.4.2 Targeted ablation of WWOX in the liver alters triglyceride serum levels

While studies on the full Wwox KO mice shed light on the *in vivo* Wwox requirement in HDL metabolism, previous studies have shown that whole body Wwox ablation leads to severe metabolic defects and growth retardation, resulting in early death by 4 weeks of age (Aqeilan et al., 2007; Ludes-Meyers et al., 2009), thus precluding additional adult lipoprotein analyses. Given the majority of HDL liver production, we generated a Wwox liver-specific KO mouse model (Wwox hep KO) (**Supplementary Figure 4.2**) in order to further delineate the role of WWOX in HDL metabolism. Wwox hep KO mice did not show any noticeable abnormalities, had normal life span and body weight, and presented no evidence of tumor development.

To assess the effect of liver-specific WWOX ablation on lipid metabolism, lipid profile analyses were performed on young and mature Wwox hep KO mice (**Supplemental Figure 4.3**) where total serum cholesterol, HDL-C, triglycerides (TG) and LDL-C were measured. While the absence of Wwox did not appear to result in a substantial change in cholesterol, HDL-C and LDL-C serum levels (**Supplementary Figure 4.3A-D, G-H**), a significant increase in TG levels was observed in older female mice (P=0.0025, **Supplementary Figure 4.3F**), as compared to WT. Separation of serum lipoproteins by HPLC in Wwox WT and Wwox hep KO females (n=10) and male (n=5) mice, further confirmed no significant differences in VLDL, LDL and HDL cholesterol profiles in the absence of WWOX in the livers of both genders (**Supplemental Figures 4.4A-B**). VLDL-associated TG levels were however significantly elevated in females hep KO mice (**Supplemental Figures 4.4C**), consistent with previous lipid serum measurements (**Supplementary Figure 4.3F**). ApoA-I serum levels assessed by western blot also showed no change in the liver-specific KO male and female mice, as compared to WT (**Supplementary Figure 4.4E, F**). These data suggest that liver-specific ablation of Wwox has no apparent effect on HDL serum levels, but rather an effect on circulating TG levels.

4.4.3 Gene expression profiling of Wwox hep$^{-/-}$ mice indicates a role of Wwox in several lipid metabolic pathways

Having targeted WWOX disruption in the liver, we wished to specifically assess its effects on liver tissues. To determine the effect of hepatic *Wwox* ablation on global gene expression in the liver, we performed microarray analyses on RNA from ~100 days old mice (8 males and 8 females, 4 Wwox WT and 4 Wwox hep$^{-/-}$ per group). Statistical analysis of Agilent genechip expression profiling data identified 699 probes differentially expressed ($P<0.05$, |fold change (FC)|>1.5) between Wwox hep$^{-/-}$ and WT females and 424 for the males ($P<0.05$ with |FC|>1.5), corresponding to 473 and 311 annotated genes, respectively. Wwox was differentially regulated with $P = 6.01x10^{-5}$ and FC = -2.53 in females and $P = 7.87x10^{-5}$, FC = -1.92 in males. Hepatic deletion of *WWOX* resulted primarily in a significantly different gender-specific gene expression pattern, with only 24 commonly regulated genes between males and females (**Supplementary Figure 4.5**). Heatmap representations of top 100 genes in both genders are shown in **Supplementary Figure 4.6A and B.** Significant genotype differences were also observed by hierarchical cluster analyses (**Supplementary Figure 4.6A and B)**. The Ingenuity resource was employed for pathways, network and functional analyses of significantly regulated probes (699 females and 424 males with $P<0.05$, |FC|>1.5) between Wwox WT and liver-specific KO mice. We identified the *lipid metabolism* function as top statistically significant annotated molecular and cellular function in females ($P=4.86x10^{-4}$ - $1.61x10^{-1}$ Benjamini-Hochberg FDR, **Figure 4.2A**), containing 64 molecules (**Supplementary Table 4.1A**) associated with 31 different sub-cellular functions (**Supplementary Table 4.2A**). In males, the *lipid metabolism* function was ranked as 7th significant function (Benjamini-Hochberg $P=2.74x10^{-3}$ - $9.24x10^{-2}$, **Figure 4.2B**) associated with the Wwox hep$^{-/-}$ differential expression profile, containing 56 genes (**Supplementary Table 4.1B**) associated with 16 subcellular functions **(Supplementary Table 4.2B)**. Furthermore, top canonical pathways differentially regulated between Wwox WT and hep KO female mice included pathways involved in FXR/RXR activation (P=0.0126), atherosclerosis signaling (P=0.0147), fatty acid

biosynthesis (P=0.0173), glycerolipid metabolism (P=0.019) and LXR/RXR (P=0.0477) activation (**Figure 4.2C**). The latter two were also common to male canonical pathways (**Figure 4.2D)**.

To further compare similarities and differences in differential expression between Wwox WT and hep$^{-/-}$ mice, all genes found to be associated with the *lipid metabolism* function in females (64 genes, **Supplementary Table 4.1A**) and males (56 genes, **Supplementary Table 4.1B**) were displayed in heatmaps (**Figure 4.3A and B**). We obtained evidence that genes such as apoA-I (P=0.0028, FC=-1.80), neutral cholesterol ester hydrolase 1 (NCHE1, P=0.003, FC=1.59), fatty acid synthase (FAS, P=0.003, FC=1.98), glycerol-3-phosphate acyltransferase (GPAM, P=0.00426, FC=1.51), endothelial lipase (LIPG, P=0.0045, FC=1.94), SREBP chaperone (SCAP, P=0.0093, FC=-1.80), lipoprotein lipase (LPL, P=0.014, FC=-2.08), phospholipid transfer protein (PLTP, P=0.028, FC=1.93), angiopoietin-like 4 (ANGPTL4, P=0.032, FC=1.64), choline kinase-α (P=0.042, FC=-1.70) and insulin induced gene 2 (INSIG2, P=0.049, FC=-1.75), among others, where significantly deregulated in females. A differentially expressed fold change decrease in ABCA1 levels was also observed in both males (FC= -1.14) and females (FC= -1.04), although not statistically significant at the mRNA level. Further classification of all *lipid metabolism* genes by subcellular functions in both genders confirmed a considerably greater number of lipid-related functions in females (31 functions, **Supplementary Table 4.2A**) than in males (16 functions, **Supplementary Table 4.2B**). These include, among others, regulation of cholesterol, TG, and fatty acid concentration, synthesis and transport, suggesting that the differentially expressed lipid-related genes between Wwox WT and hep KO females were strongly associated with different lipid metabolic pathways, including HDL, TG and fatty acid metabolism (**Supplementary Table 4.2A and B**). This was confirmed by network pathways analysis of the 64 female and 56 male genes involved in *lipid metabolism*, where we observed, through different pathways, a predicted overall decrease in HDL metabolism in both sexes, and a predicted TG increase in females (**Figure 4.4A and B**), consistent with previous observations.

Figure 4.2 Top 15 Functions and Canonical Pathways associated with Wwox hepatic deletion in mice

Lipid metabolism was the top most significant molecular and cellular function associated with differentially expressed genes ($P < 0.05$, $|FC| > 1.5$) in females (A, green) and seventh in males (C, green). Overall in females, there were more lipid- and cardiovascular-associated canonical pathways (B, orange) than in males (D, orange). Fatty acid metabolism and LXR/RXR activation were pathways found in both males and females. Ingenuity Systems Inc. software was used to clusters genes by function or pathways. Significance threshold $P < 0.05$ was calculated using Fisher's exact test.

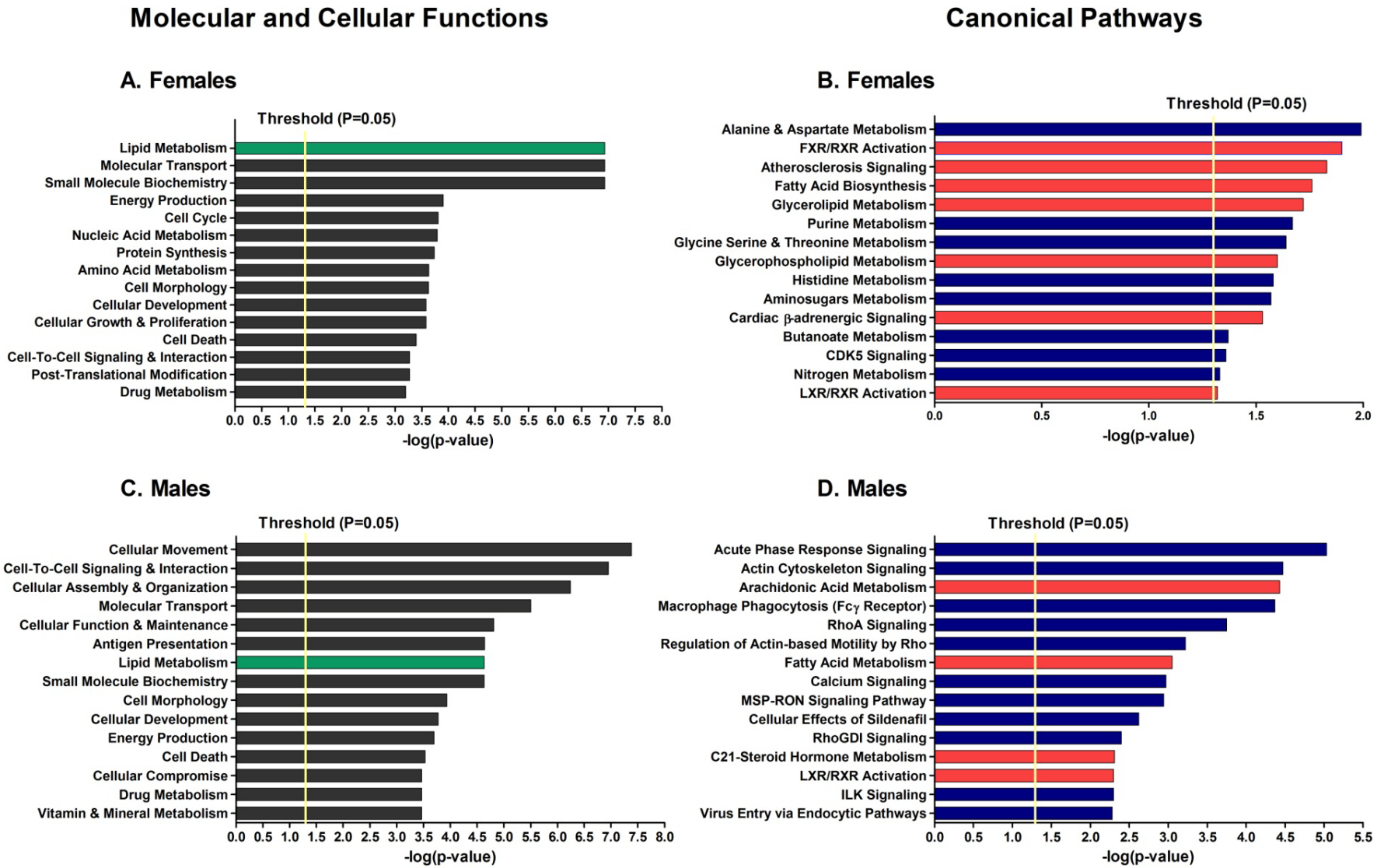


Figure 4.2. Top 15 Functions and Canonical Pathways associated with Wwox hepatic deletion in mice

Figure 4.3 Hierarchical cluster analysis of genes involved in lipid metabolism
Among the differentially expressed genes ($P < 0.05$, $|FC| > 1.5$) between Wwox WT and Wwox hep KO mice, 64 genes in females (A) and 56 genes in males (B) were significantly associated with the lipid metabolism (a top function in females). Heat map representations of these genes are shown below, with expression values being standardized within-gene and mapped to a color scale going from green (significantly down-regulated) to red (significantly up-regulated). The cluster correlation is shown on the right and the numbers at the bottom indicate individual mice.

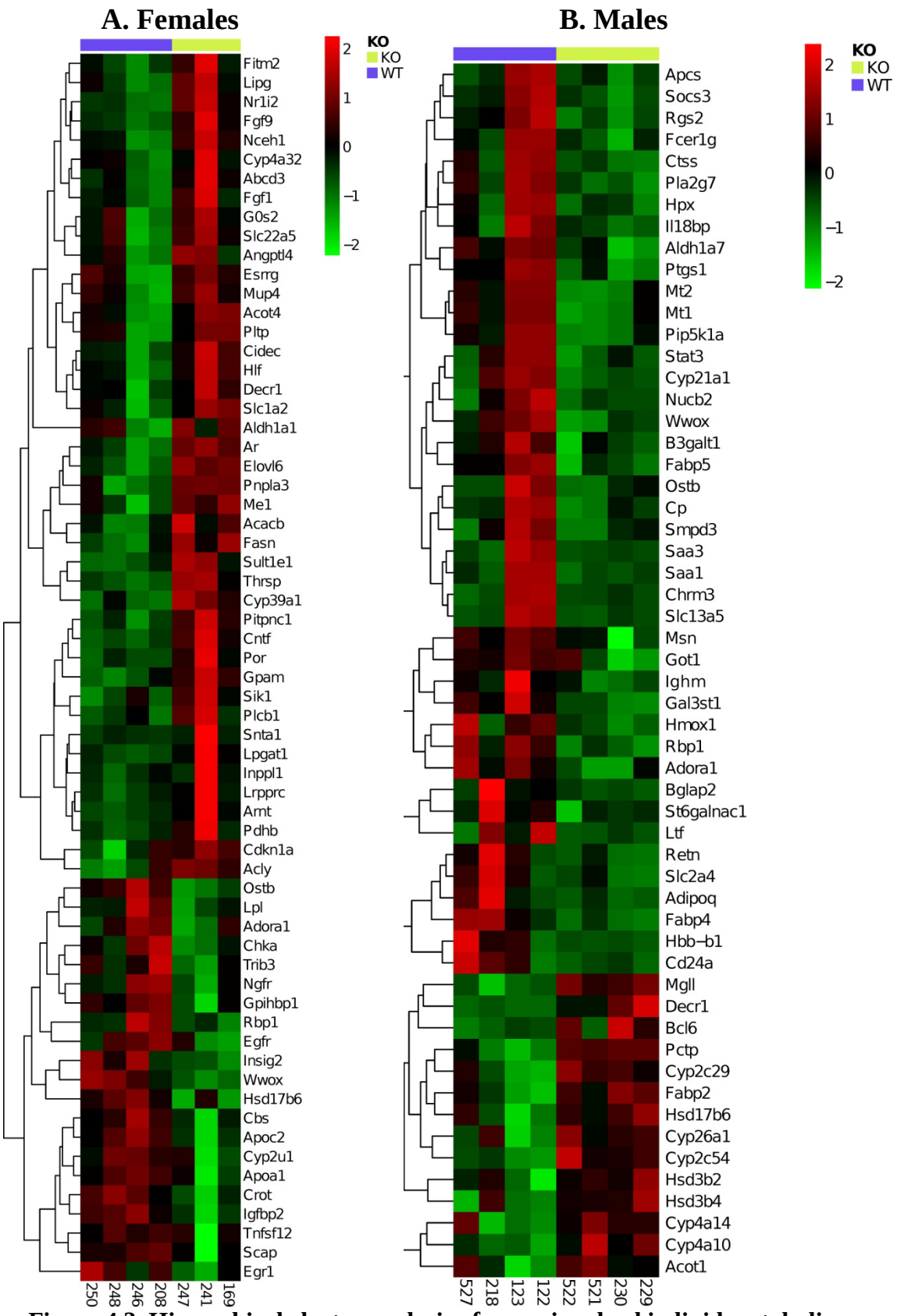
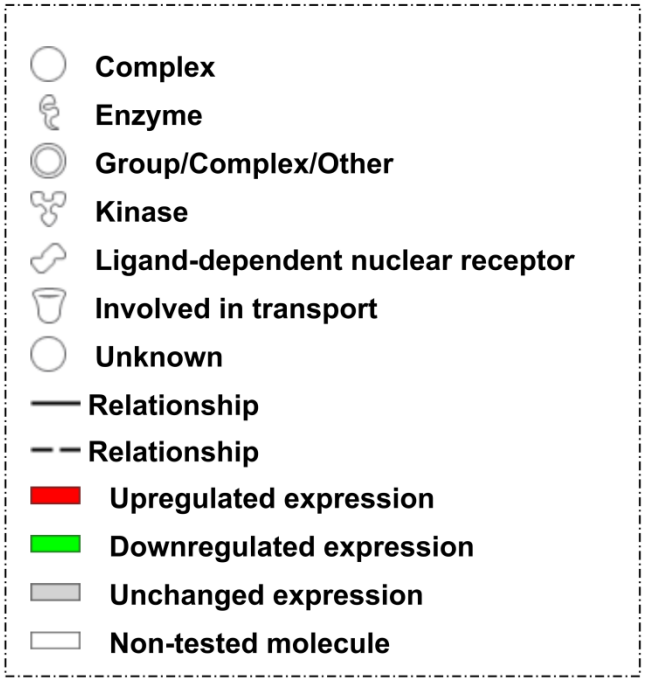


Figure 4.3 Hierarchical cluster analysis of genes involved in lipid metabolism

Figure 4.4 Proposed effect of hepatic Wwox deletion on lipid metabolic pathways

To see the net effect of all significantly regulated ($P < 0.05$, $|FC| > 1.5$) lipid genes (64 in females and 56 in males) on lipid metabolism, Ingenuity Network analysis was performed. 21 molecules in females and 19 in males had the highest score associated with the lipid metabolism having an effect of HDL-C. Pathways analyses suggest that HDL levels may be lower in both female (A) and male (B) hepatic Wwox-deficient mice. Red color indicates upregulated genes or molecules, green color denotes downregulated signature genes and gray color represents genes with unchanged expression. Molecules indicated with empty white icons were not probed by the microarray but are associated with the network based on pathway analysis. Lines between molecules indicate direct interactions and dotted lines indicate indirect relationships. Full names of genes is provided in **Supplementary Table 4.1 A** (females) and B (males).

Legend



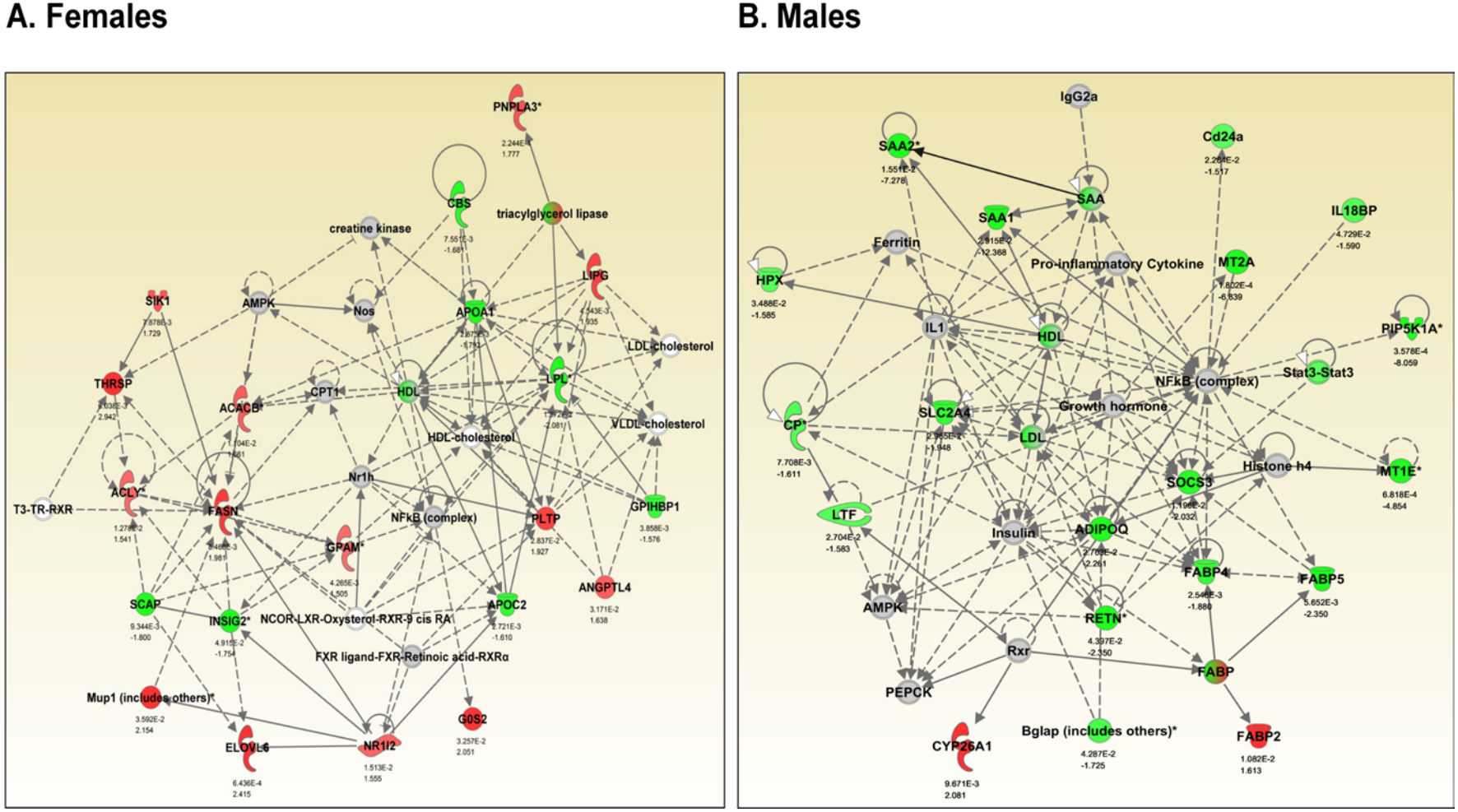


Figure 4.4 Proposed effect of hepatic Wwox deletion on lipid metabolic pathways

4.4.4 Validation of gene expression changes on HDL- and Tg-regulators in Wwox liver-specific knock-out mice

We next sought to validate findings from our microarray analyses by assessing protein levels of key regulators of HDL and TG metabolism. One of the most significantly downregulated lipid-related genes in the *Wwox hep KO female mice was ApoA-I (*P=0.0028, FC=-1.8), which interacts with ABCA1 for nascent HDL formation. We therefore determined levels of these proteins in mice hepatic tissues, similarly to total KO mice. In both male and female tissues, a significant reduction in ApoA-I (males 55% reduction, P=0.0068, **Figure 4.5A;** females, 50% reduction, P=0.00073, **Figure 4.5B**) levels was found, with a concomitant decrease in males ABCA1 (50% reduction, P=0.035, **Figure 4.5C**), but no significant change in females ABCA1 (**Figures 4.5D**). Next, having identified several genes involved in modulating TG levels, we focused on ANGPTL4 (FC=1.64, P=0.032), recently established as a potent modulator of blood plasma TG and HDL levels, and found to be upregulated 1.64 fold in Wwox hep KO females (**Supplementary Table 4.1A**). Consistent with mRNA findings, Wwox liver-specific KO mice displayed an increase in ANGPTL4 protein expression in both males (60% increase, P=0.0023, **Figure 4.5E**) and females (35% increase, **Figures 4.5F**). These results indicate that the microarray collection of data may provide substantial insight into the regulation of lipid metabolism by Wwox.

To further validate gene expression findings, we investigated how liver-specific Wwox KO mice handle a Western diet (WD). Male and female Wwox WT and hepatic KO mice were fed a high-fat/cholesterol diet (42% calories from fat with 0.2% cholesterol content**)** for 46 weeks and weighed at various time points. Throughout the study, both male and females Wwox hep KO mice on WD weighed significantly more (P<0.0001) than their age-matched WT counterparts (**Figures 4.6A, B**). When sacrificed, livers, intraperitoneal fat and hearts were measured and expressed as percent of body weight (**Figure 4.6C**). Interestingly, female Wwox hep KO mice had a significantly higher amount of percent body fat (45% increase, P=0.009, **Figure 4.6D**) as compared to WT, while male hepatic knock-outs showed no change. These results suggest that hepatic deletion of WWOX might activate TG- and fatty acid related pathways in female mice, consistent with microarray findings.

Figure 4.5 Validation of gene expression changes on HDL- and Tg-related proteins in Wwox liver-specific knock-out mice

(A-D) *Effect of WWOX liver-specific knock-out (Wwox hep KO) on ABCA1 and ApoAI protein levels*. Total protein extracts were isolated from liver samples from male (**A**) and female (**B**) WT and Wwox hep KO mice as described in Methods. Equivalent quantities of lysate were analyzed by western blot, using antibodies against ABCA1, ApoAI and GAPDH (loading control). Band intensities were quantified, and relative levels of ABCA1 and ApoAI were graphed for both male (**C**) and female (**D**) samples. Graphs represent the average values of three different mice samples, with error bars representing the SEM. Blots shown are representative of 5 independent experiments. **(E-F)** *Effect of Wwox hep KO on ANGPTL4 expression*. (**E**) Lysates were prepared as described in panel *A* and subjected to western blot, using antibodies against ANGPTL4 and GAPDH (loading control). Relative levels were quantified and values graphed represent means ± SEM of three independent samples (**F**). Statistical analyses were performed using Student's *t*-test.

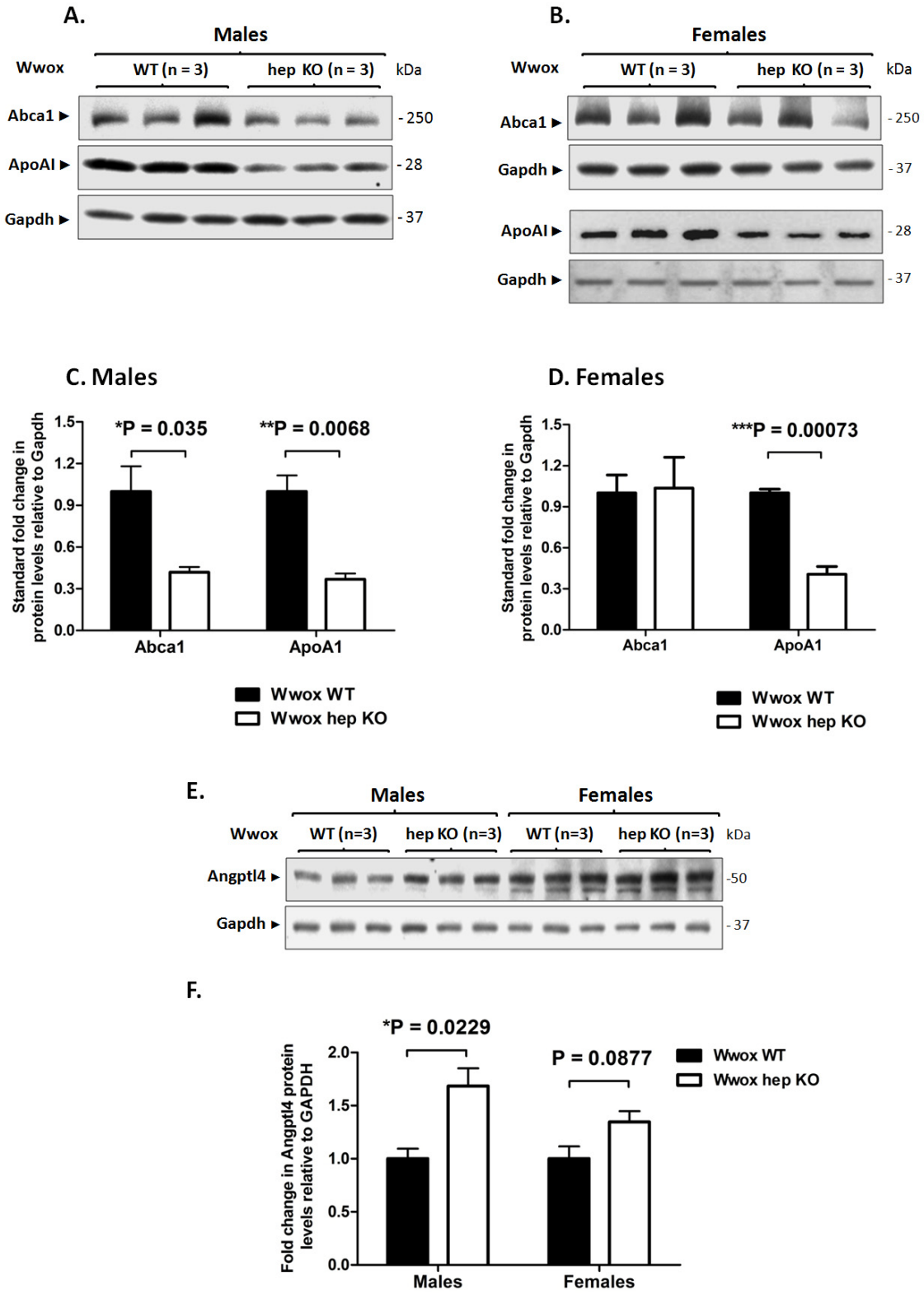
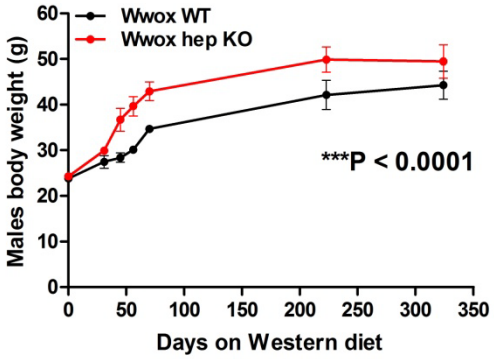


Figure 4.5 Validation of gene expression changes on HDL- and Tg-related proteins in Wwox liver-specific knock-out mice

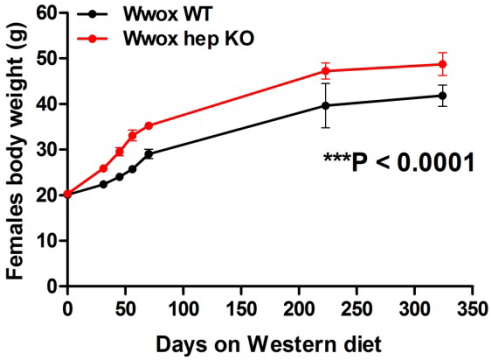
Figure 4.6 Effect of Western diet on Wwox liver-specific knock-out mice

(A-B) E*ffect of Western diet on body weight of Wwox liver-specific knock-out (Wwox hep KO) mice.* Wwox mice (3 male WT, 4 male hep KO, 3 female WT, 5 female hep KO) were put on a high-fat high-cholesterol diet (TD-88137) for 46 weeks, after which they were sacrificed. Average mice body mass weighted at the indicated time points is graphed for males (**A**) and females (**B**) with error bars representing ± SEM. Two-way Anova was used for statistical analyses between body weight curves of Wwox WT and hep KO mice. Liver-specific Wwox mice gain weight significantly faster than the Wwox WT mice under a Western diet, in both genders. **(C**, **D)** *Effect of Western diet on fat content in Wwox hep KO mice.* When sacrificed, body weight, liver, heart and total intraperitoneal fat content of *Wwox hep KO mice* on Western diet was determined as described in Methods. The percentage of liver (% liver), heart (% heart) and fat (% fat) of body weight was calculated for each mouse, and means ± SEM are shown for both genders. Females Wwox hep KO had a significantly higher % fat content as compared to WT. Statistical analyses were performed using Student's *t*-test.

