THE IMPACT OF GENETIC VARIATION IN ABCA1 ON CHOLESTEROL METABOLISM, ATHEROSCLEROSIS AND DIABETES

by

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Abstract

The ATP-binding cassette transporter, sub-family A, member 1 (ABCA1) mediates the major pathway for cholesterol exit from non-hepatic cells and thereby controls the rate-limiting step in the biogenesis of high density lipoprotein (HDL) particles. In humans, ABCA1 deficiency results in Tangier disease, characterized by low levels of HDL cholesterol, cellular cholesterol accumulation and increased risk for atherosclerosis. More than 100 coding variants have been described in the ABCA1 gene. We attempted to understand how both naturally occurring and engineered mutations in ABCA1 impact its role in cholesterol transport in a variety of in vitro and in vivo systems. We attempted to correlate specific genetic variants in ABCA1 with phenotypes in patients carrying these variants, and used an evolutionary approach to predict which specific variants in ABCA1 would impact its function. We then turned to the study of tissue-specific genetic deletion of ABCA1 in mice to study its role in HDL biogenesis, atherosclerosis and glucose metabolism. We found that intestinal ABCA1 is an important site of HDL biogenesis and that activation of intestinal ABCA1 raises HDL levels in vivo. Hepatic ABCA1, which is a major site of HDL biogenesis, was shown to significantly contribute to susceptibility to atherosclerosis. Finally, we show that ABCA1 plays an unsuspected role in ß-cell function and insulin secretion. These studies have contributed to our understanding of the impact of genetic variation in ABCA1 on diverse biological and pathological processes, and have identified novel aspects of ABCA1 function in specific cell types.

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List of Abbreviations

ABCA1	ATP-binding cassette transporter subfamily A member 1
ABCG1	ATP-binding cassette transporter subfamily G member 1
ABCG5/8	ATP-binding cassette transporter subfamily G members 5 and 8
ACAT	Acyl-CoA:Cholesterol Acyltransferase
Apo	apolipoprotein
CAD	coronary artery disease
CE	cholesterol ester
CETP	cholesterol ester transfer protein
EL	endothelial lipase
FFA	free fatty acid
HDL	high density lipoprotein
HL	hepatic lipase
HMGCR	HMG-CoA reductase
HMGCS	HMG-CoA synthase
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LPL	lipoprotein lipase
LXR	liver X receptor
NPC1L1	Niemann-Pick C1 Like 1
PL	Phospholipid
PLTP	Phospholipid transfer protein
RCT	reverse cholesterol transport

SNP	single nucleotide polymorphism
SR-B1	Scavenger receptor class B type 1
subPSEC	substitution position-specific evolutionary conservation score
T2D	Type 2 diabetes
TD	Tangier disease
TG	triglyceride
TGRL	triglyceride rich lipoprotein
VLDL	very low density lipoprotein

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Co-Authorship Statement

All studies described in this thesis were designed and performed by the author with the following exceptions:

ApoA-I western blot in Figure 4.8H was performed by Janine Kruit. FPLC analysis and murine apolipoprotein measurements were performed by Catherine Fievet. Enterocyte isolations and cholesterol secretion in Figure 4.7 were performed by Jahangir Iqbal. Southern blot in Figure 4.1a was performed by Jenelle Timmins. The islet perifusion experiemt in Figure 7.6 was performed by Jim Johnson, and the filipin staining in Figure 7.6 was performed by Zainisha Vasanji. The methodology for calculating substitution position-specific evolutionary conservation scores described in Chapter 3 was developed by Paul Thomas.

Chapter 1 - Introduction

1.1. Cholesterol in Health and Disease

All living cells require rigid, planar molecules to focally modulate the fluidity of phospholipid bilayers in cellular membranes [1]. In eukaryotes this role is filled by sterols, structural lipids characterized by four fused carbon rings which do not allow rotation around the C-C bonds. Vertebrates preferentially use cholesterol as their sterol of choice. Even animals that do not themselves synthesize cholesterol rely on its acquisition through diet [2]. Cholesterol and its metabolites and biosynthetic intermediates also play critical roles in nutrient absorption, reproductive physiology and hormone signaling [3]. The central role of cholesterol in biology is evident in view of the nearly 100 genes dedicated to its biosynthesis, transport and metabolism.

Notwithstanding these essential functions, cholesterol, the "most highly decorated small molecule in biology" [4], is also associated with the majority of death and disease on the planet [5]. Indeed one might well counsel cholesterol be not proud, for it "dost with poison, war and sickness dwell" [6]. The first experimental evidence for the pathological potential of cholesterol was provided by Nikolai Anitschkow, a pathologist working at the St. Petersburg military academy. At the age of 28, Anitschow demonstrated that feeding rabbits purified cholesterol induced vascular lesions that were grossly and microscopically similar to the atherosclerostic lesions he observed in humans [7].

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Ueber experimentelle Cholesterinsteatose und ihre Bedeutung für die Entstehung einiger pathologischer Prozesse.

Von Dr. med. **N. Anitschkow** und Dr. **S. Chalatow.**
(Aus dem pathologisch-anatomischen Institut der Kaiserlichen militär-medizinischen Akademie zu St. Petersburg.)

Es kommt in der Geschichte mancher wissenschaftlichen Fragen vor, daß Forscher, die in ihren Versuchen von ganz verschiedenen Grundideen geleitet werden und verschiedene Methoden angewandt haben, zu Resultaten gelangen, deren unerwartete Gleichwertigkeit nicht uninteressante Schlüsse zu ziehen und neue Ansichten in der Wissenschaft zu fordern veranlaßt. Die Mannigfaltigkeit der solchen Ansichten zu Grunde gelegten Beobachtungen garantiert schon an und für sich ihre Richtigkeit und verleiht ihnen das Bürgerschaftsrecht in der Wissenschaft.

Eine derart glückliche Stellung beginnt sich scheinbar in den letzten Jahren die Lehre über den Cholesterinstoffwechsel und seine Störungen zu schaffen, indem die Angaben verschiedener medizinischen Disziplinen und zwar der pathologischen Histologie, Klinik und physiologischen Chemie zu einer erfreulichen Uebereinstimmung in Bezug auf die Wichtigkeit des genannten Stoffes im Haushalte des Organismus kommen.

Nur eine Seite dieser äußerst interessanten Lehre ist noch bisher außer genügender Beachtung geblieben, nämlich die Möglichkeit, die aufgeworfenen Fragen auf experimentell-pathologischem Wege zu studieren, was gewiß auch zur weiteren Entwicklung der ganzen Frage über den Cholesteringehalt unserer Organe und die Bedeutung von dessen Schwankungen für die Entstehung mancher pathologischen Prozesse außerordentlich beitragen könnte.

Gegenwärtig glauben wir, dank den Arbeiten einer Reihe russischer Forscher schon ein genügendes Material zu besitzen, um die in der Rede stehende Lehre gerade vom experimentell-pathologischen Standpunkte aus beurteilen zu können, wobei unsere eigenen sich noch im Gange befindenden Experimente einen sozusagen letzten Strich in dieser Reihe von Versuchen entwerfen dürfen.

Wie es oft zu geschehen pflegt, gingen die Autoren der ersten hierzu gehörenden Arbeiten von einem scheinbar ganz anderen, in keiner Beziehung zur Lehre von Cholesterinstoffwechsel stehenden Standpunkte aus. Im Jahre 1908 veröffentlichte Ignatowski[1]) seine

[1]) Zur Frage über den Einfluß der animalischen Nahrung auf den Kaninchenorganismus. Ber. der Kaiserlichen militär-mediz. Akademie zu St. Petersburg, Bd. 16, 1908.
Derselbe. Ueber die Veränderungen der parenchymatösen Organe unter dem Einflusse des animalischen Eiweißes. Ebenda, Bd. 17, 1908.

Figure 1.1 Anitschow's original article [7] describing experiments in which cholesterol feeding of rabbits induced vascular lesions resembling human atherosclerosis.

More than 50 years later the Seven-Country study [8] led by Ancel Keys examined the relationship between cultural factors such as diet with heart attacks and strokes in Japan, Finland, Yugoslavia, Greece, the Netherlands, Italy and the United States. This study documented a striking correlation between diet-related differences in plasma cholesterol levels and cardiovascular disease risk over a range of 10-fold, thus establishing for the first time a general underlying factor contributing to the differences in coronary artery disease (CAD) rates in different populations. The Framingham and subsequent large epidemiological studies further established the strong correlation between elevated low density lipoprotein (LDL) cholesterol and low high density lipoprotein (HDL) cholesterol with coronary disease [9,10]. Although initially met with controversy [11], the central role for cholesterol as a cause of heart disease and stroke is now supported by a compelling body of evidence [12,13].

1.2. Epidemiology and Pathogenesis of Atherosclerosis

Atherosclerosis and its manifestation in cardiovascular disease are among the world's leading causes of death. In 1998 cardiovascular disease was responsible for 30.9% of all reported deaths worldwide [5]. While the incidence of cardiovascular disease has been declining in western countries over the past 3 decades [14], the worldwide incidence is projected to increase along with an aging population and economic development, especially in current low- to middle-income countries [15]. Examining the relationship between plasma cholesterol levels and body-mass index (BMI) with average national income reveals that these risk factors rise rapidly as income level rises, suggesting that these important risk factors for CVD will quickly become more prevalent and concentrated in current low-income countries as their economies grow [16]. In addition, within high-income countries, CVD burden is disproportionately carried amongst the lowest socio-

economic groups [17]. Rather than being a "disease of affluence", CVD is therefore projected to join infectious diseases as a disease burden disproportionately afflicting the poor and the marginalized, and in the absence of improved methods of prevention and treatment will contribute to widening inequities in global health.

Atherosclerosis is a multifactorial disease process influenced by diverse genetic and environmental factors, and interactions among these factors [18]. The principal risk factors include disturbances of plasma lipid levels, hypertension, smoking, type 2 diabetes, and family history. Secondary risk factos are a pro-coagulable state, systemic inflammation, sedentary lifestyle and infectious agents. The INTERHEART study identified nine potentially modifiable risk factos that accounted for more than 90% of the population attributable risk of myocardial infarction across different countries. These were, in descending order of odds ratio: raised ApoB/ApoA1 ratio, smoking, psychosocial factors, diabetes, hypertension, abdominal obesity, regular alcohol consumption, exercise and daily consumption of fruits and vegetables [19]. The earliest atherosclerotic lesion is the fatty streak, which consists of subendothelial accumulation of cholesterol engorged macrophage "foam cells" and can be observed as early as the first decade of life [18]. These lesions tend to occur at sites of disturbed shear stress [20] such as the abdominal aorta, the coronary arteries, popliteal arteries, descending thoracic aorta, internal carotid arteries and the circle of Willis. Fatty streaks can either remain stable over long periods of time, or progress to more complicated fibrous plaques with increased accumulation of necrotic debris and smooth muscle cells. In advanced atherosclerotic lesions, smooth muscle cells migrate to the intimal layer, foam cells begin to infiltrate the media, and the centre of the lesion becomes necrotic. At these advanced stages, the lesion can result in significant occlusion of vessels, although the more significant complication is plaque rupture leading to thrombus formation. While atherosclerosis was previously viewed as primarily a mechanical problem of obstruction

of arteries by fatty plaques, it is now clear that atherosclerosis is also an inflammatory disease [21], and that inflammation is a major factor in promoting plaque rupture.

Although many advances have been made in the surgical and medical treatment of the complications of atherosclerosis, there has been only partial success in developing therapeutic approaches to stop or reverse the pathological processes involved in atherosclerosis. This is made clear by the high prevalence of residual disease burden even in the most aggressively treated populations [22]. Specifically, we lack therapies that would directly mobilize cholesterol from atherosclerotic plaques and thus reverse the disease process. For these reasons, inquiry into the fundamental mechanisms of atherogenesis and how HDL can stop or reverse this process remains an important approach to treating atherosclerosis.

1.3. Atherosclerosis, Type 2 Diabetes and the Metabolic Syndrome

The Metabolic Syndrome is a constellation of metabolic risk factors including dyslipidemia, hypertension, hyperglycemia, abdominal obesity and a pro-inflammatory and pro-coagulable state. The main lipid abnormalities observed are a reduction in HDL cholesterol, an elevation of triglycerides, and an increase in small dense LDL particles. The predominant underlying risk factors in the syndrome appear to be obesity and insulin resistance [23]. The utility of the syndrome as an entity is the high risk for atherosclerosis and type 2 diabetes mellitus (T2D) with which it is associated. The specific mechanisms by which the underlying risk factors combine to contribute to the pathogenesis of the metabolic syndrome are unclear.

The incidence of T2D is increasing at an astonishing rate along with the worldwide explosion in obesity [24]. T2D is a disease of insulin resistance and relative insulin deficiency. Insulin resistance is a critical component of the pathogenesis of T2D, and many of the molecular details of insulin signaling and insulin resistance have been elucidated [25]. The impairment of the endocrine pancreas in T2D has been recognized since before the discovery of insulin, Osler having commented as early as 1906 that diabetes could be caused by "functional or organic disease by the islands of Langerhans in the pancreas" [26]. Indeed, a reduction in glucose-stimulated insulin secretion is a virtually uniform finding among patients with symptomatic T2D [27]. Even amongst intensively treated diabetic patients, data from the UK Prospective Diabetes Study (UKPDS) indicate that there is an almost inevitable progression of the disease towards ß-cell failure and worsening hyperglycemia [28]. Interestingly, the UKPDS indicated that the progression of diabetes involves a continuing deterioration of ß-cell function with no change in insulin sensitivity [29].

A hyperbolic relationship exists between insulin secretion and insulin sensitivity in euglycemic individuals [30] such that insulin secretion increases rapidly with increasing insulin resistance. Insulin sensitivity varies widely in the general population [31], and in the presence of normal ß-cell function euglycemia can be maintained even in the face of profound insulin resistance. The onset of clinical diabetes therefore appears to require both an insulin resistant state as well as impairment in ß-cell function or mass.

The reasons for ß-cell failure in T2D are not known. The deficit appears to be partially a reduction in ß-cell mass [32]. However, type 2 diabetic islets are also functionally impaired in vitro as shown by their reduced glucose-stimulated insulin secretion even when islet mass is corrected for [33]. Several lines of evidence have been advanced to explain the deterioration in insulin

secretory output. These include ß-cell exhaustion, desensitization to glucose stimulation, deposition of islet amyloid, and lipotoxicity [29].

Lipotoxicity is an emerging theme in which toxic lipids are postulated to accumulate in ß-cells leading to dysfunction and apoptosis [34]. The ability of triglycerides and saturated free fatty acids (FFAs), such as palmitate to induce ß-cell dysfunction is well documented [35]. The toxic effects of FFAs on ß-cells are in fact synergistic with those of high glucose levels [36], and the combined effects of hyperglycemia and hyperlipidemia, or gluco-lipotoxicity, is one recognized component of ß-cell loss in type 2 diabetes. In contrast, the role of cholesterol in ß-cell lipotoxicity has received little attention. Free cholesterol is known to be toxic to cells in general, and specifically in macrophages [37] via the induction of the unfolded protein response [38]. The role of cholesterol in modulating ß-cell function, however, has not been assessed.

1.4. Cholesterol and Lipoprotein Metabolism

Because cholesterol is insoluble in water, it must be transported through the circulation in lipoproteins, macromolecules consisting of an amphipathic lipid monolayer covering a core of hydrophobic lipids. In humans the major lipoproteins can be separated by ultracentrifugation and electrophoretic mobility. Each class of lipoprotein contains characteristic apolipoproteins in their lipid monolayer which directs their transport and function.

Chylomicrons, the largest and least dense lipoprotein, are synthesized in the epithelium of the small intestine following food intake and consist predominantly of triglycerides (TG) and cholesterol esters (CE). Chylomicrons are secreted by the intestine into the lymphatics, and eventually enter the circulation through the left subclavian vein. Once in the circulation, the

triglycerides in chylomicrons are available for lipolysis and release as free fatty acids (FFA) by lipoprotein lipase (LPL) which is activated by apolipoprotein C-II (apoC-II) on the surface of chylomicrons. Chylomicrons also transfer TG to HDL particles in exchange for cholesterol ester, a process that is mediated in humans by the enzyme cholesterol ester transfer protein (CETP). The chlyomicron remnants are subsequently taken up in the liver by the low density lipoprotein receptor (LDLR) that recognizes apoE and by the LDLR-related protein [39].

The endogenous lipoprotein pathway begins with the synthesis and secretion of very low density lipoproteins (VLDL) by the liver. VLDL particles consist of TGs that are either synthesized in the liver or derived from FFAs from chylomicron remnant uptake or from adipose tissue. As with chylomicrons, VLDL can exchange TGs for CEs with HDL, and undergo TG hydrolysis by LPL. Further modification by hepatic lipase results in remodeling to low density lipoprotein (LDL) particles, characterized by the presence of a single molecule of apoB-100. LDL particles, which carry about 70% of the plasma cholesterol in humans, transport cholesterol to peripheral tissues where they are taken up by the LDL receptor in a process of receptor-mediated endocytosis [40].

HDL particles are a heterogeneous population of lipoprotein particles, defined by the density range 1.063 - 1.21 g/ml. HDL2 and HDL3 are the major forms in human plasma [41]. HDL4 is identical to newly secreted apo-AI—phospholipid particles, and is also called nascent HDL, lipid-poor HDL, or pre-β1 HDL. As the pre-β1 particle acquires lipid and undergoes esterification it increases in size and decreases in density to HDL3, HDL2 and HDL1 (the α particles).

1.5. HDL Metabolism and Genetics

The history of the study of HDL metabolism, and indeed of lipoprotein metabolism in general, is a testament to the utility of studying rare genetic diseases in order to gain insight into fundamental biological processes. The study of rare diseases as an opportunity to gain far-reaching insight was established early-on by Garrod Archibald, a physician whose observations of patients with black urine established the concept that a single gene could interfere with a biochemical pathway [42]. Fifty years later in 1951, chemist Linus Pauling demonstrated that a single amino acid change in a protein could lead to disease and in so doing established the paradigm of molecular medicine, prior even to the elucidation of the structure of DNA [43]. So too has the study of HDL been a story of observations of patients with rare disorders as an entry point into biological processes. Perhaps this was the intention of William Bateson, the father of modern genetics, when he counseled beginners to "Treasure your exceptions!" for they are "like rough brickwork of a growing building which tells that there is more to come" [44], or of Jose Saramago:

> *we would know far more about life's complexities if we applied ourselves to the close study of contradictions instead of wasting so much time on similarities and connections, which should, anyway, be self-explanatory.* [45]

Approximately 50% of the variation in HDL levels is thought to be genetically determined [46]. Of all plasma lipoproteins including levels of LDL, TG and total cholesterol, HDL cholesterol levels show by far the highest heritability across multiple populations [47]. Human genetic diseases associated with changes in HDL metabolism are shown in Table 1 and are discussed below.