A. Males



B. Females



C.

	Males		Females	
Gene	WT	Wwox hep KO	WT	Wwox hep KO
Number	n = 2	n = 4	n = 3	n = 5
Age (weeks)	52 ± 1.5	53± 1	50 ± 1.9	53 ± 0.2
Body weight (g)	44 ± 3.8	49 ± 3.7	42 ± 2.4	49 ± 2.5
Liver (% of weight)	6.5 ± 2	5.9 ± 0.8	4.9 ± 0.6	5.5 ± 0.7
Heart (% of weight)	0.44 ± 0.01	0.42 ± 0.02	0.42 ± 0.02	0.37 ± 0.01
Fat (% of weight)	6.7 ± 0.1	6.8 ± 1.2	8.7 ± 0.1	**13.1 ± 0.9

D.

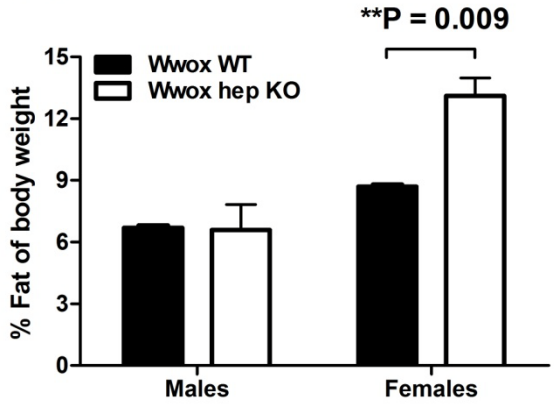


Figure 4.6 Effect of Western diet on Wwox liver-specific knock-out mice

4.4.5 Resequencing of WWOX in low HDL-C families

In light of the observed physiological significance of WWOX in HDL and TG metabolism in mice models, we next sought to validate its role in HDL metabolism in families with low HDL-C.

We selected two family members (HDL-C $\leq 5^{th}$ age-sex percentile, **Table 4.1**) from two French Canadian families with linkage on chr16q23-24, and four unrelated controls (HDL-C $\geq 30^{th}$ percentile, **Table 4.1**) for targeted resequencing of the entire WWOX region +/- 100kb in search for regional variants causing low HDL-C. Lipid levels and other clinical characteristics of all family members from both French-Canadian families are indicated in **Supplementary Table 4.3**. We identified 19 variants that were shared by all the sequenced cases in family 1 and 2 and absent in the normal controls. We selected 7 SNPs and an indel based on their frequency and linkage disequilibrium to avoid typing redundant SNPs (**Table 4.2**). These variants were then investigated for co-segregation with the low HDL-C affection status in the extended low HDL-C families (**Figure 4.7A, B**). All 16 affected family members except one shared the variant allele haplotype of rs72790052, rs4462603, rs5818121, rs16948856, and rs11647906 (**Figure 4.7A, B**). Using the Mendel software, we also observed statistically significant evidence for association between rs72790052, rs4462603, and rs5818121 and serum HDL-C levels (P=0.0013-0.0005; Beta=-0.3544 - -0.3333; and SE=0.1016-0.1035) in the families (**Table 4.2**). These three variants are located in intron 5 and are relatively rare (MAF=~0.1-9.0%), whereas the variants rs16948856 and rs11647906 located in intron 8 are common (MAF=18% and 27% respectively), and they may thus be shared by chance alone. Additionally, we observed a borderline gender effect (P=0.06) between these segregating WWOX variants and low HDL. Among the 8 variants there is only one nonsynonymous variant (rs289723) in the NLRC5 gene which did not co-segregate with low HDL-C status in the families (**Figure 4.7A, B**). Regarding the two remaining variants rs67003684 and rs9938194, the haplotype co-segregating to the affected subjects in family 2 contains the wildtype allele of these two variants rs67003684 and rs9938194, making them less likely candidates.

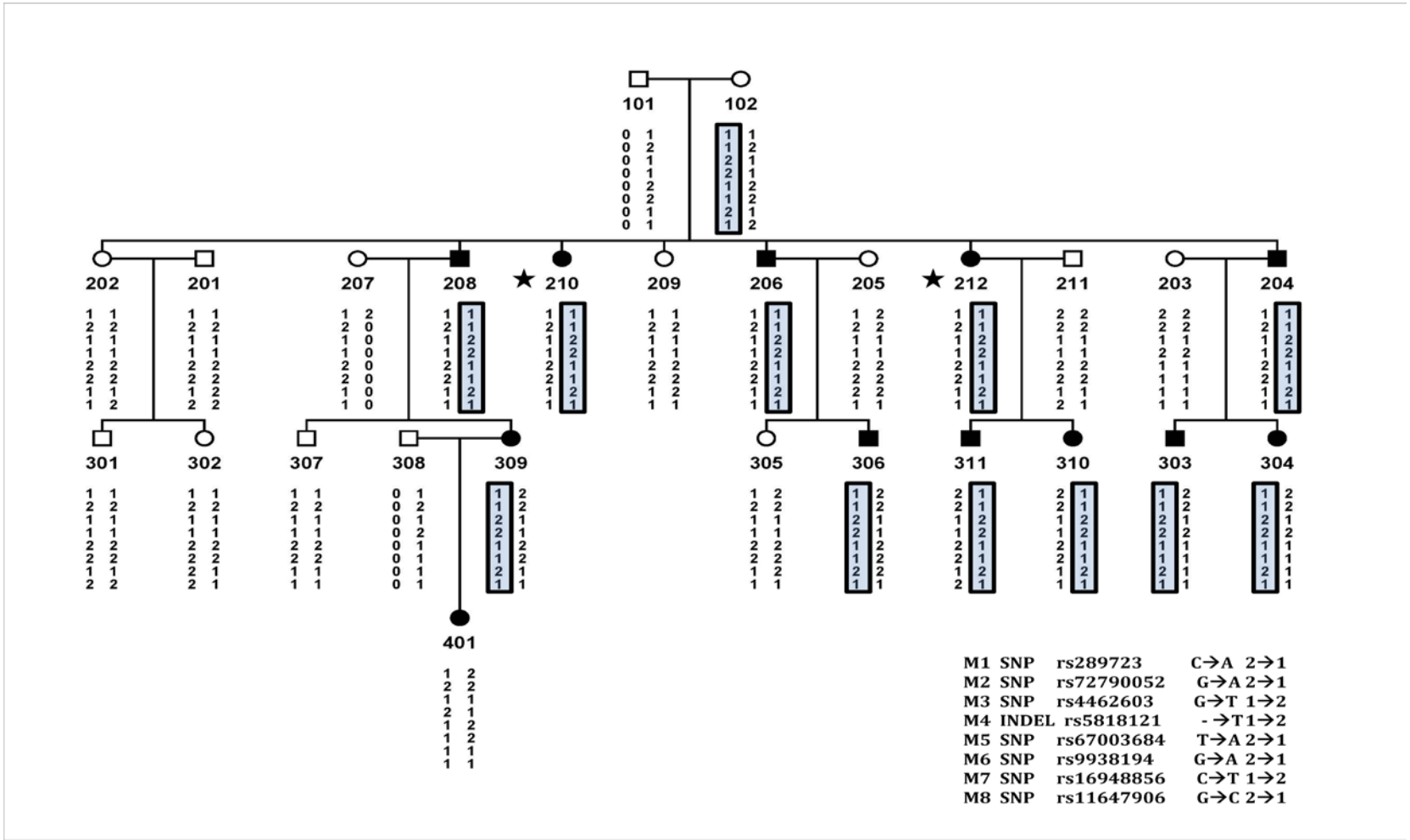


Figure 4.7 A and B. Haplotypes of the variants identified in the targeted sequence analysis in the two French Canadian low HDL-C families

Fifteen of the 16 affected subjects with HDL-C < 5th age-sex specific population percentile have the risk alleles for the WWOX variants rs72790052, rs4462603, and rs5818121. The subjects whose samples were resequenced are indicated by a star. M, marker; Completely filled symbols indicate < 5% HDL-C; Unfilled symbols indicate >10% HDL-C

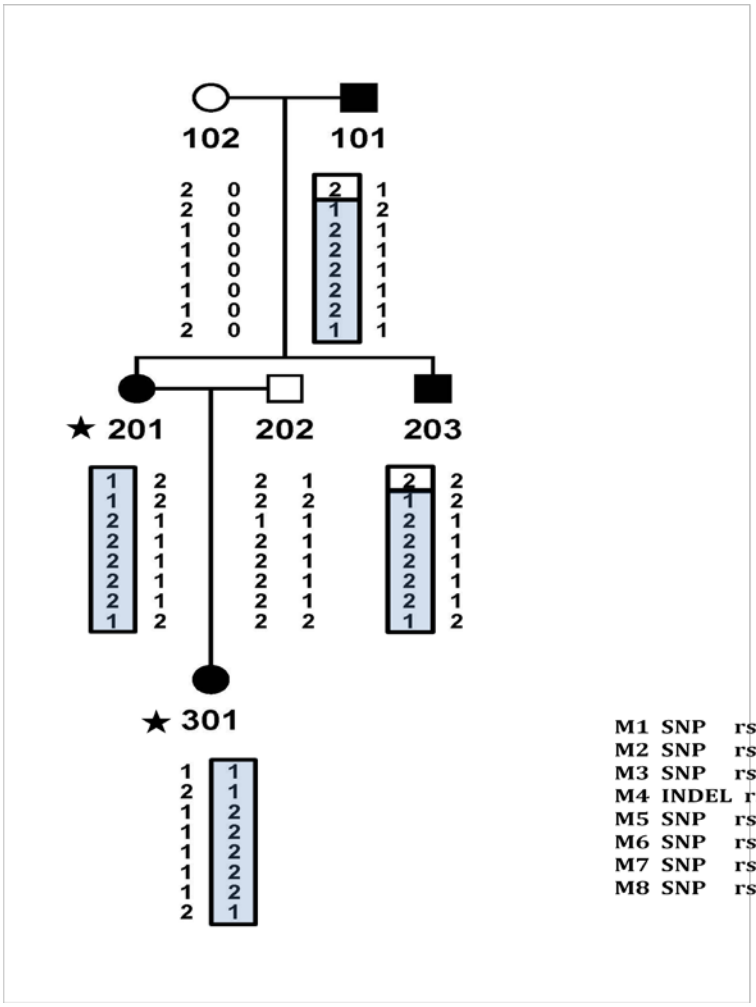


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Table 4.1 Lipid levels and other clinical characteristics of the four affected and four controls that were resequenced

FAM	Ind_ID	Sex	Status	Age	HDL-C	HDL%	BMI	TG	TC	LDL-C
1	210	2	2	69	0.67	<5%	23.8	5.31	5.71	2.65
1	212	2	2	48	0.66	<5%	21.5	3.78	6.22	3.86
2	201	2	2	40	0.47	<5%	29.2	3.67	3.98	1.86
2	301	2	2	13	0.46	<5%	25.6	1.34	3.82	2.76
Control	101	2	1	50	1.41	33	27.6	1.41	6.2	4.15
Control	102	2	1	50	1.58	49	18.9	1.35	4.84	2.64
Control	103	2	1	28	1.44	52	27	0.54	3.73	2.05
Control	104	1	1	39	1	33	19	0.66	5.03	3.73

Lipid levels are shown in millimoles per liter.
FAM, family; Ind ID, individual ID; HDL-C, high-density lipoprotein-cholesterol; BMI, body mass index, TG, triglycerides; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol

Table 4.2 Association analysis in the two families with low HDL-C (mmol/L)

SNP	Position	Ref Allele (Ref Allele code)	Var allele (Var allele code)	MAF	Gene	Location	Effect*	SE†	P-value
rs289723	57080528	C (=2)	A (=1)	0.267	NLRC5	Exon21	-0.1973	0.1956	0.3131
rs72790052	78305527	G (=2)	A (=1)	0.025	WWOX	Intron5	-0.3544	0.1016	0.0005
rs4462603	78311633	G (=1)	T (=2)	0.092	WWOX	Intron5	-0.3544	0.1016	0.0005
rs5818121	78320704	-- (=1)	T (=2)	--	WWOX	Intron5	-0.3333	0.1035	0.0013
rs67003684	78321698	T (=2)	A (=1)	0.175	WWOX	Intron5	-0.3213	0.1099	0.0035
rs9938194	78322348	G (=2)	A (=1)	0.158	WWOX	Intron5	-0.3213	0.1099	0.0035
rs16948856	78946587	C (=1)	T (=2)	0.183	WWOX	Intron8	-0.2801	0.1208	0.0205
rs11647906	79084889	G (=2)	C (=1)	0.275	WWOX	Intron8	-0.5195	0.2229	0.0198

*Effect size shown is beta-coefficient per copy of the rare allele after adjustment for age and sex.
†SE denotes standard error; Ref, reference; Var, variant; MAF, minor allele frequency

4.5 Discussion

In this study, we show that WWOX is involved in HDL and lipoprotein metabolism, using a combination of *in vivo* functional studies, by means of total Wwox knock-out and Wwox liver-specific mouse models, gene microarray and next generation resequencing analyses in HDL-deficient French Canadian families.

WWOX has been previously established as a tumour suppressor (Aqeilan et al., 2007) found to be associated with HDL-C levels in several human and mouse genetic association studies (Lee et al., 2008b; Leduc et al., 2011; Saez et al., 2010), as well as online databases (Willer et al., 2008). Given its biological plausibility in HDL metabolism, we were interested in investigating its role in cellular lipid homeostasis and nascent HDL biogenesis.

Using two Wwox deficient mouse models (Wwox total KO and Wwox liver-specific KO), we determined that in the absence of WWOX, mRNA and protein levels of key regulators of HDL metabolism are altered. Both models demonstrated a reduction in ApoA-I and ABCA1 levels, critical components in RCT and generation of nascent HDL (Iatan et al., 2011) (**Figure 4.1 and Figure 4.5**), suggesting that it may be through these targets that WWOX acts on regulating HDL-C levels. Interestingly however, in the Wwox hep KO, while we observed a small ABCA1 mRNA fold change decrease by microarray analyses in both genders (males FC= -1.14 and females FC= -1.04), only in males did we notice a significant reduction in ABCA1 protein levels (50% reduction, P=0.035, **Figure 4.5A, C**), with unchanged levels in the females. This might be due to gender specific translational regulation and warrants further investigations. ApoA-I protein levels were observed to be reduced in both genders in the Wwox hep KO (males 55% reduction, P=0.0068, **Figure 4.5A, C**; females 50% reduction, P=0.00073, **Figure 4.5B, D**), similarly to total KO mice (**Figure 4.1**). To date however, limited information has been found on WWOX interactions partners in lipid metabolism, though it has been shown to alter the activity of transcription factors through binding to PPxY rich motifs (Aqeilan et al., 2005; Aqeilan and Croce, 2007). Further studies would be needed to assess if WWOX directly

promotes ABCA1 and ApoA-I transcription, or if other intermediates are involved, to determine its specific mechanisms on HDL metabolism.

Despite consistent results in liver tissues of both mice models, differences in serum lipid levels between total and liver-specific Wwox knockout models were observed. It was first determined that total Wwox knockout mice had significantly lower HDL and serum ApoA-I levels (**Supplementary Figure 4.1 A, B, C**), consistent with previous observations by Aqeilan and others that Wwox knockout mice exhibit marked reductions in total lipid serum levels and impaired expression of key steroidogenic enzymes (Ludes-Meyers et al., 2009; Aqeilan et al., 2009; Aqeilan et al., 2007). Furthermore, analyses of HDL sub-species in Wwox total KO mice showed an absence of larger α-LpAI and pre-β migrating particles (**Supplementary Figure 4.1D**), consistent with human studies (Marcil et al., 2003) where similar 2D-PAGGE patterns are frequently secondary to disorders of HDL biogenesis. In contrast, liver-specific WWOX deletion did not result in a substantial change in total serum cholesterol, HDL or ApoAI levels (**Supplementary Figure 4.3, 4.4**). Instead, a significant increase in overall TG levels and VLDL-TG content in serum lipoproteins was observed in older Wwox hep KO female mice, in agreement with previous genetic studies documenting association between Wwox and TG (Saez et al., 2010). It was further determined that circulating HDL-C levels were subject to variations between individual Wwox hep KO mice (**Supplementary Figure 4.3**). This was not however reported with TG levels, consistently seen to be elevated in the female Wwox hep KO mice, as demonstrated by both serum lipid measurements (**Supplementary Figure 4.3**) and HPLC lipoprotein profiling (**Supplementary Figure 4.4**).

Specific disruption of WWOX in the liver revealed important roles in lipid metabolism. In addition to identifying the *lipid metabolism* function as the most significantly regulated function in females, and 7th for male Wwox hep KO mice **(Figure 4.2A, C)**, microarray analyses identified several lipid-related canonical pathways differentially regulated between Wwox WT and hep KO female and male mice (**Figure 4.2B, D**). Furthermore, when specifically examining the 64 female genes and 56 male genes ($P<0.05$, $|FC|>1.5$) associated

with the *lipid metabolism* (**Figure 4.3A, B and Supplementary Table 4.1A, B**), we observe genes involved in cholesterol homeostatis, hydrolysis and biosynthesis of triglycerides and fatty acid biosynthesis. As demonstrated through network analyses **(Figure 4.4**), upregulation of genes such as ANGPLT4, FASN, PLTP, GPAM, LIPG and downregulation of genes such as ApoAI, LPL, INSIG2 and SCAP, suggest that a global effect on several pathways in lipid metabolism **(Supplementary Table 4.2A, B)** exists by the loss of Wwox in the livers of both males and females. Importantly, these point towards a decrease in HDL metabolism in both males and females (**Figure 4.6A, B**).

While we focused on lipid metabolism-related genes and pathways, it is important to mention that WWOX deletion affected a multitude of other metabolic pathways, such as those involved in molecular transport, energy production, cellular assembly and cell death, among others. In particular, in males it was observed that canonical pathways associated with acute phase response signaling and immune response genes were affected. (**Figure 4.4 and Supplementary Figure 4.6**). This is in concert with previous findings where WWOX was found to associate with a spectrum of biological partners, and thus play a central role in various signal transduction pathways (Del Mare S. et al., 2009).

Our microarray studies led us to pursue several genomic targets involved in lipoprotein metabolism. In addition to downregulated mRNA levels of ApoAI and ABCA1, which we subsequently validated through protein analyses (**Figure 4.5A, B**), we also investigated ANGPTL4, an inhibitor of lipoprotein lipase, which hydrolyzes and facilitates clearance of plasma TG. Consistent with our array data, depletion of WWOX in liver tissue caused a significant 1.6 fold increase in ANGPTL4 protein expression (P=0.023, **Figure 4.5E, F**). In combination with other TG-regulating genes, such as reduction in LPL (FC=-2.08), ANGPTL4 upregulation might explain the increase in circulating TG levels and VLDL-TG content in Wwox hep KO female mice. In recent studies by Lichtenstein *et al*, ANGPTL4 overexpression was shown to increase plasma TG by decreasing LPL activity, supported by the -2.08 FC decrease in LPL from our

data, converting LPL from a catalytically active dimer to an inactive monomer, and thus increasing VLDL-associated TG levels (Lichtenstein et al., 2007). ANGPTL4 overexpression thus impairs LPL-dependent plasma TG and cholesteryl ester clearance and subsequent uptake of fatty acids and cholesterol into tissues, which might explain the upregulation of fatty acid biosynthesis pathways and TG-related genes in Wwox hep KO mice. Additionally, while ANGPTL4 primarily affects plasma levels of TG, it was recently shown in GWAS studies to affect other related metabolic parameters, such as HDL metabolism (Kathiresan et al., 2009).

To further validate metabolic pathways identified through microarray analyses, Wwox WT and liver-specific KO mice were fed a Western diet for 46 weeks. A significant weight gain was observed in both males and female hepatic KO mice ($P<0.0001$, **Figure 4.6A, B**), as compared to WT, supporting findings that hepatic WWOX disruption affects genes involved in lipid and fatty acid metabolism, such as upregulation of FAS (FC 1.98) and ANGPTL4. This was also strengthened by observations that female Wwox hep KO mice had a significantly higher body fat% (45% increase, $P=0.009$, **Figure 4.6D**), than their WT counterparts. While it is well-established that ANGPTL4 expression is associated with diet-induced conditions, given that Wwox-deficient high fat-fed mice are heavier than their WT counterpart, one can thus postulate that WWOX may play an important role in diet-induced pathways.

Another intriguing observation was the significant gender difference between the role of WWOX in male and female mice. While both genders present the WWOX-mediated effect of ABCA1, ApoAI and ANGPTL4 proteins, genes found to be up- and down-regulated in each differ greatly, as do their lipid profiles. As such, the gender effect observed, first suggested by elevated serum TG levels exclusively in Wwox hep females, was further supported by differentially expressed pathways in microarray analyses and fat accumulation in female mice under a high-fat diet. As evidenced in our studies, it is likely that WWOX plays a more pronounced role in female mice in relation to HDL, TG and fatty acid metabolism, than it does in males. While it may be suggested that the

SDR domain of WWOX might influence a gene- gender effect, as recent data shows interaction between 17β-estradiol and this domain (Sze et al., 2004) it remains to be determined the exact physiological role of WWOX in gender differences associated with lipid metabolism.

In addition to the physiological significance of WWOX in HDL metabolism in mice models, we report here a genetic association between WWOX variants and the low HDL trait in French Canadian families with HDL-C $\leq 5^{th}$ age-sex percentile (**Figure 4.7, Table 4.2**). We have previously identified the WW-domain-containing oxidoreductase (WWOX) gene region to be associated with low serum HDL-C levels in a study sample comprising 9,798 subjects (Lee et al., 2008b). The same region has also been implicated in multiple linkage studies for HDL-C (Dastani et al., 2006c; Mahaney et al., 2003; Shearman et al., 2000; Soro et al., 2002). In the current study, we hypothesized that rare or low frequency HDL-C variants in WWOX may co-segregate with the low HDL-C trait in the linked families. As the exact role of WWOX in lipid metabolism remains to be established, rare WWOX variants may help reveal the underlying disease mechanism(s). The rare variant allele haplotype 'ATT' (rs72790052, rs4462603 and rs5818121) co-segregated with the low HDL-C in the families (P=0.0013-0.0005; beta= -0.3544 - -0.3333), suggesting intron 5 of WWOX as the location of the functional variant. Additionally, the observed borderline gender effect (P=0.06) between segregating WWOX variants and low HDL, might be due to the otherwise small number of individuals investigated, suggesting that larger and diverse study samples are needed to identify a more pronounced effect.

Moreover, our human genetic observations on region-wide association between WWOX variants and low HDL-C are consistent with total WWOX knock-out findings, where a reduction in HDL serum levels was also determined, as well as impairment of key regulators of HDL biogenesis. In contrast, sole ablation of WWOX in the liver does seem to be not sufficient to impair total HDL-C circulating levels, as evidence by the unchanged levels between WT and Wwox liver-specific KO mice. This supports the notion that the effects of WWOX extend beyond the liver, as evidenced by its involvement in multiple

genetic pathways (**Figure 4.4, Figure 4.6, Supplementary Table 4.2A, B**), and its *in vivo* requirement for mice survival, as demonstrated by early death of total KO mice (Aqeilan et al., 2007; Ludes-Meyers et al., 2009). The effect of hepatic WWOX disruption may also be diluted by the fact that our knockout was hepatocyte-specific, though livers contain other cells including stellate cells, sinusoidal endothelial cells and Kupffer cells, which may well express WWOX in the livers that were isolated.

Collectively, our data has established a physiological significance for WWOX in lipoprotein metabolism in mouse models and human genetic studies. Findings suggest a novel role for the *WWOX* gene in HDL metabolism, which may be mediated through the ABCA1/ApoA-I pathway, raising the possibility that WWOX may be involved in the complex network of cellular cholesterol homeostasis. This report is therefore a first line of functional evidence and comprehensive examination of a novel gene in lipoprotein metabolism, implicating WWOX as an important and influential modulator of HDL-C and TG levels, both in mice and humans. These findings thus emphasize the need to further elucidate the mechanisms of action of WWOX in lipoprotein metabolism, which may have important implications in preventing and treating atherosclerotic cardiovascular disease.

4.6 Acknowledgements

We thank the family members who participated in the study. This research was supported by CIHR grant MOP 97752 and Heart and Stroke Foundation and grants HL095056 and HL-28481 from the National Institutes of Health (PP). Iulia Iatan is supported by the CIHR's Frederick Banting and Charles Best Canada Graduate Doctoral award and MV Prasad Linga Reddy is supported by the American Heart Association grant 11POST7380028. Please include CIHR grants and other affiliations

4.7 Materials and Methods

4.7.1 Ethics and animal protocols

Mice used in these studies were maintained in a clean, modified-barrier animal facility, fed regular commercial mouse diet (Harlan Lab, Indianapolis, IN), unless otherwise mentioned, under controlled light (12L:12D) and temperature (68–74°F). All animal research was approved by the University of Texas M. D. Anderson Cancer Center Institutional Animal Care and Use Committee (Animal Welfare Assurance Number A3343-01) and the McGill University Animal Care Committee.

4.7.2 Generation of total Wwox-deficient mice

Total Wwox knockout (KO) mice were generated as previously described (Aqeilan et al., 2007). Briefly, embryonic stem (ES) cells previously transfected by electroporation with WWOX gene-targeting constructs were isolated from SVJ129 mice, injected into C57Bl/6J blastocysts and implanted into foster female mice. Male chimeras (B6-129) derived from ES clones were selected and intercrossed with C57Bl/6J females to identify mice with germ-line transmission.

4.7.3 Generation of C57BL/6-Wwoxflox;AlbCre KO/transgenic mice (Wwox liver-specific KO)

Female $Wwox^{flox/flox}$ mice (129SV/C57Bl/6 background, previously generated (Ludes-Meyers et al., 2009) were bred with male Alb-Cre (Tg/Tg) transgenic mice that have the Cre-recombinase gene under the control of the Albumin promoter (pure C57Bl6/J inbred from the Jackson Labs (Postic et al., 1999). The $Wwox^{flox/WT}$;AlbCre (Tg/0) mice were intercrossed to obtain $Wwox^{flox/flox}$;AlbCre (Tg/0) mice. Genotypes were determined by PCR using the oligonucleotide primers: Cre F: *5′-GCCTGCATTACCGGTCGATGCAACG-3′* Cre R: *5′-GTGGCAGATGGCGCGGCAACACCAT-3′* Wwox-N1: *5′-ATGGGCCGAAACTGGAGCTCAGAA-3′* Wwox-N2: *5′-TCAGCAACTCACTCTGGCTTCAAC-3′* Wwox-L:*5′-GCATACATTATACGAAGTTATTCGAG-3′.*

4.7.4 Study design of mice on Western diet

Mice were bred and allowed to mature to 7 weeks of age at which point Wwox WT and Wwox liver-specific KO (hep KO) males and females mice were weighted and put on a high fat- high cholesterol diet (Western diet) for a period of 46 weeks. The high fat diet (TD-88137, Harlan Teklad) consisted of 42% calories from fat with 0.2% cholesterol content. Mice body weight was measured at 0, 31, 45, 56, 70, 223 and 324 days, at which point the animals were sacrificed. At the end of the study period, mice were fasted for 4 hours, serum isolated and livers, intraperitoneal fat and hearts were harvested, weighted and snap frozen in liquid nitrogen.

4.7.5 Lipid measurements and lipoprotein separation assays

Serum was isolated by centrifugation (3000 rpm, 15 min, 4C) from blood extracted by aortic perfusion from fasting mice (4-6 hours). Samples used for lipid measurements (150ul) were shipped on dry ice to LipoScience Inc. (Raleigh, North Carolina) where serum levels of total cholesterol, HDL-C, LDL-C, triglycerides, apoA-I, apoB and glucose were measured on an Olympus AU400e immunoautoanalyzer. Liposcience measurement assays were validated in humans and lipid values falling below human range linearity were excluded from analyses (apoB, ApoA-I, LDL-C). Serum lipoproteins (250ul) were separated by high-performance liquid chromatography with a Superose 6 10/300 GL column (GE Healthcare) attached to a Beckman Coulter System Gold™ apparatus. A 150 mM NaCL mobile phase with a flow rate of 0.4 ml/ml was used for separation of samples into 72 400 ul-HPLC fractions that were collected in a 96-well plate using the ProteomeLabTM automated fraction collector (Beckman Coulter). Cholesterol and triglycerides content was determined enzymatically (Infinity™ kit; Thermo Scientific) following manufacturer's instructions. ApoA-I-containing particles were separated by two-dimensional polyacrylamide gradient gel (2D-PAGGE) electrophoresis as described previously (Krimbou et al., 2001; Krimbou et al., 2005). Briefly, samples (25 μl) were separated in the first dimension

(according to their charge) by 0.75% agarose gel electrophoresis (100 V, 3 h, 4°C) and in the second dimension (according to the size) by 5–23% polyacrylamide concave gradient gel electrophoresis (125 V, 24 h, 4°C). Iodinated high molecular weight protein mixture (7.1–17.0 nm; Pharmacia) was run as a standard on each gel. Electrophoretically separated samples were electrotransferred (30 V, 24 h, 4°C) onto nitrocellulose membranes (Hybond ECL; Amersham). ApoA-I-containing particles were detected with immunopurified polyclonal anti-apoA-I antibody (Biodesign) labeled with ^{125}I. The presence of labeled ^{125}I-apoA-I was detected directly by autoradiography using Kodak XAR-2 film.

4.7.6 RNA isolation and analysis of gene expression by RT-PCR

Total RNA was isolated from liver tissues using the RNeasy mini RNA extraction kit (Qiagen), according to manufacturer's instructions. Total RNA (200 ng) was reverse-transcribed using the QuantiTect Reverse Transcription kit (Qiagen). Real-time quantitative PCR was carried out using the Quantitect SYBR Green PCR kit and QuantiTect Primer assays (Qiagen): WWOX, (QT00147735), ApoA-I (QT00110663), and ABCA1 (QT00165690). All reactions were performed on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems). Amplifications were carried out in a 96-well plate with 50 µl reaction volumes and 40 amplification cycles (94°C, 15s; 55°C, 30s; 72°C, 34s) were performed. Experiments were carried out in triplicate, and the mRNA expression was taken as the mean of three separate experiments. The expression of each gene was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH # QT01020908) expression. Fold changes relative to controls were determined using the ΔΔCt method.

4.7.7 Western blot analyses

Protein isolation from liver tissues was performed on ice with lysis buffer containing 20 mM Tris (pH 7.5), 5 mM EDTA, 5 mM EGTA and 0.5% n-dodecylmaltoside (Roche) in the presence of a protease inhibitor cocktail (Roche Diagnostics), using a rotor stator. Protein concentrations were measured with

Bradford reagent (Bio-Rad) according to manufacturer's instructions. Total protein was subsequently separated on SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked in 5% dry milk and incubated in 1% dry milkTBS with various anti-mouse primary antibodies (ABCA1 (Novus Biologicals), ApoAI (Biodesign), ANGPTL4 (Novus Biologicals), WWOX (in house made antibody (Ludes-Meyers et al., 2003) and Cell Signaling)) and horseradish peroxidase-conjugates secondary antibodies (Jackson Biolabs). Chemiluminescence was detected using Western lighting plus ECL reagents (Pierce Thermo Scientific) as described by the manufacturer. Quantification of bands on Western blots was done using Biorad Imager software.

4.7.8 Microarray data analysis

Output from the Agilent Feature Extraction software were read into R, preprocessed and tested for differential expression using functions from the Bioconductor (Gentleman et al., 2004) package *Limma (Smyth et al., 2005).* Specifically, the function *read.maimages* was employed to read raw intensities into R. Quality control was performed by inspecting various diagnostic plots of the intensity distribution and correlation structure of control and regular probes. One array, JGT059, was discarded and removed from the data set prior to preprocessing. The *normexp* method with an offset value of 16 was used for global background adjustment, followed by quantile normalization and a log2 transformation (functions *backgroundCorrect* and *normalizeBetweenArrays*). Within-array duplicate spots were summarized by averaging using the function *avereps*. The annotation for probes was retrieved from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), platform id GPL10787. This annotation was last updated on Apr 6 2012 by Agilent Technologies.

Using the appropriate Limma functions, a linear model was fit to each gene separately. This linear model included the mice gender and the Wwox hepatic knockout (hep KO) status as independent categorical variables. Moderated *t*-tests on various coefficients of interest were used to test the differences between Wwox hep KO and Wwox WT mice, or male and female mice. The differential

response and the male/female average response to the Wwox hep KO were also tested. False discovery rate (FDR) estimation was carried out using the Benjamini-Hochberg method.

IPA (Ingenuity® Systems, www.ingenuity.com) was utilized for pathway, network and functional analysis. For this purpose, gene-level statistical significance for differential expression was reached at $P<0.05$ and |Fold Change (FC)|>1.5. The over-representation of pathways and biological functions in the lists of significantly differentially expressed genes was tested using Fisher's exact test with the Benjamini-Hochberg method used for FDR estimation.

4.7.9 Human study samples

Human study samples consist of two French Canadian families collected at the Cardiovascular Genetics Laboratory, McGill University Health Centre, Royal Victoria Hospital, Montreal, Canada, as described previously (Dastani et al., 2006c). The affection status in all families was determined using the 5^{th} age-sex specific population percentiles of HDL-C (Dastani et al., 2006c). Lipids and lipoproteins were measured using standardized techniques as described previously (Brooks-Wilson et al., 1999; Marcil et al., 1999b). The research protocol was approved by the Research Ethics Board of the McGill University Health Center, and all subjects gave informed consent.

4.7.10 Library construction and resequencing

We selected two closely related family members per family with HDL-C levels $\leq$5th age-sex percentile and 4 unrelated controls with HDL-C levels $\geq 30^{th}$ percentile for targeted sequencing. The selected families also demonstrated both shared haplotypes and linkage to low HDL-C on chromosome 16q23-q24 (Dastani et al., 2010; Lee et al., 2008b). We performed targeted re-sequencing +/- 100kb of the WWOX as well as two other regional genes previously known to influence HDL-C levels (LCAT and CETP +/- 100kb) to search for other possible regional causes of low HDL-C. We used Agilent SureSelect Target Enrichment System, and masked the repeat regions to avoid read alignment errors.

4.7.11 Resequencing data analysis

Each sample was sequenced on one lane of Illumina GA2 Analyzer. We used Burroughs-Wheeler Aligner (BWA) (Langmead et al., 2009) to align sequence reads to the reference sequence (hg19) and SAMtools to make SNP calls. Duplicates were removed and a pileup file was generated using SAMtools (Li et al., 2009). After quality control, the mean coverage was 110X. In the sequenced HDL-C cases and controls, we found 1965 SNPs, 1.02% of which were not present in the HapMap (Altshuler et al., 2010), the 1000 Genomes Project (The 1000 Genomes Project Consortium, 2010), and the dbSNP135 databases. Next we filtered those variants that were shared only by the affected subjects of the two low HDL-C families and not present in the normal controls resequenced. Annovar was used for functional annotation (Wang et al., 2010). Association analysis in the extended low HDL-C families was performed using a measured genotype approach utilizing the 'Polygenic-QTL' option of Mendel (Lange et al., 2005), using continuous HDL-C levels with age and sex as covariates.

4.7.12 Statistical analysis

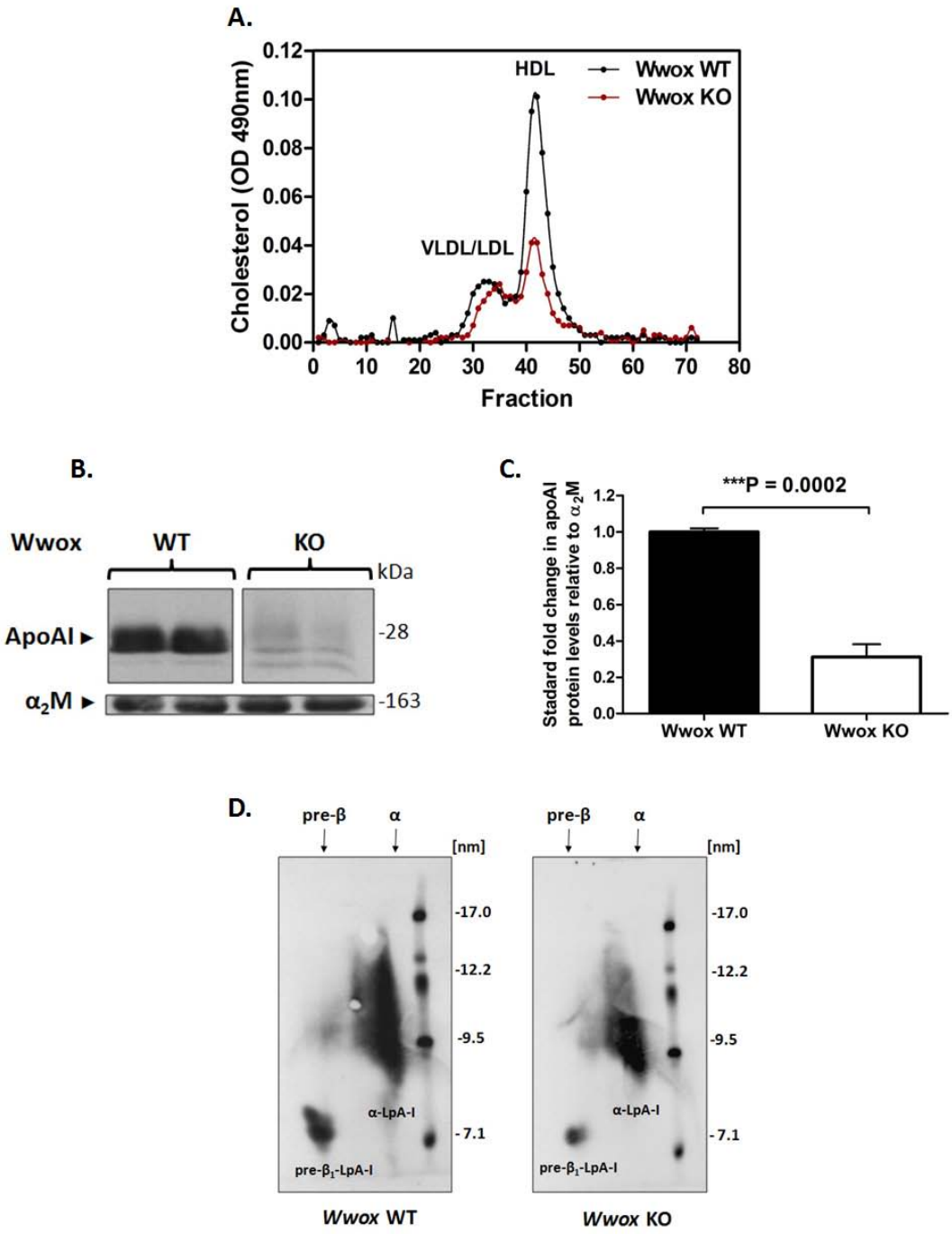
Statistical analyses were performed with GraphPad statistical software 5.0 (GraphPad Softwares Inc). Data were expressed as mean ± SEM, as indicated in figure legends. Two tailed Student's t-test was used for comparisons between two groups as applicable, where indicated, and a level of significance of $P<0.05$ was considered statistically significant. Two-way ANOVA was performed to study the differences in mouse body weight with time for Wwox-liver specific and control mice, as indicated. For microarray analyses, moderated t-tests on various coefficients of interest were used to test the differences between Wwox hep KO and Wwox WT mice, or male and female mice. The over-representation of pathways and biological functions in the lists of significantly differentially expressed genes was tested using Fisher's exact test with the Benjamini-Hochberg method used for FDR estimation, as previously described (Microarray data analysis).

4.8 Supplementary Figures and Tables

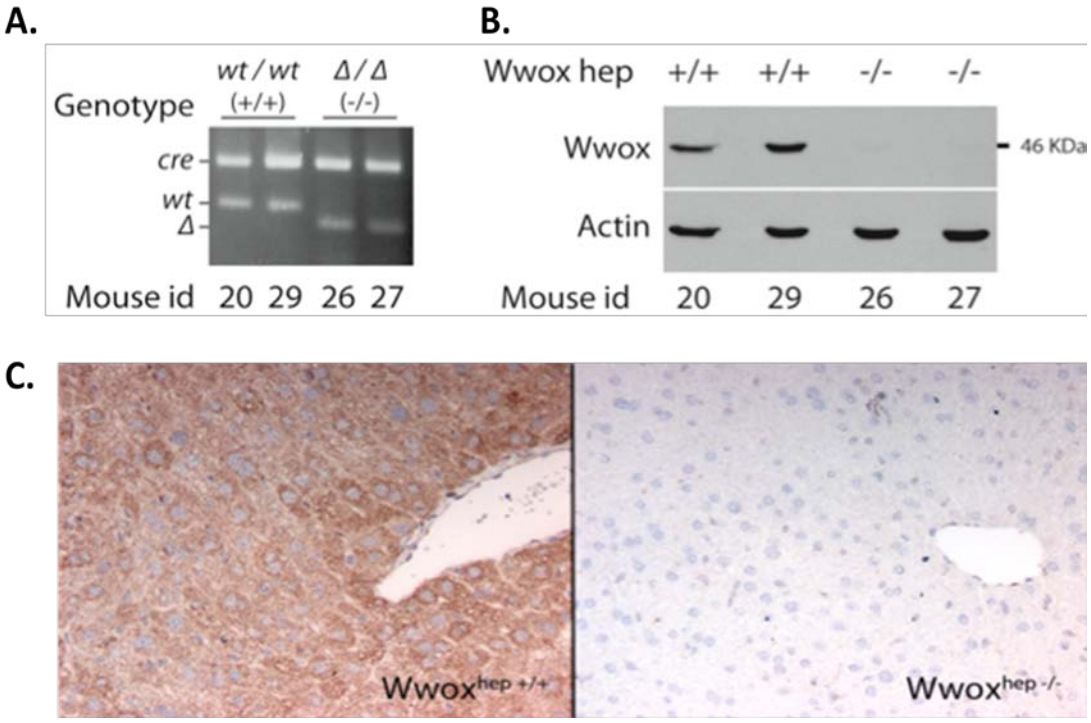
Supplementary Figure 4.1. Characterization of apoA-I- containing particles in serum levels from Wwox null mice

A. Serum (80 μl) from Wwox wild-type (WT) and KO mice was separated using HPLC, and total cholesterol was measured enzymatically as described in Methods. Results are representative of four independent experiments. **B**. ApoA-I from duplicate serum samples (5ul) from a Wwox WT and a Wwox KO mouse were analysed by western blot using antibodies against apoA-I and alpha-2-macroglobulin (loading control). **C**. Band intensities were quantified and relative apoA-I levels were determined. Average values shown are means ± SEM of four independent experiments. **D.** Serum (25 μl) from individual Wwox null and WT mice was separated by 2D-PAGGE and apoA-I-containing particles were detected by immunopurified polyclonal anti-apoA-I antibody labeled with ^{125}I. Molecular size markers are indicated on the right side of the gel. Results are representative of three independent experiments.

Supplementary Figure 4.1 Characterization of apoA-I- containing particles in serum levels from Wwox null mice.



Supplementary Figure 4.2 Characterization of liver specific knock-out mice (Wwox hep$^{-/-}$)

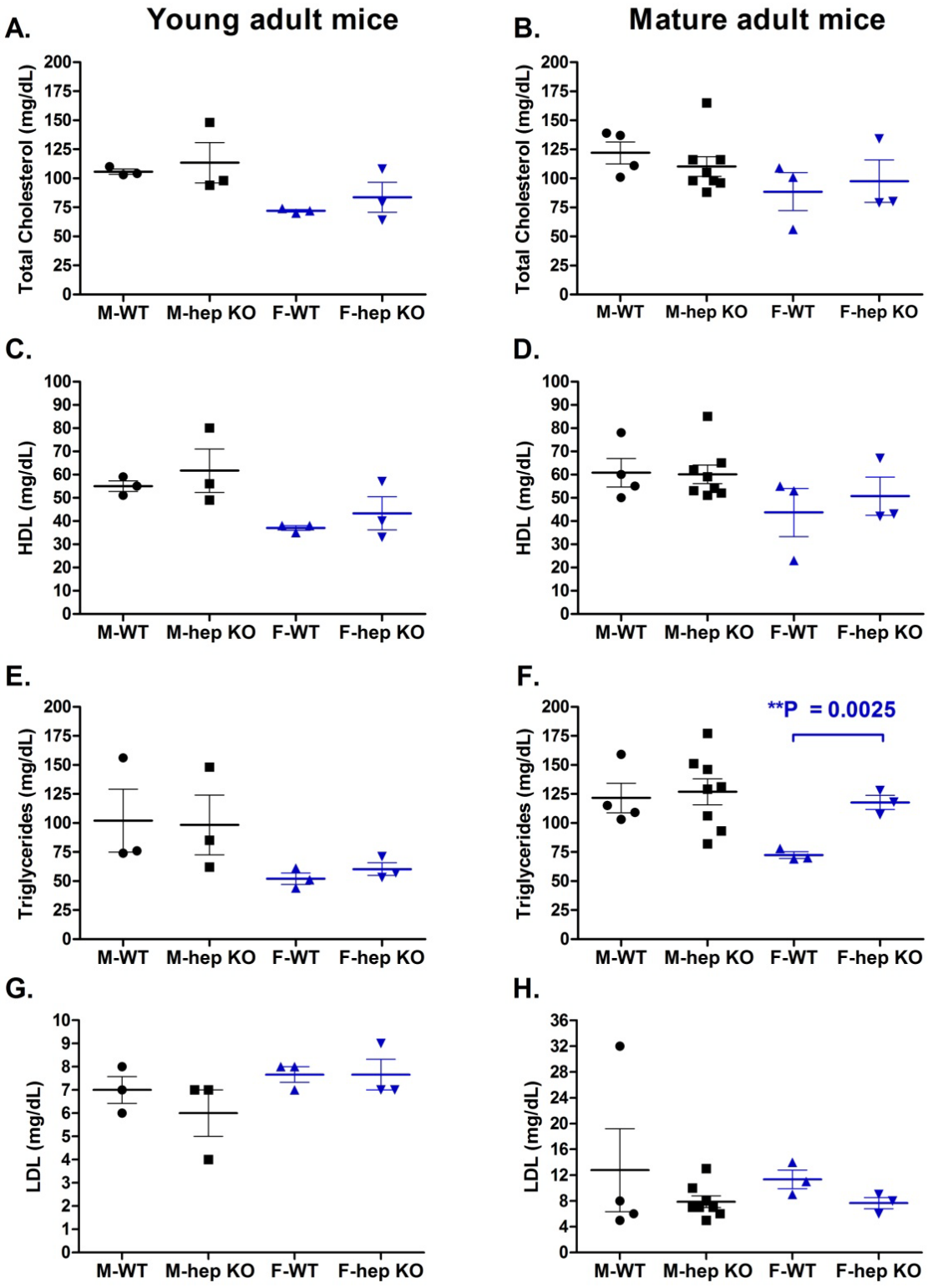


(A) Multiplex PCR of liver samples from 6 week-old Wwox hep+/+ and Wwox hep$^{-/-}$ littermates, assessing the presence of Cre. Lower PCR products, amplified using Wwox specific primers flanking exon 1, show the (WT) PCR product (homozygous WT mice 20 and 29) and the deleted allele (Δ; homozygous deletion, mice 26 and 27). **(B)** Protein extracts from the livers of the mice described in *A* were analyzed by immunoblot using anti-Wwox antibodies and anti-actin (loading control), demonstrating complete ablation of WWOX protein expression in Wwox hep$^{-/-}$ mice 26 and 27. **(C)** Immunohistochemistry was performed on liver samples obtained from Wwox hep+/+ and Wwox hep$^{-/-}$ mice (left and right respectively) using an anti-Wwox antibody. Note the strong immunopositivity of the Wwox hep+/+ hepatocytes (left) and complete lack of immunoreactivity on samples from the liver specific conditional knock-out mice (right). Images are 20x magnification.

Supplementary Figure 4.3. Serum lipid levels in Wwox liver-specific knockout mice

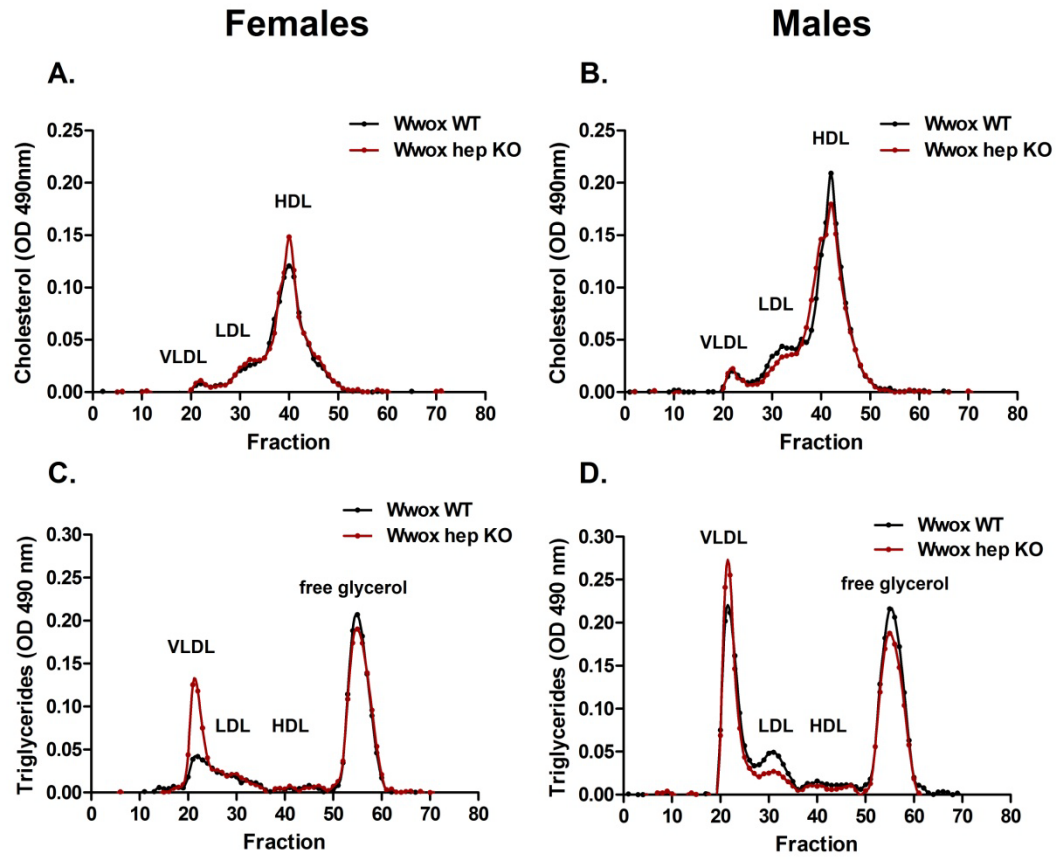
Serum samples (150 μl) from Wwox WT and liver-specific knockout (hep KO) young adult (172-183 days of age) or mature adult (364-475 days of age) mice were analysed by LipoScience Inc. to assess lipid levels of total cholesterol, HDL-C, triglycerides and LDL-C. Mice were separated according to gender and genotype as indicated. For young adult mice (panels **A**, **C**, **E**, **G**), 3 mice/ group were used, while for mature adult mice (panels **B**, **D**, **F**, **H**) there were 4 and 8 WT and Wwox hep KO male mice, respectively, and 3 mice/group for females. Triglyceride levels were significantly increased in the females Wwox hep KO mice as compared to WT (panel **F**). Of note, LDL-C measurement levels in both young and older mice were below the Liposcience human linearity range. **P = 0.0025 by Student's *t*-test.

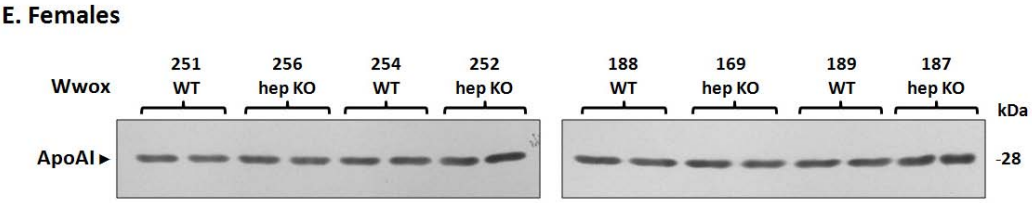
Supplementary Figure 4.3 Serum lipid levels in Wwox liver-specific knockout mice.

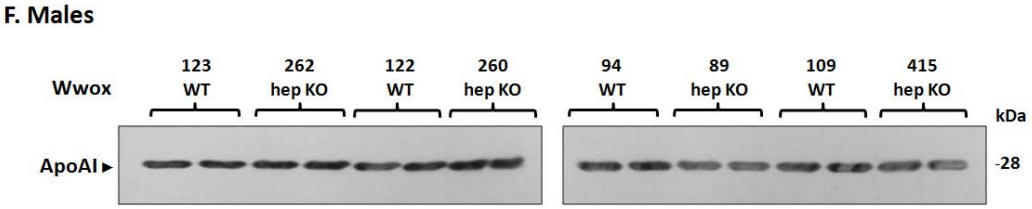


Supplementary Figure 4.4 Lipoprotein fractions separation in serum from Wwox liver-specific knockout mice

Serum (250 ul) from Wwox WT and liver-specific knockout (hep KO) mice was separated by HPLC and total lipoprotein cholesterol (panels **A** and **B**) and triglyceride (panels **C** and **D**) content was determined as described in Methods. Profiles shown are averages of total HPLC cholesterol and TG profiles for females (n=10/genotype, panels **A** and **C**) and males (n=5/genotype, panels **B** and **D**). The elution position of VLDL, LDL, HDL and free glycerol are shown. VLDL-associated Tg levels were significantly elevated in the females Wwox hep KO mice as compared to WT. ApoA-I from duplicate serum samples (5ul) from Wwox WT (n=4) and Wwox hep KO (n=4) mice were analysed by western blot using mouse anti-apoAI antibody, in both female (**E**) and male (**F**) mice.



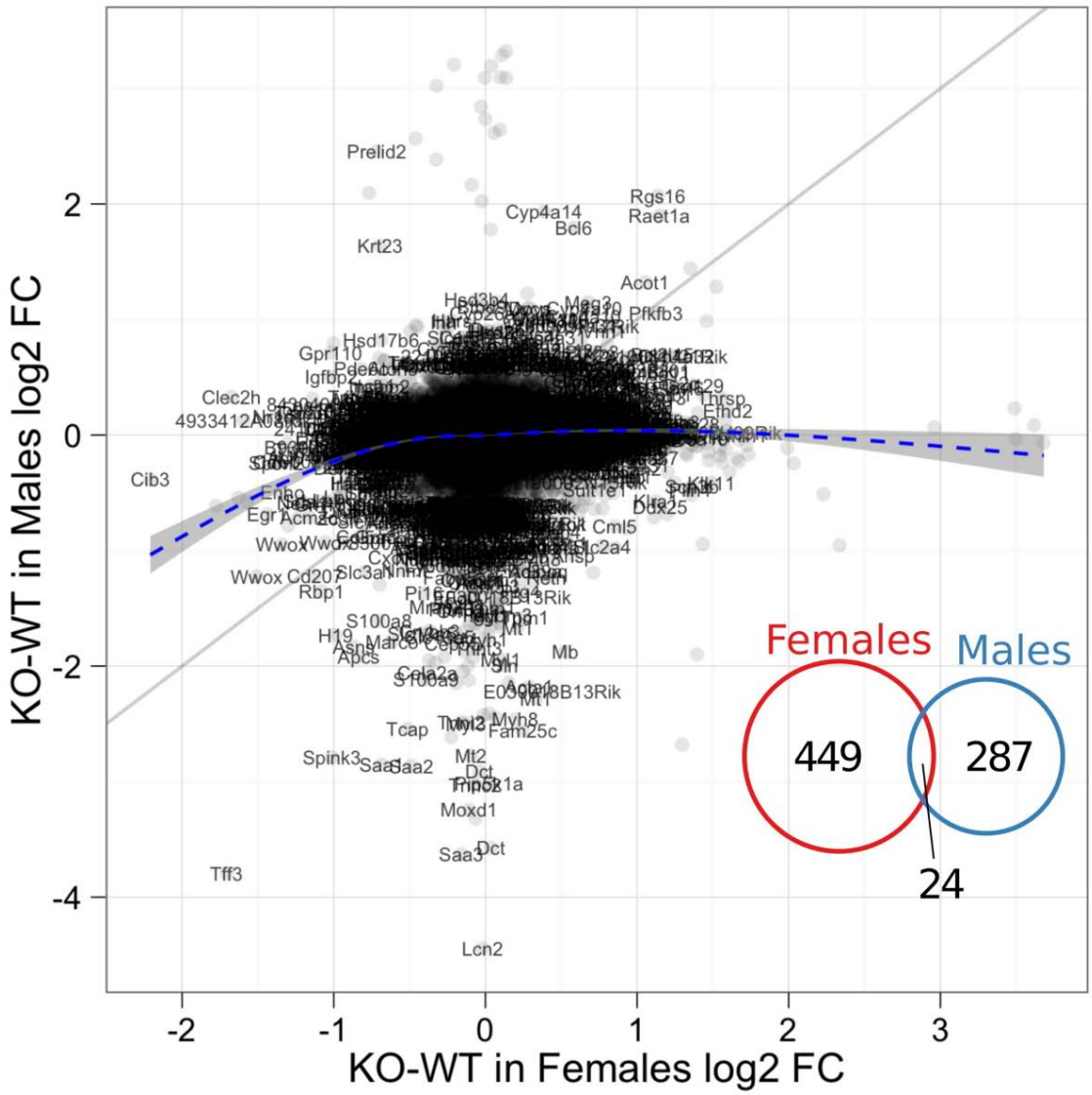




Supplementary Figure 4.4 Lipoprotein fractions separation in serum from Wwox liver-specific knockout mice

Supplementary Figure 4.5 Gender effect of differentially expressed genes in Wwox WT and Wwox hep KO mice

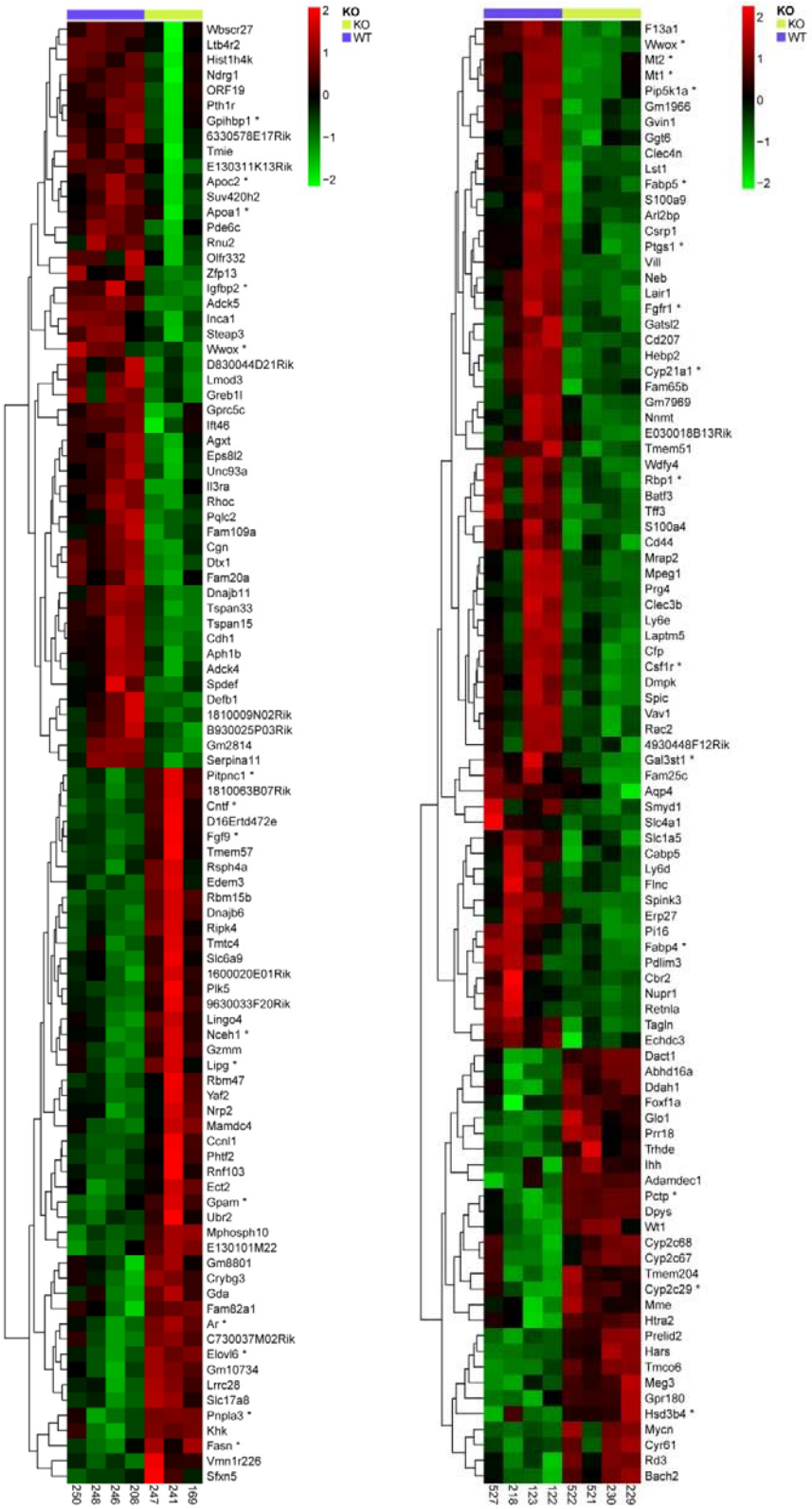
Scatter plot comparing the Wwox hep KO vs. WT log2(FC) values in females (X-axis) to the Wwox hep KO vs. WT log2(FC) values in males (Y-axis). Probes equally regulated in females and males would lie along the identity line, while genes differentially responding to KO between males and females would lie away from the identity line. A text label appears over points only if the corresponding probe meets statistical significance, defined as $P < 0.05$ and $|FC| > 1.5$, in either males or females. The inset Venn diagram counts the number of uniquely and commonly differentially expressed genes. Scatter plot shows that the effect of hepatic Wwox deletion on liver gene expression is strongly influenced by gender. Venn diagram illustrates that 449 annotated genes in females and 287 in males are differentially expressed in hepatic Wwox-deficient mouse liver compared to WT counterpart ($P<0.05$, $|FC|> 1.5$), and that only 24 genes are common between females and males.