Table 1.1 Human diseases affecting HDL metabolism

Human Disease	Gene	OMIM#	Clinical Features	Frequency / # of patients
ApoA-I deficiency	*APOA1*	107680	near absent HDL, premature CAD	~25 patients worldwide
ApoA-II deficiency	*APOA2*	107670	reduced apoA-II, normal HDL	2 patients worldwide
LCAT deficiency / Fish eye disease	*LCAT*	606967	reduced/abnormal HDL	~70 patients worldwide
CETP deficiency	*CETP*	607322	increased HDL, longevity?	~1 in 100 in Japanese
Familial chylomicronemia	*LPL*	238600	increased TG, reduced HDL	1 in 500,000
ApoC-II deficiency	*APOC2*	608083	increased TG, reduced HDL	~10 patients worldwide
HL deficiency	*LIPC*	151670	increased HDL-TGs	~10 patients worldwide
Tangier disease / familial HDL deficiency	*ABCA1*	205400	near absent HDL, premature CAD	~100 TD patients worldwide

Apolipoprotein A-I (apoA-I), the principal protein constituent of HDL, is synthesized and secreted by the liver and intestine [48,49]. Norum and colleagues reported two sisters who lack apoA-I, resulting in very low HDL cholesterol levels (0-7 mg/dl) and premature coronary artery disease [50]. The presence of apoA-I was therefore shown to be essential for the biogenesis of HDL particles, as well as for the anti-atherogenic effect of HDL. Concentrations of other lipoprotein classes, notably LDL cholesterol, are not significantly impacted [51]. ApoA-I based therapies have recently attracted attention, as infusion of a recombinant apoA-I variant, apoA-I_{Milano}, was shown to significantly reduce atheroma volume as detected by vascular ultrasound after only 5 weeks of treatment [52]. Whether apoA-I_{Milano} represents a beneficial variant compared to native apoA-I is still unclear as lipid-free apoA-I_{Milano} has been demonstrated to have reduced capacity to deplete cholesterol from cholesterol-loaded human fibroblasts compared to the native protein [53].

Apolipoprotein A-II (apoA-II) is the second most abundant apolipoprotein in HDL particles and is synthesized predominantly in the liver [54]. ApoA-II deficiency is an exceedingly rare condition, associated with no detectable apoA-II protein, and a ~30% reduction in apoA-I [55]. Interestingly, HDL cholesterol levels are not significantly reduced in these patients, indicating that the presence of apoA-II is not necessary for the biogenesis and maintenance of plasma HDL. The physiological roles of apoA-II remain ambiguous, but may be involved in the activation of hepatic lipase (HL) and the inhibition of lecithin:Cholesterol Acyltransferase (LCAT) [51].

Mutations in the enzyme LCAT result in two allelic disorders, familial LCAT deficiency and Fish Eye Disease. LCAT deficiency was first observed in 1967 in 4 families from the same region of western Norway [56]. These patients presented with corneal opacities, lipemia, anemia and proteinuria. The most striking lipoprotein abnormality is a 70-80% reduction in plasma HDL cholesterol levels. Remaining HDL particles are abnormal, and resemble "nascent HDL" particles observed in liver perfusion experiments and in the mesenteric lymph of rats [57]. Plasma LDL cholesterol ester is also markedly reduced. An increase in coronary artery disease is not generally observed among patients with LCAT deficiency [58].

Mutations in LCAT can manifest in a separate clinical entity characterized by corneal opacities, but without the other hematological abnormalities observed in familial LCAT deficiency. This entity is referred to as Fish Eye Disease because of the observation that the eyes of these patients resemble those of boiled fish. Patients with Fish Eye Disease have significantly reduced HDL cholesterol, similar to patients with familial LCAT deficiency, although other lipoprotein classes are relatively normal with the exception of increased LDL triglycerides.

These observations of patients lacking LCAT activity teach us a good deal as regards the physiological role of LCAT in HDL metabolism. LCAT, which is secreted predominantly by the liver and circulates in plasma bound to HDL and LDL, catalyzes the esterification of free cholesterol to cholesterol ester. The esterfication of free cholesterol in HDL is necessary to partition the so-formed CE to the centre of the maturing HDL particle and to maintain a gradient of free cholesterol between cells and HDL particles, thus allowing the continued efflux of cholesterol from peripheral cells to HDL. The existence of a physiologically significant plasma cholesterol esterification reaction was the initial basis on which Glomset postulated the reverse cholesterol transport (RCT) hypothesis, in which cholesterol can be returned by HDL from peripheral tissues to the liver [59]. Reverse cholesterol transport is generally thought to be among the most important anti-atherogenic functions of HDL [60].

In the absence of LCAT, HDL particles do not mature and their cholesterol content is dramatically reduced. The differences between familial LCAT deficiency and Fish Eye Disease are thought to reflect impairment of global LCAT activity in the case of the former, and of HDL-specific LCAT activity in the latter [51]. The fact that LDL-TG are elevated in Fish Eye Disease may be secondary to the reduction in HDL-CE which would normally be available for exchange with LDL-TG by the action of CETP.

Human deficiency for CETP was first reported in 1990 by Akihiro and colleagues in 4 Japanese families [61]. Family members homozygous for the CETP mutation had markedly elevated HDL cholesterol and reduced LDL cholesterol and apoB. Anecdotal evidence exists that these beneficial changes in plasma lipids are associated with increased lifespan. CETP mediates the transfer of CE from HDL for TG from LDL, VLDL or chylomicrons. Theoretically, this transfer should de-stabilize HDL particles and increase their catabolism by the kidney. The increased

HDL cholesterol in patients deficient for CETP supports this concept. Such reasoning motivated the development of compounds to inhibit CETP in humans. These compounds effectively raise HDL cholesterol levels [62] though their effect on clinical endpoints remains to be determined.

Patients with milky lipemic plasma have been recognized since the late 18th century and familial chylomicronemia was first reported in the early 20th century [63]. This disorder, which manifests as severe hypertriglyceridemia, eruptive xanthomas and pancreatitis, was subsequently shown to be due to an absence of lipoprotein lipase activity [64]. The first mutations in the LPL gene in familial chylomicronemia were reported in 1989 in Vancouver [65]. Patients with LPL deficiency also display markedly reduced plasma LDL and HDL cholesterol levels, in particular a near absence of HDL2 and reduced HDL3. These observations therefore indicate that plasma HDL cholesterol levels, and in particularly HDL2 are dependent on the activity of LPL, potentially by LPL releasing apoproteins from chylomicrons and VLDL particles necessary for the generation and maturation of HDL. These findings also provide a molecular underpinning for the known inverse relationship between plasma TG and HDL levels.

Apolipoprotein C-II deficiency is a rare genetic disease and presents clinically very similarly to LPL deficiency [66]. ApoC-II is the physiological activator of LPL, thus explaining the clinical overlap in these disorders. In particular the effect on HDL levels is equivalent in these two disorders again indicating the importance of LPL activity for HDL levels.

Genetic deficiency for HL is another rare disorder, in which patients have increased levels of total plasma cholesterol and triglycerides [67]. HDL levels, and in particular HDL-TGs tend to be increased in these patients. Despite the increase in HDL cholesterol, these patients exhibit a trend

towards increased coronary artery disease. HL is thought to play an important role in the hydrolysis of HDL-TGs.

A number of other genes are known to be involved in HDL metabolism because of findings in animal models, but for which no human mutation has been identified. These genes are shown in Table 2 and are discussed below.

Table 1.2 Genes involved in HDL metabolism for which no human mutations have been reported

Protein	Gene	OMIM#	Clinical Features
Phospholipid transfer protein	*PLTP*	172425	reduced HDL in ko mice
Endothelial lipase	*LIPG*	603684	reduced HDL in transgenic mice, increased HDL with inhibitory antibody
Scavenger receptor, B1	*SCARB1*	601040	increased HDL and increased atherosclerosis in ko mice

Phospholipid transfer protein (PLTP) is a key molecule for the remodeling and maturation of HDL particles. Humans with mutations in PLTP have not been reported, but mice lacking PLTP display markedly reduced HDL cholesterol and phospholipids levels and reduced plasma apoA-I concentrations [68]. These observations indicate that the PLTP-mediated transfer of phospholipids from triglyceride rich lipoproteins (TGRLs) to HDL is crucial for the maintenance of HDL particles.

Endothelial lipase (EL) is the most recently identified member of the lipase family. There have thus far been no patients described with mutations in EL, though studies in mice have shown that overexpression of EL results in reduced HDL cholesterol and apoA-I [69]. EL has relatively greater PL than TG hydrolytic activity compared to HL. Inhibition of EL activity in mice using an inhibitory antibody results in increased HDL levels and slowed catabolism of HDL-PLs [70]. EL-mediated hydrolysis of HDL-PL therefore appears to facilitate the removal of HDL particles

from the circulation, suggesting that inhibition of EL activity may be a useful therapeutic approach to raise HDL levels. In support of an important role of EL in HDL metabolism in humans, specific polymorphisms in the gene encoding EL have been found to exist at different frequency in individuals with high HDL compared to a control population [71].

HDL particles have long been known to be the preferential source of plasma cholesterol that ultimately appears in bile [72]. While this provides strong evidence for a physiological reverse cholesterol transport pathways in vivo, the mechanisms by which HDL cholesterol reaches the liver were long unclear. The identification of the scavenger receptor class B member I (SR-BI) as a high-affinity HDL receptor in the liver [73] that mediates the selective uptake of HDL cholesterol but not protein [74] therefore provided a mechanism by which HDL cholesterol could be taken up by the liver for removal into bile. Overexpression of hepatic SR-BI substantially lowers plasma HDL cholesterol levels [75], while targeted inactivation of SR-BI results in increased HDL [76]. Notably, mice lacking SR-BI have increased susceptibility to atherosclerosis despite having elevated HDL cholesterol [77], indicating the SR-BI-mediated uptake of HDL cholesterol is necessary for the anti-atherogenic effect of HDL. Recently, overexpression of SR-BI was shown to increase macrophage-to-feces reverse cholesterol transport using an in vitro RCT model [78].

The pathways of HDL metabolism are represented in Figure 1.2.

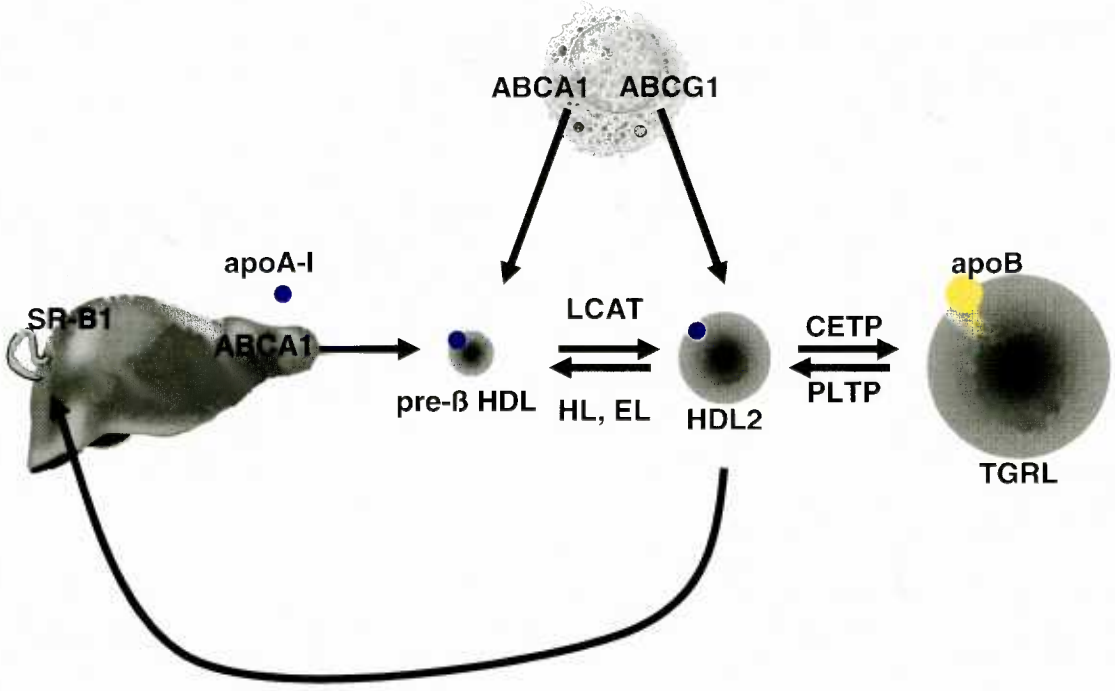

Figure 1.2 Pathways of HDL metabolism. Nascent or pre-ß HDL is assembled by hepatic ABCA1 and released into the circulation. The pre-ß particle undergoes further maturation by the action of LCAT. CETP and PLTP mediate the exchange of lipids between HDL and the triglyceride-rich lipoproteins (TGRL). Hepatic lipase (HL) and endothelial lipase (EL) hydrolyze HDL-TGs and -PLs, respectively, with the effect of reducing the size and enhancing the catabolism of HDL. HDL lipids are taken up by the liver through the action of SR-B1. ABCA1 and ABCG1 in peripheral tissues can promote efflux to apoA-I and HDL particles, respectively. The contribution of ABCA1 in other tissues to HDL biogenesis is not known.

1.6. ABCA1 and Cholesterol Efflux

Tangier Disease is another rare disorder of HDL metabolism, first recognized in the Chesapeake Bay area of the United States of America by Donald Fredrickson [79], and presents clinically as a near absence of HDL cholesterol, reduced LDL cholesterol, increased incidence of coronary artery disease, and yellow lipid-engorged tonsils [80,81]. Other manifestations include histiocyte accumulation in the rectal mucosa, and a multifocal demyelinating or syringomyelia-type neuropathy [82-85]. The demyelinating type neuropathy is characterized morphologically by peripheral nerve demyelination and remyelination, with lipid vacuoles in Schwann cells [86,87]. The syringomyelia type, in contrast, is notable for neuronal lipid inclusions and loss of motor neurons [88].

Pioneering studies in cultured fibroblasts identified the metabolic defect in TD as an inability for cells to efflux cholesterol and phospholipids in response to apoA-I, as well as diminished binding of apoA-I [89]. This biochemical defect readily explained many of the clinical manifestations observed in TD: by failing to efflux cholesterol to apoA-I, HDL particles are either not formed or are unable to mature, leading to their rapid clearance from the circulation, while cells that take up large amounts of cholesterol such as macrophages become lipid engorged, leading to the characteristic orange tonsils, hepatosplenomegaly and peripheral neuropathy.

The molecular defect in TD was identified in 1999 as mutations in both alleles of the *ABCA1* gene [90-93]. This established ABCA1 as the crucial cholesterol efflux regulatory protein responsible for the export of cellular cholesterol. The *ABCA1* gene consists of 50 exons spanning a genomic region of ~150 kb on chromosome 9q31.1. Translation begins at an ATG in exon 2. ABCA1 is a typical ABC "full transporter" consisting of 2 large extracellular loops, each followed by a nucleotide binding fold with Walker A and B motifs [94]. To date, more than 100 coding variants have been described in the *ABCA1*. Aspects of ABCA1 genetics are discussed in Chapter 2.

Studies in cells transfected with ABCA1 showed that ABCA1 specifically binds to apoA-I, and facilitates the efflux of cholesterol and phospholipids to lipid-poor apoA-I [95]. In contrast, HDL was shown to be a poor acceptor for ABCA1-mediated efflux. ABCA1-mediated lipid efflux was shown to be tightly associated with binding of apoA-I [96]. ABCA1 has been shown to be expressed both on the plasma membrane [97] and in intracellular compartments [98]. Within cells,

ABCA1 is present in early and late endosomes and lysosomes, and ABCA1-containing vesicles recycle between the plasma membrane and intracellular sites [98].

From the studies of ABCA1 in cultured cells there emerged a model in which ABCA1 was suggested to mediate the efflux of cholesterol and phospholipids to a newly synthesized, or newly released apolipoprotein to generate a nascent HDL particle. The HDL particles so produced could acquire additional lipids from other cells in either an ABCA1-dependent or independent manner, and could also undergo the various remodeling pathways described above. The mechanism by which ABCA1 elicits lipid efflux appears to be complex. Initially it was proposed that ABCA1 acts as a phospholipid transporter [96], and that cholesterol efflux occurs to phospholipidated particles in an ABCA1-independent manner. However, further studies shed doubt on this hypothesis [99] and rather suggested that ABCA1 mediates the efflux of both phospholipids and cholesterol. A portion of ABCA1-mediated cholesterol efflux is thought to involve the direct mobilization of cholesterol from the late endosome compartment [100].

The lipidation of apoA-I by ABCA1 may occur both intracellularly and at the plasma membrane [101,102]. Within the cell, early lipidation of apoA-I at the endoplasmic reticulum (ER) may occur in an ABCA1-independent manner [103], with subsequent bulk lipidation at the Golgi and plasma membrane requiring ABCA1. This may explain why Tangier Disease patients have only pre-ß1 HDL [104], representing an early particle that is lipidated independently of ABCA1, but which requires ABCA1 for further lipidation and maturation.

1.7. ABCA1 Physiology

ABCA1 is widely expressed throughout the body [97]. Until recently, the specific contributions of ABCA1 in discrete tissues to plasma HDL cholesterol levels were not known. Although

macrophages are important sites of lipid accumulation in TD [51], ABCA1 in macrophages was shown to not contribute significantly to plasma HDL levels [105]. ABCA1 is particularly abundant in the liver [97], and overexpression of ABCA1 in the liver significantly raises HDL levels [106], thus suggesting an important contribution of hepatic ABCA1 to plasma HDL cholesterol levels.

This hypothesis was directly tested by the generation of mice which specifically lack ABCA1 in hepatocytes. Such mice display a ~80% reduction in plasma HDL cholesterol, thereby indicating that the liver is the primary site of HDL biogenesis in vivo [107]. Similarly, a 50% knockdown of hepatic ABCA1 expression by adenovirus-mediated RNA interference in mice is associated with a 40% decrease in HDL cholesterol [108]. The finding that plasma HDL cholesterol and apoA-I levels were significantly reduced in the absence of hepatic, but in the presence of extraheptic ABCA1, further indicates that the lipidation of newly secreted apoA-I must occur at or near the hepatocyte surface, prior to the release of the apoA-I particle into the circulation.