Supplementary Figure 4.5 Gender effect of differentially expressed genes in Wwox WT and Wwox hep KO mice.

Supplementary Figure 4.6 Hierarchical cluster analysis of top 100 genes significantly regulated by hepatic Wwox deletion in male and female mice

Top 100 differentially regulated genes ($P<0.05$, $|FC|> 1.5$) between Wwox WT and Wwox hep KO females (A) and males (B) are shown. Genes marked with an asterisk represent genes involved in lipid metabolism (14 in females and 15 in males respectively). Color scale reflects the magnitude of the log ratios (Wwox hep KO/WT): up-regulated genes are in red, and down-regulated genes are in green. The cluster correlation is shown on the right and the numbers at the bottom indicate individual mice.



Supplementary Figure 4.6 Hierarchical cluster analysis of top 100 genes significantly regulated by hepatic Wwox deletion in male and female mice.

Supplementary Table 4.1 (A). Differentially regulated lipid metabolism genes in females (n=64)

Probe Name	Gene ID	Symbol	Entrez Gene Name	P-value	FDR	FC	Location	Type(s)
A_55_P201589	80707	WWOX	WW domain containing oxidoreductase	1.46E-04	0.65	-2.85	Cytoplasm	enzyme
A_55_P202551	116939	PNPLA3	patatin-like phospholipase domain containing 3	2.24E-04	0.65	1.78	Cytoplasm	enzyme
A_55_P205672	16008	IGFBP2	insulin-like growth factor binding protein 2, 36kDa	2.41E-04	0.65	-2.02	ES	other
A_51_P463440	170439	ELOVL6	ELOVL fatty acid elongase 6	6.44E-04	0.65	2.42	Cytoplasm	enzyme
A_55_P195855	11835	AR	androgen receptor	9.72E-04	0.65	1.52	Nucleus	LDNR
A_55_P208694	12803	CNTF	ciliary neurotrophic factor	1.54E-03	0.65	1.69	ES	cytokine
A_55_P201599	14180	FGF9	fibroblast growth factor 9 (glia-activating factor)	1.97E-03	0.65	1.95	ES	growth factor
A_51_P334979	11813	APOC2	apolipoprotein C-II	2.72E-03	0.65	-1.61	ES	transporter
A_51_P408082	11806	APOA1	apolipoprotein A-I	2.88E-03	0.65	-1.79	ES	transporter
A_51_P369862	320024	NCEH1	neutral cholesterol ester hydrolase 1	3.00E-03	0.65	1.59	PM	enzyme
A_52_P609109	71795	PITPNC1	phosphatidylinositol transfer protein, cytoplasmic1	3.08E-03	0.65	1.56	Cytoplasm	transporter
A_52_P100252	14104	FASN	fatty acid synthase	3.47E-03	0.65	1.98	Cytoplasm	enzyme
A_51_P331328	68453	GPIHBP1	glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1	3.86E-03	0.65	-1.58	PM	transporter
A_55_P205077	14732	GPAM	glycerol-3-phosphate acyltransferase,mitochondrial	4.26E-03	0.65	1.51	Cytoplasm	enzyme
A_52_P344424	16891	LIPG	lipase, endothelial	4.54E-03	0.65	1.94	ES	enzyme
A_52_P394395	19299	ABCD3	ATP-binding cassette,sub-familyD(ALD),member3	4.96E-03	0.65	1.62	Cytoplasm	transporter
A_55_P217331	18795	PLCB1	phospholipase C, beta1 (phosphoinositide-specific)	5.81E-03	0.65	1.72	Cytoplasm	enzyme
A_55_P201866	21835	THRSP	thyroid hormone responsive	6.04E-03	0.65	2.94	Nucleus	other
A_55_P200975	217082	HLF	hepatic leukemia factor	6.21E-03	0.65	1.65	Nucleus	TR
A_55_P204718	14164	FGF1	fibroblast growth factor 1 (acidic)	7.31E-03	0.65	1.73	ES	growth factor
A_55_P207867	12411	CBS	cystathionine-beta-synthase	7.55E-03	0.65	-1.68	Cytoplasm	enzyme
A_52_P172441	228859	FITM2	fat storage-inducing transmembrane protein 2	7.87E-03	0.65	1.69	Cytoplasm	other
A_55_P206127	17691	SIK1	salt-inducible kinase 1	7.88E-03	0.65	1.73	Cytoplasm	kinase
A_55_P210089	11863	ARNT	aryl hydrocarbon receptor nuclear translocator	8.50E-03	0.65	1.54	Nucleus	TR
A_55_P208614	56050	CYP39A1	cytochromeP450,family39,subfamilyA,polypeptid	8.61E-03	0.65	1.57	Cytoplasm	enzyme
A_55_P195541	20511	SLC1A2	solute carrier family 1 (glial high affinity glutamate transporter), member 2	8.79E-03	0.65	1.69	PM	transporter
A_52_P485971	235623	SCAP	SREBF chaperone	9.34E-03	0.65	-1.80	Cytoplasm	other
A_55_P210453	100705	ACACB	acetyl-CoA carboxylase beta	1.10E-02	0.65	1.66	Cytoplasm	enzyme
A_55_P196977	68263	PDHB	pyruvate dehydrogenase (lipoamide) beta	1.17E-02	0.65	1.78	Cytoplasm	enzyme
A_55_P208291	104112	ACLY	ATP citrate lyase	1.28E-02	0.65	1.54	Cytoplasm	enzyme
A_55_P202957	18984	POR	P450 (cytochrome) oxidoreductase	1.36E-02	0.65	1.86	Cytoplasm	enzyme
A_51_P259296	16956	LPL	lipoprotein lipase	1.37E-02	0.65	-2.08	Cytoplasm	enzyme
A_55_P196984	72416	LRPPRC	leucine-rich pentatricopeptide repeat containing	1.43E-02	0.65	1.60	Cytoplasm	other
A_66_P101879	71519	CYP2U1	cytochromeP450,family2,subfamilyU,polypeptide1	1.47E-02	0.65	-1.63	Cytoplasm	enzyme
A_55_P200995	17436	ME1	malic enzyme 1, NADP(+)-dependent, cytosolic	1.48E-02	0.65	2.04	Cytoplasm	enzyme
A_51_P266958	18171	NR1I2	nuclear receptor subfamily 1, group I, member 2	1.51E-02	0.65	1.56	Nucleus	LDNR
A_51_P453351	226856	LPGAT1	lysophosphatidylglycerol acyltransferase 1	1.60E-02	0.65	1.57	Cytoplasm	other
A_51_P165060	20520	SLC22A5	solute carrier family 22 (organic cation/carnitine transporter), member 5	1.68E-02	0.65	1.50	PM	transporter
A_52_P229943	330962	OSTBETA	organic solute transporter beta	1.72E-02	0.65	-1.79	PM	transporter
A_51_P489153	74114	CROT	carnitine O-octanoyltransferase	1.85E-02	0.65	-1.66	Cytoplasm	enzyme
A_55_P200998	228775	TRIB3	tribbles homolog 3 (Drosophila)	1.96E-02	0.65	-1.86	Nucleus	kinase
A_51_P493649	20860	SULT1E1	sulfotransferase family 1E,estrogen-preferring, member 1	2.01E-02	0.65	1.65	Cytoplasm	enzyme
A_55_P205901	19659	RBP1	retinol binding protein 1, cellular	2.20E-02	0.65	-2.11	ES	transporter
A_51_P208555	67460	DECR1	2,4-dienoyl CoA reductase 1, mitochondrial	2.22E-02	0.65	1.50	Cytoplasm	enzyme
A_55_P200649	26381	ESRRG	estrogen-related receptor gamma	2.58E-02	0.65	1.75	Nucleus	LDNR
A_55_P199395	18830	PLTP	phospholipid transfer protein	2.84E-02	0.65	1.93	ES	other
A_55_P195686	13649	EGFR	epidermal growth factor receptor	2.89E-02	0.65	-1.67	PM	kinase
A_52_P215170	171282	ACOT4	acyl-CoA thioesterase 4	2.90E-02	0.65	1.95	Cytoplasm	enzyme
A_55_P198762	100040843	CYP4A11	cytochrome P450, family 4, subfamily A, polypeptide 11	3.04E-02	0.65	2.40	Cytoplasm	enzyme
A_52_P236448	18053	NGFR	nerve growth factor receptor	3.16E-02	0.65	-1.61	PM	TrR
A_51_P338443	57875	ANGPTL4	angiopoietin-like 4	3.17E-02	0.65	1.64	ES	other
A_51_P363947	12575	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	3.26E-02	0.65	1.52	Nucleus	kinase
A_52_P681391	14373	G0S2	G0/G1switch 2	3.26E-02	0.65	2.05	Cytoplasm	other
A_55_P196913	14311	CIDEC	cell death-inducing DFFA-like effector c	3.53E-02	0.65	2.07	Cytoplasm	other

Probe Name	Gene ID	Symbol	Entrez Gene Name	P-value	FDR	FC	Location	Type(s)
A_55_P206110	17844	Mup1(othe	major urinary protein 1	3.59E-02	0.65	2.15	ES	other
A_52_P590154	27400	HSD17B6	hydroxysteroid (17-beta) dehydrogenase 6 homolog (mouse)	3.60E-02	0.65	-1.61	unknown	enzyme
A_51_P367866	13653	EGR1	early growth response 1	4.02E-02	0.65	-2.71	Nucleus	TR
A_55_P212218	20648	SNTA1	syntrophin, alpha 1 (dystrophin-associated protein A1, 59kDa, acidic component)	4.08E-02	0.65	1.62	PM	other
A_55_P195622	12660	CHKA	choline kinase alpha	4.19E-02	0.65	-1.70	Cytoplasm	kinase
A_51_P334942	11668	ALDH1A1	aldehyde dehydrogenase 1 family, member A1	4.38E-02	0.65	1.51	Cytoplasm	enzyme
A_55_P210177	11539	ADORA1	adenosine A1 receptor	4.67E-02	0.65	-1.50	PM	GPCR
A_55_P217804	16332	INPPL1	inositol polyphosphate phosphatase-like 1	4.83E-02	0.65	1.52	Cytoplasm	hosphatase
A_55_P195302	21944	TNFSF12	tumor necrosis factor (ligand) superfamily, member 12	4.84E-02	0.65	-1.58	ES	cytokine
A_51_P439452	72999	INSIG2	insulin induced gene 2	4.91E-02	0.65	-1.75	Cytoplasm	other

Supplementary Table 4.1 (B). Differentially regulated lipid metabolism genes in males (n=56)

Probe Name	Gene ID	Symbol	Gene Name	P-value	FDR	FC	Location	Type(s)
A_51_P246317	17750	MT2A	metallothionein 2A	1.80E-04	0.84	-6.84	Cytoplasm	other
A_55_P1999902	18720	PIP5K1A	phosphatidylinositol-4-phosphat5-kinase,typeI,alpha	3.58E-04	0.84	-8.06	Cytoplasm	kinase
A_51_P279100	19224	PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	4.81E-04	0.84	-1.57	Cytoplasm	enzyme
A_55_P2015892	80707	WWOX	WW domain containing oxidoreductase	4.97E-04	0.84	-2.34	Cytoplasm	enzyme
A_52_P423810	17748	MT1E	metallothionein 1E	6.82E-04	0.84	-4.85	Cytoplasm	other
A_55_P2013823	53897	GAL3ST1	galactose-3-O-sulfotransferase 1	2.29E-03	0.94	-1.50	Cytoplasm	enzyme
A_51_P336833	11770	FABP4	fatty acid binding protein 4, adipocyte	2.55E-03	0.94	-1.88	Cytoplasm	transporter
A_55_P1969665	15495	Hsd3b4 (& others)	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 4	3.00E-03	0.94	2.26	Cytoplasm	enzyme
A_55_P2140107	13079	CYP21A2	cytochrome P450, family 21, subfamily A, polypeptide 2	3.03E-03	0.94	-1.97	Cytoplasm	enzyme
A_55_P2059010	19659	RBP1	retinol binding protein 1, cellular	3.50E-03	0.94	-2.56	ES	transporter
A_52_P136709	18559	PCTP	phosphatidylcholine transfer protein	4.35E-03	0.94	1.60	Cytoplasm	transporter
A_55_P1953387	16592	FABP5	fatty acid binding protein 5 (psoriasis-associated)	5.65E-03	0.94	-2.35	Cytoplasm	transporter
A_66_P119034	27226	PLA2G7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	7.06E-03	0.94	-1.93	ES	enzyme
A_55_P2111302	12870	CP	ceruloplasmin (ferroxidase)	7.71E-03	0.94	-1.61	ES	enzyme
A_55_P2036547	15493	HSD3B1	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	7.74E-03	0.94	1.86	Cytoplasm	enzyme
A_55_P2024888	13040	CTSS	cathepsin S	8.21E-03	0.94	-1.58	Cytoplasm	peptidase
A_55_P2101776	11539	ADORA1	adenosine A1 receptor	8.47E-03	0.94	-1.69	PM	GPCR
A_55_P2011111	100040843	CYP4A11	cytochrome P450, family 4, subfamily A, polypeptide 11	8.66E-03	0.94	2.14	Cytoplasm	enzyme
A_51_P263965	15368	HMOX1	heme oxygenase (decycling) 1	9.36E-03	0.94	-1.72	Cytoplasm	enzyme
A_52_P590154	27400	HSD17B6	hydroxysteroid (17-beta) dehydrogenase 6 homolog (mouse)	9.51E-03	0.94	1.77	unknown	enzyme
A_52_P382149	13082	CYP26A1	cytochrome P450, family 26, subfamily A, polypeptide 1	9.67E-03	0.94	2.08	Cytoplasm	enzyme
A_51_P201480	20848	STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	9.82E-03	0.94	-1.53	Nucleus	TR
A_52_P563825	26877	B3GALT1	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 1	9.83E-03	0.94	-1.77	Cytoplasm	enzyme
A_51_P313581	14079	FABP2	fatty acid binding protein 2, intestinal	1.08E-02	0.94	1.61	Cytoplasm	transporter
A_51_P238576	13119	Cyp4a14	cytochrome P450, family 4, subfamily a, polypeptide 14	1.17E-02	0.94	3.82	Cytoplasm	enzyme
A_51_P208555	67460	DECR1	2,4-dienoyl CoA reductase 1, mitochondrial	1.18E-02	0.94	1.53	Cytoplasm	enzyme
A_55_P2109857	19735	GS2 EG:19735)	regulator of G-protein signaling 2, 24kDa	1.20E-02	0.94	-1.76	Nucleus	other
A_51_P474459	12702	SOCS3	suppressor of cytokine signaling 3	1.20E-02	0.94	-2.03	Cytoplasm	other
A_51_P405476	14127	FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	1.22E-02	0.94	-1.57	PM	TrR
A_51_P193336	53322	NUCB2	nucleobindin 2	1.28E-02	0.94	-1.79	Nucleus	other
A_51_P383399	26358	Aldh1a7	aldehyde dehydrogenase family 1, subfamily A7	1.33E-02	0.94	-1.51	Cytoplasm	enzyme
A_52_P161495	12053	BCL6	B-cell CLL/lymphoma 6	1.50E-02	0.94	3.47	Nucleus	TR

Probe Name	Gene ID	Symbol	Gene Name	P-value	FDR	FC	Location	Type(s)
A_55_P2063312	23945	MGLL	monoglyceride lipase	1.55E-02	0.94	1.52	PM	enzyme
A_55_P1994807	20209	SAA2	serum amyloid A2	1.55E-02	0.94	-7.28	ES	other
A_51_P354165	20219	APCS	amyloid P component, serum	1.61E-02	0.94	-3.78	ES	other
A_55_P1958434	14718	GOT1	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	1.76E-02	0.94	-1.77	Cytoplasm	enzyme
A_55_P2034655	16019	IGHM	immunoglobulin heavy constant mu	1.80E-02	0.94	-1.80	PM	TrR
A_55_P1963508	237831	SLC13A5	solute carrier family 13 (sodium-dependent citrate transporter), member 5	1.91E-02	0.94	-3.35	PM	transporter
A_52_P537545	58994	SMPD3	sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase II)	1.92E-02	0.94	-2.12	Cytoplasm	enzyme
A_55_P2072373	17698	MSN	moesin	2.17E-02	0.94	-1.63	PM	other
A_52_P244193	12484	Cd24a	CD24a antigen	2.26E-02	0.94	-1.52	PM	other
A_55_P1973809	15129	HBB	hemoglobin, beta	2.48E-02	0.94	-1.97	Cytoplasm	transporter
A_55_P2038358	26897	Acot1	acyl-CoA thioesterase 1	2.64E-02	0.95	2.50	Cytoplasm	enzyme
A_51_P458451	11450	ADIPOQ	adiponectin, C1Q and collagen domain containing	2.70E-02	0.95	-2.26	ES	other
A_52_P15388	17002	LTF	lactotransferrin	2.70E-02	0.95	-1.58	ES	peptidase
A_55_P1953169	20210	SAA1	serum amyloid A1	2.91E-02	0.95	-12.37	ES	transporter
A_55_P2118609	20445	ST6GALNAC1	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1	2.91E-02	0.95	-1.72	Cytoplasm	enzyme
A_51_P217498	20528	SLC2A4	solute carrier family 2 (facilitated glucose transporter), member 4	2.96E-02	0.96	-1.95	PM	transporter
A_55_P1964483	404195	CYP2C19	cytochrome P450, family 2, subfamily C, polypeptide 19	3.10E-02	0.96	1.67	Cytoplasm	enzyme
A_51_P325836	15458	HPX	hemopexin	3.49E-02	0.96	-1.59	ES	transporter
A_55_P2116272	13095	Cyp2c29 (& others)	cytochrome P450, family 2, subfamily c, polypeptide 29	3.93E-02	0.97	1.68	Cytoplasm	enzyme
A_55_P2106803	12097	Bglap (& others)	bone gamma carboxyglutamate protein	4.29E-02	0.97	-1.73	ES	other
A_55_P2428514	57264	RETN	resistin	4.40E-02	0.97	-2.35	ES	other
A_52_P229943	330962	OSTBETA	organic solute transporter beta	4.50E-02	0.97	-1.55	PM	transporter
A_52_P577384	16068	IL18BP	interleukin 18 binding protein	4.73E-02	0.97	-1.59	ES	other
A_55_P1955681	12671	CHRM3	cholinergic receptor, muscarinic 3	4.86E-02	0.97	-1.53	PM	GPCR

Legend

LDNR, ligand-dependent nuclear receptor; GPCR, G-protein coupled receptor; PM, Plasma Membrane; ES, Extracellular Space; TR, Transcription regulator

Supplementary Table 4.2 (A) 31 Functions associated with lipid metabolism in females

Functions	Functions Annotation	p-val	Predicted Activation state/z-scor	Mol	Up-Regulated Molecules	Down-Regulated Molecules
concentration	concentration of triacylglycerol	1.17E-07	increased/ 2.47	23	ACACB, ACLY, ANGPTL4, ARNT, CIDEC, CNTF, FASN, FITM2, GPAM, LIPG, LPGAT1, NCEH1, NR1I2, PLTP, POR, SIK1, THRSP	APOA1, CBS, CHKA, EGR1, GPIHBP1, LPL
	concentration of acylglycerol	2.16E-07	increased/ 2.28	24	ACACB, ACLY, ANGPTL4, ARNT, CIDEC, CNTF, FASN, FITM2, GPAM, LIPG, LPGAT1, NCEH1, NR1I2, PLTP, POR, SIK1, THRSP	ADORA1, APOA1, CBS, CHKA, EGR1, GPIHBP1, LPL
	concentration of sterol	2.17E-05	0.92	19	ANGPTL4, ARNT, CDKN1A, CNTF, GPAM, LIPG, LPGAT1, NCEH1, NR1I2, PLTP, POR, SLC1A2	APOA1, CBS, CHKA, EGR1, GPIHBP1, LPL, SCAP
	concentration of cholesterol	3.64E-05	0.55	18	ANGPTL4, ARNT, CDKN1A, CNTF, GPAM, LIPG, LPGAT1, NCEH1, NR1I2, PLTP, POR, SLC1A2	APOA1, CBS, EGR1, GPIHBP1, LPL, SCAP
	concentration of lipid	1.11E-04	1.10	37	ACACB, ACLY, ALDH1A1, ANGPTL4, AR, ARNT, CDKN1A, CIDEC, CNTF, DECR1, FASN, FITM2, GPAM, INPPL1, LIPG, LPGAT1, NCEH1, NR1I2, PLTP, POR, SIK1, SLC1A2, SULT1E1, THRSP	ADORA1, APOA1, CBS, CHKA, EGFR, EGR1, GPIHBP1, LPL, NGFR, RBP1, SCAP, TNFSF12, WWOX
	concentration of tretinoin	6.34E-04	-	3	ALDH1A1, POR	RBP1
	concentration of fatty acid	4.82E-03	0.50	14	ACACB, ACLY, ANGPTL4, CIDEC, CNTF, DECR1, GPAM, NCEH1, PLTP	ADORA1, CBS, EGFR, LPL, SCAP
	concentration of acyl-coenzyme A	5.52E-03	-	3	ACACB, ACLY, GPAM	
	concentration of cholesterol ester	6.11E-03	1.73	4	PLTP	APOA1, CBS, CHKA
	concentration of 9-cis-retinol	2.32E-02	-	1		RBP1
modification	modification of palmitic acid	2.29E-05	0.00	7	ABCD3, ACACB, FASN, GPAM, LRPPRC, SLC22A5	APOA1
quantity	quantity of non-esterified fatty acid	5.33E-05	-	4	ANGPTL4, CNTF, PLTP	CBS
	quantity of steroid	3.47E-04	1.16	23	ANGPTL4, AR, ARNT, CDKN1A, CNTF, GPAM, LIPG, LPGAT1, NCEH1, NR1I2, PLTP, POR, SLC1A2, SULT1E1	APOA1, CBS, CHKA, EGFR, EGR1, GPIHBP1, LPL, SCAP, TNFSF12
	quantity of lysophosphatidic acid	3.72E-03	-	3	GPAM, LIPG	LPL
release	release of cholesterol	5.33E-05	0.51	4	FGF1, PLTP, SNTA1	APOA1
synthesis	synthesis of lipid	9.93E-05	increased/ 2.18	34	ACACB, ACLY, ALDH1A1, AR, CNTF, CYP39A1, CYP4A11, DECR1, ESRRG, FASN, FGF1, FGF9, FITM2, GPAM, PDHB, PLCB1, PLTP, PNPLA3, POR, SULT1E1, THRSP	APOA1, APOC2, CHKA, EGFR, EGR1, IGFBP2, INSIG2, LPL, NGFR, RBP1, SCAP, TNFSF12, TRIB3
	synthesis of acyl-coenzyme A	6.71E-04	-	4	ACACB, ACLY, FASN, PDHB	
	synthesis of acylglycerol	8.43E-04	increased/ 2.40	8	ESRRG, FASN, FITM2, GPAM, PLCB1, PNPLA3, THRSP	LPL
	synthesis of triacylglycerol	8.94E-04	1.95	6	FASN, FITM2, GPAM, PNPLA3, THRSP	LPL
	synthesis of cholesterol	1.50E-03	0.50	6	FGF1, PLTP, POR	APOA1, INSIG2, SCAP
	synthesis of malonyl-coenzyme A	1.58E-03	-	2	ACACB, FASN	
	synthesis of acetyl-coenzyme A	1.77E-03	-	3	ACLY, FASN, PDHB	
	synthesis of phosphatidylcholine	1.77E-03	-	4	GPAM	APOA1, CHKA, SCAP
	synthesis of terpenoid	2.36E-03	1.92	14	ALDH1A1, AR, CYP39A1, FGF1, FGF9, PLTP, POR, SULT1E1	APOA1, EGFR, IGFBP2, INSIG2, SCAP, TNFSF12
	synthesis of steroid	2.49E-03	1.66	13	AR, CYP39A1, FGF1, FGF9, PLTP, POR, SULT1E1	APOA1, EGFR, IGFBP2, INSIG2, SCAP, TNFSF12
	synthesis of fatty acid	6.77E-03	0.36	14	ACACB, ACLY, CNTF, CYP4A11, DECR1, FASN, PDHB	APOC2, EGFR, EGR1, INSIG2, LPL, SCAP, TRIB3
oxidation	oxidation of lipid	1.24E-04	-0.29	14	ABCD3, ACACB, ALDH1A1, CYP4A11, DECR1, FITM2, GPAM, LRPPRC, POR, SLC22A5	APOA1, HSD17B6, LPL, RBP1
	oxidation of fatty acid	1.60E-04	-0.29	12	ABCD3, ACACB, CYP4A11, DECR1, FITM2, GPAM, LRPPRC, POR, SLC22A5	APOA1, LPL, RBP1
	oxidation of palmitic acid	1.60E-04	-0.37	6	ABCD3, ACACB, GPAM, LRPPRC, SLC22A5	APOA1
metabolism	metabolism of acyl-coenzyme A	1.60E-04	-	6	ACACB, ACLY, ACOT4, FASN, GPAM, SLC22A5	
	fatty acid metabolism	2.17E-04	0.20	28	ABCD3, ACACB, ACLY, ACOT4, CNTF, CYP4A11, DECR1, FASN, GPAM, HLF, ME1, PDHB, PITPNC1, PLTP, SLC22A5	APOA1, APOC2, CHKA, CROT, EGFR, EGR1, GPIHBP1, INSIG2, LPL, NGFR, OSTBETA, SCAP, TRIB3
	metabolism of acylglycerol	5.07E-04	1.60	10	ESRRG, FASN, FITM2, GPAM, PLCB1, PNPLA3, THRSP	APOC2, INSIG2, LPL
	metabolism of acetyl-coenzyme A	6.34E-04	-	3	ACACB, ACLY, FASN	
	metabolism of triacylglycerol	6.66E-04	0.95	8	FASN, FITM2, GPAM, PNPLA3, THRSP	APOC2, INSIG2, LPL
	metabolism of long chain fatty acid	1.77E-03	-	4	ACOT4, CYP4A11, HLF, SLC22A5	
	metabolism of terpenoid	3.52E-03	1.12	15	ALDH1A1, CYP39A1, FGF1, FGF9, NR1I2,	APOA1, EGFR, INSIG2,