Because of the strong influence of hepatic ABCA1 on HDL cholesterol levels, it is of interest to determine the contribution of hepatic ABCA1 to atherosclerosis. Studies in mice overexpressing ABCA1 in the liver and macrophages [109,110] have led to the hypothesis that hepatic ABCA1 may actually be proatherogenic [111,112] due to an effect on apoB containing lipoproteins. Macrophage ABCA1 is clearly anti-atherogenic [113] but whether ABCA1 in other tissues also modulates susceptibility to atherosclerosis directly through HDL levels remains to be determined. Interestingly, absence of macrophage ABCA1 has been shown to influence atherogenesis independently of changes on lipid levels [114,115], indicating that ABCA1 may contribute to susceptibility to atherosclerosis without appreciably impacting steady-state HDL cholesterol levels. Whether hepatic ABCA1, the major source of plasma HDL, impacts atherogenesis will be addressed herein.

Studies in mice lacking hepatic ABCA1 indicated that the liver is essential for the production of the majority of HDL in plasma. However the finding that HDL cholesterol levels were not completely reduced in the absence of hepatic ABCA1, as they are TD patients and mice lacking ABCA1 globally, also suggested the existence of important, extrahepatic sites of HDL biogenesis. One potential extrahepatic site for HDL biogenesis is the small intestine. The intestine, along with the liver, produces apoA-I, the principal apolipoprotein constituent of HDL [116]. The relative contributions of the intestine and liver to plasma apoA-I levels is not known. The intestine is a key organ in overall body cholesterol homeostasis and in recent years many of the molecular mechanisms of intestinal cholesterol metabolism have been elucidated. Cholesterol that enters the intestinal lumen from either dietary or biliary sources is available for absorption into the enterocyte through the action of the recently identified protein NPC1L1 [117]. Once taken up into the intestine, free cholesterol is esterified into cholesterol ester by the action of ACAT2 [118], or effluxed back into the intestinal lumen by ABCG5 and ABCG8 [119], which transport both cholesterol and plant sterols into the intestinal lumen and thereby prevent the accumulation of plant sterols. ABCG5/8 also mediate the flux of cholesterol from hepatocytes into bile [120]. Recently, it has been proposed that the intestine directly secretes cholesterol, establishing a route of cholesterol loss from the body independent of the traditional hepatobiliary pathway [121].

Cholesterol transport into the body is thought to occur via the lymphatics in the form of chylomicrons. However, studies in rats identifying a form of nascent HDL in mesenteric lymph suggest that a portion of cholesterol is secreted from the intestine as HDL [57,122-124]. In support of this concept, ABCA1 is known to be expressed on the basolateral membrane of enterocytes,

where it effluxes cholesterol to apoA-I [125-127]. It is therefore of interest to determine the contribution of intestinal ABCA1 to HDL biogenesis in vivo.

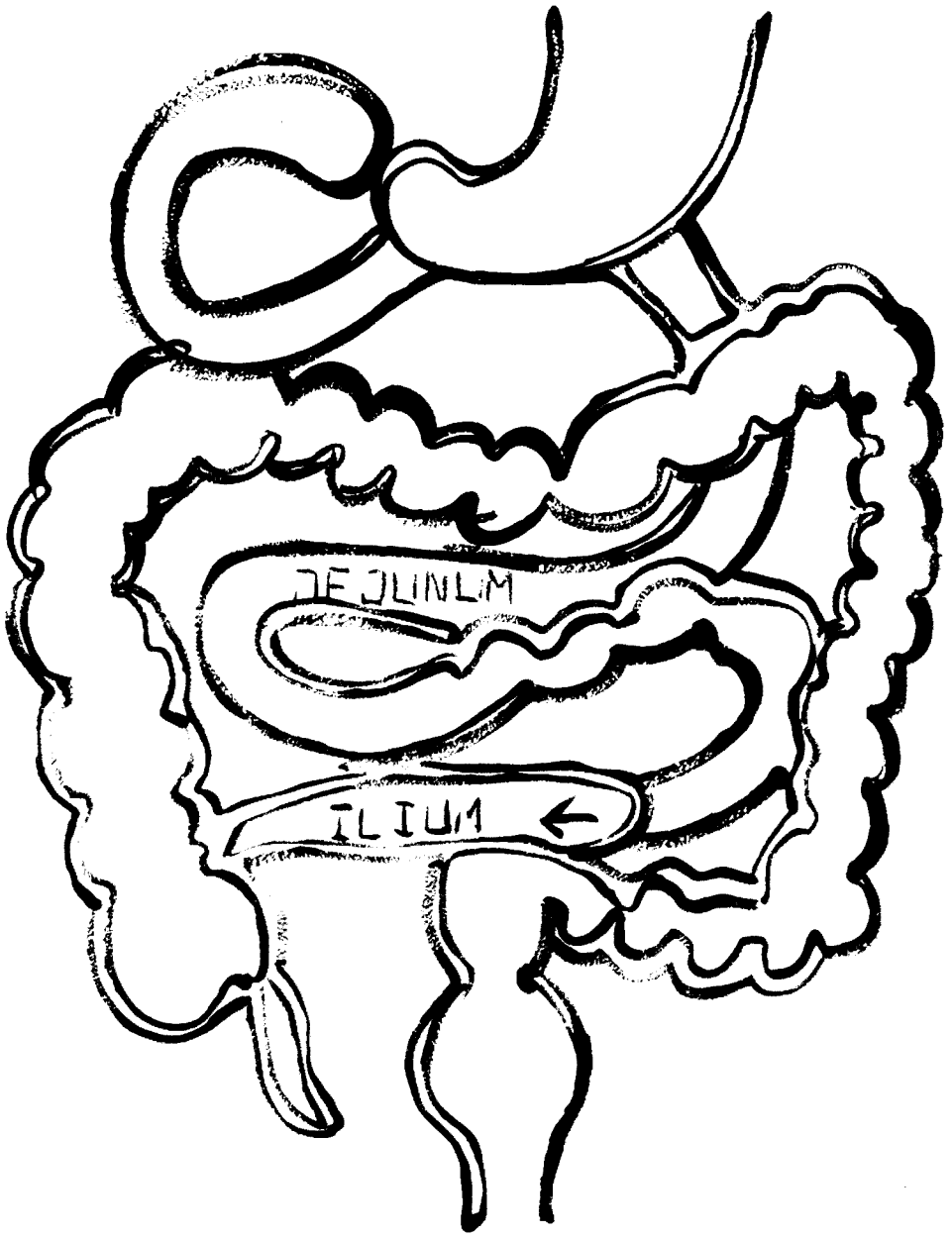

Figure 1.3 Andy Warhol's Physiological Drawing (date unknown). Reprinted with permission. © Andy Warhol Foundation for Vidsual Arts / SODRAC (2007).

Although ABCA1 is expressed in many tissues [97], it is likely that HDL biogenesis occurs in a limited number of tissues in the body. It is therefore of interest to investigate the function of

ABCA1 in other tissues which may not contribute to plasma lipid levels but in which ABCA1 may be an important regulator of cellular cholesterol homeostasis. As described above, cholesterol metabolism may play an important role in pancreatic islets. Lipoprotein receptors, in particular the low density lipoprotein receptor (LDL) and LDL-related protein (LRP) are expressed in mouse islets [128], and atherogenic lipoproteins such as LDL and VLDL induce apoptotic death in isolated islets and transformed ß-cell lines [128-130]. This effect is blocked by high density lipoproteins [128]. In addition, depletion of ß-cell cholesterol by incubation with ß-methyl cyclodextrin alters the distribution of ion channels in membrane lipid rafts and enhances insulin exocytosis [131], thus suggesting that cellular cholesterol levels are an important component of normal ß-cell function. However, the role of ABCA1 in ß-cell cholesterol homeostasis and glucose metabolism in vivo has not been directly assessed.

1.8. Experimental Approaches to Understanding the Role of Genetic Variation in ABCA1 in Cholesterol Metabolism, Atherosclerosis and Glucose Homeostasis

The study of rare genetically determined diseases has provided insight into lipoprotein metabolism which would otherwise have been impossible. This approach continues to bear fruit, and to whatever extent possible the studies described herein use knowledge of the study of human patients to guide experimental design. In particular, in the study of ABCA1 variants we have focused on those naturally occurring variants for which there exists substantial data on the phenotype of humans harbouring the variants.

The second major component of the heuristic of the present work involves the study of genetically-altered mice. Unlike chemistry or physics, biology as a science lacks grand over-arching theories, and major conceptual revolutions tend to be rare. More often than not, advances

in our knowledge are due to the development of new technologies that allow us to ask and answer questions with more precision. The ability to precisely manipulate the genome of a mammal in which more than 99% of genes are homologous to those of humans is one such development [132].

Figure 1.4 Katharina Fritsch "Mann und Maus". The giant mouse towers over the patient who calmly looks to the mouse with hopefulness that it will lead to new treatments for his ailments. 1991-92 Polyester and paint. Courtesy Matthew Marks Gallery, New York, Nic Tenwiggenhorn photographer.

While substantial differences exist in lipoprotein metabolism between mice and humans -- significantly the lack of a CETP protein in mice and the use of HDL, rather than LDL as the

major cholesterol carrier -- the importance of studies in mice to our understanding of lipoprotein and HDL metabolism and atherosclerosis cannot be overemphasized. Of particular relevance to this work, the faithful recapitulation of the Tangier disease phenotype in mice which lack ABCA1 [113] provides powerful evidence for the use of mouse models to study ABCA1 function.

One of the important developments of ABCA1 research in recent years is the generation by Drs. John Parks and Nobuyo Maeda of ABCA1 floxed mice [107], which allow for both spatial- and temporal-specific deletion of ABCA1 by introducing the Cre transgene under specific promoters. The availability of mice with targeted deletion of ABCA1 globally as well as in specific tissues allows for a precise dissection of the specific functions of ABCA1 in certain cell types, in isolation of its effects elsewhere in the body. We have therefore made special use of these mouse models in the studies described herein.

1.9. Objectives and Hypotheses

The overall goals of these studies are to understand how genetic variation in ABCA1 influences its function in cholesterol metabolism in different tissues, and to gain insight into the role of cell-specific lipid transport in HDL biogenesis, atherogenesis, and glucose homeostasis and insulin secretion.

The specific objectives of this work are:

1. **to investigate how naturally occurring genetic variation in ABCA1 influences its function in cholesterol transport (Chapter 2).**

2. **to assess the hypothesis that the functional consequence of genetic variation in ABCA1 can be predicted by comparing the specific sites at which the variants occur in the sequences of related protein in different species (Chapter 3).**
3. **to assess the role of intestinal ABCA1 in HDL biogenesis (Chapter 4).**
4. **to evaluate the hypothesis that specific induction of intestinal ABCA1 could raise plasma HDL levels (Chapter 5).**
5. **to assess the contribution of hepatic ABCA1, the major contributor to plasma HDL levels, to susceptibility to atherosclerosis (Chapter 6).**
6. **to examine the role of ABCA1 in cholesterol homeostasis in ß-cells, and its impact on glucose metabolism (Chapter 7).**

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Chapter 2 - Variations on a Gene[1]

2.1. Introduction

Atherosclerosis, and its manifestation in coronary artery disease (CAD), are the leading causes of death worldwide (1). CAD is a multifactorial disease influenced by smoking, hypertension, obesiy, diabetes mellitus, and alterations of plasma lipid levels (2). The central role of elevated low density lipoprotein (LDL) cholesterol as an important risk factor for and cause of CAD is well established. Dietary saturated and trans fats are critical drivers of plasma hyperlipidemia (3), and indeed have a significantly greater hyperlipidemic effect than does dietary cholesterol, partially through their ability to activate lipogenic genes in the liver (4). The development of HMG-CoA Reductase inhibitors (statins), which reduce LDL cholesterol by inhibiting hepatic cholesterol synthesis, have been a critical development in the treatment and prevention of CAD (5). However, despite significant reductions in mortality with statin treatment, recent studies continue to document a high incidence of residual disease burden even in aggressively treated populations (6), highlighting the need for new therapeutic approaches to prevent CAD.

A low level of high density lipoprotein (HDL) cholesterol is the most common lipoprotein abnormality among men with CAD (7;8). HDL is thought to prevent atherosclerosis by selectively returning cholesterol from peripheral tissues to the liver for excretion, in a process termed reverse cholesterol transport (9;10). Other properties of HDL that may contribute to its atheroprotective role include modulation of endothelial function through nitric oxide production (11), suppression of monocyte adhesion molecules and inhibition of LDL oxidation (12). Data from epidemiological studies suggest that for every 1 mg/dL increase in HDL cholesterol, CAD

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risk is reduced by 2-3% (13;14). The Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial further demonstrated that gemfibrozil treatment, which increased HDL cholesterol modestly by 6%, was associated with a 22% reduction in coronary events (15). Dietary modification (16) and high-intensity exercise (17) can achieve modest increases in HDL cholesterol, but there are currently no approved therapies that substantially raise HDL levels. HDL cholesterol therefore represents an attractive, but largely underexploited avenue for therapeutic intervention in CAD (18).

2.2. Identification of Genes that Regulate HDL Cholesterol

About half of the variation in HDL cholesterol levels is under genetic control (19;20). As with most common phenotypes, the search for the genetic components of HDL levels has been difficult. Both whole-genome linkage analysis and candidate gene association studies have been undertaken to identify the genetic factors that influence HDL levels (21;22). However, in general these approaches have been unable to identify major loci involved in HDL regulation in humans. Linkage analysis is generally underpowered to identify all but the most powerful loci, and candidate gene studies necessarily examine only a small number of potential targets out of the entire genome. With the completion of the phase I HapMap (23), whole genome association studies may soon become a reality. To date, however, one extremely fruitful approach for identifying the genetic factors involved in HDL metabolism has been the study of rare, Mendelian disorders of HDL metabolism.

Tangier Disease (TD) (Online Mendelian Inheritance in Man no. 205400) is one such rare disease, affecting approximately 100 patients worldwide. TD was first identified by Donald Fredrickson on Tangier Island in the Chesapeake Bay area of the United States (24). While

performing routine tonsil examinations, Fredrickson discovered two siblings with massively enlarged orange tonsils and virtual absence of alpha migrating (HDL) lipoprotein, thus identifying the two salient features of TD: reduced HDL cholesterol, and cholesterol accumulation in cells of the reticuloendothelial system. With remarkable foresight, Frederickson noted that "patients with rare genetically determined diseases offer ... an occasional view of normal processes obtainable in no other way", and that TD may "provide us with just such a view into some now-clouded aspects of fat transport and metabolism" (24).

In 1999, the molecular cause of TD was identified as mutations in both alleles of the *ABCA1* gene (25-28). Soon after, mutations in *ABCA1* were also described in familial HDL deficiency, a more common and relatively milder disorder than TD which results from heterozygous loss of *ABCA1* function (29). To date, more than 100 common and rare variants have been described in the *ABCA1* gene, with a wide range of biochemical and clinical phenotypes (30). Here we review genetic variation in *ABCA1* and how it influences cholesterol transport, HDL metabolism, and risk for atherosclerosis.

2.3. ABC Gene Superfamily

The transport of specific molecules against gradients across cellular membranes is a fundamental feature of biological systems. The ABC transporter superfamily encodes proteins that transport a wide variety of substances including sterols, metabolic products and drugs across both intra- and extracellular membranes. The ABC gene superfamily is highly diverse, and well conserved between species, hinting at the evolutionarily ancient history and critical importance of this gene family (31). ABC transporters are the largest known membrane transporter family, consisting of 49 members in humans (32). The mammalian ABC gene superfamily is divided into seven

subfamilies, A through G, based on similarity in gene structure, order of domains, and sequence homology in the nucleotide binding folds and transmembrane regions. Given the fundamental importance of this class of transporters, it is perhaps not surprising that mutations in 16 of the 49 human ABC genes are known to be associated with genetic disease, shown in Table 2.1, including cystic fibrosis, Stagardt disease, Sistosterolemia, Dubin-Johnson Syndrome, and others.

Table 2.1 ABC Transporters associated with diseases in humans

Gene	Associated Disease	Reference
ABCA1	Tangier Disease	(25)
ABCA3	Surfactant deficiency	(33)
ABCA4 (ABCR)	Stargardt Disease	(34)
ABCA12	harlequin ichthyosis	(35)
ABCB1 (MDR1)	multi-drug resistance	(36)
ABCB2 (TAP1)	Defective antigen presentation	(37)
ABCB3 (TAP2)	Immune deficiency	(38)
ABCB4 (MDR3)	Progressive familial intrahepatic cholestasis type 3	(39)
ABCB7	X-linked sideroblastosis and anemia	(40)
ABCB11	Progressive familial intrahepatic cholestasis type 2	(41)
ABCC2	Dubin-Johnson Syndrome	(42)
ABCC6	Pseudoxanthoma elasticum	(43)
ABCC7 (CFTR)	Cystic Fibrosis	(44)
ABCC8 (SUR1)	persistent hyperinsulinemic hypoglycemia of infancy	(45)
ABCD1	Adrenoleukodystrophy	(46)
ABCG5/ABCG8	Sitosterolemia	(47)

2.4. Molecular Evolution of ABCA1

ABCA1 is highly conserved between species, showing over 90% identity with mouse *ABCA1* at the protein level. The recent availability of genome sequences from many different organisms has made it possible to infer the ancestral state of DNA sequences, and thereby determine lineage-specific changes for specific genes. Such an approach was recently employed in a large set of sequences from humans, mice and chimps (48). To identify genes that have undergone adaptive evolution, this group used a statistical test, the model 2 *P* value, which assesses the likelihood that specific sites in the human lineage display a ratio of non-synonymous (amino acid sequence is altered) to synonymous (no change in amino acid sequence) substitutions of greater than 1, indicative of positive selection. Interestingly, the model 2 *P* value for *ABCA1* was P_2=0.00211, putting it in the top 0.6% of the nearly 8000 genes assessed in terms of adaptive evolution since the divergence of the last common ancestor between humans and chimps (48). By comparison, the model 2 *P* value for *FOXP2*, a transcription factor involved in language for which there exists considerable evidence of positive selection (49;50), is P_2=0.0027. Therefore *ABCA1* is among the genes with the strongest evidence for having undergone adaptive evolution in the human lineage.

We examined the rates of synonymous and non-synonymous substitutions that have occurred in mouse, human and chimp *ABCA1* sequences. Figure 2.1 shows that the ratio of non-synonymous to synonymous substitutions (d_N/d_S) is significantly elevated in the human (d_N/d_S=0.86), compared to mouse (d_N/d_S=0.18) and chimp (d_N/d_S=0.33) lineages ($P \leq 0.05$, Fishers exact test). An elevation of d_N/d_S indicates either a relaxation of selective pressure or positive selection for amino acid substitution. This analysis therefore provides further support for the concept that human *ABCA1* has undergone adaptive evolution, and suggests that the

ABCA1 gene product may have played a critical functional role during the evolution of modern humans, leading to the observed positive selection for amino acid changes in this protein.

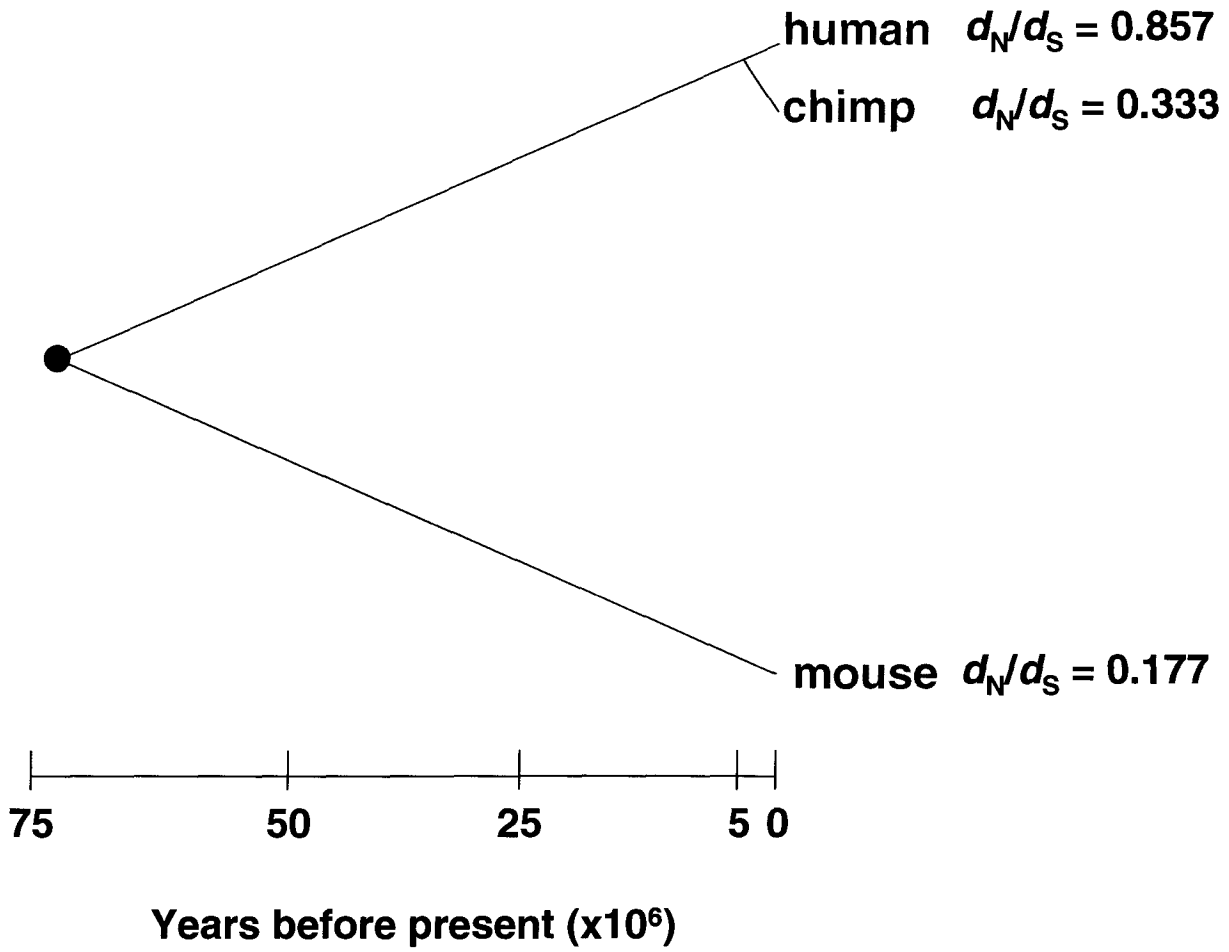

Figure 2.1 Lineage Specific Evolution of *ABCA1*. The ratio of non-synonymous to synonymous substitutions (d_N/d_S) in each lineage is shown. The lineage leading to modern humans shows a significant acceleration in (d_N/d_S), with 6 non-synonymous and 7 non-synonymous substitutions having occurred since the divergence of the last common ancestor between humans and chimps.

2.5. Mutations in ABCA1

To date, 73 mutations have been described in the *ABCA1* gene (25-29;29;51-57;57-75) (Table 2.2). These include 44 missense mutations, 18 nonsense mutations, and 11 insertions and deletions. The distribution of mutations in *ABCA1* is non-random (Figure 2.2). By far, the

majority of mutations occur in the large extracellular loops and the areas surrounding the nucleotide binding folds. Twenty-five mutations occur in the two large extracellular loops, and 28 occur in the intracellular regions, for a total of 53/74 = 72% of mutations occurring in 55% of the protein. In contrast, only 3/74 = 4% of mutations occur in transmembrane regions, which make up 12% of the protein (P=0.03, Fisher's exact test).

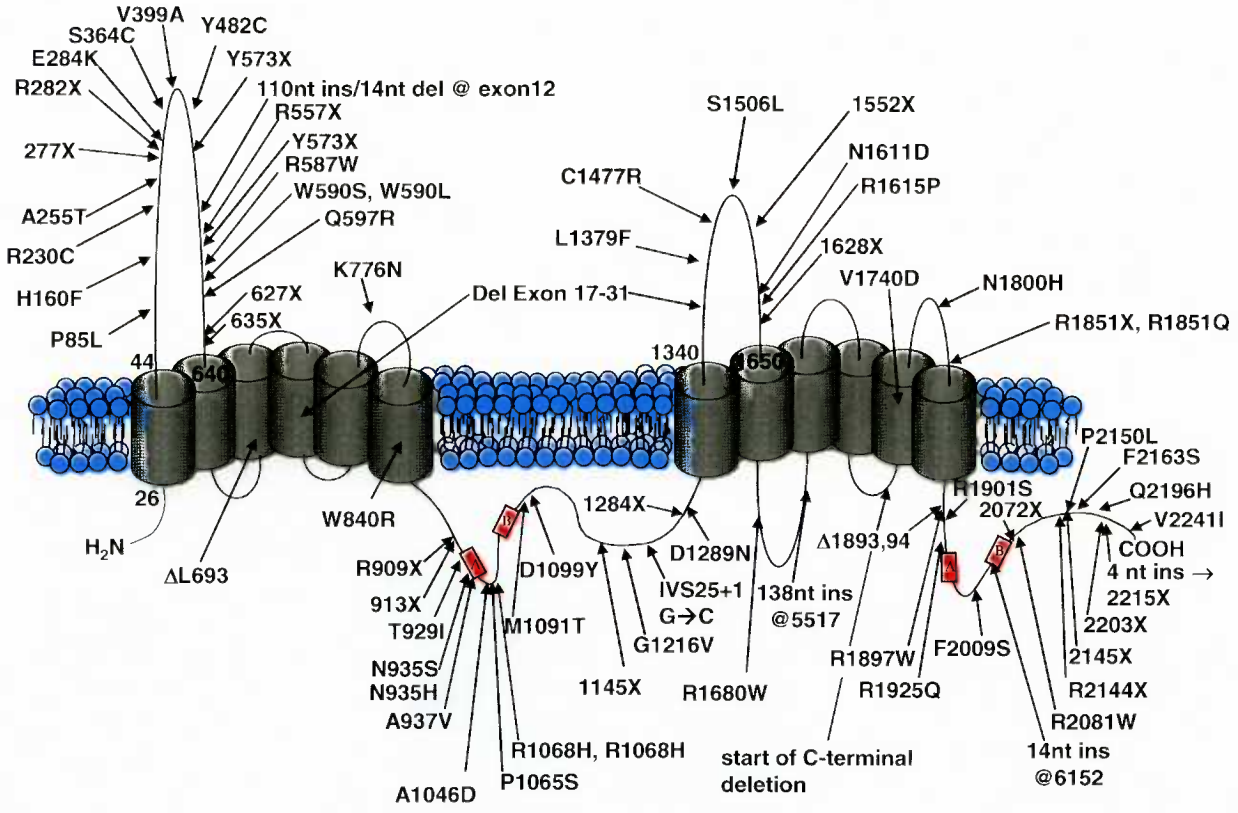

Figure 2.2 Location of Mutations in *ABCA1*. The location of all reported mutations in *ABCA1* are shown in a diagrammatic representation of the ABCA1 protein.

Table 2.2 Mutations in ABCA1

Missense			
Nucleotide Change	**Amino Acid change**	**Exon**	**Reference**
C648T	P85L	4	(51)
--	H160F	6	(52)
C1083T	R230C	7	(53)
G1158A	A255T	8	(54)
	E284K	9	(52)
C1090G	S364C	10	(55)
T1519C	V399A	11	(27)
	Y482C		(52)
C2154T	R587W	14	(28)
G2265T	W590L	14	(56)
G2164C	W590S	14	(27)
A2185G	Q597R	14	(28)
	K776N		
--	W840R		(57)
C3181T	T929I	19	(58)
A3199G	N935S	19	(27;59)
A3198C	N935H	19	(59)
C3205T	A937V	19	(27)
C3532A	A1046D	22	(53)
C3192T	P1065S	22	(55)
	R1068C	22	(57)
G3516A	R1068H	22	(60)
T3667C	M1091T	23	(29)
G3690T	D1099Y	23	(70)
G3646T	G1216V	25	(55)
G4260A	D1289N	27	(61;62)
C4425T	L1379F	28	(63)
T4824C	C1477R	31	(25)
C4912T	S1506L	32	(64)
A5226G	N1611D	36	(54)
--	R1615P	35	(65)
C5433T	R1680W	37	(66)
T5401A	V1704D	36	(63)
A5793C	N1800H	40	(62)
G5947A	R1851Q	41	(67)
C5689T	R1897W	42	(68)
--	R1901S	42	(52)
G6064A	R1925Q	42	(69)
T6361C	F2009S	45	(70)
C6636T	R2081W	47	(61)
C6844T	P2150L	49	(71)
T6801C	F2163S	49	(57)
--	Q2196H		(52)
G7043A	V2244I	50	(57)
Nonsense			
1210_1217del	277X	9	(62)

C1240T	R282X	9	(72)
--	R557X		(52)
C2033A	Y573X	12	(73)
C2276G	Y627X	14	Assman
G2219del	635X	14	(26;27)
C3120T	R909X	19	(29)
C2265del	913X	19	(64)
3680insG	1145X	23	
3738_3739del	1145X	23	(74)
4242_4245del	1284X	27	(61)
4943insA	1552X		(53)
C5946T	R1851X	41	Assman
IVS46delT	2072X		(67)
C6825T	R2144X	49	(29)
C6825del	2145X	49	(71)
CTC6952-4TT	2203X	49	(75)
6968ins 4nt	2215X	49	(62)
Insertion/deletion			
IVS2+1G>C			(72)
IVS25+1G>C			(25)
110 nt ins/14nt del exon 12		13	(26)
IVS16_IVS31del		17-31	(59)
5517ins 138 nt		38	(28)
IVS39→del		39	(27)
6152ins 14 nt		42	(28)
IVS12_IVS14del			(59)
GG5277,8C	1628Frameshift	36	
6073_6078del	E1893_D1894del	42	(29)
2472-2474del	L693del	15	

Several mutations have been described in the C-terminal region of ABCA1, while there are no common coding SNPs reported in this region, suggesting that it is a functionally critical domain. The C-terminus of ABCA1, like that of the related CFTR protein (76), has been reported to contain a PDZ domain (62;77) that could mediate protein-protein interactions. In addition, a VFVNFA motif was recently identified in the C-terminus of ABCA1, and deletion of this domain resulted in diminished apoA-I binding and lipid efflux (78). The C-terminus of ABCA1 therefore appears to be a crucial functional domain, potentially by recruiting other proteins in the

efflux pathway. The specific protein interactions necessary for efflux to occur remain to be determined.

Although certain regions of the ABCA1 protein do appear to be more susceptible to deleterious mutations, there are no "common", recurrent mutations in *ABCA1* associated with Tangier Disease. A small number of mutations have been reported in more than 1 unrelated individual, such as N1800H (55;62;79), and the K776N variant which was recently reported to occur in a Danish population at a frequency of 0.4% and to be associated with a 2 to 3-fold increased risk for ischemic heart disease (80). The K776N variant was initially described as a SNP (81), however, the Danish study reporting that it occurs at a frequency less than 1% and is associated with a strong phenotypic effect would suggest that this is a relatively "common" *ABCA1* mutation, at least in the Danish population, influencing atherosclerosis susceptibility. However, by far the majority of mutations in *ABCA1* are private mutations occurring only in individual families. This indicates that mutations in *ABCA1* associated with Tangier Disease have occurred relatively recently, and are efficiently purged from the population. Interestingly, mice lacking *ABCA1* have reduced fertility and placental defects (82). Although it is not clear if loss of *ABCA1* activity is associated with reduced fertility in humans, this offers one hypothetical mechanism by which newly arising mutations are removed from the population, thus explaining the observed high frequency of new mutations in *ABCA1* despite the small number of individuals with TD.

An additional 24 rare variants in *ABCA1* have been described in a population based cohort with low levels of HDL cholesterol (79). It is likely that many of these variants are pathogenic mutations, but it is not yet clear to what extent they impair *ABCA1* function (83), or if they

segregate with the low HDL phenotype. We therefore have not included them in the list of validated *ABCA1* mutations in Table 1.

2.6. Phenotypic Impact of Mutations in ABCA1

Prior to the identification of *ABCA1* as the molecular defect in TD, patients were identified based on their clinical phenotype, that is, extremely low HDL cholesterol in homozygotes, with the offspring and parents of homozygotes being obligate heterozygotes. There was therefore potential for heterozygotes with more mild phenotypes to be missed, and conversely for severely affected heterozygotes to be misclassified as suffering from TD. The possibility to determine an individual's *ABCA1* genotype has now allowed for more unambiguous genetic diagnosis.

Such a genetic analysis has now revealed that mutations in *ABCA1* are associated with a broad range of biochemical defects. Table 2.3 shows mean plasma HDL cholesterol levels and mean percentage HDL of age- and sex-matched controls for *ABCA1* heterozygotes. Although HDL levels are an insensitive marker, and will reflect a variety of other environmental and genetic factors besides *ABCA1* genotype, they do allow for some assessment of the severity of each *ABCA1* mutation. A remarkably broad range of phenotypes associated with heterozygous *ABCA1* mutations is evident, ranging from 30 to 100% of control HDL cholesterol. The majority of *ABCA1* mutations are associated with HDL levels approximately 50% of control. This indicates that most of these mutations are complete loss of function alleles; removal of one copy of *ABCA1* results in half of normal efflux activity, underlying the observed phenotype of an approximately 50% reduction in HDL cholesterol.

Table 2.3 Patient phenotypes associated with heterozygous ABCA1 mutations

Mutation	HDL (mmol/L)	HDL (% of control)	Number of patients
M1091T	0.48±0.5	30±30	4
G1216V	0.50	40	1
R2144X	0.56±0.2	41±18	12
R282X	0.52	41	1
R909X	0.59±0.3	42±19	5
K776N	0.55±0.1	47±5	2
R587W	0.61±0.1	47±8	7
S364C	0.60	48	1
P1065S	0.80	51	1
c-ter deletion	0.75	53	1
N1800H		56.5	33
P85L	0.72±0.4	57±33	5
Del693L	0.79±0.2	57±15	8
D1289N	0.80±0.1	59±12	4
R2081W	0.80±0.1	59±12	4
2203X	0.80±0.2	59±20	4
DelED1893, 4	0.77±0.2	59±18	8
2145X	0.82±0.1	59±9	4
A1046D	0.70±0.1	60±8	2
Q597R	0.82±0.1	60±5	5
C1477R	0.82±0.2	61±15	9
IVS25+1G>C	0.78±0.1	62±12	4
D1099Y	0.83±0.3	63±21	5
1552X	1.00±	64	1
F2009S	0.82±0.2	64±19	6
R587W	0.86±0.1	65±17	2
R1068H	0.90±0.3	67±26	9
N935S	1.00±0.3	74±16	7
T929I	1.01±0.2	76±7	8
1284X	1.11±0.2	83±14	5
A937V	1.15±0.6	85±28	2
R1680W	1.22±0.2	87±17	3
635X	1.24±0.5	90±32	7
W590S	1.32±0.6	103±46	15

However, a subset of mutations are associated with greater than 50% of control HDL levels, specifically T929I, A947V, R1680W and W590S. Therefore, these mutations are likely not complete loss of function mutations, but may retain some residual activity. In this case, overall efflux is reduced, but not by 50%, thus resulting in HDL levels greater than 50% of controls.

Consistent with this concept, cholesterol efflux from fibroblasts of an individual carrying the T929I mutation have been reported to be approximately 75% of control levels (71).

Conversely, a small number of mutations are associated with less than 50% of control HDL cholesterol, specifically M1091T, G1216V, and the truncation mutations R2144X, R282X and R909X. Since a complete loss of function allele would be expected to result in a 50% reduction in HDL levels, a greater than 50% reduction in HDL is most likely explained by a dominant negative allele, in which the mutant protein actually interferes with the activity of the remaining wildtype protein. Recent biochemical data helps to explain how this could occur. ABCA1 was recently shown to exist in dimeric and tetrameric forms in human fibroblasts (84). Therefore, a dysfunctional ABCA1 protein that is still able to oligomerize may sequester wildtype ABCA1 proteins, thus reducing the activity of the wildtype protein. The ability of ABCA1 truncation mutations to suppress the activity of wildtype ABCA1 has been previously shown in transfected cells (85).

Other factors could also explain the wide-range of phenotypic effects associated with *ABCA1* mutations seen in Table 2.3. In addition to environmental factors and the influence of other genes, the genetic background with respect to *ABCA1* on which the mutation occurs could influence the phenotypic expression. Support for this concept comes from a recent study showing that HDL cholesterol levels in carriers of the R1068H mutation are significantly influenced by the *ABCA1* promoter haplotype associated with the mutation (60). It will be of interest to examine in greater detail whether specific *ABCA1* haplotypes play an important role in influencing the manifestation of mutations in this gene and therefore explain part of the variation amongst individuals with identical mutations in *ABCA1*.