Functions	Functions Annotation	p-val	Predicted Activation state/z-scor	Mol	Up-Regulated Molecules	Down-Regulated Molecules
					PLTP, POR, SULT1E1	RBP1, SCAP, TNFSF12, WWOX
	steroid metabolism	5.09E-03	0.73	13	CYP39A1, FGF1, FGF9, NR1I2, PLTP, POR, SULT1E1	APOA1, EGFR, INSIG2, SCAP, TNFSF12, WWOX
	metabolism of palmitic acid	5.12E-03	-	2	HLF, SLC22A5	
	metabolism of cholesterol	6.17E-03	0.50	7	CYP39A1, FGF1, PLTP, POR	APOA1, INSIG2, SCAP
homestasis	homeostasis of acylglycerol	2.36E-04	-	4	ANGPTL4	APOC2, GPIHBP1, LPL
	homeostasis of lipid	6.31E-03	-	10	ACACB, ANGPTL4, GPAM, LIPG, NCEH1, THRSP	APOA1, APOC2, GPIHBP1, LPL
	homeostasis of phospholipid	1.04E-02	-	2	GPAM, LIPG	
sereodogenesis	steroidogenesis	1.16E-03	1.73	12	FGF1, FGF9, PLTP, POR, SULT1E1	APOA1, EGFR, IGFBP2, INSIG2, SCAP, TNFSF12, WWOX
hydrolysis	hydrolysis of acylglycerol	1.54E-03	0.44	5	ANGPTL4, LIPG, PLCB1	APOC2, LPL
	hydrolysis of triacylglycerol	4.26E-03	0.14	4	ANGPTL4, LIPG	APOC2, LPL
	hydrolysis of 1,2-dipalmitoylphosphatidylcholine	5.12E-03	-	2	LIPG	LPL
	hydrolysis of triolein	7.56E-03	-	2	LIPG	LPL
	hydrolysis of N-acetylsphingosine	2.32E-02	-	1		NGFR
conversion	conversion of acyl-coenzyme A	1.77E-03	-	3	ACLY, FASN	CROT
	conversion of palmitic acid	2.32E-02	-	1	FASN	
beta-oxidation	beta-oxidation of palmitic acid	2.32E-03		3	ABCD3, LRPPRC, SLC22A5	
transmission	transmission of phospholipid	3.12E-03	-	2	PLTP	APOA1
	transmission of lipid	4.57E-03	-	3	PLTP	APOA1, LPL
	transmission of cholesterol	5.12E-03	-	2	PLTP	APOA1
degradation	degradation of acylglycerol	3.72E-03	-	3	PLCB1, PNPLA3	LPL
accumulation	accumulation of triacylglycerol	3.88E-03	increased/ 2.41	6	GPAM, Mup1 (includes others), NR1I2	INSIG2, LPL, RBP1
clearance	clearance of lipid	4.26E-03	0.15	4	ALDH1A1, NR1I2	APOA1, LPL
	clearance of corticosterone	2.32E-02	-	1	NR1I2	
depletion	depletion of sterol	5.12E-03	-	2		APOA1, SCAP
	depletion of phosphatidylcholine	2.32E-02	-	1		APOA1
	depletion of sphingomyelin	2.32E-02	-	1		APOA1
hydroxylation	hydroxylation of eicosanoid	5.12E-03	-	2	CYP4A11	CYP2U1
	hydroxylation of 20-hydroxyeicosatetraenoic acid	2.32E-02	-	1		CYP2U1
	hydroxylation of arachidonic acid	2.32E-02	-	1		CYP2U1
	hydroxylation of docosahexaenoic acid	2.32E-02	-	1		CYP2U1
efflux	efflux of phospholipid	1.37E-02		3	PLTP	APOA1, APOC2
transport	transport of lipid	1.71E-02	-0.60	9	ABCD3, PITPNC1, PLTP	APOA1, CHKA, CROT, GPIHBP1, LPL, OSTBETA
deposition	deposition of triacylglycerol	1.73E-02	-	2	GPAM, NR1I2	
catabolism	catabolism of triacylglycerol	2.13E-02	-	2	PNPLA3	LPL
import	import of lipid	2.13E-02	-	2	ABCD3	APOA1
	import of long chain fatty acid	2.32E-02	-	1	ABCD3	
lipolysis	lipolysis	2.18E-02	-0.85	5	ACACB, ANGPTL4, CIDEC, G0S2, LIPG	
breakdown	breakdown of diacylglycerol	2.32E-02	-	1	PLCB1	
	breakdown of phosphatidylinositol 3,4-diphosphate	2.32E-02	-	1	PLCB1	
distribution	distribution of triacylglycerol	2.32E-02	-	1		CBS
down regulation	downregulation of prostaglandin E2	2.32E-02	-	1	CNTF	
elongation	elongation of lauroyl-coenzyme A	2.32E-02	-	1	ELOVL6	
	elongation of myristoyl-coenzyme A	2.32E-02	-	1	ELOVL6	
	elongation of palmitic acid	2.32E-02	-	1	ELOVL6	
	elongation of palmitoleoyl-coenzyme A	2.32E-02	-	1	ELOVL6	
	elongation of palmitoyl-coenzyme A	2.32E-02	-	1	ELOVL6	
formation	formation of retinyl ester	2.32E-02	-	1		RBP1
glucuronidation	glucuronidation of beta-estradiol	2.32E-02	-	1	NR1I2	

Functions	Functions Annotation	p-val	Predicted Activation state/z-score	Mol	Up-Regulated Molecules	Down-Regulated Molecules
inactivation	inactivation of beta-estradiol	2.32E-02	-	1	SULT1E1	

Supplementary Table 4.2 (B) 16 Functions associated with lipid metabolism in males

Functions	Functions Annotation	p-val	Predicted Activation state/z-score	Mol.	Up-Regulated Molecules	Down-Regulated Molecules
regulation	regulation of lipid	2.32E-05		6	MGLL	ADIPOQ, PLA2G7, PTGS1, RBP1, SOCS3
efflux	efflux of cholesterol	1.76E-04	-0.86	8	PCTP	ADIPOQ, APCS, CTSS, FABP4, MSN, SAA1, SAA2
oxidation	oxidation of lipid	2.02E-04	0.76	12	CYP2C19, CYP4A11, Cyp4a14, DECR1, FABP2, HSD17B6	ADIPOQ, Aldh1a7, NUCB2, PTGS1, RBP1, SLC2A4
	oxidation of fatty acid	1.68E-03	0.67	9	CYP4A11, Cyp4a14, DECR1, FABP2	ADIPOQ, NUCB2, PTGS1, RBP1, SLC2A4
conversion	conversion of lipid	2.35E-04	increased/ 2.26	10	CYP2C19, CYP4A11, Cyp4a14, Hsd3b4 (includes others)	FABP4, HMOX1, HPX, MT1E, MT2A, PTGS1
homeostasis	homeostasis of lipid	3.30E-04		11	Cyp4a14, FABP2	ADIPOQ, Cd24a, CP, FABP4, GOT1, MT1E, MT2A, RETN, SAA2
hydroxylation	hydroxylation of tretinoin	3.43E-04		2	CYP26A1, Cyp2c29 (includes others)	
	hydroxylation of 11,12-epoxyeicosatrienoic acid	1.02E-03		2	CYP4A11, Cyp4a14	
	hydroxylation of 14,15-epoxyeicosatrienoic acid	1.02E-03		2	CYP4A11, Cyp4a14	
	hydroxylation of 8,9-epoxyeicosatrienoic acid	1.02E-03		2	CYP4A11, Cyp4a14	
	hydroxylation of lipid	1.08E-03	1.95	4	CYP26A1, Cyp2c29 (includes others), CYP4A11, Cyp4a14	
metabolism	fatty acid metabolism	5.38E-04	-0.60	23	Acot1, CYP2C19, CYP4A11, Cyp4a14, DECR1, FABP2, PCTP	ADIPOQ, B3GALT1, FABP4, FABP5, FCER1G, GAL3ST1, HBB, HMOX1, IGHM, LTF, OSTBETA, PTGS1, SAA2, SLC13A5, SMPD3, ST6GALNAC1
	metabolism of eicosanoid	7.10E-03	0.54	10	CYP2C19, CYP4A11, Cyp4a14	ADIPOQ, FCER1G, HBB, HMOX1, IGHM, LTF, PTGS1
synthesis	synthesis of lipid	5.63E-04	-0.46	27	CYP26A1, CYP4A11, Cyp4a14, DECR1, HSD3B1, Hsd3b4 (includes others), PCTP	ADIPOQ, Aldh1a7, B3GALT1, CHRM3, CYP21A2, FABP5, FCER1G, GAL3ST1, HBB, HMOX1, IGHM, LTF, PIP5K1A, PTGS1, RBP1, RETN, RGS2 (includes EG:19735), SMPD3, SOCS3, ST6GALNAC1
	synthesis of 20-hydroxyeicosatetraenoic acid	1.02E-03		2	CYP4A11, Cyp4a14	
	synthesis of eicosanoid	9.35E-03	0.17	9	CYP4A11, Cyp4a14	ADIPOQ, FCER1G, HBB, HMOX1, IGHM, LTF, PTGS1
transport	transport of palmitic acid	1.23E-03		3	FABP2	FABP4, FABP5
peroxidation	peroxidation of lipid	1.27E-03	increased/ 2.19	5	CYP4A11	HMOX1, HPX, MT1E, MT2A
cleavage	cleavage of fatty acid	2.17E-03	-0.69	4	MGLL	FABP4, FABP5, PTGS1
utilization	utilization of fatty acid	3.30E-03		2	FABP2	PTGS1
release	release of prostaglandin	3.85E-03		4	BCL6	ADIPOQ, FCER1G, PTGS1
concentration	concentration of lipid	6.61E-03	decreased/ -2.17	26	CYP26A1, DECR1, FABP2, MGLL, PCTP	ADIPOQ, ADORA1, Bglap (includes others), CHRM3, CTSS, FABP4, FABP5, FCER1G, GAL3ST1, HMOX1, IL18BP, NUCB2, PIP5K1A, PTGS1, RBP1, RETN, SAA2, SLC2A4, SMPD3, STAT3, WWOX
	concentration of tretinoin	8.91E-03		2	CYP26A1	RBP1
lipolysis	lipolysis of fatty acid	6.77E-03		2		FABP4, FABP5
hydrolysis	hydrolysis of fatty acid	7.48E-03		3	MGLL	FABP4, FABP5

Supplementary Table 4.3 Lipid levels and other clinical characteristics of all family members from both French-Canadian families

FAM	Ind ID	Sex	Status	Age	HDL-C	%HDL	BMI	TG	TC	LDL-C
1	101	1	1	NA	NA	NA	NA	NA	NA	NA
1	102	2	1	NA	NA	NA	NA	NA	NA	NA
1	210	2	2	69	0.67	<5%	23.8	5.31	5.71	2.65
1	208	1	2	64	0.61	<5%	35.2	2.68	4.13	2.31
1	207	2	1	NA	NA	NA	NA	NA	NA	NA
1	206	1	2	59	0.57	<5%	26.3	2.06	3.22	1.72
1	204	1	2	55	0.7	<5%	30	4.66	4.17	1.37
1	202	2	1	50	1.04	10%	29.8	3.12	6.56	4.12
1	211	1	1	52	0.9	0.21	23	1.06	4.97	3.59
1	205	2	1	52	1.4	0.32	23.4	0.77	4.31	2.56
1	203	2	1	53	1.75	0.66	23.5	0.66	3.99	1.94
1	201	1	1	48	1.01	0.28	35.3	1.81	5.92	4.1
1	311	1	2	26	0.59	<5%	30.7	1.17	3.43	2.31
1	310	2	2	24	1	12%	27.2	1.61	3.87	2.15
1	309	2	2	40	0.99	9	22.3	1.19	5.03	3.5
1	308	1	1	NA	NA	NA	NA	NA	NA	NA
1	307	1	1	39	0.96	28	30.4	1.35	4.63	3.06
1	306	1	2	33	0.45	<5%	34.1	2.36	4.23	2.72
1	303	1	2	32	0.61	<5%	26.9	2.43	3.68	1.98
1	304	2	2	31	0.93	<5%	25.9	1.65	4.01	2.34
1	302	2	1	25	1.01	10	25.9	1.56	3.95	2.24
1	401	2	2	14	0.95	<5%	20.3	1.53	4.99	3.35
1	301	1	1	16	1.41	82	19.7	0.6	4.58	2.9
1	209	2	1	52	1.52	43	25.8	1.72	6.75	4.46
1	305	2	1	29	1.15	20	25	1.01	4.16	2.56
1	212	2	2	48	0.66	<5%	21.5	3.78	6.22	3.86
2	102	2	1	NA	NA	NA	NA	NA	NA	NA
2	203	1	2	64	0.59	<5%	24.7	1.44	4.39	3.15
2	201	2	2	40	0.47	<5%	29.2	3.67	3.98	1.86
2	202	1	1	44	0.93	25	25.8	1.32	4.76	3.24
2	101	1	2	35	0.7	<5%	21.1	1.31	3.5	2.21
2	301	2	2	13	0.46	<5%	25.6	1.34	3.82	2.76

Lipid levels are shown in millimoles per liter. FAM, family; Ind ID, individual ID; HDL-C, high-density lipoprotein-cholesterol; BMI, body mass index, TG, triglycerides; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol

CHAPTER 5. MEMBRANE MICRODOMAINS MODULATE OLIGOMERIC ABCA1 FUNCTION: IMPACT ON APOAI-MEDIATED LIPID REMOVAL AND PHOSPHATIDYLCHOLINE BIOSYNTHESIS

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This research was originally published in Journal of Lipid Research. Membrane microdomains modulate oligomeric ABCA1 function: impact on apoAI-mediated lipid removal and phosphatidylcholine biosynthesis. Iulia Iatan, Dana Bailey, Isabelle Ruel, Anouar Hafiane, Steven Campbell, Larbi Krimbou and Jacques Genest. *J Lipid Res.* 2011;52(11):2043-2055.

5.1 Preface and Rationale

In the previous sections (Chapters 2, 3 and 4) we identified several genes that modulate HDL-C plasma concentrations, each acting at different sites and in different ways on the metabolism of HDL. This is evidence of the genetic complexity of HDL regulation, and supports the concept that HDL particles are regulated by a multitude of cellular and extracellular factors. Likewise, in addition to genetic modulations, molecular and biochemical regulations are also central to the generation of HDL particles. Of particular importance is elucidating the cellular mechanisms by which nascent HDL particles are formed via the lipidation of apoAI and its binding to the ABCA1/HCBS complex within plasma membrane microdomains. We were hence interested in providing a biochemical basis for the nascent HDL genesis pathway, by examining the apoAI/ABCA1/HCBS interactions within membrane microdomains and their impact on excess cholesterol removal. Specifically, we characterized the properties of the HCBS, and examined the distribution of the ABCA1/HCBS complex between plasma microdomains in various cell lines, while also exploring the role of membrane lipids, particularly cholesterol and phosphatidycholine, in mediating ApoAI/cellular interactions and nascent HDL composition and formation.

5.2 Abstract

Recent studies have identified an ABCA1-dependent phosphatidylcholine-rich microdomain, called the "high-capacity binding site" (HCBS), that binds apoA-I and plays a pivotal role in apoA-I lipidation. Here, using sucrose gradient fractionation, we obtained evidence that both ABCA1 and [^{125}I]-apoA-I associated with the HCBS were found localized to nonraft microdomains. Interestingly, phosphatidylcholine (PtdCho) was selectively removed from nonraft domains by apoA-I, whereas sphingomyelin and cholesterol were desorbed from both detergent-resistant membranes and nonraft domains. The modulatory role of cholesterol on apoA-I binding to ABCA1/HCBS was also examined. Loading cells with cholesterol resulted in a drastic reduction in apoA-I binding. Conversely, depletion of membrane cholesterol by methyl-β-cyclodextrin treatment resulted in a significant increase in apoA-I binding. Finally, we obtained evidence that apoA-I interaction with ABCA1 promoted the activation and gene expression of key enzymes in the PtdCho biosynthesis pathway. Taken together, these results provide strong evidence that the partitioning of ABCA1/HCBS to nonraft domains plays a pivotal role in the selective desorption of PtdCho molecules by apoA-I, allowing an optimal environment for cholesterol release and regeneration of the PtdCho-containing HCBS. This process may have important implications in preventing and treating atherosclerotic cardiovascular disease.

5.3 Introduction

Factors affecting cellular cholesterol homeostasis play a pivotal role in preventing the accumulation of excess cholesterol. Cellular cholesterol accumulation is believed to be cytotoxic to many cell types and is thought to contribute to foam cell death and lesional necrosis in advanced ACVD as documented by Feng and Tabas *et al* (Tabas, 2002; Feng et al., 2003). A substantial body of evidence from studies conducted in vitro and in vivo have shown that the ABCA1 transporter orchestrates cellular phospholipid and cholesterol removal to lipid-poor apolipoprotein acceptors by an active process.

The loss of ABCA1 function in humans leads to Tangier disease, which is associated with severe HDL deficiency and increased risk of ACVD (Brooks-Wilson et al., 1999).

It is well accepted that apoA-I interaction with ABCA1 has important implications in the reverse cholesterol transport (RCT) process. This pathway is not only a mode of excess cholesterol removal from peripheral cells that are unable to catabolize cholesterol, including macrophages in the vessel wall, but also it is crucial for the optimal lipidation of newly synthesized apoA-I within hepatic cells in a process called nascent HDL biogenesis (Krimbou et al., 2006; Lee and Parks, 2005). The pioneering studies by Rothblat's and Phillips' groups (Rothblat et al., 1992; Yancey et al., 2003) have proposed that the initial transfer of cholesterol from peripheral cells to HDL occurs via a number of mechanisms, including the aqueous diffusional/bidirectional exchange of lipids between the cell membrane and phospholipid-containing acceptors. This process has been shown to be accelerated when scavenger receptor class-B type I (SR-BI) is present (Yancey et al., 2003). In contrast, cholesterol and phospholipid efflux mediated by ABCA1 transporter is unidirectional and the primary acceptors of this pathway are lipid-poor apolipoproteins (Cavelier et al., 2006; Hassan et al., 2007; Vedhachalam et al., 2007a). Other ABC transporters, such as ABCG1 and ABCG4 have been shown to mediate cholesterol efflux to mature HDL particles (Cavelier et al., 2006).

Several models of ABCA1-mediated cholesterol efflux have been proposed, including a two-step model in which ABCA1 acts by flipping phospholipids to the outer leaflet of the plasma membrane (PM) bilayer. Subsequently, apoA-I is proposed to bind to these translocated phospholipid molecules, followed by acquisition of cholesterol and membrane solubilization (Krimbou et al., 2006; Rothblat et al., 1992; Yancey et al., 2003). This concept is strongly supported by recent studies from our laboratory and Phillips' (Hassan et al., 2007; Hassan et al., 2008; Vedhachalam et al., 2007a; Vedhachalam et al., 2007b), in which we identified an ABCA1-dependent, phosphatidylcholine-rich, PM apoA-I binding site having a near 10-fold higher capacity to bind apoA-I as

compared to ABCA1. As such, we have called this site the “high-capacity binding site” (HCBS) required for apoA-I lipidation.

There is an increasing interest in understanding the role of lipid-protein interactions within specialized membrane microdomains in vascular biology and atherogenesis. Indeed, sphingolipid/ cholesterol-rich rafts, known to be implicated in signal transduction, intracellular trafficking of lipids and proteins, and translocation of solutes across the membrane, have been shown to be involved in nitric oxide regulation and the RCT process (Mason and Jacob, 2003). Earlier studies by (Fielding and Fielding, 1995) have documented that PM caveolae represent a major site of efflux of both newly synthesized and low density lipoprotein-derived free cholesterol (FC) in the cells. More recently, Storey et al. (Storey et al., 2007) demonstrated selective cholesterol dynamics between lipoproteins and caveolae/lipid rafts.

Although (Mendez et al., 2001) have shown that membrane lipid domains distinct from cholesterol rich rafts are involved in the ABCA1-mediated lipid secretory pathway, the nature and specifics of apoA-I/ABCA1 interactions with membrane microdomains and their impact on excess cholesterol removal remain poorly understood. In this report, we examined the distribution of ABCA1 and the HCBS between membrane microdomains, explored the modulatory role of the lipid environment on ABCA1 function, and examined the potential role of apoA-I in the regulation of the phosphatidylcholine (PtdCho) biosynthesis pathway.

5.4 Results

5.4.1 Localization of ABCA1- and HCBS-associated apoA-I to nonraft fractions

Using detergent extraction with 0.2% Triton X-100 followed by sucrose equilibrium density gradient, we examined the localization of ABCA1, ABCA1-associated apoA-I, and HCBS-associated apoA-I to detergent-resistant membranes (DRMs) and/or non-raft fractions. We chose to use 0.2% instead of 1% Triton X-100 because the latter is known to produce artifacts in the distribution of phospholipids and cholesterol between DRMs and non-raft

domains, as reported by (Gaus et al., 2005). To ascertain the integrity of the DRMs and non-raft domains, the sucrose fractions were separated by SDS-PAGGE and monitored by appropriate antibodies for the co-localization of specific PM markers. As shown in **Figure 5.1A**, extraction with 0.2% Triton X-100 followed by sucrose fractionation enabled clear separation of the DRMs (raft) marker proteins caveolin-1 and flotillin-1 (fractions 1-5) from the detergent soluble/non-raft marker transferrin receptor (TfR; fractions 6-10), as reported previously (Gaus et al., 2005). As shown in **Suppl. Figure 5.1A**, ABCA1 complexes were localized exclusively to non-raft fractions (fractions 7-10). We found that ABCA1 migrated primarily in monomeric (≈250 kDa) and dimeric (~500 KDa) forms, as we have documented previously (Denis et al., 2004a). DTT reduced the dimeric forms to monomeric ABCA1.

Next, we examined whether the HCBS was located along with ABCA1 complexes within non-raft fractions. Fibroblasts or THP-1 cells were treated or not with 22OH/9CRA and incubated with 10 µg/ml of [125I]-apoA-I for 45 min at 37°C. After washing to remove unbound [125I]-apoA-I, cross-linking with DSP was performed and the samples were subjected to sucrose fractionation. The efficiency of DSP cross-linking was verified by cross-linking [125I]-transferrin to the transferrin receptor, as we have reported previously (Hassan et al., 2007). To quantify the amount of HCBS- or ABCA1-associated [^{125}I]-apoA-I present in each fraction, [^{125}I]-apoA-I/ABCA1 complexes were directly immunoprecipitated from sucrose fractions with an anti-ABCA1 antibody, as we have reported previously (Hassan et al., 2007). As shown in **Figure 5.1 (B, E)**, in both fibroblasts and THP-1, [^{125}I]-apoA-I was distributed between two densities, an intermediate density (fractions 5-7), and a high density (fractions 8-10). Fractions 9 and 10 may potentially contain some apoA-I dissociated in vitro. Similar results were obtained using BHK cells stably overexpressing ABCA1 and HepG2 (data not shown). An ABCA1 mutant (Q597R) used as a negative control for binding specificity did not show significant [^{125}I]-apoA-I association with any sucrose fraction (**Figure 5.1B**). When [^{125}I]-apoA-I was co-immunoprecipitated by an ABCA1-antibody, less than 10% of [^{125}I]-apoA-I was found associated with

ABCA1, while ~90% was found associated with the HCBS (**Figures 5.1 C, F, G**), consistent with our previous analysis of apoA-I distribution between ABCA1 and the HCBS (Hassan et al., 2007; Hassan et al., 2008). Furthermore, [^{125}I]-apoA-I associated with ABCA1 was localized exclusively to non-raft domains, while [^{125}I]-apoA-I associated with the HCBS was found to partition between two densities: an intermediate density (fractions 5-7) and a high density (fractions 8-10) in both fibroblasts and THP-1 (**Figures 5.1 B, C, E, F)**. A significant proportion of HCBS-associated [^{125}I]-apoA-I was found associated with fractions 5 and 6, without any detectable ABCA1 as assessed by SDS-PAGGE (**Suppl. Figure 5.1 A, B)**. Upregulation of ABCA1 with 22OH/9CRA in THP-1 increased the association of [^{125}I]-apoA-I with both ABCA1 and the HCBS in the non-raft fractions, including the less dense fractions (5-7) (**Figures 5.1 F,G**). The partition of HCBS-associated [^{125}I]-apoA-I between fractions 5-7 and 8-10, and the absence of ABCA1 in fraction 5 and 6, as well as poor protein content (**Figure 5.1 D**), suggests that the HCBS is heterogeneous in nature. We speculate that the HCBS region that excludes ABCA1 may represent exovesiculated domains or "mushroom-like protrusions" as reported by the groups of Phillip and Oram (Lin and Oram, 2000; Vedhachalam et al., 2007a).

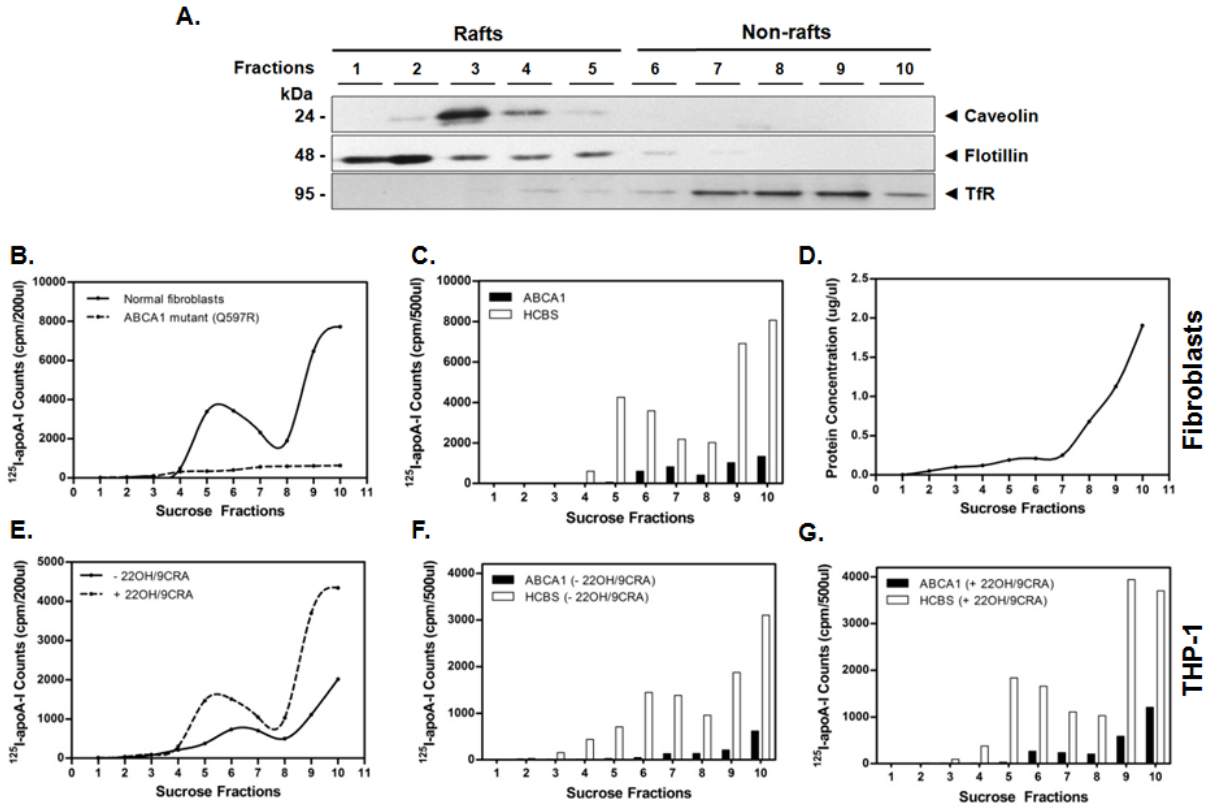


Figure 5.1 (A-G) Localization of ABCA1- and HCBS-associated apoA-I to nonraft fractions.

(A) Normal human fibroblasts were stimulated with 22OH/9CRA for 18 h and lysed at 4°C with 0.2% Triton X-100 in TNE buffer, followed by low-speed centrifugation to remove insoluble material. Supernatants were then subjected to sucrose gradient as described in Experimental Procedures, and 10 fractions of 1 ml collected. Fractions were concentrated and subsequently separated by 4-22.5% SDS-PAGE. After electrophoresis, rafts and nonraft distribution was detected with anti-caveolin and anti-flotillin antibodies protein markers for detergent insoluble membranes and with an anti-transferrin receptor antibody for nonraft membranes. Normal and ABCA1 mutant (Q597R) human fibroblasts (B–D) and THP-1 cells (E–G) stimulated or not with 22OH/9CRA were incubated with 10μg/ml [^{125}I]apoA-I for 45 min at 37°C. After washing to remove unbound [^{125}I]apoA-I, cross-linking with DSP was performed as described in Experimental Procedures. Cells were lysed at 4°C with 0.2% Triton X-100 in TNE buffer for 30 min followed by low-speed centrifugation to remove insoluble material. Supernatants were then subjected to sucrose gradient as described in Experimental Procedures, 10 fractions of 1 ml were collected, and radioactivity found in all fractions was determined by γ-counting. Cell associated [^{125}I]apoA-I profiles following gradient separation are shown for fibroblasts (B) and THP-1 cells (E). Fractions were subsequently concentrated and immunoprecipitated with 10 μl of affinity-purified polyclonal human anti-ABCA1 antibody (Novus) as described in Experimental Procedures. Radioactivity found in pellets (ABCA1-associated) and in supernatants (ABCA1-nonassociated, HCBS) was determined by γ-counting in fibroblasts (C), unstimulated THP-1 cells (F), and stimulated (G) THP-1 cells. Protein concentrations were assessed in fraction samples from fibroblasts (D). Results shown are representative of four independent experiments.

5.4.2 ApoA-I desorbs PtdCho selectively from the HCBS within nonraft domains

We have previously reported that disruption of the HCBS by PtdCho-PLC treatment impaired apoA-I-mediated cholesterol efflux (Hassan et al., 2007). The finding that the majority of apoA-I associated with the cell was found localized to the HCBS within nonraft domains (**Figure 5.1**) suggests that apoA-I may mediate efficiently PtdCho desorption from these domains. To further examine the involvement of the HCBS in apoA-I mediated PtdCho removal, fibroblasts were labeled with [^{3}H]-choline or [^{3}H]-cholesterol, stimulated with 22OH/9CRA, and incubated in the presence or absence of 15 μg/ml apoA-I for 12 h. After incubation, cells were homogenized with 0.2% Triton X-100 and subjected to sucrose fractionation. Lipids were extracted and PtdCho and SM were quantitated by TLC. Aproximatevly 36% of the cell content of PtdCho and 22% of SM respectively was found within nonraft domains, associated with the HCBS (**Figures 5.2A, B**). Incubation with ApoA-I was found to promote PtdCho desorption from nonraft domains, but not from DRMs (**Figure 5.2A**). Conversely, SM and cholesterol were found to be desorbed from both nonraft and DRMs as shown by profiles distribution (**Figures 5.2B**, **C)**. This is consistent with quantification of radioactivity appearing in DRMs (1 to 5) and nonraft (6 to 10) fractions expressed as percentage of control (100% in the absence of ApoA-I) for [^{3}H]-PtdCho (**Figure 5.2D**), [^{3}H]-SM (**Figure 5.2E**) and [^{3}H]-cholesterol (**Figure 5.2F**) between DRMs and non-rafts in the absence or presence of apoA-I.

On the basis of this result, we can assume that the rate of desorption of PtdCho from the HCBS is equivalent to the release of PtdCho to the media in the presence of apoA-I. As shown in **Suppl. Figure 5.2A**, apoA-I removes PtdCho from the HCBS within non-raft domains with an estimated K_m of 2 ± 0.35 μg/mL, which is characteristic of a high affinity process. These results indicate that the partitioning of ABCA1/HCBS to non-raft domains, as well as specific structural characteristics of these lipid microdomains, dictates the selective desorption of PtdCho from HCBS.