Two recent reports suggest that *ABCA1* mutations may be associated with additional clinical manifestations, besides the classic phenotypes of low HDL cholesterol, tissue cholesterol accumulation and atherosclerosis. Albrecht et al. reported a case of Scott Syndrome, a rare bleeding disorder characterized by defective phosphatidlyserine exposure on the surface of platelets, that was associated with a novel missense mutation in *ABCA1*, R1925Q (69). *ABCA1* is known to be expressed on platelets, and prolonged bleeding time has been previously reported in association with TD (86;87). In addition, *ABCA1* has previously been shown to mediate calcium-stimulated phosphatidlyserine exposure in red blood cells (88). The 1925Q mutation was shown to cause mislocalization of ABCA1 in transfected cells, suggesting that this is a functionally significant variant (69). Paradoxically, however, this patient had normal levels of HDL cholesterol (1.3 mmol/L). Clearly this observation needs to be extended to other cases, but the case described by Albrecht et al. does raise the intriguing possibility that defective ABCA1-mediated efflux in platelets could result in the aberrant PS externalization associated with Scott Syndrome.

Saleheen et al. reported a case of a 33 year male with type 2 diabetes and very low HDL cholesterol (0.26mmol/L) (65). This patient was found to be heterozygous for a novel R1651P missense mutation in *ABCA1*. Because Saleheen et al. did not demonstrate segregation of the diabetic phenotype with the mutation in the kindred it cannot be excluded that this is a chance finding. However, certain *ABCA1* haplotypes have been found to be over-represented in a type 2 diabetic compared to normo-glycemic population (89), consistent with a relationship between loss of ABCA1 activity and diabetes. Although the mechanism underlying a relationship between reduced ABCA1 activity and type 2 diabetes is unclear, previous studies have described specific down-regulation of ABCA1 in response to unsaturated free fatty acids (90;91) and advanced glycosylation end products (92), suggesting that the imbalances associated with the

metabolic syndrome -- such as elevated fatty acids and hyperglycemia -- may themselves reduce ABCA1 activity. Detailed studies of glucose homeostasis in TD patients would be useful to resolve these issues.

ABCA1 is expressed in a wide variety of tissues (93), but it is likely that only hepatic and intestinal ABCA1 are significantly involved in HDL biogenesis (94;95). Using tissue-specific gene-targeting, we have recently shown that the liver (94) and the intestine (96) are the major sites of HDL biogenesis, contributing to approximately 80% and 30% of the plasma HDL pool, respectively. The studies of Albrecht et al. and Saleheen et al. suggest that detailed study of phenotypes associated with *ABCA1* mutations could point to previously unexpected functions of ABCA1 in other tissues.

2.7. The Role of Rare ABCA1 Variants in the General Population

Two recent studies addressing the role of rare *ABCA1* variants in the general population provide substantial information about the role of *ABCA1* in modulating HDL levels on a population scale. Cohen et al. sequenced the entire coding region of *ABCA1* (as well as the genes encoding apolipoprotein A-I and LCAT) in 256 individuals representing the top and bottom 5% of HDL cholesterol levels from the Dallas Heart Study population (79). Remarkably, 20 of the 128 individuals with the lowest 5% of HDL cholesterol levels were found to harbor a rare amino acid variant in *ABCA1* that was not found in the high HDL cholesterol group. This finding was replicated in a second population, indicating that in two separate populations ~15% of individuals with low HDL cholesterol have rare sequence variants in *ABCA1*. In contrast, none

of the common (>10% frequency) *ABCA1* variants they identified were associated with HDL cholesterol levels across all gender and ethnic groups.

While it remains unclear what percentage of these rare *ABCA1* alleles are truly functionally significant, this study does provide evidence that rare, but not common variants in *ABCA1* may contribute significantly to low HDL cholesterol in the general population. This notion is supported by a second study in which the *ABCA1* coding region and promoter were sequenced in the highest and lowest 1% of HDL cholesterol levels (95 individuals per group) from the Copenhagen City Heart Study (80). Seven rare variants were identified which altered the amino acid sequence of *ABCA1*, and of these six were observed only in the low HDL cholesterol group, suggesting that they impart a major phenotypic effect. In total, 10% of individuals with low HDL cholesterol were found to harbor a mutation in *ABCA1*. In contrast to the study by Cohen et al., Frikke-Schmidt et al. did observe significant association between common *ABCA1* alleles and HDL levels in the entire population. This may be due to the fact that they examined a much larger population than did Cohen et al. (9259 individuals versus 2569), and that Cohen et al. restricted their association study to variants greater than 10% frequency, whereas Frikke-Schmidt et al. examined all SNPs with a frequency of 1% or greater. However, the study by Frikke-Schmidt et al. does verify the main finding from Cohen et al. that a significant proportion of individuals with low HDL cholesterol from the general population harbor rare variants in *ABCA1*.

These studies have important implications for future research into the role of *ABCA1* in modulating HDL cholesterol levels in the general population. Notably, they indicate that approximately 10-15% of individuals with low HDL cholesterol from the general population have rare *ABCA1* variants. The relative importance of mutations in *ABCA1* as a cause of low

HDL in the general population is an important question and has been the subject of some debate. Initial reports suggested that up to 40% of familial HDL deficiency (FHA) was caused by defective *ABCA1*-mediated efflux (29;97). However, later studies suggested that the frequency of *ABCA1* in FHA is much less, about 4.5% (98). However, these studies both suffered from potential selection-biases in that the patients were recruited from medical clinics. In contrast, the studies by Cohen et al. and Frikke-Schmidt et al. examined patients from the general population who were selected solely on the basis of their HDL phenotype, and in addition the *ABCA1* gene was sequenced in all individuals with low HDL (55;79). These studies, therefore, provide the strongest evidence to date that *ABCA1* is a critically important locus for modulating HDL cholesterol levels in the general population, and that loss of *ABCA1* activity underlies a significant proportion of the low HDL cholesterol seen in the population.

These studies also suggest that future work aiming to investigate the relationship of *ABCA1* variants to HDL levels and atherosclerosis should examine rare variants by sequencing, in addition to association studies using common SNPs. More broadly, these studies bring into question the popular theory that common traits, such as low HDL cholesterol, are caused by common alleles, each with moderate phenotypic effects -- the so-called common-disease common-variant hypothesis (99-101). On the contrary, the results of these two studies suggest that for the low HDL phenotype rare alleles with strong functional effects modulate the variation of HDL levels in the population to a more significant extent than common alleles. The utility of large-scale SNP projects, such as the International HapMap Project (23), rests on the assumption that genome-scale association studies of common variants will be useful in dissecting the genetics of common phenotypes. If the results of the studies by Cohen et al. and Frikke-Schmidt et al. prove generalizable to other common traits, however, sequence-based approaches to

identify rare alleles will also be necessary to identify the genetic factors important in complex traits.

2.8. Common Variants in ABCA1

Fifteen coding non-synonymous single nucleotide polymorphisms (SNPs) have been described in the *ABCA1* gene (Table 2.4). Many of these variants have been studied in relationship to their association with HDL cholesterol levels and atherosclerosis (53;55;79-81;102;103;103-116), and these results have been reviewed elsewhere (30). Most of these studies have reported significant association of *ABCA1* SNPs with lipid levels and atherosclerosis, suggesting that common variation in *ABCA1* does influence HDL cholesterol levels and risk for atherosclerosis in the general population. However, not all findings have been replicated and some findings are inconsistent, making it difficult to determine which specific variants mediate these effects.

Table 2.4 Non-synonymous SNPs in *ABCA1*

SNP id	Nucleotide[A]	Amino Acid	Amino Acid[B]	Observed Hetero-zygosity
rs2230806	G>A	969	R219K	0.488
rs9282541	C>T	1001	R230C	0.029
rs9282543	T>C	1509	V399A	0.020
rs4131108	A>C	1556	M415L	--
rs13306068	A>G	1949	I546V	--
rs2066718	G>A	2624	V771M	0.074
rs2472458	G>A	2804	D831N	--
rs4149313	A>G	2962	I883M	--
rs2482437	C>T	3326	E1005K	--
rs13306072	G>A	3473	V1054I	--
rs13306073	G>A	3599	V1096I	--
rs1997618	T>C	4977	I1555T	--
rs2230808	A>G	5073	K1587R	0.480
rs1883024	T>C	5256	L1648P	--
--	C>G	5505	S1731C	--

[A]Nucleotide position is with respect to NM_005502. [B]Amino acid position is with respect to NP_005493.

The largest study of *ABCA1* variants to date examined the relationship between 6 *ABCA1* cSNPs and HDL cholesterol in over 9000 individuals from an ethnically homogeneous population (55). This study had the added advantages that the authors considered the effect of each SNP in isolation of variation at the other sites, and assessed phenotypic data collected at two time points ten years apart (55). The only SNP associated with HDL cholesterol in both men and women at both time points was R1587K, for which the minor K allele was associated with modestly but significantly reduced HDL. The V825I and V771M SNPs were associated with increased HDL cholesterol in one, but not both, genders.

This study provides the most convincing data to date that common *ABCA1* SNPs do affect *ABCA1* activity and HDL levels in the general population. However, the magnitude of the effect on HDL cholesterol was generally small. As mentioned, HDL levels are influenced by many other factors, and therefore may be an unreliable measure of *ABCA1* activity. Indeed, several studies have documented an association of *ABCA1* SNPs with clinical endpoints independent of changes in lipid levels, suggesting that small changes in *ABCA1* activity, potentially in the important vessel-wall macrophage pool (117), can affect atherogenesis without appreciably impacting steady-state HDL levels. Unfortunately, the large study by Frikke-Schmidt et al. (55) did not examine association of these *ABCA1* SNPs with markers of atherosclerosis.

2.9. Haplotypic Architecture of ABCA1

A major limitation in examining the effects of common *ABCA1* variants in modulating HDL cholesterol levels and risk for atherosclerosis has been an incomplete understanding of linkage disequilibrium patterns at the *ABCA1* locus. Figure 2.3 displays a graphical representation of the patterns of linkage disequilibrium and major haplotype blocks across the approximately 150

kilobase genomic region containing the *ABCA1* gene, as determined by data from the HapMap project. The *ABCA1* locus displays a complex haplotypic structure, with 9 major haplotype blocks. The 5' region of *ABCA1* shows particularly complex patterns of linkage disequilibrium. Notably, only two of the 15 common *ABCA1* non-synonymous SNPs, I883M and R219K, are represented in the HapMap data set, which is an incomplete data set (the phase I project having set-out to identify 1 SNP every 5000 base pairs) (23). However, it is apparent from the haplotypes in which I883M and R219K reside that the minor alleles of each of these are unique to one particular, different haplotype, which may explain in part why these two SNPs have been consistently found to be associated with HDL levels and atherosclerosis risk. This also suggests that such associations are not necessarily due to functional effects of these sites, but could instead reflect the functional effects of linked SNPs. Significant linkage disequilibrium does not appear to exist between *ABCA1* and the nearby *NIPSNAP3A* and *NIPSNAP3B* genes.

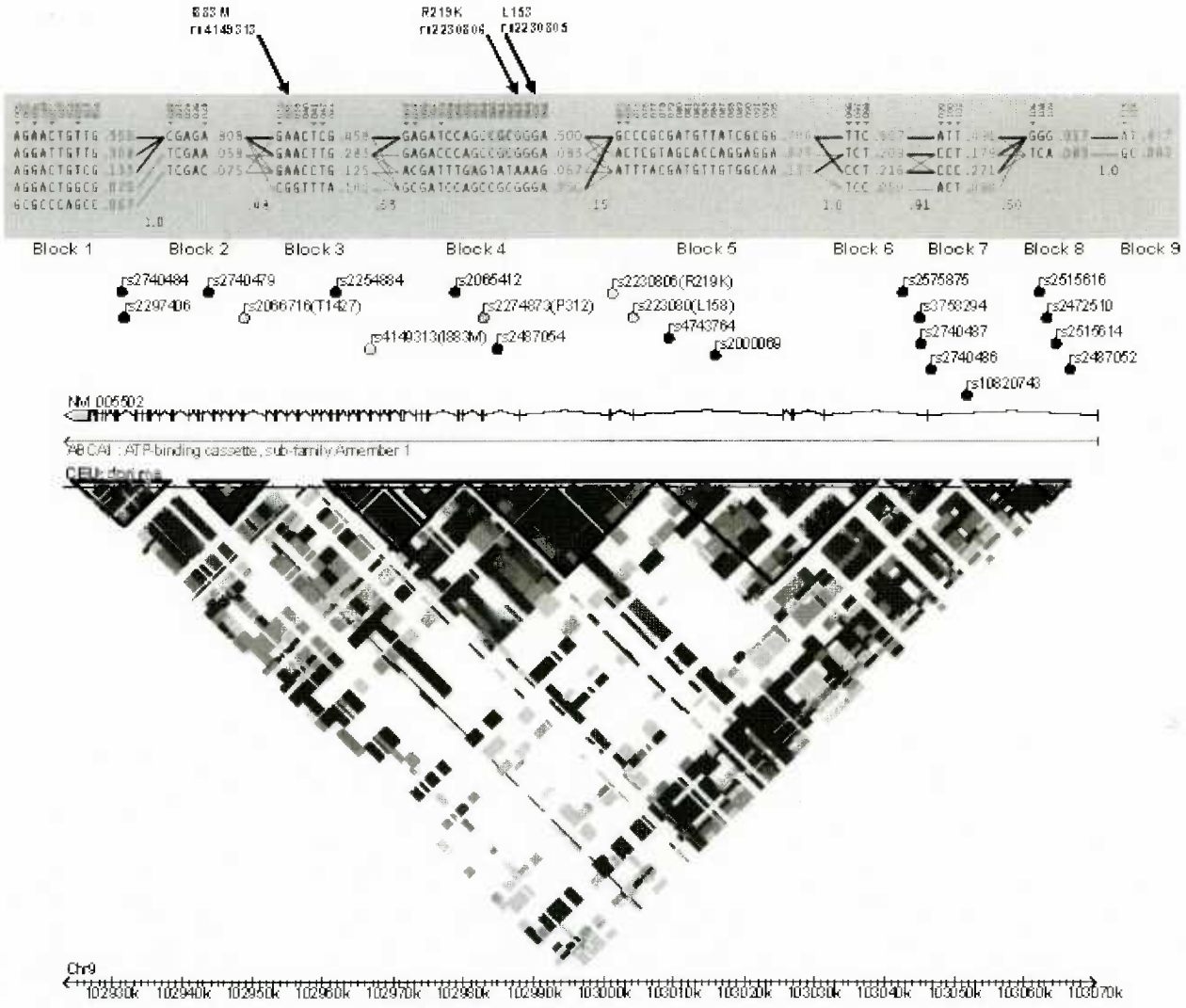

Figure 2.3 Haplotype Structure of *ABCA1* in Americans of Northern and Western European Ancestry. The nine major haplotype blocks in the *ABCA1* gene are shown. Below the haplotype blocks, *ABCA1* tag SNPs are shown as blue circles, non-synonymous coding SNPs as yellow circles and synonymous coding SNPs as red circles. Below, the patterns of linkage disequilibrium, estimated by the r^2 value is shown. Red blocks indicate regions of significant linkage disequilibrium. Adapted from the HapMap project webpage (www.hapmap.org).

Considerations of linkage disequilibrium amongst SNPs can guide selection of a more efficient set of "tag" SNPs, which provide information about nearby linked SNPs without genotyping the linked SNPs directly (118). Based on data from the HapMap project, we assembled a list of tag SNPs for *ABCA1* using the Tagger program (119), based on a correlation coefficient (r^2) between tag SNPs of 0.8. The list of *ABCA1* tag SNPs is shown in Table 2.5. These 17 SNPs capture most of the genetic variation in the *ABCA1* gene, while dramatically reducing the

number of genotypes required. These SNPs are all highly polymorphic, with observed heterozygosities ranging from 0.278 to 0.556, indicating that they are all likely to be informative. The use of a set of tag SNPs selected from an incomplete data set such as the current HapMap was recently shown to result in only minor losses in power compared to a complete data set (119). Of course, the actual selection of tag SNPs for any given association study will also be influenced by details of the population under study and the genotyping platform used. In addition, it would seem wise to include known non-synonymous SNPs in a genotyping panel, because of their higher probability of functionality.

Table 2.5 ABCA1 tag SNPs

SNP id	Observed hetero-zygosity	Position[A]	Variation	Location
rs2740484	0.456	14872385	A>G	intron 44
rs2297406	0.478	14872743	C>T	intron 44
rs2740479	0.378	14884642	C>T	intron 34
rs2254884	0.500	14902954	C>T	intron 34
rs2065412	0.422	14919945	C>T	intron 11
rs2487054	0.456	14925927	A>C	intron 8
rs4743764	0.433	14950309	C>T	intron 5
rs2000069	0.456	14957074	C>T	intron 5
rs2575875	0.389	14983699	A>G	intron 2
rs3758294	0.378	14986020	C>T	intron 2
rs2740487	0.556	14986166	C>T	intron 2
rs2740486	0.528	14987718	A>C	intron 1
rs10820743	0.393	14992864	C>T	intron 1
rs2515616	0.389	15003200	C>T	intron 1
rs2472510	0.310	15004327	A>C	intron 1
rs2515614	0.378	15005523	G>T	intron 1
rs2487052	0.278	15007610	C>T	intron 1

[A]Position is in contig NT_008470

This set of SNPs, however, illustrates the principle of selecting a highly informative and efficient set of SNPs. The confluence of data from HapMap, and the ability to predict the functional significance of non-synonymous variants in *ABCA1* (83), should aid considerably in the

prioritization of SNP selection for future association studies of *ABCA1*, as well as the entire genome.

2.10. Conclusion

The identification of *ABCA1* as the molecular defect in Tangier Disease has ushered in a new era in our understanding of HDL metabolism and reverse cholesterol transport. This discovery provides further support for the study of rare diseases as an opportunity to gain "an occasional view of normal processes obtainable in no other way" (24), and to provide insights into potential ways to raise HDL levels and protect against a common disease.

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Chapter 3 - Accurate Prediction of the Functional Significance of Single Nucleotide Polymorphisms and Mutations in the *ABCA1* Gene[1]

3.1. Introduction

The ATP-binding cassette transporter A1 (*ABCA1*) (Entrez GeneID:19) is a cholesterol and phospholipid transporter, and mutations in *ABCA1* cause Tangier Disease (TD) (Online Mendelian Inheritance in Man no. 205400) (1-3), a rare disorder characterized by reduced levels of plasma HDL cholesterol and increased risk for coronary artery disease (4). More than 70 coding variants have been reported in the *ABCA1* gene, including 30 missense mutations, 10 cSNPs, and many large and small deletions and insertions (5). Variants detected in individuals with TD have been assumed to impair the function of ABCA1. However, without functional testing of individual variants, it has not been possible to determine which of these variants directly affect ABCA1 protein function. This is a fundamental problem in human genetics, in which most DNA variants are not functionally tested, and the number of individuals with any given mutation is often small, making statistical assessment difficult or impossible.

We used an evolutionary model to predict the functional consequence of genetic variation in the *ABCA1* gene, and tested these predictions through in vitro assessments of protein function. We predicted the functional consequence of each variant in *ABCA1* using PANTHER, a collection of protein families and sub-families that allows one to ask the question, how often does a given amino acid occur at a given position in a family of evolutionarily related proteins across different species (6)? PANTHER uses as its data set the natural experiment of evolution, in which over

[1] A version of this chapter has been published. Brunham LR, Singaraja RR, Pape TD, Kejariwal A, Thomas PD, Hayden MR. Accurate prediction of the functional significance of single nucleotide polymorphisms and mutations in the ABCA1 gene. PLoS Genet. 2005 Dec;1(6):e83

time, random mutation will test every amino acid-coding nucleotide sequence in the genome, with those variants that do not impair protein function being represented in the data set of extant proteins. The probability that a given coding variant will cause a deleterious functional change is estimated by the substitution position-specific evolutionary conservation (subPSEC) score, derived from the probabilities of observing the variant amino acids in a PANTHER Hidden Markov Model.

PANTHER subPSEC scores have previously been shown to statistically distinguish Mendelian disease-associated missense mutations from random coding polymorphisms on a genomic scale (7). Here we test the hypothesis that subPSEC scores can predict which specific variants in *ABCA1* will be functionally impaired, and to what degree.

3.2. Results

3.2.1. Prediction of functional effect of ABCA1 mutations and cSNPs

We used data from the PANTHER database to predict the functional significance of each of the 30 missense mutations and 10 cSNPs reported in the *ABCA1* gene (5). The output of PANTHER, the subPSEC score, is the negative logarithm of the probability ratio of the wildtype and mutant amino acids at a particular position. PANTHER subPSEC scores are continuous values from 0 (neutral) to about –10 (most likely to be deleterious). The subPSEC scores for *ABCA1* mutations and cSNPs are shown in Table 3.1. Twenty-three of the 30 *ABCA1* mutations score below -3, the previously identified cut-off point for functional significance (6), compared to 2 of 10 cSNPs (P=0.002, Fisher's exact test). The mean subPSEC score for *ABCA1* mutations is –4.82 compared to -2.03 for SNPs. Figure 3.1 shows the distribution of subPSEC scores for *ABCA1* mutations and cSNPs. Compared to the data set of *ABCA1* cSNPs, *ABCA1* mutations

have significantly lower subPSEC scores (P<0.0001, Mann Whitney U test). Therefore the majority of *ABCA1* mutations are predicted to impair the function of the ABCA1 protein on the basis of the variability of the particular amino acid positions at which the variants occur in evolutionarily related proteins, compared to only a small fraction of cSNPs.

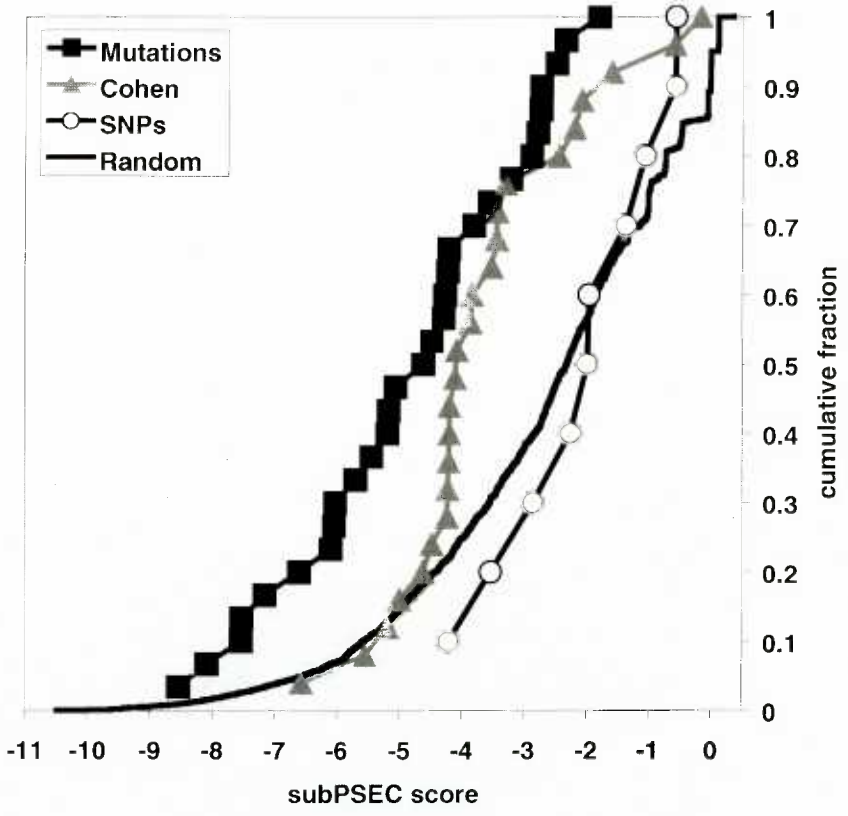

Figure 3.1 Comparison of subPSEC scores for *ABCA1* cSNPs, mutations, recently described variants in a cohort of individuals with low HDL-C from the general population, and a random distribution of low frequency alleles. *ABCA1* cSNPs (empty circles) have significantly greater subPSEC scores than do mutations (filled squares) (P<0.0001, Mann-Whitney U test). subPSEC scores for ABCA1 variants described in the general population (filled triangles) are significantly different from those of both *ABCA1* cSNPs and mutations (P<0.01, Mann-Whitney U test), as well as from the random distribution of ABCA1 variants (P<0.001), indicating that this group of variants consists of both functional and neutral variants.

3.2.2. Functional Assessment of ABCA1 variants

In order to test experimentally the bioinformatics predictions, we established stably transfected polyclonal cell lines with which to assess cholesterol efflux as a measure of ABCA1 function. We established cell lines for 18 of the *ABCA1* alleles for which we predicted modulation of

ABCA1 function using PANTHER, representing 13 mutations, 4 cSNPs, and wildtype *ABCA1* as a control. We chose at least one SNP and one mutation from each of the predictive categories, neutral and deleterious. In addition, we attempted to choose variants for which substantial clinical data are available in order to correlate our findings with patient phenotypes.

All *ABCA1* alleles expressed protein (8), with the exception of the S1731C cell line, for which we observed low levels of protein expression from 2 independently generated cell lines. To confirm that the S1731C allele was being expressed, we performed RT-PCR for *ABCA1* on reverse-transcribed RNA from untransfected 293 cells, and cells transfected with the wildtype or S1731C *ABCA1* alleles. We found that cells transfected with the S1731C allele expressed abundant *ABCA1* mRNA, at levels comparable to that of wildtype *ABCA1*. The S1731C allele therefore expresses normal *ABCA1* mRNA but fails to generate significant amounts of ABCA1 protein.

We next evaluated the biochemical deficit resulting from each sequence variant by assessing apolipoprotein A-I dependent cholesterol efflux in these cell lines. Cholesterol efflux values from cell lines expressing the *ABCA1* alleles are shown in Table 3.1. Of the five variants we tested that were predicted to be functionally neutral (subPSEC > -3), R219K, V771M, I883M, D1289N and P2150L, four had cholesterol efflux values that were not statistically different from wildtype *ABCA1*. This included two variants, D1289N and P2150L, which have been previously reported to be disease-causing mutations (4;9;10), as well as two cSNPs, R219K and V771M. One variant, I883M, was predicted to be functionally neutral but found to have cholesterol efflux modestly but significantly reduced (approximately 70% of wildtype *ABCA1*, $P<0.01$). This SNP has been reported to be associated with decreased HDL cholesterol and increased severity of

atherosclerosis in some (11;12), but not all (13) association studies, supporting the concept that this is a functional variant.

Table 3.1 Cholesterol efflux values for 293 cells transfected with ABCA1 variants and subPSEC and PolyPhen predictions of the functional impact of these variants.

Variant	Variant Type	subPSEC	Cholesterol Efflux	PolyPhen
R2081W	mutation	-7.20	21.1±21% *	Probably damaging
N935S	mutation	-6.77	29.3±13% *	Benign
A1046D	mutation	-6.65	16.8±7% *	Possibly damaging
R587W	mutation	-5.79	31.7±33% *	Probably damaging
C1477R	mutation	-4.83	20.5±10% *	Probably damaging
W590S	mutation	-4.81	47.1±13% *	Probably damaging
Q597R	mutation	-4.11	17.7±14% *	Probably damaging
S1506L	mutation	-3.99	17.8±15% *	Probably damaging
S1731C	cSNP	-3.91	12.3±10% *	Possibly damaging
T929I	mutation	-3.85	69.9±11% *	Possibly damaging
N1800H	mutation	-3.57	31.3±16% *	Possibly damaging
M1091T	mutation	-3.37	6.9±20% *	Probably damaging
P2150L	mutation	-2.51	88.4±21%	Probably damaging
D1289N	mutation	-1.87	137.7±86%	Benign
I883M	cSNP	-1.48	69.1±16% *	Benign
V771M	cSNP	-1.15	145.4±33%	Benign
wildtype	--	0.0	100%	--

*$P<0.01$ compared to wildtype ABCA1.

We generated stably-transfected cell lines expressing twelve different *ABCA1* variants that were predicted to impair ABCA1 function. All twelve of these variants had cholesterol efflux levels that were significantly reduced compared to wildtype *ABCA1* (Table 3.1), indicating that PANTHER correctly predicted the functional impact of each of these variants. The *ABCA1* cSNP, S1731C, has a subPSEC score of less than -3, predictive of a deleterious effect on ABCA1 function. Cells transfected with the S1731C allele displayed a significant reduction in cholesterol efflux, relative to wildtype *ABCA1* ($P<0.01$), indicating that this SNP significantly impairs ABCA1 function, as predicted by PANTHER. These data indicate that S1731C may be a useful SNP to use as a functional marker in association studies.

Of all *ABCA1* alleles tested functionally, M1091T displays the greatest reduction in cholesterol efflux (6.9±20% of wildtype *ABCA1*), consistent with previous reports that this is a severe mutation associated with a severe clinical presentation (4;14). However, the PANTHER score for this mutation (-3.56) is only marginally predictive of a negative impact on function, because while this position is conserved in ABCA1 proteins in other species, it is less conserved among other members of the human ABCA sub-family of proteins, the orthologous position aligning a leucine in the closely paralogous ABCA7 protein (Entrez GeneID:10347) (Figure 3.2a). To determine whether the severe phenotype conferred by the M1091T mutation is a result of the sensitivity of this site, or rather is specific to the insertion of the threonine residue, we generated and characterized cell lines transfected with plasmids bearing M1091L and M1091V alleles, both predicted to have no impact on ABCA1 function (subPSEC scores -2.65 and -2.71, respectively). Interestingly, both of these mutations dramatically impair cholesterol efflux, to a similar extent as the M1091T mutation (Figure 3.2b). Therefore, amino acid position 1091, occurring in the first nucleotide binding domain of ABCA1 (5), appears to be exquisitely sensitive to mutation and absolutely critical for ABCA1 function, despite its relatively modest conservation in related human ABCA proteins. This finding also supports the notion that any amino acid changes in the nucleotide binding region of ABCA1 are likely to have significant functional effects regardless of their evolutionary conservation.

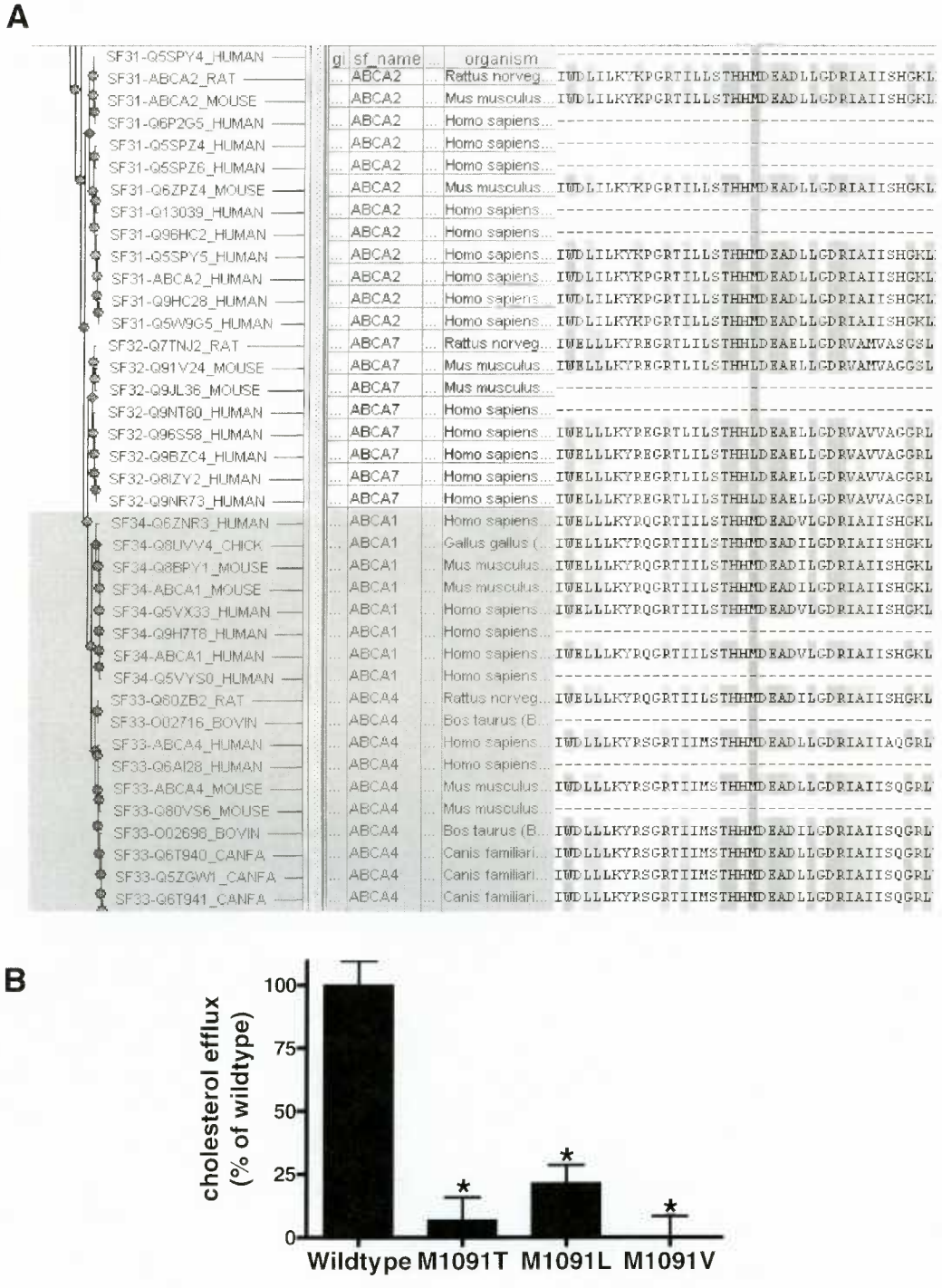

Figure 3.2 Conservation of ABCA1 amino acid position 1091 in related proteins and functional effect of mutation at this site. (A) Multiple sequence alignment adapted from the view of family PTHR19229 available on the PANTHER website, showing the ABCA1, ABCA2, ABCA4 and ABCA7 subfamilies. Human ABCA1 position 1091 is highlighted in red; other conserved positions are highlighted in blue. (B) Cholesterol efflux was assessed in 293 cells stably transfected with wildtype, M1091T, M1091L or M1091V ABCA1 alleles. *P<0.001.

Data from PANTHER can also be used to calculate the probability that a given variant will have a deleterious effect on protein function ($P_{deleterious}$), such that a subPSEC score of -3 corresponds to a $P_{deleterious}$ of 0.5 (see Methods for details). Figure 3.3 shows a plot of cholesterol efflux of individual *ABCA1* variants versus the probability of each variant being functionally impaired. The $P_{deleterious}$ value is significantly correlated with cholesterol efflux for *ABCA1* mutations, (r^2=0.62, *P*=0.0008), indicating that PANTHER can not only discriminate between neutral and functional mutants, but that those variants with a greater $P_{deleterious}$ tend to have more severe impairments in function. Inclusion of *ABCA1* SNPs in the linear regression also reveals a significant correlation (r^2=0.56, *P*=0.0004), indicating that this relationship is significant across all *ABCA1* variants.

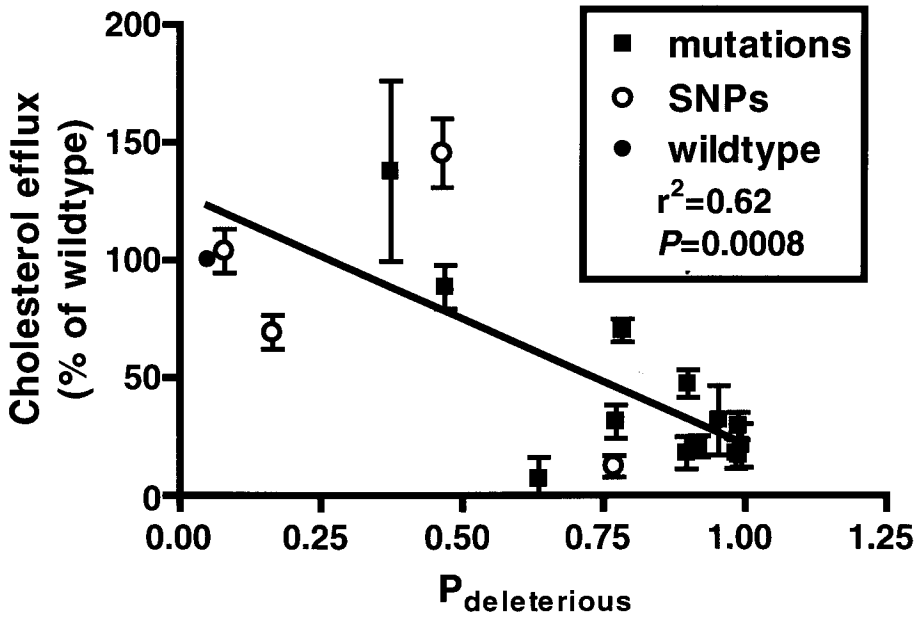

Figure 3.3 Correlation of cholesterol efflux values with the probability of a functional impairment ($P_{deleterious}$) for *ABCA1* mutations (filled squares) and SNPs (clear circles). PANTHER predictions are significantly correlated with the severity of impairment of *ABCA1* mutations (r^2=0.62, *P*=0.0008), and of all *ABCA1* variants (r^2=0.56, P=0.0004). The linear regression shown is for *ABCA1* mutations.