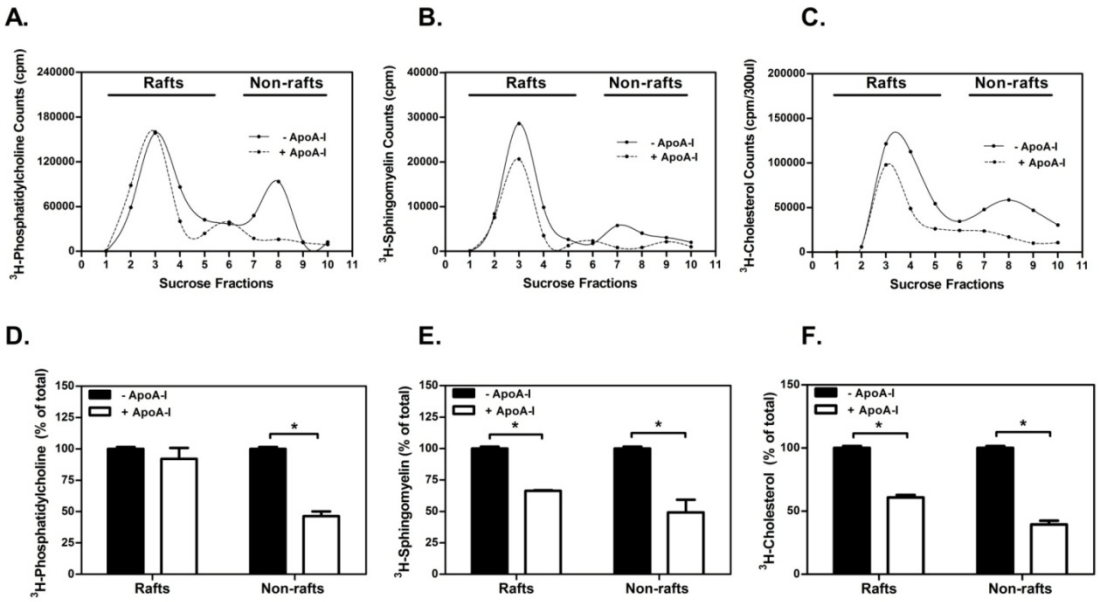
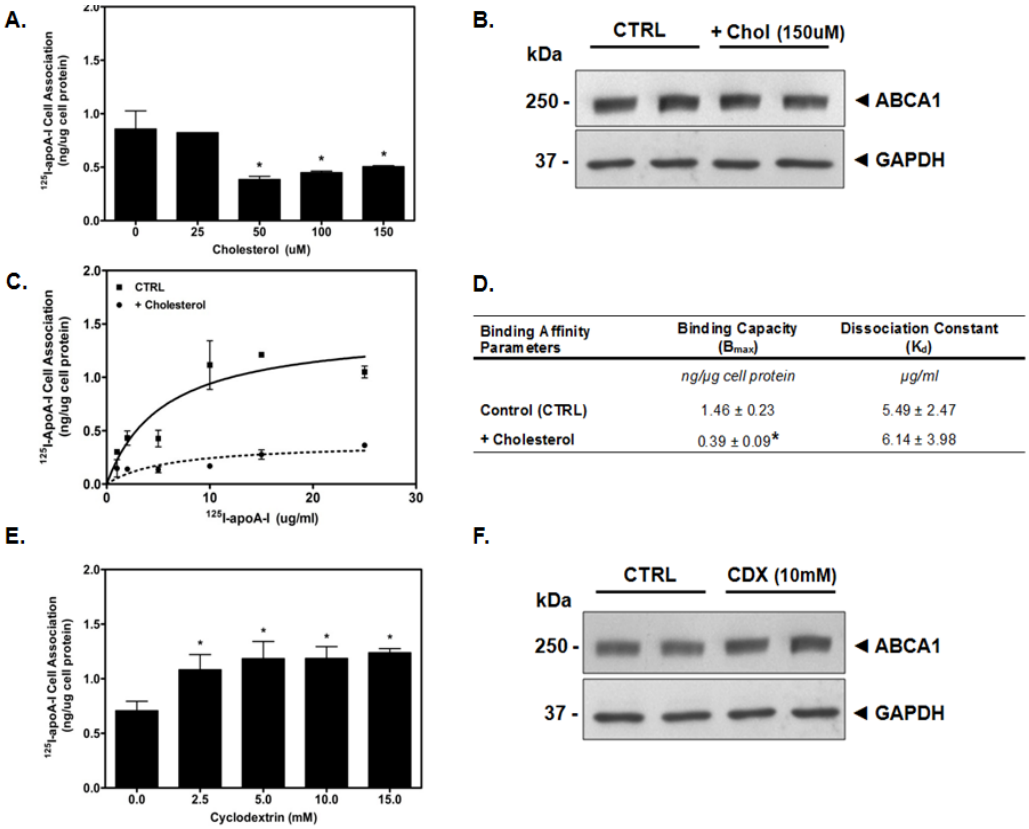


Figure 5.2 (A-F) ApoA-I desorbs PtdCho selectively from nonraft domains.
Normal human fibroblasts were labeled with [^{3}H]choline or [^{3}H]cholesterol for 48 h, followed by stimulation with 22OH/9CRA for 18 h. Cells were incubated or not with 15 μg/ml apoA-I for 12 h and lysed at 4°C with 0.2% Triton X-100 in TNE buffer for 30 min, and then supernatants were subjected to sucrose gradient as described in Figure 5.1. After lipid extraction, [^{3}H]PtdCho and [^{3}H]SM distribution in sucrose gradient fractions were assessed by TLC (A and B, respectively). [^{3}H]cholesterol (C) in each fraction was directly assessed for radioactivity. Radioactivity appearing in fractions corresponding to raft (1-5) and nonraft (6-10) material was pooled, and the desorption of [^{3}H]PtdCho (D), [^{3}H]SM (E), and [^{3}H]cholesterol (F) from raft versus nonraft in the presence of apoA-I was expressed as a percentage of control (100%, in the absence of apoA-I). Results shown are representative of three independent experiments. * $P < 0.05$ by Student's *t* -test.



Binding Affinity Parameters	Binding Capacity (B_{max})	Dissociation Constant (K_d)
	ng/µg cell protein	*µg/ml*
Control (CTRL)	1.46 ± 0.23	5.49 ± 2.47
+ Cholesterol	0.39 ± 0.09*	6.14 ± 3.98

Figure 5.3 (A-F) Effect of cellular cholesterol loading and depletion on the association of apoA-I with ABCA1/HCBS.

(A) Normal human fibroblasts were stimulated with 22OH/9CRA for 18 h and incubated with increasing concentrations of water-soluble cholesterol for 45 min, followed by 10 µg/ml [^{125}I]apoA-I for 45 min at 37°C. After washing to remove unbound [^{125}I]apoA-I, cells were lysed with 0.1N NaOH, and cellular associated [^{125}I]apoA-I was determined by γ-counting. Results shown are representative of three independent experiments. (B) Stimulated fibroblasts were loaded or not with 150 µM water-soluble cholesterol for 45 min, lysed, and separated by 4-22.5% SDS-PAGE. ABCA1 was detected using an affinity-purified monoclonal anti-ABCA1 antibody. GAPDH was used as a loading control. (C) Stimulated fibroblasts were loaded with 150 µM water-soluble cholesterol for 45 min and treated as in panel A. (D) Binding parameters of [^{125}I]apoA-I to both control and cholesterol-loaded samples (binding capacity B_{max} and dissociation constant K_d) were obtained using GraphPad Prism 5.0 software. Values shown are means ± SD of triplicate measures. * $P < 0.05$ by Student's *t* -test. (E) Normal human fibroblasts were stimulated with 22OH/9CRA for 18 h and incubated with increasing concentrations of CDX for 30 min. After washing, cells were incubated with 10 µg/ml of [^{125}I]apoA-I for 45 min at 37°C, washed, and lysed with 0.1N NaOH. Cellular-associated [^{125}I]apoA-I was determined by γ-counting. Results shown are representative of three independent experiments. * $P < 0.05$ by Student's *t* -test. (F) Stimulated fibroblasts were treated or not with 10 mM CDX for 30 min, lysed, and separated by 4-22.5% SDS-PAGE. ABCA1 was detected using an affinity-purified monoclonal anti-ABCA1 antibody. GAPDH was used as a loading control.

5.4.3 Influence of the lipid environment on the interaction of apoA-I with the ABCA1/HCBS system

The localization of ABCA1/HCBS to nonraft domains raises the possibility that the lipid constituents of the plasma membrane, namely PtdCho and cholesterol, could be important modulators of apoA-I binding.

Given that PtdCho is a major component of the HCBS, we examined whether modulation of the PtdCho content of the cell would affect the apoA-I/HCBS interaction. To test this, we used CHO-MT58 cells, a cell line with a temperature-sensitive deficiency in CTP:phosphocholine cytidylyltransferase (CCT)α, the rate-limiting enzyme in the PtdCho biosynthesis pathway (Vance et al., 1997). At 33°C, these cells contain levels of PtdCho lower than control cells; however, they are viable and grow at an almost normal rate (Lee et al., 1996). At 40°C, however, CCT-α is nonfunctional, causing a reduction in the cellular PtdCho content. CHO-MT58 and CHO-K1 cells were stimulated with T09/9CRA to induce ABCA1 expression and incubated with 10μg/mL [^{125}I]-apoA-I for 45 min at either permissive or restrictive temperatures. Consistent with previous analyses, at 40°C, the PtdCho content of CHO-MT58 was reduced by ~30% compared to control CHO-K1 (data not shown) (Lee et al., 1996). As shown in **Suppl. Figure 5.3A**, this reduction in PtdCho levels corresponded with a significant reduction in cellular associated [^{125}I]-apoA-I (62.6±5.3% vs control). Similar ABCA1 levels were found in both cell lines at 33°C and 40°C (**Suppl. Figure 5.3B**). This result supports the role of PtdCho in associating apoA-I within the HCBS microdomains.

Additionally, we hypothesized that cholesterol may influence the association of apoA-I with the ABCA1/HCBS within non-raft domains. To examine whether membrane cholesterol is involved in the binding of apoA-I to ABCA1 and the HCBS, cells were either loaded with water-soluble cholesterol or depleted of cholesterol by incubation with CDX, and the cellular association of [^{125}I]-apoA-I was determined. Conditions were chosen such that ABCA1 levels were maintained across treatments. Loading of stimulated fibroblasts with increasing concentrations of water-soluble cholesterol significantly decreased the

association of [^{125}I]-apoA-I with the cells (**Figure 5.3A**). Treatment with 150 μM water-soluble cholesterol increased the cellular FC content to 150±6% relative to control, but did not alter ABCA1 levels (**Figure 5.3B**), nor cause significant cellular toxicity as measured by an LDH-release assay (data not shown). To examine the influence of cholesterol loading on apoA-I binding properties, stimulated fibroblasts were loaded with 150 μM cholesterol for 45 min, and then incubated with increasing concentrations of [^{125}I]-apoA-I for 45min. As shown in **Figure 5.3C**, cholesterol loading significantly decreased the binding capacity of apoA-I to the cell as compared to control cells (B_{max}=0.39±0.09 vs. 1.46±0.22 ng apoA-I/μg cell protein). In contrast, the K_d was not significantly affected (K_d=6.14±3.98 vs. 5.49±2.47 μg apoA-I/ml, cholesterol-loaded vs. control) (**Figure 5.3D**). This specific effect of cholesterol loading on the capacity, but not the affinity, of [^{125}I]-apoA-I association with the cell suggests that cholesterol may directly affect the binding of apoA-I to the HCBS, owing to the fact that the HCBS is largely responsible for associating [^{125}I]-apoA-I (Hassan et al., 2007; Krimbou et al., 2006; Vedhachalam et al., 2007b). On the contrary, cholesterol depletion by CDX increased significantly [^{125}I]-apoA-I cell association at a relatively low CDX concentration (2.5 mM) (**Figure 5.3E)**. Under our experimental conditions, treatment of cells with 10 mM CDX for 30 min reduced the cellular free cholesterol content to 56±3% of control cells. CDX treatment did not affect the levels of ABCA1 (**Figure 5.3F**), nor induce any cellular toxicity.

Although manipulation of cholesterol levels had a profound effect on apoA-I cellular association independent of changes in ABCA1 protein expression, it remained possible that these treatments could affect the subcellular distribution of ABCA1, and consequently apoA-I association. To examine the effect of cholesterol loading and depletion on ABCA1 localization, distribution of ABCA1 and apoA-I between the PM and ICCs was monitored by a cell-surface biotinylation assay, as we have previously described (Hassan et al., 2008). After treatment with water-soluble cholesterol, CDX, or media alone, cells were incubated with [^{125}I]-apoA-I for 45 min at 37°C, and cell surface proteins were labeled with 0.5 mg/ml sulfo-NHS-biotin for 30 min at 4°C. PM proteins were

separated from intracellular proteins by streptavidin pull-down, as described in Methods. ABCA1 localization was monitored by western blot, while [^{125}I]-apoA-I was quantified by γ-counting. As shown in **Figure 5.4A**, the reduction or increase in [^{125}I]-apoA-I binding after cholesterol or CDX treatment, respectively, was largely mediated by association with the PM. Conversely, manipulation of cholesterol levels had, at most, only a modest effect on the distribution of ABCA1 between the PM and ICCs (**Figure 5.4B**).

5.4.4 The PtdCho biosynthesis pathway is regulated by apoA-I/ABCA1 interaction

After we determined that apoA-I selectively removes PtdCho from the HCBS within nonraft domains, the questionwas raised whether this process activates the PtdCho biosynthesis pathway. To determine the particular steps in the Kennedy pathway that were activated, ABCA1-expressing cells were incubated with apoA-I for 16 h, and then pulsed with [^{3}H]choline for 1 h. The distribution of radioactivity among the choline (Cho) metabolites [phosphocholine (Pcho), cytidine diphosphocholine (CDPcho) and PtdCho] was quantitated by TLC after separation of the organic and aqueous phases. Incubation of 22OH/9CRA-stimulated fibroblasts with apoA-I was shown to significantly increase the incorporation of [^{3}H]choline into Pcho (~130% versus control) and PtdCho (~170% versus control) (**Figure 5.5A**), with a concomitant depletion of [^{3}H]choline (~30% versus control). CDP-choline did not accumulate in fibroblasts, likely because levels and/or activity of the CDP-choline:DAG choline phosphotransferase (CPT) enzyme was in excess of CCTα. Conversely, no significant increase in [^{3}H]choline conversion was observed in TD fibroblasts (Fig. 5B). Similarly, apoA-I incubation with 22OH/9CRA-stimulated THP-1 resulted in a significant increase of [^{3}H]choline with 10% LPDS for 48 h was used as a positive control. Under lipoprotein deprivation incorporation of [^{3}H]choline into CDP-choline was significantly increased (data not shown), as reported previously (Ryan et al., 2000).

To confirm the observation that incubation of ABCA1-expressing cells with apoA-I induces PtdCho synthesis through the Kennedy pathway, we examined the mRNA expression of CCTα, the rate-limiting enzyme in PtdCho synthesis, and choline kinase (CKα) after apoA-I incubation. As shown in Fig. 5C, incubation of apoA-I with normal fibroblasts stimulated with 22OH/9CRA induced a significant increase in mRNA expression of CCTα (2-fold versus control) and CKα (1.7-fold versus control). Incubation with apoA-I did not upregulate mRNA expression of CCTαor CKα in ABCA1 mutant (Q597R) fibroblasts (**Figure 5.5D**). Additionally, because loss of cholesterol from the PM could stimulate PtdCho synthesis, we examined whether depletion of cholesterol with 10 mM CDX would stimulate mRNA expression of CCTαor CKα in normal fibroblasts. As shown in supplementary Fig. IV-A, CDX treatment had no significant effect on the expression of either of these genes. Incubation with apoA-I did not significantly affect gene expression from 22OH/9CRA-stimulated THP-1 cells (data not shown).

Taken together, these results suggest that ABCA1 is involved in an apoA-I-mediated upregulation of PtdCho biosynthesis. It is likely that the PtdCho depletion of the HCBS by apoA-I activates key enzymes in the PtdCho biosynthesis pathway or, alternatively, that apoA-I itself induces cell-signaling pathways that activate PtdCho synthesis directly. We are currently investigating these pathways in macrophages.

5.4.5 Heterogeneity of nascent LpA-I released during dissociation from HCBS

As shown in **Figure 5.1**, after 45 min incubation at 37°C, the majority of apoA-I was found associated with the HCBS, as we have previously reported (Hassan et al., 2007; Hassan et al., 2008). In an attempt to examine the nature of the nascent LpA-I particles released from the HCBS, we monitored the dissociation of the lipidated apoA-I products. [125I]-apoA-I was incubated with stimulated fibroblasts for 45 min at 37°C. After washing to remove unbound [^{125}I]-apoA-I, the plates were incubated with DMEM alone at 37°C or 4°C for the indicated time points. At each of the indicated timepoints, the medium was

removed and replaced with fresh medium. As shown in **Figure 5.6**, [^{125}I]-apoA-I dissociated from wildtype cells in two peaks (Peak I and Peak II). Dissociation of [^{125}I]-apoA-I was inhibited at 4°C, suggesting that dissociation was not an artifactual release of apoA-I after extensive washing of the cells. An ABCA1 mutant (Q597R) that has been shown to have no significant apoA-I binding was used as a negative control. Analysis of the dissociated lipidated apoA-I products by 2D-PAGGE revealed initial dissociation (Peak I) generated α-LpA-I particles with sizes of 8 nm (panel B), whereas later dissociation (Peak II) produced larger α-LpA-I with sizes between 9 nm and 17 nm (panel E). The α-electrophoretic mobility of dissociated LpA-I was determined based on a standard reference gel depicting the pre-β electrophoretic mobility of lipid-free apoA-I (panel A) (Hassan et al., 2008). To assess the lipid composition of the nascent LpA-I particles, cells were loaded with [^{32}P]orthophosphate or [^{14}C]-cholesterol to label the phospholipid and cholesterol components, respectively. Cells were stimulated and then incubated with lipid-free apoA-I. Collection fractions corresponding to Peaks I and II were pooled and washed extensively by filtration to remove free [^{32}P]-phospholipids and [^{14}C]-cholesterol. Samples were concentrated and analyzed by 2D-PAGGE followed by autoradiography. Initial dissociation of apoA-I from the HCBS was found to produce particles containing phospholipids, but no detectable cholesterol (panels C and D), whereas later dissociation was found to produce particles containing both phospholipids and cholesterol. PtdCho was a major phospholipid species found in those small particles (Peak I), as assessed by TLC (data not shown). These results suggest that apoA-I could be initially released from the HCBS as small PtdCho-apoA-I discs and that these discs interact successively with different microdomains, including DRMs (raft), to acquire cholesterol.

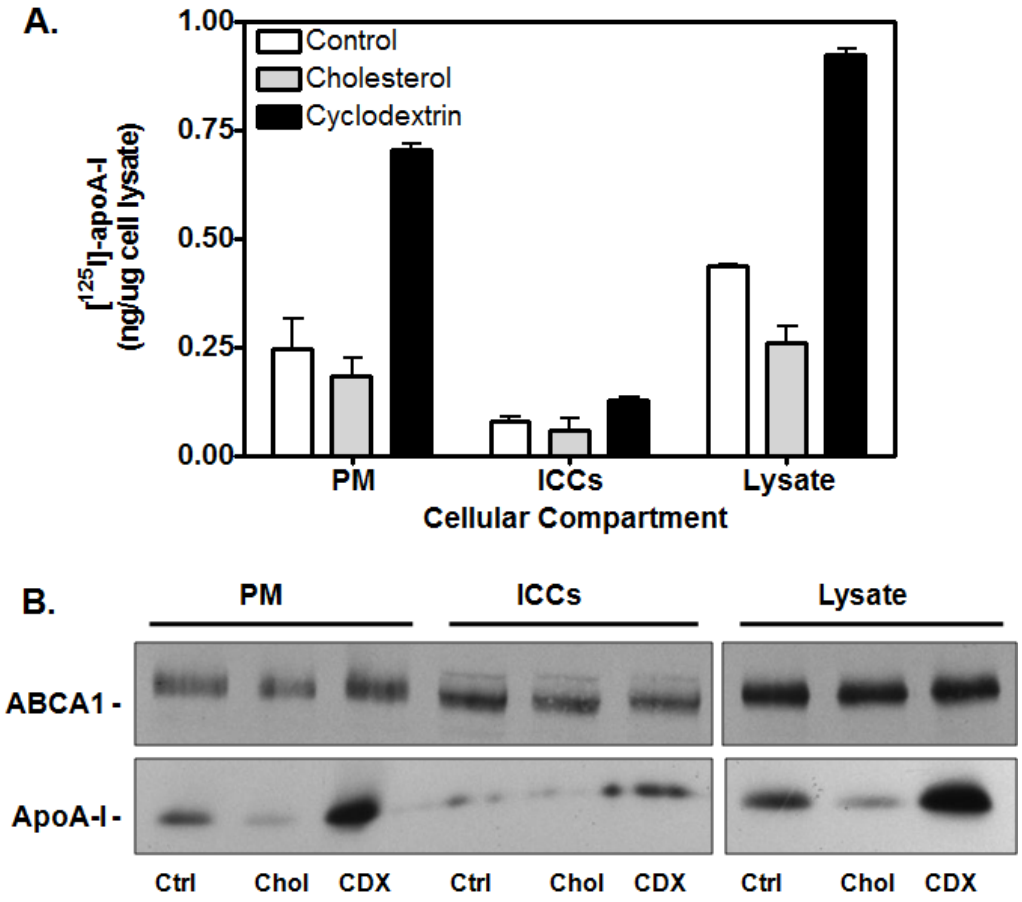


Figure 5.4 (A, B) ABCA1 compartmentalization under cellular cholesterol loading and depletion.

(A) Confluent fibroblasts were stimulated with 2.5 μg/ml 22OH and 10 μm 9CRA for 20 h. Cells were depleted of or loaded with cholesterol as described in Experimental Procedures, and then incubated with 10 μg/ml [^{125}I]apoA-I for 45 min at 37°C. PM proteins were biotinylated with 0.5 mg/ml sulfo-NHS-biotin for 30 min at 4°C, and then separated from ICC proteins by streptavidin pulldown. [^{125}I]apoA-I was detected by γ-counting (A) and autoradiography (B), and ABCA1 was monitored by Western blot (B). Results are representative of two independent experiments.

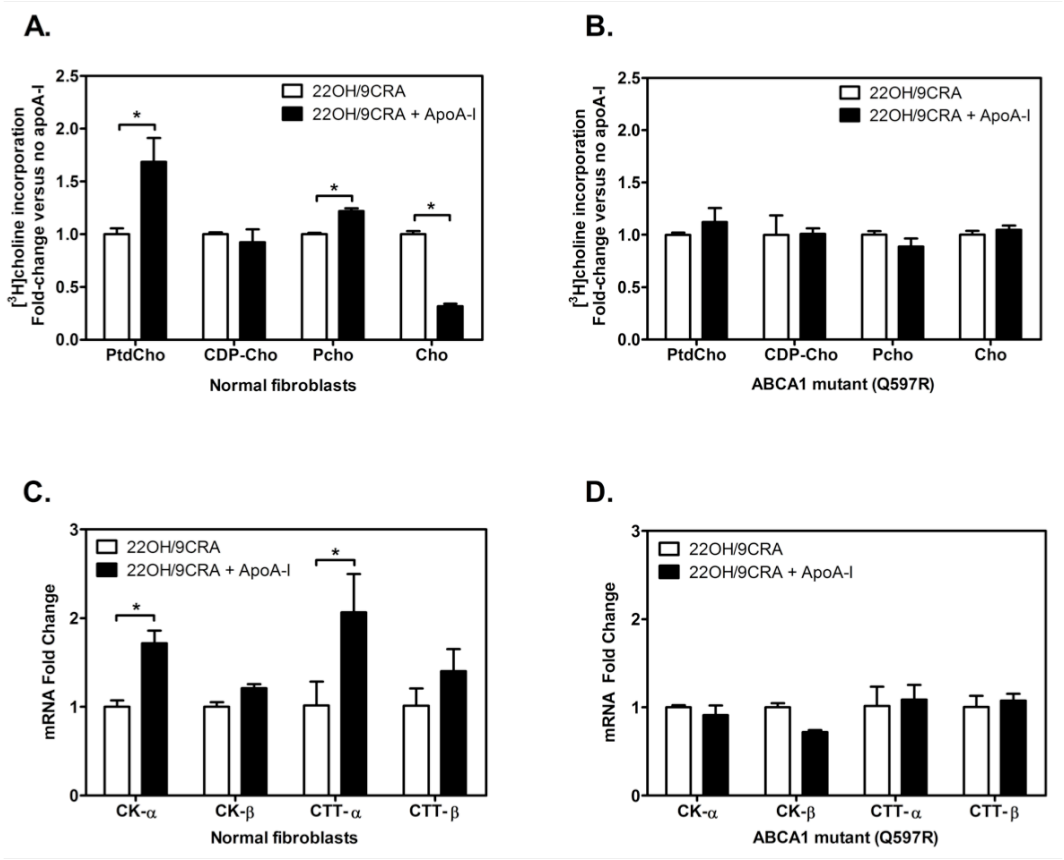


Figure 5.5 (A-D) The PtdCho biosynthesis pathway is regulated by apoA-I/ABCA1 interaction in fibroblasts.

(*A, B)* Normal and ABCA1 mutant (Q597R) fibroblasts were stimulated with 22OH/9CRA and incubated or not with 20 μg/ml apoA-I for 16 hours. Cells were then pulsed with 10 μCi/ml methyl[^{3}H]choline for 1h and the distribution of radioactivity among metabolites (Cho, Pcho and CDP-cho) and PtdCho was determined as described in Experimental Procedures. Values shown are means ± SD of triplicate measures. *$P < 0.05$ by Student's *t*-test. (C, D) Stimulated normal and ABCA1 mutant (Q597R) fibroblasts were incubated or not with 20 μg/ml apoA-I for 16 hours followed by mRNA extraction using the RNeasy mini RNA extraction kit (Qiagen). Analysis of mRNA expression of CCTα, CCTβ, CK-α and CK-β was carried by real-time quantitative PCR from 200 ng total RNA as described in Experimental Procedures. Values shown are means ± SD of triplicate experiments. The expression of each gene was normalized to GAPDH expression, and mRNA fold changes relative to controls were determined. *P < 0.05 by Student's *t*-test.

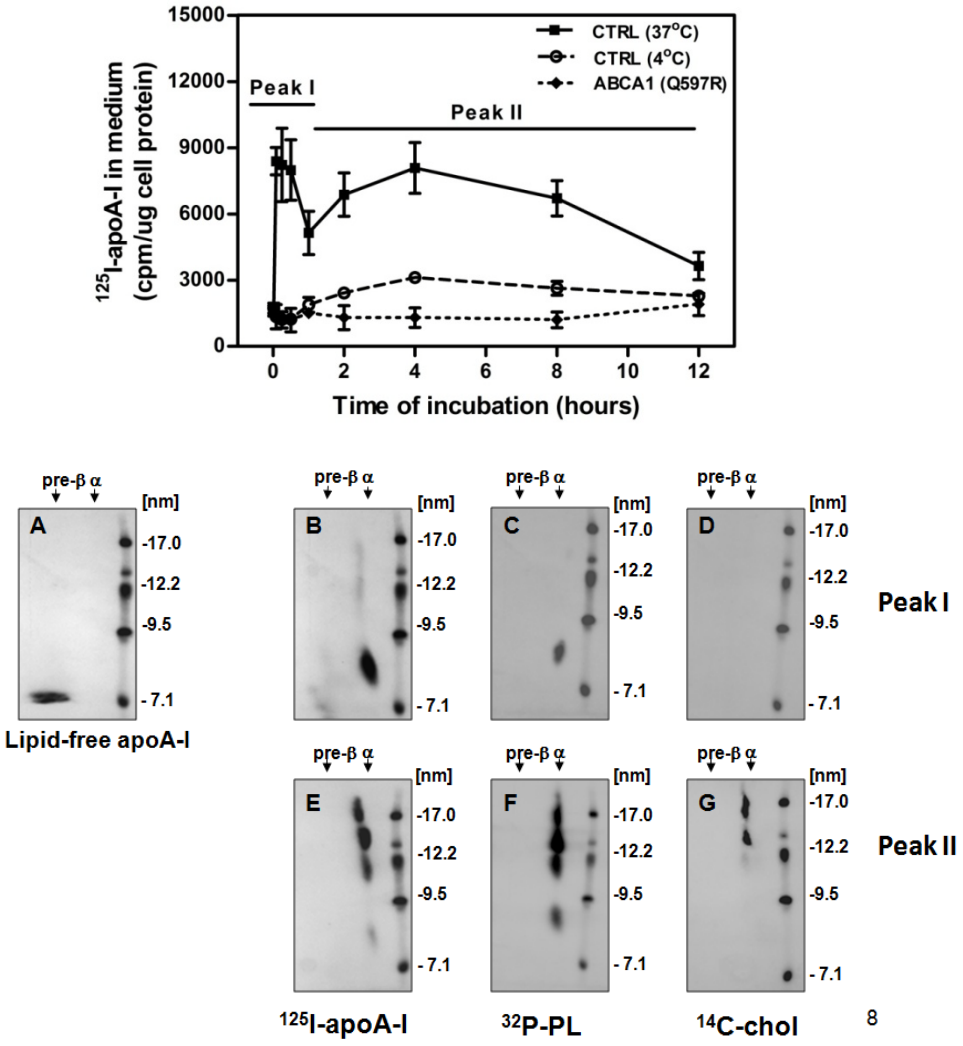


Figure 5.6 Heterogeneity of nascent LpA-I released during dissociation from HCBS.

(Top) Normal and ABCA1 mutant (Q597R) fibroblasts were stimulated with 22OH/9CRA and incubated in the presence of 10 μg/mL of [^{125}I]-apoA-I for 45 min at 37°C. After washing to remove unbound [^{125}I]-apoAI, DMEM was added, and plates were immediately incubated at 37°C or 4°C. Medium was removed and replaced with fresh medium for the indicated time periods. Dissociated [^{125}I]-apoA-I was determined by γ-counting. (Bottom) Analysis of the dissociated lipidated apoA-I products by 2D-PAGGE. The α-electrophoretic mobility of dissociated LpA-I was determined based on a standard reference gel depicting the preβ electrophoretic mobility of lipid-free apoA-I (A) (Hassan et al., 2008). To assess the lipid composition of the nascent LpA-I particles, fibroblasts were incubated with 300μCi of [^{32}P]orthophosphate for 72 hours (***C, F***) or with 15 μCi/ml of [^{14}C]cholesterol for 24 hours (***D, G***). Cells were stimulated with 22OH/9CRA and incubated with lipid-free apoA-I under similar conditions as described above. ApoA-I particles released pertaining to each peak were pooled, concentrated and analyzed for lipid content on 2D-PAGGE as described in Experimental Procedures. The presence of [^{125}I]-apoA-I, [^{32}P]phospholipids or [^{14}C]cholesterol was detected by autoradiography.

5.5 Discussion

The concept proposed by the Phillips and Rothblat groups, that apoA-I-mediated membrane solubilization pathway efficiently clears cells of excess cholesterol, has important implications in the RCT process and atherogenesis (Gillotte et al., 1999; Vedhachalam et al., 2007a). Defining the lipid microenvironment surrounding oligomeric ABCA1, which likely influences its conformation, function and regulation, is key for understanding how the cholesterol homeostasis pathway and nascent HDL biogenesis are regulated at the cellular level.

Here, we obtained evidence that both ABCA1 and the HCBS were localized to non-raft domains in fibroblasts and THP-1 stimulated with 22OH/9CRA (**Figure 5.1 and Suppl. Figure 5.1**), consistent with our previous report documenting that the ABCA1 and the HCBS were found in the Triton X-100 soluble fraction (Hassan et al., 2007). The exclusion of ABCA1 and the HCBS from raft domains could be an intrinsic property of the transporter. Indeed, Landry et al. (Landry et al., 2006), have reported that through it's ATPase-related functions, ABCA1 expression alters the general packing of the PM by generating more loosely packed microdomains. This is in agreement with a recent study by Zarubica et al. (Zarubica et al., 2009) reporting that analysis of the partitioning of dedicated probes in PM vesiculated blebs, allowed visualization of ABCA1 partitioning into the liquid disordered like phase and corroborated the idea that ABCA1 destabilizes the lipid arrangement at the PM, supporting the concept that ABCA1 plays a pivotal role in controlling transversal and lateral lipid distribution at the PM and positioned the effluxes of cholesterol from cell membrane to the redistribution of the sterol into readily extractable membrane pools.

An earlier study from our laboratory (Denis et al., 2004a) has proposed that a homotetrameric complex of ABCA1 constitutes the minimal functional unit required for apoA-I lipidation, in agreement with the work by the Chimini group (Trompier et al., 2006). It is possible that generation of the HCBS stabilizes ABCA1 clustering, which can influence both the initial association of apoA-I to ABCA1, as well as the ABCA1 oligomerization state, thereby maximizing the

number of apoA-I molecules that bind to the oligomeric ABCA1 simultaneously. This is consistent with the finding that apoA-I binds to tetrameric and dimeric, but not monomeric, forms of ABCA1 (Denis et al., 2004a).

The phospholipid translocase activity of ABCA1 appears to create two populations of HCBS microdomains within the non-raft fractions that could impact both the capacity of the membrane to associate with apoA-I and the composition of the nascent HDL products. We obtained evidence that the HCBS is heterogeneous, based on the observation that apoA-I was distributed between two physically distinct density regions (**Figure 5.1**). In the intermediate density fractions, ABCA1 was absent, whereas in the higher density fractions, ABCA1 was present, as were the majority of membrane proteins (**Suppl. Figure 5.1**). It is likely that the HCBS associated with the less dense intermediate fractions, which are relatively rich in lipids and poor in membrane proteins (**Figure 5.1D**), represent the "mushroom-like protrusions" or exovesiculated domains to which apoA-I bind on the surface of human fibroblasts and THP-1, as reported (Lin and Oram, 2000). This is in line with the idea of (Vedhachalam et al., 2007b) that "plasma membrane proteins (including ABCA1) are presumably excluded from the exovesiculated domains" and suggested that these domains are formed from regions of protein-free PM that are likely to be relatively fluid.

The structural and physicochemical characteristics that dictate the partitioning of ABCA1 and apoA-I to non-raft domains are presently unknown. We obtained evidence to support the role of PtdCho in apoA-I association (**Suppl. Figure 5.3** and **Figure 5.5**) (Hassan et al., 2007), however, the peaks of PM levels of PtdCho as separated by sucrose fractionation do not appear to correlate directly with the peaks of apoA-I binding (**Figure 5.1** vs. **Figure 5.2A**). We hypothesize that the cholesterol composition of membrane microdomains could play a major role in partitioning both ABCA1/HCBS and apoA-I to non-raft domains. It is possible that the greater lipid lateral packing density within cholesterol-rich raft domains could reduce the affinity of apoA-I to these microdomains. Here, we obtained evidence to support this concept. First, we demonstrate that cholesterol loading of cells, without altering ABCA1 levels, drastically reduced the binding

of apoA-I to ABCA1/HCBS (**Figure 5.3A, B, C, D**). Second, depletion of cholesterol from cells using a low concentration of CDX, which is known to disrupt rafts, increased significantly the association of apoA-I to ABCA1/HCBS, without significantly changing ABCA1 levels (**Figure 5.3E, F**). It is possible that excess cholesterol directly affects the conformation of the oligomeric ABCA1 or the packing of PtdCho molecules within the HCBS populations, and thereby reduces the affinity for apoA-I. These results are supported by studies using artificial membranes showing that apoA-I efficiently solubilizes multilamellar vesicles of the loosely packed L_d phase (Fukuda et al., 2007). Alternatively, cholesterol could directly modulate the activity of ABCA1. This is supported by a previous study by Ueda's group documenting that the ATPase activity of purified ABCA1 in PtdCho-liposomes was significantly decreased by enrichment with cholesterol (Takahashi et al., 2006). The disturbance of ABCA1/HCBS system by excess cholesterol accumulation within foam cells may have a direct impact on the development of atherosclerotic lesions. Indeed, (Choi et al., 2009a) have recently documented that accumulation of cholesterol by intimal arterial smooth muscle cells (SMCs) resulted in the impairment of both apoA-I-mediated cholesterol efflux and apoA-I binding to the cells.

Previous studies from our laboratory and others have not supported the existence of a clear precursor-product relationship between the various nascent LpA-I particles (Denis et al., 2004b; Krimbou et al., 2005; Liu et al., 2003). In this report, by monitoring the dissociation of apoA-I from the HCBS, we observed the production of, initially, small LpA-I particles with a high phospholipid-to-cholesterol ratio, and subsequently, larger LpA-I particles containing both phospholipids and cholesterol (**Figure 5.6**). These results are consistent with findings by Mulya and Sorci-Thomas et al.(Mulya et al., 2007; Sorci-Thomas et al., 2011). The smaller particles could represent initial PtdCho-apoA-I discs that arise from the HCBS and interact successively with different microdomains, including rafts, to acquire cholesterol and produce the larger particles. Indeed, we observed that apoA-I selectively desorbs PtdCho from HCBS within non-raft domains and cholesterol from DRMs (Fig.2). This is in agreement with a previous

study by Mendez et al. (Mendez et al., 2001), showing that membrane lipid domains distinct from cholesterol/SM rich rafts are involved in the lipidation of apoA-I, while membrane rafts are involved in efflux to lipidated particles. Additionally, small HDL particles enriched in phospholipid but depleted of cholesterol have been shown to be one of the most efficient acceptors of cellular cholesterol (Castro and Fielding, 1988). Alternatively, the complexity of the larger particles could require a longer incubation period for production. This is in agreement with a previous study by Jessup group demonstrating that two kinetically distinguishable pathways of cholesterol transfer to apoA-I exist (Gaus et al., 2001), and a study by the Parks laboratory demonstrating that larger nascent particles are produced only with prolonged apoA-I incubation (Mulya et al., 2008). In this case, the heterogeneity of the LpA-I particles may reflect simultaneous microsolubilization of lipids from distinct microdomains, consistent with our observation that apoA-I associated with the HCBS was found in two physically distinct densities (**Figure 5.1**), that likely reflect heterogeneity in membrane lipid composition. This hypothesis is in accordance with the microsolubilization mechanism supported by Vedhachalam et al. (Vedhachalam et al., 2007a).

The interaction of apoA-I with the ABCA1/HCBS system was found to significantly increase PtdCho synthesis (**Figure 5.5**). Indeed, we obtained evidence that both CK-α and CCT-α gene expression were significantly upregulated following incubation of the cell with apoA-I. This is in agreement with an earlier study by Mallampalli's group (Ryan et al., 2000) documented that lipoprotein deprivation upregulates the gene expression and activity of CCT-α in alveolar type II epithelial cell line. Furthermore, Shiratori et al. have reported that FC-loading of macrophages upregulates CCT-α activity by a post-translational mechanism and the increased of PtdCho biosynthesis is an adaptive response to accommodate the excess cholesterol (Shiratori et al., 1994). The activation of the PtdCho biosynthetic pathway by apoA-I may be physiologically important in the regeneration of the HCBS, allowing the removal of excess cholesterol from PM microdomains. Whether apoA-I directly induces a cell-signaling pathway that

stimulates PtdCho biosynthesis, or activates PtdCho production via an adaptive response to PtdCho depletion is currently under investigation.

As summarized in **Figure 5.7,** our current working model of apoA-I lipidation is as follows. The lipid translocase activity of oligomeric ABCA1 creates a heterogenous HCBS population, including the exovesiculated HCBS that excludes ABCA1 and the HCBS that associates with oligomeric ABCA1 complexes. Initial interactions of apoA-I with the oligomeric ABCA1 facilitates its translocation to the HCBS populations from which apoA-I desorbs PtdCho, becoming an efficient acceptor of cholesterol. These small PtdCho-containing particles may interact successively or simultaneously with different microdomains, including cholesterol rich-rafts, allowing the formation of larger heterogeneous, cholesterol-containing nascent LpA-I particles. The desorption of PtdCho molecules by apoA-I induces an adaptive response to upregulate the PtdCho biosynthesis pathway, thereby allowing replenishment of the lost membrane PtdCho and regeneration of HCBS populations. The model presented in **Figure 5.7** is a simple illustration of our current findings and previous investigations on the ABCA1/HCBS system (Hassan et al., 2007; Hassan et al., 2008; Krimbou et al., 2006; Vedhachalam et al., 2007a; Vedhachalam et al., 2007b). Our findings are in agreement with the model proposed recently by Phillips'group (Vedhachalam et al., 2007b), in which the initial event consists of binding of a small pool of apoA-I to ABCA1 at the PM, stimulating the net phospholipid translocation to the exofacial leafleat. This situation leads to unequal lateral packing densities in the two leaflets of the phospholipid bilayer, creating exovesiculated lipid domains. The formation of a highly curved membrane surface promotes high affinity binding of apoA-I to these domains. This pool of bound apoA-I spontaneously solubilizes the exovesiculated domain to create discoidal nascent HDL particles. The identification and isolation of HCBS populations as reported here (**Figure 5.1**), will certainly aid the analysis of the lipidome of these domains and the determination of their impact on the assembly of nascent particles, their composition and speciation. The detailed mechanisms

underlying apoA-I interactions with membrane microdomains require more extensive investigations, which are currently ongoing.

The present report provides a biochemical basis for apoA-I-mediated plasma microdomain excess cholesterol removal that involves both the oligomeric ABCA1 and the HCBS population. This process may have important implications in preventing and treating atherosclerotic cardiovascular disease.

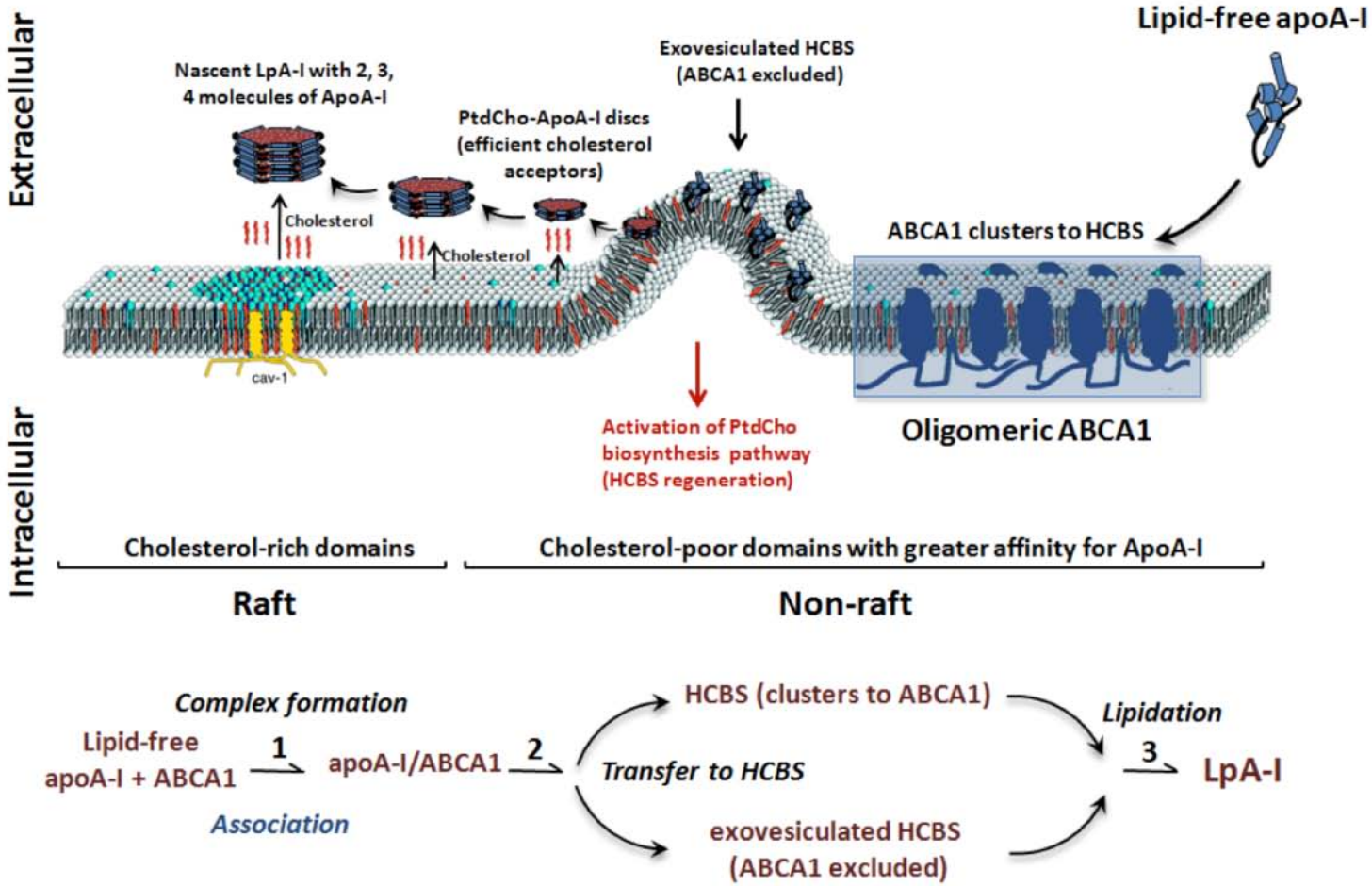


Figure 5.7 A proposed model for apoA-I interaction with ABCA1 within PM microdomains.
Lipid free apoA-I initially interacts with oligomeric ABCA1 through a rapid, but transient association. The phospholipid translocase activity of ABCA1 creates a heterogenous HCBS microdomains populations within the nonrafts, composed of an ABCA1-excluded exovesiculated HCBS domain and a HCBS that clusters with oligomeric ABCA1 complexes. ABCA1 subsequently mediates the transfer of apoA-I to these HCBS populations, from which apoA-I selectively desorbs PtdCho to become PtdCho-apoA-I discs. Subsequently, apoA-I obtains PtdCho and cholesterol to generate heterogenous nascent LpA-I particles with two, three, and four molecules of apoA-I. The desorption of PtdCho by apoA-I activates the PtdCho biosynthesis pathway, thereby allowing replenishment of the lost membrane PtdCho and regeneration of the HCBS populations.

5.6 Acknowledgements

The authors wish to express their gratitude to the late Dr. John F. Oram and to Dr. Ashley M. Vaughan for generously providing BHK cells overexpressing ABCA1 and to Dr. Dennis Vance for kindly providing CHO-MT58 cells. The authors are grateful for Tudor Iatan's assistance in editing the proposed model.

This work was supported by grants MOP 15042 from the Canadian Institutes of Health Research (CIHR), and the Heart and Stroke Foundation of Quebec. J. Genest holds the McGill University-Novartis Chair in Cardiology.

5.7 Materials and Methods

5.7.1 Cell culture

Human skin fibroblasts were obtained from 3.0 mm punch biopsies of the forearms of patients and healthy control subjects. Fibroblasts obtained from a TD (homozygous for Q597R at the ABCA1 gene) were used as a negative control throughout the study, as previously described (Denis et al., 2004a; Hassan et al., 2007). Cells were cultured in DMEM supplemented with 0.1% nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS. THP-1 macrophages were cultured under standard conditions (ATCC) and treated with 150 nM PMA for 72 h prior to use. When indicated, cells were stimulated with 2.5 μg/ml 22-(R)-hydroxycholesterol (22OH) and 10 μm 9-cis-retinoic acid (9CRA) for 20 h to induce ABCA1 expression. BHK cells stably transfected with an ABCA1 expression vector that is inducible by treating the cells with mifepristone and cells transfected with the same vector lacking the ABCA1 cDNA insert (mock-transfected) were generously provided by the late Dr. John F. Oram from the Department of Medicine, University of Washington and were characterized and cultured as described previously (Oram et al., 2001). These BHK cells do not normally express ABCA1.

5.7.2 Human plasma apoA-I

Purified plasma apoA-I (Biodesign) was resolubilized in 4M guanidine-HCl and dialyzed extensively against PBS buffer. Freshly resolubilized apoA-I was iodinated with ^{125}Iodine by IODO-GEN® (Pierce) to a specific activity of 3000 to 3500 cpm/ng apoA-I and used within 48 h.

5.7.3 Sucrose gradient fractionation

Sucrose density gradient was performed as described previously (Gaus et al., 2005). Cells were lysed at 4°C with TNE buffer [50 mM Tris-HCl (pH 7.5), 140 mM NaCl, 5 mM EDTA] containing 0.2% (v/v) Triton X-100 and protease inhibitor cocktail (Roche) for 30 min on ice followed by low-speed centrifugation to remove insoluble materials. Samples were mixed with an equal volume of 90% (w/v) sucrose in MBS [25 mM MES (pH 6.5), 150 mM NaCl] and overlaid with 35, 30, 25, and 5% (w/v) sucrose. The gradient was spun at 198,000 g (average) in a Beckman SW41 rotor for 16 h. Ten fractions of 1.0 ml were collected from the top and analyzed for protein, radioactivity, and lipid content.

5.7.4 Quantitative chemical cross-linking and immunoprecipitation assay

Quantitative immunoprecipitation was performed as we have described previously (Hassan et al., 2007). Briefly, fibroblasts or PMA-treated THP-1 were incubated with 10 μg/ml of [^{125}I]apoA-I, washed with PBS, and cross-linked with 500 μM dithiobis-(succinimidylpropionate) DSP (Pierce) for 30min/RT. The cross-linker was inactivated by the addition of 20 mM Tris (pH 7.5) (final concentration). Cells were washed with PBS, lysed at 4°C with TNE-0.2% Triton X-100 buffer containing protease inhibitor cocktail, and subjected to sucrose fractionation. [^{125}I]apoA-I associated with ABCA1 in sucrose gradient fractions was coimmunoprecipitated with 10 μl of anti-ABCA1 antibody (Novus) for 18 h at 4°C, followed by the addition of protein A bound to Sepharose (30 μl). Radioactivity found in pellets (ABCA1-associated) and in supernatants (ABCA1-non associated, HCBS) was determined by γ-counting Protein concentration was determined by standard assay (Bio-Rad).

5.7.5 Lipid labeling and efflux

Lipid labeling and efflux were performed as described previously (Denis et al., 2004b; Hassan et al., 2007; Krimbou et al., 2004), with minor modifications. Briefly, fibroblasts were labeled with either 15 μCi/ml [^{3}H]choline (Perkin-Elmer) for 48h or 3 μCi/ml [^{3}H]cholesterol (Perkin-Elmer) for 24 h, and then stimulated as described above. Cells were subsequently incubated with lipid-free apoA-I and fractionated by sucrose gradient in the presence of 0.2% Triton X-100. For each fraction, lipids were extracted by Folch and [^{3}H]choline-labeled PtdCho and SM were separated by TLC. [^{3}H]cholesterol was quantified directly. Alternatively, for experiments investigating the lipid composition of nascent apo-A-I-conaining (LpA-I) particles, fibroblasts were labeled with 300 μCi/mL [^{32}P]orthophosphate or 15 μCi/mL [^{14}C]cholesterol, stimulated, and incubated with lipid-free apoA-I. LpA-I particles were analyzed by two-dimensional polyacrylamide nondenaturing gradient gel electrophoresis (2D-PAGGE0 and autoradiography.

5.7.6 Cholesterol depletion and loading

For cholesterol depletion, cells were incubated for 30 min at 37°C in DMEM containing 50 mM HEPES (pH 7.2) and 0.2% BSA (DMEM/BSA) and the indicated concentration of of methyl-β-cyclodextrin (CDX). Control cells were incubated in the same medium lacking cyclodextrin. Cells were loaded with cholesterol using the above media containing the indicated concentration of water-soluble cholesterol·cyclodextrin complex for 45 min.

5.7.7 Cellular [^{125}I]-apoA-I binding assay

22OH/9CRA stimulated cells were incubated with 10 μg/ml of ^{125}I-apoA-I, or with increasing concentrations of ^{125}I-apoA-I for 45min at 37°C. For nonspecific binding determination, cells were incubated with a 30-fold excess of unlabeled apoA-I. ABCA1 mutant (Q597R) associated with TD was used as a negative control for binding specificity. This mutant did not show significant [^{125}I]-apoA-I association with cells as we have previously described (Hassan et

al., 2007). After washing to remove unbound [^{125}I]-apoA-I, cells were lysed in 0.1 N NaOH, and the radioactivity was determined by γ-counting.

5.7.8 Cell surface biotinylation assay

Confluent fibroblasts were stimulated with 2.5 μg/ml 22(R)-hydroxycholesterol and 10 μm 9-cis-retinoic acid for 20 h. Cells were depleted of or loaded with cholesterol, as described above, and incubated with 10 μg/ml [125I]-apoA-I for 45 min at 37°C. Cells were washed three times with PBS, and surface proteins were biotinylated with 0.5 mg/ml sulfosuccinimidobiotin (sulfo-NHS-biotin; Pierce) for 30 min at 4 °C. The biotinylation reaction was quenched by removal of the biotin solution and addition of 20 mm Tris-HCl (pH 7.5). Cells were washed twice with ice-cold PBS, lysed, and homogenized. 100 μg of protein was added to 50 μl of streptavidin-Sepharose beads and incubated overnight on a platform mixer at 4 °C. The PM pellet or intracellular compartment (ICCs) supernatant was washed with lysis buffer. Localization of ABCA1 was monitored by Western blot and [^{125}I]-apoA-I by γ counting. Results are representative of two independent experiments.

5.7.9 Dissociation of [^{125}I]-apoA-I from intact cells

The dissociation of apoA-I was performed as we have previously described (Denis et al., 2004b; Hassan et al., 2008). Stimulated fibroblasts were incubated with 10 μg/mL of [^{125}I]-apoA-I for 45 min/37°C. After washing to remove unbound [^{125}I]-apoA-I, DMEM was added, and the plates were immediately incubated at 37°C or 4°C for the indicated time periods. At each time-point, the medium was removed and replaced with fresh medium. Dissociated [^{125}I]-apoA-I was determined by γ-counting. LpA-I particles were analyzed by 2D-PAGGE and autoradiography.

5.7.10 Analysis of gene expression by RT-PCR

Total RNA was prepared from fibroblasts and THP-1 cells using the RNeasy mini RNA extraction kit (Qiagen), according to the manufacturer's

instructions. Total RNA (200 ng) was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen). Real-time quantitative PCR was carried out using the Quantitect SYBR Green PCR kit and QuantiTect Primer assays (Qiagen): PCYT1A (#QT00051835), PCYT1B (#QT00023380), CHKA1 (#QT00013405) and CHKB1 (#QT00218435). All reactions were performed on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems). Amplifications were carried out in a 96-well plate with 50 µl reaction volumes and 40 amplification cycles (94ºC, 15s; 55ºC, 30s; 72ºC, 34s). Experiments were carried out in triplicate and the mRNA expression was taken as the mean of three separate experiments. The expression of each gene was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Fold changes relative to controls were determined using the ΔΔCt method.

5.7.11 Analysis of labeled choline metabolites

The analysis of the incorporation of [^{3}H]choline into phosphocholine (Pcho), cytidine diphosphate choline (CDP-cho), and PtdCho, were performed as described previously (Gasull et al., 2003). Stimulated cells were incubated or not with 20 µg/ml apoA-I for 16hr at 37°C. Cells were then pulsed with 10 µCi/ml methyl[^{3}H]choline for 1h. After labeling, cells were washed with PBS and methanol was added. Cells were harvested by scraping and were sonicated on ice. To extract lipids, chloroform was added to the methanol-cell suspension for 15 min/RT (1:1 v/v). Water was added to separate the aqueous and lipid phases (6:6:5 methanol:chloroform:water, v/v/v). The aqueous and lipidic phases were transferred to new tubes and dried under nitrogen gas. Water-soluble metabolites were dissolved in 40µl ethanol/water (1:1), spotted onto Whatman K5 Silica Gel Adsorption Plates (Whatman), and developed in methanol/0.9%NaCl/ammonia (50:50:5, v/v/v). Lipids were dissolved in 40µl chloroform/methanol (2:1), spotted onto Whatman K5 Silica Gel Adsorption Plates (Whatman), and developed in chloroform/methanol/acetic acid/water (75:45:3:1, v/v/v/v). Choline, Pcho, CDP-cho, and PtdCho were identified by comigration with standards. Spots

corresponding to each metabolite were revealed by iodine vapour, scraped, and the radioactivity determined.

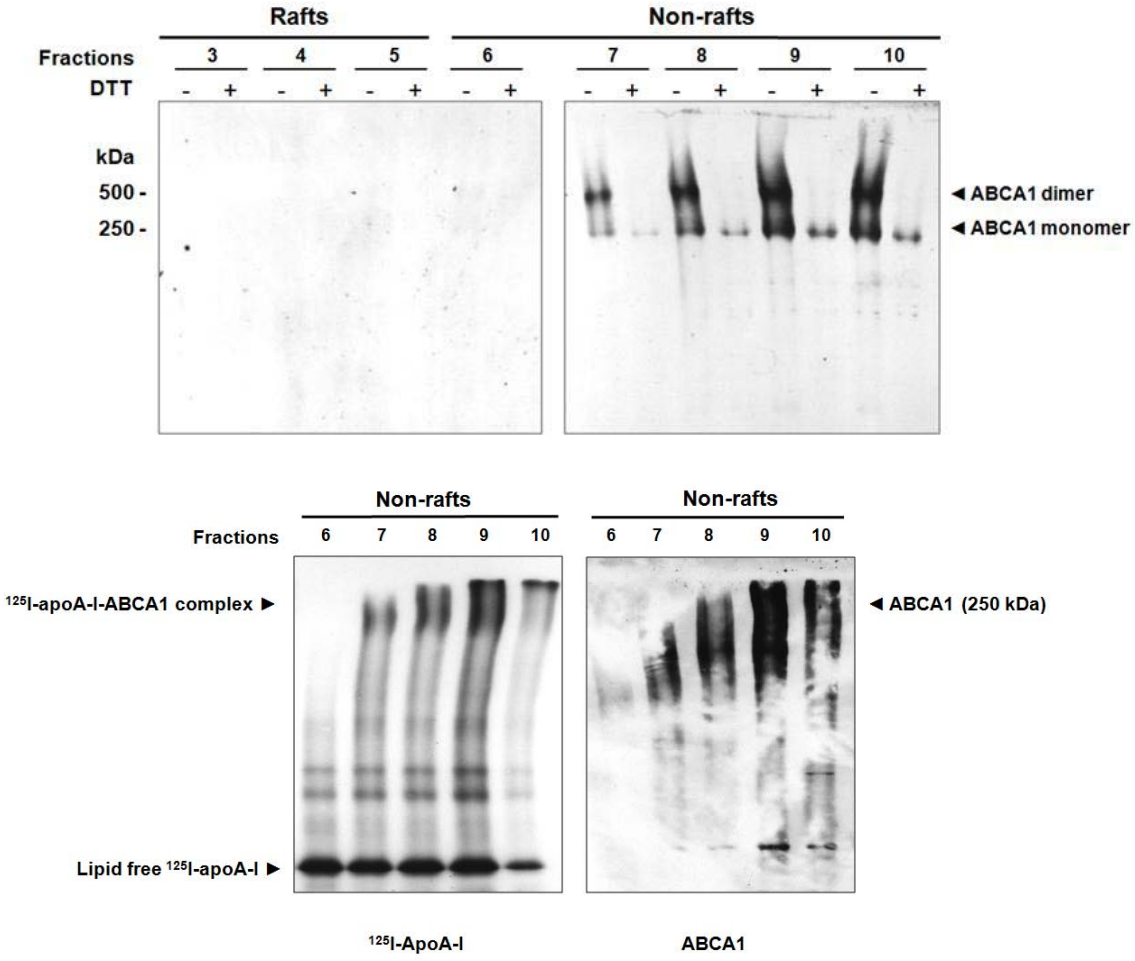
5.7.12 Lipid and lipoproteins analysis

Cellular free and esterified cholesterol mass, as well as PtdCho were quantified by TLC. ApoA-I-containing particles were separated by 2D-PAGGE, as we have previously described (Bailey et al., 2010b; Krimbou et al., 1997).

5.7.13 Statistical analysis

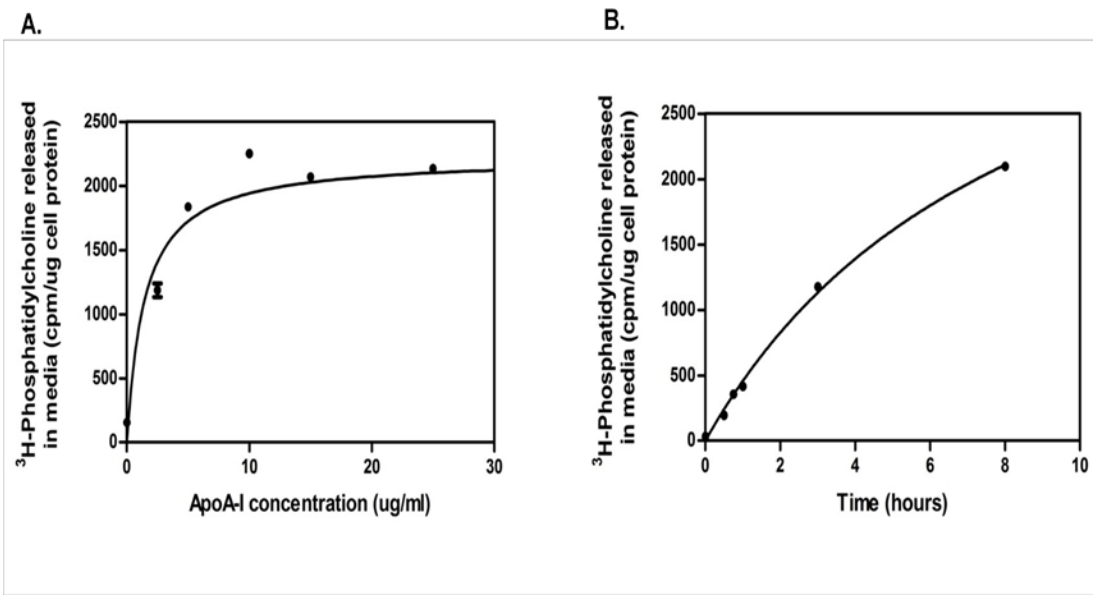
Results were compared statistically by paired *t*-test. Two-tailed *P* values < 0.05 were considered as significantly different.