3.2.3. Assessment of rare ABCA1 variants identified in the general population

Recently Cohen et al. reported that a significant proportion (~16%) of individuals with low HDL cholesterol from the general population have rare sequence variants in *ABCA1* (15). An important unanswered question from these data is what functional deficit results from these rare *ABCA1* alleles. We used data from PANTHER to predict the functional consequence of each of the 24 *ABCA1* alleles found in individuals with low HDL cholesterol by Cohen et al. (Table 3.2). This set of variants is shifted towards lower scores compared to the set of common *ABCA1* cSNPs ($P<0.01$, Mann Whitney U test) (Figure 3.1). The subPSEC scores for the Cohen et al. set of variants are also significantly greater than those observed for *ABCA1* mutations ($P<0.01$, Mann Whitney U test), indicating that this set of alleles is significantly different from both *ABCA1* mutations and SNPs. Fourteen of these 24 alleles (58%) have subPSEC scores less than -3, compared to 77% of ABCA1 mutations and 20% of *ABCA1* cSNPs.

Table 3.2. subPSEC and PolyPhen scores for ABCA1 variants described in a cohort of individuals with low HDL cholesterol from the general population. Macrophage efflux values are as reported by Cohen et al. (15).

Variant	subPSEC score	Macrophage efflux	PolyPhen
D1706N	-6.15	0.38 *	Possibly damaging
C1477F	-4.89	0.34 *	Probably damaging
W590S	-4.81	--	Probably damaging
H551D	-4.43	0.32 *	Probably damaging
R965C	-4.16	0.59	Probably damaging
W590L	-4.06	0.31 *	Probably damaging
S1731C	-3.91	0.28 *	Possibly damaging
A1670T	-3.83	--	Possibly damaging
R638Q	-3.72	--	Possibly damaging
E815G	-3.71	--	Probably damaging
T459P	-3.60	0.28 *	Possibly damaging
N1800H	-3.57	0.27 *	Possibly damaging
S1181F	-3.13	--	Possibly damaging
T2073A	-3.01	0.28 *	Possibly damaging
L1026P	-2.99	0.25 *	Benign
R1615Q	-2.70	--	Possibly damaging
R306H	-2.56	--	Benign
S1376G	-2.35	--	Benign
R1341T	-2.22	--	Possibly damaging
D2243E	-2.20	--	Benign

P85L	-1.82	0.80	Probably damaging
K401Q	-1.26	--	Benign
E1386Q	-1.19	0.51	Benign
P248A	-0.70	--	Benign

*efflux value is 2 standard deviations or more below control levels of 0.52±0.07.

In order to control for the possible confounding effect of comparing subPSEC scores of variants with very different frequencies, we compared the distribution of subPSEC scores for the Cohen et al. variants to the distribution of random single-nucleotide missense changes in *ABCA1*. Recent low-frequency mutations that have not yet been subject to significant selection pressures would be expected to approximate such a random model. To estimate the resulting distribution of subPSEC scores, we calculated the scores for all single-nucleotide missense variants in *ABCA1*, weighting the contribution of each variant by transition/transversion ratios. Figure 3.2 shows that the distribution of subPSEC scores for Cohen et al. variants is shifted significantly toward lower scores than the random distribution ($P<0.001$, Mann-Whitney U test). The Cohen et al. set of variants is therefore predicted to be enriched in deleterious alleles, but likely to also include neutral variants.

3.2.4. Comparison with PolyPhen

PolyPhen (Polymorphism Phenotyping) is a web-based program used to predict allele function based on homology and 3-D structural models where available (16), and predicts alleles as being "probably damaging", "possibly damaging", or "benign". In the data set of variants for which we assessed cholesterol efflux, the predictions made by PANTHER and PolyPhen were significantly different for two mutations: N935S and P2150L. PolyPhen predicted N935S to be benign, while PANTHER predicted it to be deleterious. Conversely PolyPhen predicted P2150L to be probably damaging while PANTHER predicted it to be neutral. In both of these cases the PANTHER predictions were correct. Using the set of twelve *ABCA1* variants described by

Cohen et al. for which macrophage efflux is reported (15) as a second data set, the PANTHER prediction differs significantly from PolyPhen in one case, where PANTHER correctly predicts that L1026P will be deleterious (efflux rates less than or equal to two standard deviations below control levels), while PolyPhen predicts the substitution will be benign. Therefore, while PANTHER predictions correlate quite closely with those of PolyPhen overall, the PANTHER predictions are correct in the 3 instances in which they differ significantly, representing a significant difference in the ability of these two approaches to correctly identify functionally significant alleles ($P<0.05$, Fisher's exact test).

3.3. Discussion

By combining bioinformatics predictions with direct biochemical assessment, we have shown that it is possible to accurately predict the functional consequence of amino acid variation on protein function using an evolutionary model. We demonstrated that it is possible to differentiate cSNPs from mutations in *ABCA1* using data from PANTHER (Figure 3.1), indicating that *ABCA1* mutations tend to occur at much more highly conserved positions in evolutionarily related proteins compared to cSNPs. This finding within a single gene is in agreement with the genome-wide finding that mutations from the Human Gene Mutation Database have lower subPSEC scores than the randomly collected SNPs from the dbSNP database (6).

We used an in vitro test of ABCA1 function in stably transfected polyclonal cell lines to determine the biochemical impact of the *ABCA1* sequence variants, and to evaluate the predictions made by PANTHER. Overall PANTHER correctly predicted the functional impact of >94% (16/17) of the naturally occurring sequence variants that we examined. The subPSEC

score cutoff of –3 suggested in the initial characterization of subPSEC scores (6) worked well for predicting functional variants in *ABCA1*, and no other cutoff would have improved the prediction accuracy. In addition, cholesterol efflux was significantly correlated with the probability of a deleterious effect for *ABCA1* mutations, as well as across all variants, indicating that PANTHER scores are a significant and reliable predictor of the degree of functional impact of ABCA1 amino acid variants.

Both the D1289N and P2150L mutations are reported as pathogenic, and causative of disease in the TD patients in which they were identified (4;9;10). However, we predicted that these variants would not impair ABCA1 function based on the variability of the sites at which they occur in evolutionarily related proteins. We were able to confirm this prediction in vitro, indicating that these mutations are benign sequence variants and are unlikely to be causal of disease. The TD patient described with the D1289N variant was also homozygous for a second mutation, R2081W (10), and our results strongly suggest that it is this second mutation, and not D1289N that causes the phenotype observed in that patient. The molecular cause of the phenotype in patients carrying the P2150L variant remains to be determined and it is possible that these patients harbor a second, yet unidentified coding or non-coding variant.

The amino acids at positions 1289 and 2150 are conserved among all ABCA1 orthologs we examined, but not among the closely paralogous ABCA7 and ABCA4 (Entrez GeneID: 24) subfamilies. Because conservation patterns in ABCA1 proteins have persisted for only a relatively short time in evolutionary history, it is difficult to determine if the conservation at a given position among ABCA1 orthologs is due to functional constraint, or simply reflects random chance. Accordingly, the amino acid probability profiles for these positions are determined only from ABCA1 orthologs (see Materials and Methods), which do not contain

enough sequence variability to conclusively assume functional constraint. Our efflux data showing that the D1289N and P2150L mutations are functionally neutral confirm the prediction that the conservation of these residues amongst ABCA1 proteins is not due to functional constraint, but rather reflects their recent common ancestry.

One *ABCA1* cSNP, S1731C, had a subPSEC score less than -3. Interestingly, this SNP has been described in a French-Canadian family which also carries the 2144X stop mutation on a separate *ABCA1* allele (11). Individuals carrying both the 2144X mutation and S1731C had significantly lower HDL cholesterol than individuals with only the 2144X mutation, although the number of patients in each group was small (11). Cells transfected with the S1731C allele expressed *ABCA1* mRNA at levels comparable to wildtype *ABCA1*; however, this cell line expressed low levels of ABCA1 protein and was markedly deficient in cholesterol efflux. Substitution of cysteine for serine at this residue therefore significantly impairs ABCA1 function, as predicted by PANTHER analysis, by interfering with protein expression. The mechanism by which this SNP inhibits protein expression remains to be determined, but may involve expression of an unstable protein which is rapidly degraded, or interference with protein translation. The identification of S1731C as a functionally significant variant indicates that it may be a useful DNA marker to be used in association studies. These results also demonstrate that PANTHER may be a useful tool in general to identify functional SNPs that would be most useful in association studies.

Recently it has been reported that rare amino acid variants in ABCA1 occur in a significant percentage of individuals from the general population with low HDL cholesterol (15). We predicted the functional consequence of these 24 *ABCA1* sequence variants using PANTHER. The subPSEC scores of this group of variants are intermediate between and significantly

different from those of both *ABCA1* mutations and SNPs. We show that the subPSEC score distribution for these variants is not due to random rare variants, which is consistent with the conclusion that these variants are not simply randomly sampled but are biased toward deleterious functional effects. Our data are therefore in agreement with Cohen et al. in that many (~58%) of these variants are predicted to impair ABCA1 function and could therefore underlie the low HDL phenotype in these patients. However, subPSEC scores for these variants are also significantly greater than the subPSEC scores for mutations involved in the Mendelian disorder Tangier Disease. This suggests that a substantial proportion of these variants may not impair ABCA1 function, and therefore suggest that other genes, or other undetected ABCA1 variants could be responsible for the low HDL phenotype in these individuals. Consistent with this hypothesis, ~33% of the variants functionally tested by Cohen et al. were not functionally impaired (15).

It is equally informative to consider the incorrect predictions made by our evolutionary conservation-based method, in order to understand the limitations of this method and to suggest how it might be improved. The incorrect predictions occurred at two positions in ABCA1, 883 and 1091. The subPSEC score for the naturally occurring M1091T mutation (-3.56) is only marginally predictive of a negative impact on function, but this variant resulted in a severe reduction in ABCA1 function, consistent with the severe phenotype observed in patients harboring this mutation (4;14). In addition, both the M1091V and M1091L substitutions severely impaired the function of ABCA1, yet they were predicted to be functionally neutral. Among closely related ABCA1 homologs, ABCA2 (Entrez GeneID:20) and ABCA4 both share a methionine at this position, while ABCA7 substitutes a leucine. From the evolutionary tree, it is apparent that the ancestral amino acid at this position is likely to have been methionine, with ABCA7 diverging from the ancestral sequence (Figure 2A). Therefore, when calculating amino

acid probabilities for position 1091, the subPSEC method includes sequences from only ABCA1 and ABCA4, which represents enough sequence variability to predict that a relatively radical mutation such as M1091T will likely be deleterious, but not enough to predict that relatively conservative mutations such as M1091L or M1091V will be deleterious. Our experimental finding that M1091L severely impairs ABCA1 cholesterol efflux suggests that substitution of leucine for methionine at this position may have played an important role in the functional divergence of ABCA7 from ABCA1. Consistent with this hypothesis, ABCA1 and ABCA7 have recently been demonstrated to be functionally divergent, with ABCA7 facilitating the efflux of phospholipids but not cholesterol (17). In addition, the two proteins mediate the formation of distinct HDL particle subpopulations (18). Taken together, our efflux data and the functional divergence of ABCA1 and ABCA7 suggest that human ABCA1 position 1091 is a critical functional site despite the relatively modest conservation at this position.

The I883M substitution results in a milder phenotype, with a modest but significant reduction in ABCA1-mediated cholesterol efflux. This variant is interesting, as both alleles are found in the human population and the minor allele, methionine, is the likely to be the ancestral allele at this position (Figure 4). Among the human ABCA1 orthologs, murine ABCA1 aligns valine at this position and the chimpanzee sequence aligns methionine. This divergence explains why a simple conservation-based approach predicts that I883M is a neutral substitution. However, *ABCA1* has recently been shown to be among the genes most likely to have been under positive selection since the divergence of humans with chimpanzees (19). Our experimental results showing increased efflux activity of the I883 versus M883 allele suggest that the M883→I883 mutation may have been one of the adaptive changes in ABCA1 which occurred during the evolution of modern humans. This also suggests that measures of positive selection, an approach complementary to measures of negative selection such as amino acid conservation, may be

useful for identifying functionally important residues in proteins, thereby improving algorithms for predicting the functional effect of amino acid substitution.

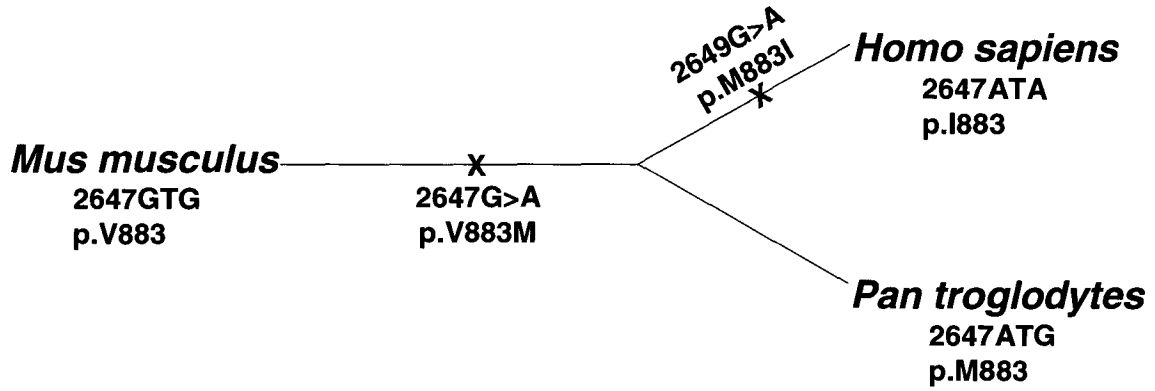

Figure 3.4 Graphical representation of the evolutionary relationship between mouse, human and chimpanzee ABCA1 proteins. ABCA1 amino acid position 883 genotype is displayed under the species name. An "X" represents the likely point in evolutionary history at which the V883→M883 and M883→I883 mutation events occurred. The M883→I883 mutation likely occurred since the divergence of the last common ancestor between humans and chimpanzees, and the increased activity of the I883 allele suggests that this may have been one of the adaptive changes that occurred during the evolution of modern humans.

The assumption of functional equivalence amongst homologs is fundamental to simple amino acid conservation analysis in general: the functional constraints on a position that lead to the observed conservation pattern are assumed to be constant (or approximately so) in all of the related sequences. However, there are many documented cases of missense substitutions whose phenotypic effect is highly dependent on genetic background. Among the most dramatic of these are human disease mutations that are fixed in the mouse genome with no similar phenotypic effect (20), as well as alleles that have different phenotypic effects in closely related mouse strains (21). In addition, the degree of functional constraint on a gene is dependent on details of the population, such as effective population size, and these effects are not accounted for in simple conservation-based approaches.

It is therefore perhaps not surprising that both cases for which PANTHER analysis failed to correctly predict the functional consequence of amino acid substitution occur at positions that

have apparently played a key role in the functional divergence of homologs, either paralogs (ABCA1 vs. ABCA7) or even orthologs (human vs. chimpanzee ABCA1). The lack of strict conservation at these positions is therefore not indicative of the lack of functional constraints; it is instead due to divergence of protein function, either to play a different role in the same organism (paralogs), or to adjust to different selective pressures in a different genetic, environmental or population background (orthologs). Other methodologies, such as those involving measures of positive selection, will be required to recognize when an amino acid change contributes to the divergence of function.

In summary, by combining a bioinformatics approach with biochemical functional assays we have been able to define the functional significance of genetic variation in the *ABCA1* gene, and validate the use of PANTHER as a robust approach to predicting allele function. These data have contributed to our understanding of the functional effect of *ABCA1* DNA variants, and in addition, suggest that PANTHER may be a useful tool in general for predicting the functional consequence of DNA variation.

3.4. Methods

3.4.1. Data Sets

We assembled a list of 30 missense mutations and 10 cSNPs reported in the *ABCA1* gene [5]. In classifying a variant as a "SNP" or "mutation" we have relied solely on their description in the literature, with mutations being reported as variants which segregate with Tangier Disease in a kindred, and SNPs being more common variants (>1% frequency) not associated with this disorder. We also examined the group of *ABCA1* variants recently reported to be associated with low concentrations of HDL cholesterol in a population based cohort (15).

3.4.2. Calculation of Substitution Position Specific Evolutionary Conservation Scores

We used data from PANTHER (6) to predict the functional consequence of each of the *ABCA1* variants described above. The ABCA1 protein (Genbank accession no. NP_005493) was aligned to the highest-scoring PANTHER (version 6.0, October 2005) Hidden Markov Models (HMMs): accession numbers PTHR19229 (ABCA transporter family) and PTHR19229:SF34 (ABCA1 subfamily). Substitution position-specific evolutionary conservation score calculations were modified from previous descriptions (6;7) by using the equation subPSEC=$0.88\ln(P_{min})$-$0.89\ln(P_{max})$-$0.94\ln(n_{ic})$, where P_{min} and P_{max} are the probabilities of the lower and higher probability amino acids that are being evaluated, respectively, and n_{ic} (number of independent counts) is the number of observations used to calculate the probabilities (B. Lazareva, A. Kejariwal, P.D. Thomas, manuscript in preparation). We discuss this method in more detail below. Reported subPSEC scores for nearly all missense variants are available on-line at the PANTHER database cSNP scoring page (22). A small number of variants occur at positions that do not align to the PANTHER library Hidden Markov Model (because these positions are not present in most sequences that are members of the ABC transporter subfamily A). To calculate subPSEC scores at these positions, we built an HMM according to the method described in (6), using human ABCA1 as the seed sequence. Briefly, this method forces each position in the seed sequence to be modeled as a "match state" (so that a probability vector will be calculated for every position), aligns all other sequences in PTHR19229 to the seed, and then re-estimates the probability vectors using all of the aligned sequences. The alignment is not as accurate overall as the PANTHER library alignment, but we verified that it is accurate for the few positions that were not modeled by the PANTHER library alignment.