5.8 Supplementary Figures



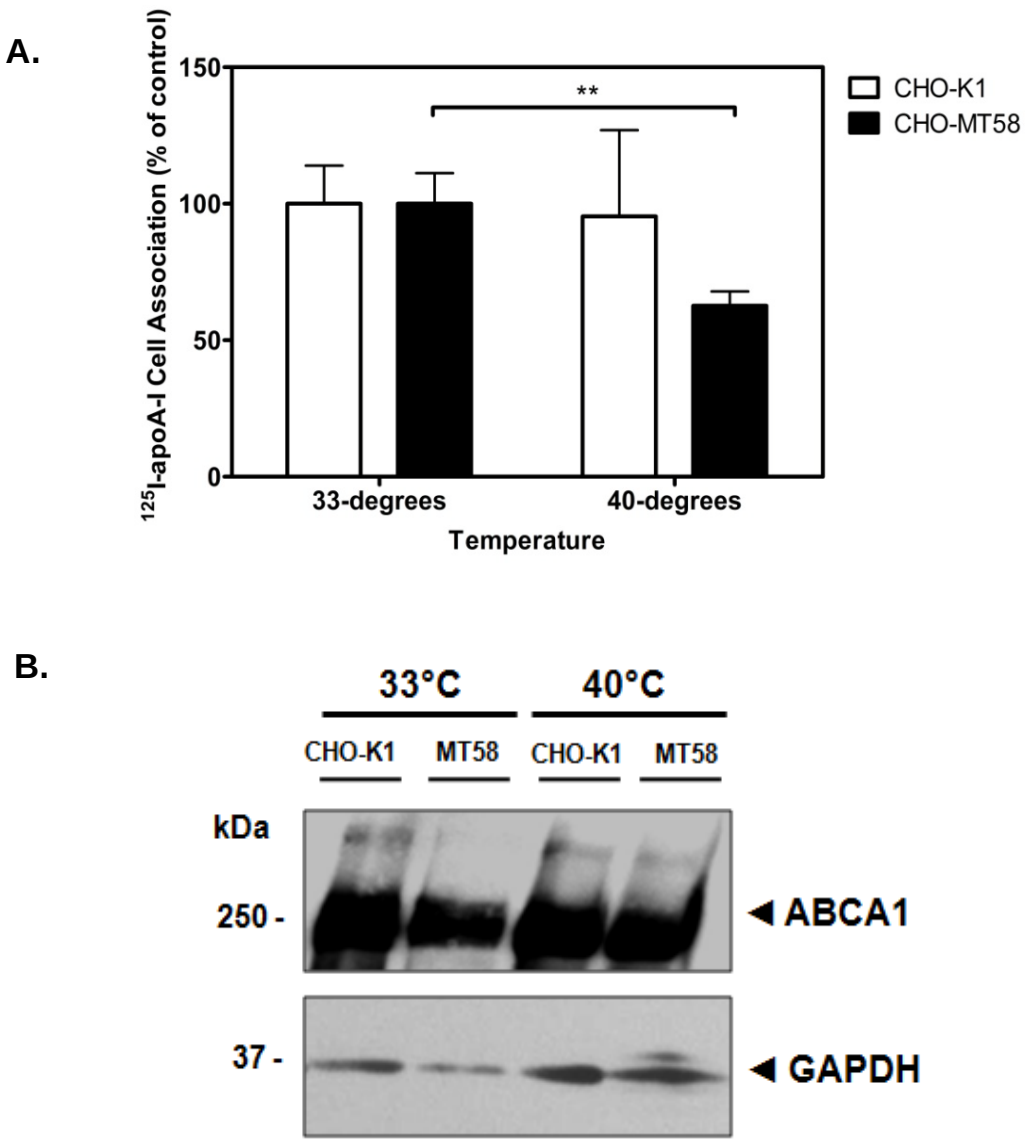
Supplementary Figure 5.1 Distribution of [125I]-apoA-I and oligomeric ABCA1 between HCBS populations within non-raft domains.

A, Stimulated fibroblasts were directly lysed (without being previously cross-linked with DSP), subjected to gradient fractionation and 10 fractions of 1 ml were collected as described in Methods. After sample concentration, fractions 3 to 10 were treated or not with 20 mM DTT for 15 min at 37°C. Samples were then separated by 4-22.5% SDS-PAGE and ABCA1 was detected with an affinity purified monoclonal anti-ABCA1 antibody (Millipore). Results shown are representative of four independent experiments. B, Normal human fibroblasts were stimulated with 22OH/9CRA and incubated with 10 ug/ml [125I]-apoA-I for 45 min at 37°C. After washing to remove unbound [125I]-apoA-I, cells were subjected to DSP cross-linker (500µM, 30 min), lysed at 4°C with 0.2% Triton X-100 in TNE buffer for 30 min and separated by sucrose gradient as described in Methods. Sucrose fractions were concentrated and samples fractions corresponding to non-rafts domains (fractions 6 to 10) were separated on a 4-22.5% SDS-PAGE, transferred to nitrocellulose, and [125I]-apoA-I was directly revealed by autoradiography while ABCA1 was detected with an affinity-purified monoclonal anti-ABCA1 antibody.



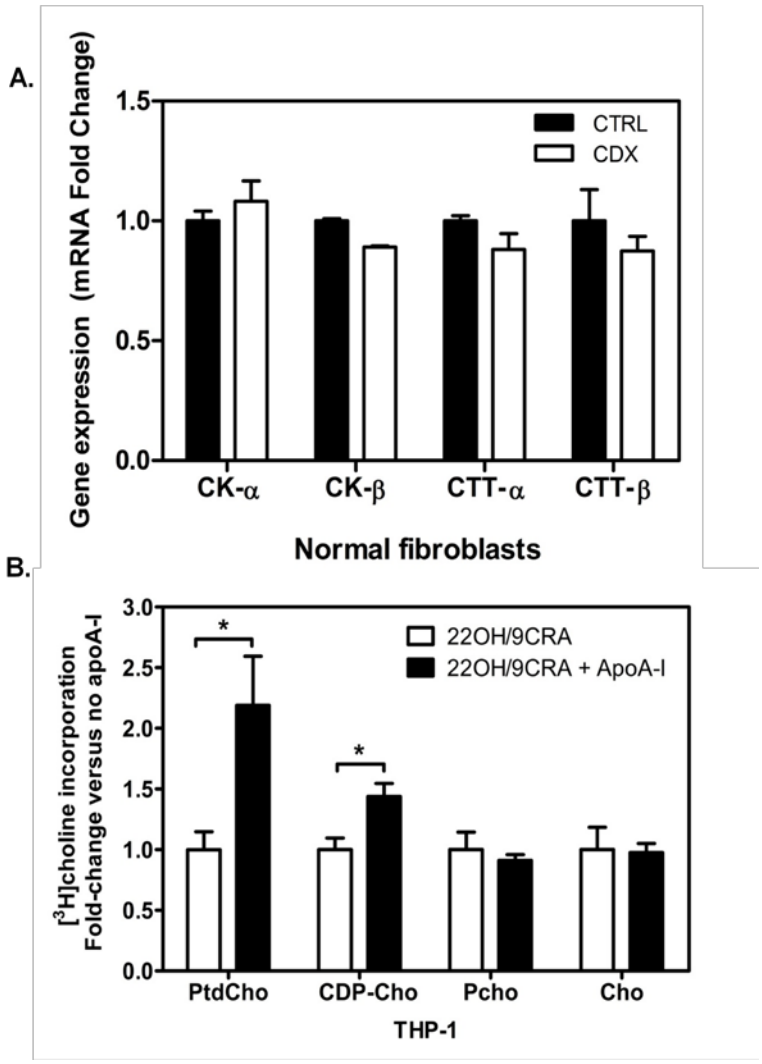
Supplementary Figure 5.2 Kinetics of apoA-I-mediated phosphatidylcholine efflux.

Normal human fibroblasts were labelled with [3H]-choline for 48 hours, stimulated with 22OH/9CRA for 18 hours and incubated with increasing concentrations of apoA-I for 8 hours at 37°C (A). At the same time, cells were incubated with 10 µg/ml of apoA-I for different time points at 37°C (B). After lipid extraction, [3H]-phosphatidiylcholine released into media was quantitated by TLC. Values shown are means ± SD of triplicate measures. Michaelis constant Km was obtained using GraphPad Prism 5.0 software. Values shown are means ± SD of triplicate measures.



Supplementary Figure 5.3 Effect of phosphatidylcholine depletion on apoA-I association with ABCA1/HCBS.

A, Normal CHO-K1 and temperature-sensitive CHO-MT58 cells were stimulated with T09/9CRA for 18h at 33°C. Cells were subsequently incubated with 10 ug/ml [125I]-apoA-I for 45 min at either 33°C or 40°C. Unbound [125I]-apoA-I was removed by washing, and cells were lysed with 0.1N NaOH. Cellular associated [125I]-apoA-I was subsequently determined by γ-counting. Results shown are representative of three independent experiments. *P < 0.05 by Student's t-test. B. Stimulated CHO-K1 and CHO-MT58 cells grown at either 33°C or 40°C were lysed and separated by 4-22.5% SDS-PAGE. ABCA1 was detected using an affinity-purified monoclonal anti-ABCA1 antibody (Millipore). GAPDH was used as a loading control.



Supplementary Figure 5.4 The phosphatidylcholine biosynthesis pathway is not affected by cellular cholesterol depletion in fibroblasts but is regulated by apoA-I/ABCA1 interaction in THP-1.

A, Stimulated normal fibroblasts were incubated or not with 10 mM methyl-β-cyclodextrin (CDX) for 30 min. Analysis of mRNA expression of CTP:phosphocholine cytidyltransferase (CCT-α, CCT-β) and choline kinase (CK-α and CK-β) was carried by real-time quantitative PCR from 200 ng total RNA as described in Methods. Values shown are means ± SD of triplicate experiments. The expression of each gene was normalized to GAPDH expression and mRNA fold changes relative to controls were determined. *P < 0.05 by Student's t-test. *B,* Differentiated THP-1 were stimulated with 22OH/9CRA and incubated or not with 20 µg/ml apoA-I for 18 hours. Cells were then pulsed with 10 µCi/ml methyl[^{3}H]choline for 1h and distribution of radioactivity among choline metabolites (choline (Cho), phosphocholine (Pcho), cytidine diphosphocholine (CDP-cho) and phosphatidylcholine (PtdCho)) was performed as described in Methods. Values shown are means ± SD of triplicate measures. *P < 0.05 by Student's *t*-test.

CHAPTER 6. GENERAL DISCUSSION

6.1 Summary

Low levels of HDL-C are known to constitute a major independent risk factor for CAD, influenced by a combination of environmental and genetic factors. Indeed, plasma HDL-C is known to have a strong inherited basis, warranting a comprehensive search for novel genes that modulate HDL-C using different integrative approaches. Furthermore, while it is well accepted that the interaction of apoA-I with ABCA1 is essential for the generation of nascent HDL particles and removal of excess cholesterol from peripheral cells, the molecular mechanisms underlying this process remain poorly understood. Collectively, the studies herein have allowed us to identify novel genetic determinants and molecular mechanisms of regulation involved in the RCT, with significant interrelated effects on HDL metabolism (**Figure 6.1**).

In Chapter 2, we demonstrated that genetic variation at the *PCSK5* gene locus regulates plasma HDL-C levels in two different populations; French Canadian and low HDL-C Finnish families. We further observed an association of significant SNPs with other lipoprotein traits, including TG, VLDL, and apoB_{tota}l (**Table 2.4**). These findings have important contributions on the remodeling of HDL_3 particles, as PC5/6 has been determined to inactivate EL (**Figure 2.3**) and act on ANGPTL3, which also inhibits EL and LPL (Shan et al., 2009).

In Chapter 3, using novel genetic approaches, we identified two rare damaging functional variants in ABCA1 and LPL, causing low HDL-C in a multigenerational French Canadian family. The importance of our findings is highlighted by the significant roles that both ABCA1 and LPL are known to play in lipoprotein metabolism, and specifically in HDL biogenesis and function. LPL catalyses the hydrolysis of TG from circulating chylomicrons and VLDL, and mutations at the gene cause severe hypertriglyceridemia (**Table 1.3**) (Klos and Kullo, 2007). Abnormalities in LPL function have also been associated with a number of other pathophysiological conditions, including atherosclerosis, chylomicronaemia, obesity, Alzheimer's disease, and dyslipidaemia associated with diabetes, insulin resistance, and infection (Mead et al., 2002). Our findings

on LPL complement our data from Chapter 2, as PC5/6 inactivates both EL and LPL, and ANGPTL3 is a potent inhibitor of LPL (Liu et al., 2010).

While exome sequencing has been established as a novel strategy for identifying rare variants behind Mendelian disorders, (Ng et al., 2010) its use for delineating genetic causes of complex traits has not been completely elucidated. Our work highlights therefore the utility of exome sequencing in identifying two variants for a multifactorial trait, HDL-C, which act in combination to produce a significant phenotypic sex-effect while lowering HDL-C plasma concentrations. As such, the ABCA1 S1731C and the LPL P207L variants were found to explain 60% of HDL-C genetic variance in the multigenerational French Canadian family investigated and 26% of the total (genetic and environment) variance, as well as 46 % of heritability in that family.

In Chapter 4, we established a physiologically significant role for a novel gene, *WWOX*, and its protein, in lipoprotein metabolism. Our results validate previous genetic studies where *WWOX* was found to be associated with low HDL-C levels in 10 000 subjects (Lee et al., 2008b). They are also a first line of functional evidence that WWOX might be an important modulator of HDL biogenesis, through its effects on ABCA1 and ApoA-I. Our findings may only be the tip of the iceberg with respect to the role of WWOX in lipoprotein metabolism however, as it was demonstrated through the network and microarray analyses (**Figure 4.4**) that disruption of WWOX in liver-specific KO mice alters multiple pathways. As such, WWOX might play a more significant role in health and disease than it was previously thought. Prior to our studies, WWOX had been firmly associated to cancer, given its role as a tumor suppressor (Aqeilan et al., 2007), but evidence suggested that it might also play a role in steroidogenesis (Aqeilan et al., 2009). We therefore believe that our findings point towards a role for WWOX in both cardiovascular and cancer-related states, which warrant further investigation.

Furthermore, our study also highlights the value of using multiple strategies in characterizing a gene, and its protein, namely by using a combination of *in vivo* functional studies, in both mice and humans, as well as gene

microarrays and next generation resequencing. This also underlines the importance and validity of identifying novel genes implicated in a particular disease by different genetics strategies, i.e. by linkage and genetic associations studies, candidate gene approach and resequencing.

In Chapter 5, we characterized the molecular mechanism of nascent HDL formation. We demonstrated that both ABCA1 and the HCBS are localized to non-raft domains and that when altering the lipid environment of ABCA1 through loading or depleting cells with cholesterol, Apo-I binding to ABCA1/the HCBS resulted in a reduction or increase, respectively. We also observed that the apoA-I/ABCA1 interaction promotes the activation and gene expression of key enzymes in the PC biosynthesis pathway. These results suggest that the partitioning of ABCA1/HCBS to nonraft domains play a pivotal role in the selective desorption of PC molecules by apoA-I, allowing an optimal environment for HDL formation and cholesterol release.

Collectively, this work has identified novel regulators of HDL-C metabolism and genetic means of influencing reverse cholesterol transport **(Figure 6.1**), providing insight into the complexities of HDL metabolism.

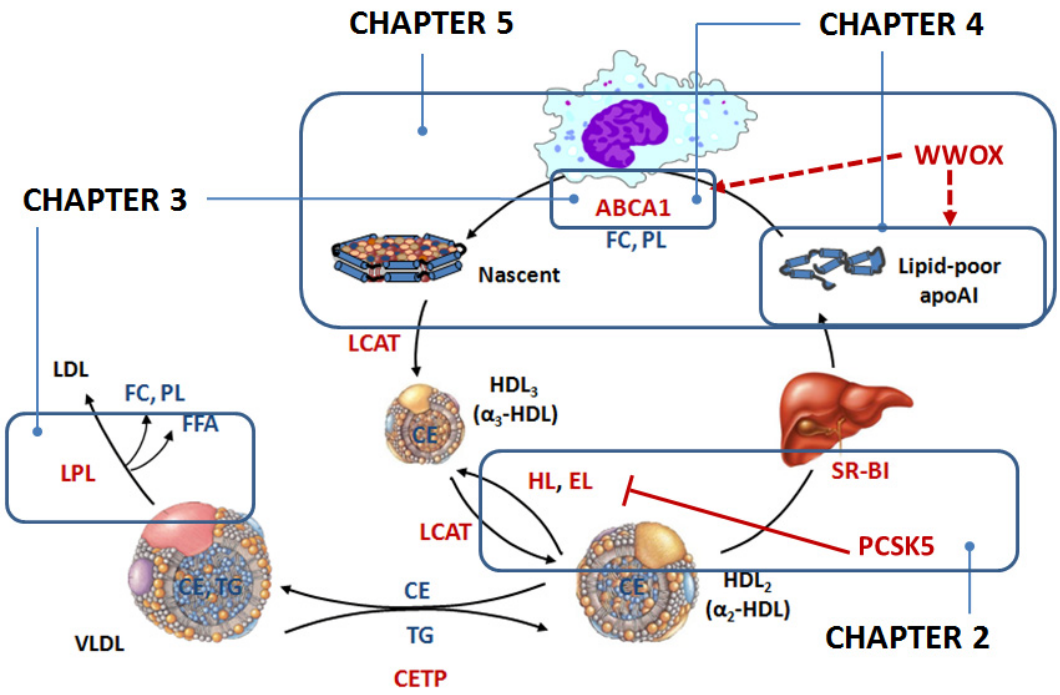


Figure 6.1 Summary of main findings in thesis

6.2 Insights gained from genetic studies

This work offers important advances in characterization of HDL regulation. The identification of novel factors that regulate HDL levels, such as *PCSK5* and WWOX, offer new potential targets for therapeutic intervention, as well as proof of principle for the importance of genetic studies. Indeed, since identifying *PCSK5* as a regulator of HDL levels, several other studies have implicated it and other related members of the subtilisin/kexin-like proprotein convertase family in regulation of cardiovascular function. *In vivo* inhibition of the PC5/6 protein products of the *PCSK5* gene in endothelial cells caused cardiovascular hypotrophy, with these mice showing much smaller mass and wall-thickness of their left ventricles (Marchesi et al., 2011). Endothelial cell knockout mice were generated given that full knockout of PC5/6 is lethal at birth. These mice also displayed decreased collagen deposition and vascular stiffness, supporting a role of endothelial PC5/6 in cardiovascular function. Furin, another related member of the proprotein convertase family, was found to be overexpressed in immune cells during the formation of atherosclerotic plaques,

and is the primary convertase present in plaques (Turpeinen et al., 2011; Stawowy et al., 2005). *PCSK9* had previously been shown to regulate plasma LDL-C levels by cleaving LDL receptors, preventing uptake of LDL-C (Maxwell and Breslow, 2004b; Park et al., 2004; Qian et al., 2007). Gain-of-function mutations in the *PCSK9* gene hence enhance the degradation of LDLR in the liver, elevating serum cholesterol (Davignon et al., 2010). With observations such as these, a new use for inhibitors of proprotein convertases may be on the horizon. Limited efforts have been dedicated to the use of such peptide inhibitors to date (reviewed by (Fritzsche and Stawowy, 2011) but as proprotein convertases are further implicated in cardiovascular disease, studies such as those by Sluijter et al. (Sluijter et al., 2005), in which an inhibitor interfered with Furin function, will become more and more important.

While WWOX has not been previously implicated in cardiovascular disease, several reports have linked it to cancer development as a tumor suppressor (Kuroki et al., 2002; Driouch et al., 2002; Paige et al., 2001), and even to Alzheimer's disease (Sze et al., 2004; Teng et al., 2012). These roles are thought to depend on the pro-apoptotic role of WWOX, though the specific mechanism by which WWOX promotes death is unclear (Zhang et al., 2012). Intriguingly, at the same time the PCSK5/6 related-family member PACE4 has also been implicated in carcinogenesis as an oncogenic factor (Bassi et al., 2005). Inhibition of proprotein convertases with the general PC inhibitor α1-PDX inhibited invasiveness of melanoma cells, further implicating this family of enzymes in tumorigenesis (Lalou et al., 2010). Even ABCA1 and HDL-C were recently suggested to be linked to the proliferation of cancer cells, as HDL was found to induce proliferation and migration of prostate cells (Sekine et al., 2010). Observations such as these present the interesting question of whether or not regulators of cardiovascular disease are also linked to cancer development, and if so, in what ways. Relationships between these two leading causes of mortality in the world certainly require further investigation, but genetic approaches facilitate the discovery of novel regulators that may be linked to a phenotype associated to either, or both, of these diseases.

The work described in Chapter 3 emphasizes the potential for discovering novel genetic regulators of physiological function using next generation techniques. Exome sequencing allows for large effect, penetrant variants to be identified, which can reveal the diversity of genetic variance and the associated phenotype (Musunuru and Kathiresan, 2010b; Ng et al., 2010). We found two such mutations, in ABCA1 and LPL, which confer significant alterations on HDL-C levels in carriers of these SNP mutants. With advances in DNA sequencing technology and increasing knowledge of the nonprotein coding regions of the genome, one expects a rapid shift from whole exome to whole genome sequencing as the desirable approach to identify disease-causing or disease-associated variants (Singleton, 2011; Marian and Belmont, 2011).

The strength of exome sequencing was further illustrated by the finding that the S1731C ABCA1 variant has a more profound effect in males than females. This higher degree of complexity for genetic regulation would normally be difficult to identify, particularly given the rarity of the mutation. As we have identified in our study (**Figure 3.3**), it has been clearly established that gender differences exist for ABCA1 expression (Zhang et al., 2011), and our work with exome sequencing and with WWOX knockout emphasize that male and female genders have alternative means of regulating HDL-C metabolism, as well as other lipid metabolic pathways. Our work in Chapter 3 illustrates that hormones, such as 17β-estradiol, can compensate for gene gender effects, and so it would be interesting to use a similar approach to determine how gender differences may be overcome. For example, it would be interesting to treat liver specific Wwox deficient mice with hormones to mimic alternate genders, and assess if the gender differences we have reported can be reduced. Such a follow-up study could greatly expand our understanding of how WWOX mediates its effects on cardiovascular health. Additionally, given the clearly-established role of WWOX in tumorigenesis, it would be valuable to determine if the gender-based differences in WWOX impairment extend beyond effects on lipid metabolism.

Our work has employed a variety of genetic approaches to further our knowledge of how HDL-C levels can be influenced, and in the process, have

identified novel regulatory factors, such as PC5/6 and WWOX, and variants of previously established regulators, such as ABCA1 and LPL. Discovering such novel modulators of HDL-C is only one part of the challenge, however, as biological and mechanistic insight is also valuable to determine the potential of such genes to be exploited therapeutically.

6.3 Molecular insight from biochemical studies

Our genetic investigation on the relationship between *PCSK5* variants and HDL-C plasma levels revealed several SNPs that had significant effects on HDL-C concentrations. Though this study did not pursue the biochemical mechanism(s) by which such variants may influence HDL-C levels, their locations in the *PCSK5* gene have allowed us to hypothesize about their mechanism of action. The SNPs rs11144766 and rs11144782 are both intronic, and it is hence likely that their effect on PC5/6 activity is mediated through splicing events. Further studies on the identity and abundance of mRNA transcripts derived from such mutated genes would allow for greater biochemical understanding of variability at the *PCSK5* locus.

Rather than focus on mutants of the *WWOX* gene, our study of this novel lipoprotein regulator focused on nascent WWOX itself. Strongly established as a tumor suppressor (Ludes-Meyers et al., 2009; Aqeilan et al., 2007) we found several lines of evidence that implicate WWOX in regulating HDL-C and lipid levels. This may be via influences on expression of ABCA1, ApoA-I and ANGPTL4, among others, though the characterization of how WWOX mediates its effects will be the subject of future studies. Interestingly, our findings on ANGPTL4 and its upregulation in Wwox liver specific KO mice correlates with previous studies where it was found to be implicated in TG and HDL metabolism (Teslovich et al., 2010; Shan et al., 2009). This raises the question if ANGPTL3, which is also a member of the angiopoietin-like protein family investigated in Chapter 2, could play a concerting role with ANGPTL4 and regulate WWOX or PC5/6 expression.

Furthermore, the broad spectrum of targets our microarray analysis identified as being significantly influenced by WWOX expression, as well as the early fatality in mice of total WWOX knockout (Ludes-Meyers et al., 2009; Aqeilan et al., 2007), strongly suggest that WWOX plays an upstream regulatory role. This may be by interfering with transcription factors, as WWOX has been shown to do in other systems (Aqeilan et al., 2005; Aqeilan and Croce, 2007). The influence of WWOX repression on transcription would have to be assessed, and could reveal details on how WWOX plays its part in controlling lipid levels.

The potential of WWOX to regulate ABCA1 levels leads directly into the RCT process, which is of great importance for atherosclerosis. As described in Chapter 5, ABCA1 plays a central role in nascent HDL formation, though the exact lipidation models by which it does this have been subject to debate (Sorci-Thomas et al., 2012; Krimbou et al., 2006; Rothblat et al., 1992; Yancey et al., 2003; Vedhachalam et al., 2007b). Previous studies from our laboratory showed that in a two-step model, ABCA1 flips phospholipids to the outer leaflet of the PM and subsequently, apoA-I binds to these translocated phospholipids and the PC-rich region formed, acquires cholesterol, and solubilises the membrane (Hassan et al., 2007). Our results here shed further light on this mechanism, as we propose this process actually occurs in a three-step mechanism. ABCA1 creates a heterogenous HCBS microdomain populations within the nonrafts composed of domains that cluster to oligomeric ABCA1, but also of ABCA1-excluded exovesiculated mushroom-like protrusions, as earlier studies by Oram and colleagues have demonstrated (Lin and Oram, 2000). Subsequently, binding of ApoA-I to these domains occurs in cholesterol-poor regions, PC is selectively desorbed, and PC-rich ApoA-I discs are formed, which then continue to absorb cholesterol from cholesterol-rich lipid rafts. We also observed that simultaneously, a regulatory biochemical mechanism occurs, whereby the PC Kennedy pathway is activated and the HCBS populations are regenerated. As such, we obtained evidence that through ABCA1/HCBS, apoA-I activates key enzymes and induces the expression of genes involved in PC biosynthesis. This finding indicates that interaction of apoA-I with the HCBS induces profound

remodeling of membrane constituents and coordinates the activity of ABCA1 through several pathways that are likely to involve a novel network of molecules that may influence the structure and function of the ABCA1/HCBS system. This network might include phospholipids, their related phospholipases, and genes encoding enzymes responsible for phospholipid signalling. Future studies should therefore address the composition of the HCBS microdomains through use of lipidomics and gene expression analyses. Specifically, it would be interesting to define the networks that coordinate apoA-I-mediated excess cholesterol removal, by examining the effect of ApoA-I on genes encoding enzymes involved in choline phospholipid and sphingolipid metabolism, as well as phospholipid signalling. Likewise, there would be value in investigating the lipid composition of the PM microdomains, including the HCBS populations within nonraft domains, as well as the impact on the composition of nascent HDL particles and their speciation into pre-β and α-LpA-I particles. This might thus offer novel insights into how *in vivo* cholesterol efflux may occur.

A recent study (Sorci-Thomas et al., 2012) found data that supported our model for the generation of HDL-C particles. This group studied the lipid composition of various sized HDL subspecies that are formed during HDL-C biosynthesis. They made several observations, including that larger nascent HDL particles contain a high cholesterol-to-phosphatidylcholine ratio, reminiscent of the raft-like domain associated with the HCBS (Sorci-Thomas et al., 2012). They show composition of each different size of HDL particle, which correlates with the organization of the lipid bilayer. This study supports the conclusions reached in our work, and collectively offers a growth of mechanistic detail for how HDL-C is formed.

As genetic variations of ABCA1 are discovered, such as by our exome sequencing study, it is also important to understand how such mutations impact a biological process. The role of ABCA1 in HDL-C formation has been the target of investigation for some time, and our work contained here provides meaningful advances to understand how ABCA1 and ApoAI contribute to the formation of specific lipoprotein particles. Establishing an understanding of how HDL-C

biosynthesis takes place in normal conditions will serve as a foundation for charting how mutations influence this process, bridging genetic and molecular characterization of the process of HDL-C metabolism.

6.4 Conclusions

For the past three decades, multiple lines of evidence have emerged supporting the concept that raising HDL-C levels may provide protection against the development of atherosclerosis, highlighting the role of HDL as a potential therapeutic target for treatment of cardiovascular disease. Taken together, we believe that the results of the aforementioned studies will help us better elucidate the molecular and genetic basis of HDL biogenesis, regulation and function. In particular, Chapter 5 of this thesis helped us understand the mechanisms of HDL formation by providing a biochemical basis for the nascent HDL genesis pathway. The identification of the 'two binding' sites working in tandem has prompted further examination into the functions of each binding site as well as their structural properties and a re-evaluation of current concepts of HDL biogenesis. Furthermore, the identification of the HCBS as a major PM microdomain required for the lipidation of ApoA-I has opened new avenues for a better understanding of the ABCA1 transporter network and its role in cardiovascular disease. Additionally, the results obtained through Chapters 2, 3 and 4 of this thesis shed light on novel genes governing plasma HDL levels in humans, and highlight the need to use multiple integrative genetic approaches to identify causal common and rare variants conferring susceptibility to low HDL-C. Because of their importance in lipoprotein metabolism and vascular endothelial function, the identification of novel genes and characterisation of their genomic pathways involved in cholesterol efflux, is warranted. Together, this work has combined genetic and biochemical approaches in order to provide a more comprehensive understanding of the complexities of the HDL metabolism and cellular cholesterol transport, which may lead to the development of novel therapeutic targets for CAD.

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APPENDIX