The rationale and methodology for calculating substitution position-specific evolutionary conservation (subPSEC) scores has been reported previously [6,7], but we briefly describe it here, as well as describing recent improvements to the method (B. Lazareva, A. Kejariwal & P.D. Thomas, manuscript in preparation). The goal is to predict the functional effect of single amino acid substitutions in proteins. From genomic and cDNA sequencing there exists a great deal of data about related protein sequences in extant organisms. To the extent that these related sequences perform the same function, they are under similar evolutionary constraints. Some of the random mutations that occur during protein sequence evolution are functionally neutral and can be fixed in extant sequences, while most mutations are selected against and will not appear in any of the extant sequences. The effect of this negative selection is apparent in the pattern of amino acids that appear in the equivalent positions in related proteins. First order HMMs have proved to be an excellent method for generating statistics on amino acid probabilities for modeling protein families (23). In these HMMs, each equivalent position in related proteins is treated as a series of observations that were "generated" by a "hidden" model represented as a Markov chain. Each position is modeled as a vector (or "profile") of 20 probabilities, one for each amino acid type. This profile is derived using a Bayesian method that weights prior knowledge (e.g. of physico-chemical similarities between amino acids) more heavily when there are few observations. The method also weights different sequences depending on their relatedness: for example, given a human sequence, the yeast ortholog will be weighted more heavily in deriving the probability vector than the chimp sequence because the human and chimp sequences have had very little time to diverge compared to human and yeast, and their mutually conserved positions are due more to recent common ancestry than to negative selection. It also allows a more general definition of "conservation pattern", as a profile can represent conservation of a single amino acid (high probability for only methionine, for example), or conservation of a class of amino acids (e.g. high probabilities for only hydrophobic amino acids).

To score the substitution of amino acid *b* by amino acid *a*, the subPSEC score uses the position-specific probabilities of *a* and *b* in the profile, according to the equation: subPSEC = $\mathbf{ln}(P_a/P_b)$, so that as P_a becomes smaller compared to P_b, subPSEC becomes increasingly negative. Smaller subPSEC scores therefore predict a higher probability of a deleterious functional effect.

The critical assumption in the subPSEC method is that the evolutionary constraints are the same across the sequences used to build the amino acid profile. This is generally accepted to be approximately true for orthologous sequences (sequences related by a speciation event), and is the basis for complementation experiments. Whether orthologs or paralogs, more closely related sequences are more likely to have similar functional constraints on their evolution, but as described above are less useful statistically because of their recent common descent. We have therefore modified the original subPSEC method to perform position-specific phylogenetic sampling (6). In this method, the set of sequences used to build the amino acid profile can be different for each position. If the position is variable among orthologs, or conserved among orthologs but not paralogs, then only orthologs are used to calculate the profile because a sequence divergence from other subfamilies of paralogous proteins may be due to functional divergence. However, if the same amino acid is conserved in all the orthologous sequences as well as in the closest paralogous subfamily, we can add the paralogous sequences to the set used to calculate the amino acid profile. In this case, we can assume that the evolutionary constraints are similar, at least for that position. We can therefore use statistics from paralogous proteins when the same amino acid is conserved at the same position. This is done iteratively until either a position is found to be divergent in a subtree, or to be conserved across all paralogs in the tree. We find that the diversity of sequences over which a position has been conserved (n_{ic}, or number of independent counts) is a useful term to add to the subPSEC score, in addition to the probabilities of the amino acids P_a and P_b. We performed log-linear logistic regression to obtain

the best discrimination between human disease-causing variation and normal human variation (HGMD vs. dbSNP, as described in (7)), obtaining estimates and standard deviations for the coefficients in the equation:

$$\ln(P_{deleterious}/(1-P_{deleterious})) = C_a \ln P_a + C_b \ln P_b + C_n \ln n_{ic} + C, \quad [1]$$

where *a* is the less probable amino acid and *b* is the more probable. The coefficients and standard deviations in their estimates were: $C_a = 0.89 \pm 0.03$, $C_b = -0.88 \pm 0.03$, $C_n = 0.94 \pm 0.04$, $C = 3.00 \pm 0.13$. For this study, subPSEC was calculated as:

$$\text{subPSEC} = 0.89 \ln P_a - 0.88 \ln P_b + 0.94 \ln n_{ic} \quad [2]$$

meaning that a cutoff of –3 corresponds to a 50% probability that the score came from HGMD (presumably mostly deleterious) or dbSNP (presumably mostly neutral). This not only allows us to calculate a subPSEC score, but to convert that score into a probability of deleterious functional effect $P_{deleterious}$ from equation [1] above.

The distribution of random subPSEC scores for ABCA1 were calculated as described previously (7). Briefly, all single-nucleotide substitutions in the coding sequence of ABCA1 were generated, and those that led to amino acid substitutions were assigned subPSEC scores. The distribution was obtained by weighting each amino acid substitution according to the transition/transversion probabilities of the corresponding nucleotide change.

3.4.3. Generation of Stable Cell Lines

Polyclonal stable cell lines expressing ABCA1 sequence variants were generated using the Flp-in system (Invitrogen) as previously described (24). The generation and detailed biochemical characterization of many of these cell lines is described elsewhere (8). Briefly, the nucleotide mutations were incorporated into a human *ABCA1* cDNA using PCR based site-directed mutatgenesis as previously described (25) (primer sequences and PCR protocols available on request) and cloned into the pcDNA5/FRT expression vector (Invitrogen). All plasmids were

completely sequenced prior to transfection. Stable cell lines were generated by co-transfecting human embryonic kidney 293 Flp-in cells (Invitrogen) with the mutation-harbouring plasmid and the pOG44 plasmid (Invitrogen). Transfected cells were maintained in DMEM (Gibco) supplemented with 10% FCS, L-glutamine and penicillin and streptomycin. Hygromycin-resistant colonies were selected for in 75 μg/ml of Hygromyocin (Invitrogen), trypsinized and pooled to generate polyclonal cell lines.

3.4.4. Western Blotting and RT-PCR

ABCA1 expression was determined by western blotting, as previously described (26). Briefly, cells were lysed in 20 mm Hepes, 5 mm KCl, 5 mm $MgCl_2$, 0.5% (v/v) Triton X-100, and complete protease inhibitor (Roche), and protein concentration was determined by the Lowry assay. Equivalent amounts of total protein were separated by SDS-PAGE, transferred to PVDF membranes and probed with anti-ABCA1 (26) or anti-GAPDH (Chemicon) antibodies.

RNA was isolated from cells using Trizol reagent (Life Technologies), and 3 μg of total RNA was reverse-transcribed using Superscript II (Life Technologies). RT-PCR was performed using previously described primers and protocols (27).

3.4.5. Cholesterol Efflux

Efflux experiments were performed as previously described (24). Briefly, cells were loaded overnight with 1 μCi [^{3}H] cholesterol (Amersham) in DMEM supplemented with 10% FCS, L-glutamine and penicillin and streptomycin. The following day, the media was removed and replaced with serum-free media containing 0.2 % delipidated bovine serum albumin (Sigma). After a one hour incubation 10 μg/ml human apolipoprotein A-I (Athens) was added. After 4 hours media was removed and centrifuged and cells were lysed in 0.2 % SDS. The amount of [^{3}H] cholesterol in supernatant and cells was determined by liquid scintillation spectroscopy.

Cholesterol efflux values are the mean of at least 3 separate assays each performed in triplicate, and are presented as mean ± standard deviation of the difference between efflux in the presence and absence of apoA-I. Each assay was performed together with wildtype ABCA1 and values are expressed as percent of wildtype efflux. Significance was calculated using a one-way ANOVA test with a Newman-Keuls post-test using Graphpad Prism 4 software.

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Chapter 4 - Intestinal ABCA1 Directly Contributes to HDL Biogenesis In Vivo[1]

4.1. Introduction

High density lipoprotein (HDL) particles mediate the transport of cholesterol from peripheral tissues to the liver in a process termed reverse cholesterol transport (1;2), which is postulated to explain, at least in part, their ability to protect against foam cell formation and atherosclerosis. Despite the widespread interest in HDL as a potential therapeutic target (3), the origins of plasma HDL are still elusive. The ATP-binding cassette transporter A1 (ABCA1) mediates the rate-controlling step in HDL particle formation by promoting the efflux of cholesterol and phospholipids to apolipoprotein A-I (apoA-I) (4;5). Mutations in ABCA1 cause Tangier Disease (6-8), characterized by near absence of HDL cholesterol and increased risk for atherosclerosis (9-11). ABCA1 is widely expressed throughout the body (12;13); however, the contributions of ABCA1 in specific tissues to HDL levels and reverse cholesterol transport are still being unraveled, and only recently the role of hepatic ABCA1 in homeostasis of HDL levels was elucidated.

Overexpression of hepatic ABCA1 raises HDL cholesterol levels (14;15) and liver-specific deletion of ABCA1 results in a substantial (~80%) decrease in plasma HDL cholesterol in chow-fed mice (16). Similarly, a 50% knockdown of hepatic ABCA1 expression by adenovirus-mediated RNA interference in mice is associated with a 40% decrease in HDL cholesterol (17). These results indicate that the liver is the single most important source of plasma HDL in vivo, but also suggest the existence of additional, extrahepatic sites of HDL biogenesis.

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[1] A version of this chapter has been published. Brunham LR, Kruit JK, Iqbal J, Fievet C, Timmins JM, Pape TD, Coburn BA, Bissada N, Staels B, Groen AK, Hussain MM, Parks JS, Kuipers F, Hayden MR. Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. J Clin Invest. 2006 Apr;116(4):1052-62

The intestine, along with the liver, is an important site for the synthesis and secretion of apolipoprotein A-I (apoA-I), the principal apoprotein of HDL, and contributes approximately 50% of total plasma apoA-I (18). In humans, approximately 1.5 grams of dietary and biliary cholesterol enters the intestinal lumen each day (19). Cholesterol that is absorbed or synthesized locally in the enterocytes (20) is thought to be either expelled into the intestinal lumen through the actions of the ABCG5/ABCG8 heterodimeric transporter for eventual excretion via the feces (21), or esterified by the action of ACAT and packaged into chylomicrons and very low density lipoprotein particles for transport into the body (22). Cholesterol transport from the intestine is thought to occur almost exclusively by the lymphatic system (23).

A potential role for the intestine in HDL particle assembly was initially inferred from studies in hepatectomized dogs (24) and studies describing the presence of HDL of distinct composition and morphology in mesenteric lymph of rats (25-28). More recent studies in cultured cells have identified an apoB-independent pathway of cholesterol secretion from intestinal epithelial cells (29-31). However, the physiological contribution of the intestine to HDL levels is unknown, and the molecular mechanisms involved in intestinal HDL assembly are obscure. In addition, the origin of mesenteric lymph HDL has been a subject of considerable controversy (32-36), raising the question of whether HDL in mesenteric lymph is in fact derived from the intestine.

To specifically assess the role of intestinal ABCA1 in HDL metabolism in vivo, we have generated mice that lack ABCA1 exclusively in the intestine (*Abca1*$^{-i/-i}$ mice). Our results show that intestinal ABCA1 is importantly involved in HDL biogenesis and contributes approximately 30% to steady-state plasma HDL cholesterol levels in mice. Furthermore, our data suggest that intestinal ABCA1 participates in HDL assembly via a direct mechanism into the circulation.

4.2. Results

To evaluate the role of intestinal ABCA1 in HDL biogenesis in vivo, we generated mice that specifically lack ABCA1 in enterocytes by crossing *Abca1* floxed mice (16) to mice expressing Cre recombinase under the control of the intestinal epithelium-specific *Villin* promoter (37). The *Villin* promoter drives robust expression of Cre through the length of the small and large intestine from 12.5 days post coitum (37). $Abca1^{-i/-i}$ mice were born at the expected Mendelian frequencies, were fertile, and did not differ significantly in weight from littermate controls. Figure 4.1a shows Southern blot analysis of EcoRV-digested genomic DNA from livers and intestines of $Abca1^{+/+}$ and $Abca1^{-i/-i}$ littermates probed with a DNA fragment corresponding to intron 44. Cre-mediated deletion of *Abca1* is evident in the intestine of $Abca1^{-i/-i}$ mice, but not in the liver of these mice, or in the intestine or liver of $Abca1^{+/+}$ controls (Figure 4.1a).

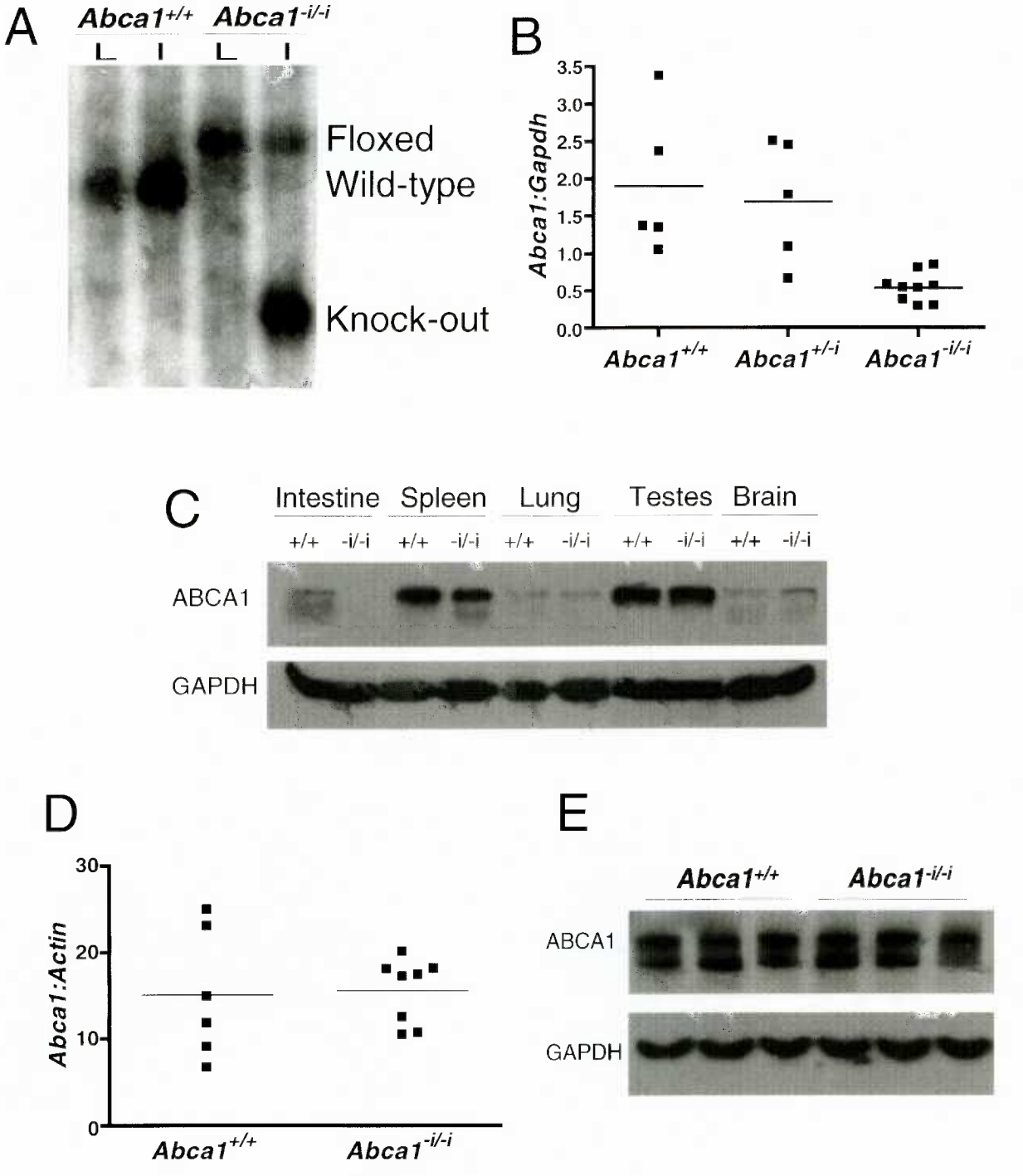

Figure 4.1 Generation of ABCA1 intestinal specific knockout mice (*Abca1*$^{-i/-i}$). (A) Southern blot of genomic liver (L) and intestine (I) DNA from mice with wildtype (+/+) or floxed (-i/-i) alleles in the presence of Cre recombinase. DNA was digested with EcoRV and hybridized with a probe to the genomic region between exon 44 and 45 in the *Abca1* gene to produce the 6 kb wildtype, 7.3 kb floxed or 4.2 kb knock-out bands. (B) Quantitative real-time PCR of RNA isolated from mouse intestine. Reverse-transcribed RNA was amplified with oligos specific for *Abca1* and *Gapdh*. (C) Western blot of tissue lysates from control (+/+) and *Abca1*$^{-i/-i}$ (-i/-i) mice with antibodies to ABCA1, and GAPDH as loading control. (D) Quantitative real-time PCR of RNA isolated from livers of *Abca1*$^{+/+}$ and *Abca1*$^{-i/-i}$ mice. Reverse-transcribed RNA was amplified with oligos specific for *Abca1* and *Actin*. (E) Representative western blot of liver lysates from *Abca1*$^{+/+}$ and *Abca1*$^{-i/-i}$ mice.

Recombination of the floxed *Abca1* allele resulted in a significant decrease in intestinal ABCA1 mRNA (Figure 4.1b) and protein levels (Figure 4.1c). ABCA1 protein expression in other tissues was unaltered (Figure 4.1c), indicating that *Abca1*$^{-i/-i}$ mice specifically lack ABCA1 in the intestine.

Hepatic ABCA1 levels are a critical determinant of plasma HDL cholesterol concentration (16). Figure 4.1d shows hepatic *Abca1* mRNA levels as determined by quantitative real-time PCR, revealing no alteration in expression of hepatic ABCA1 in *Abca1*$^{-i/-i}$ mice compared to controls. Figure 1E shows a representative immunoblot of liver lysates from *Abca1*$^{-i/-i}$ mice and controls, indicating that hepatic ABCA1 protein expression is normal. Therefore, hepatic ABCA1 levels are unaltered by deletion of intestinal ABCA1, and do not exhibit compensatory changes.

Figure 4.2 shows ABCA1 expression in mouse small intestine as detected by immunofluorescence. Ten micron sections of fixed mouse intestine were stained with a polyclonal antibody to ABCA1 (12) (green), and counterstained with DAPI for cell nuclei (blue). ABCA1 protein was found to be highly expressed in enterocytes of the ileum (Figure 4.2a, b, and c) and jejunum (Figure 4.2d). ABCA1 expression was primarily observed in the intestinal villi, with less expression in crypts, consistent with a role in the absorptive regions of the intestine. ABCA1 expression was not detectable in the duodenum, cecum or colon of wildtype mice (data not shown). ABCA1 appeared to be present both intra-cellularly and at the plasma membrane, including the basolateral membrane (Figure 4.2d), consistent with previous reports of ABCA1 localization (38;39). *Abca1*$^{-i/-i}$ mice had undetectable ABCA1 protein expression in all sections analyzed (Figure 4.2e, f and g), indicating complete ablation of ABCA1 protein

expression in these mice. Figure 4.2h shows a section of wildtype jejunum stained with a random IgG to control for non-specific staining.

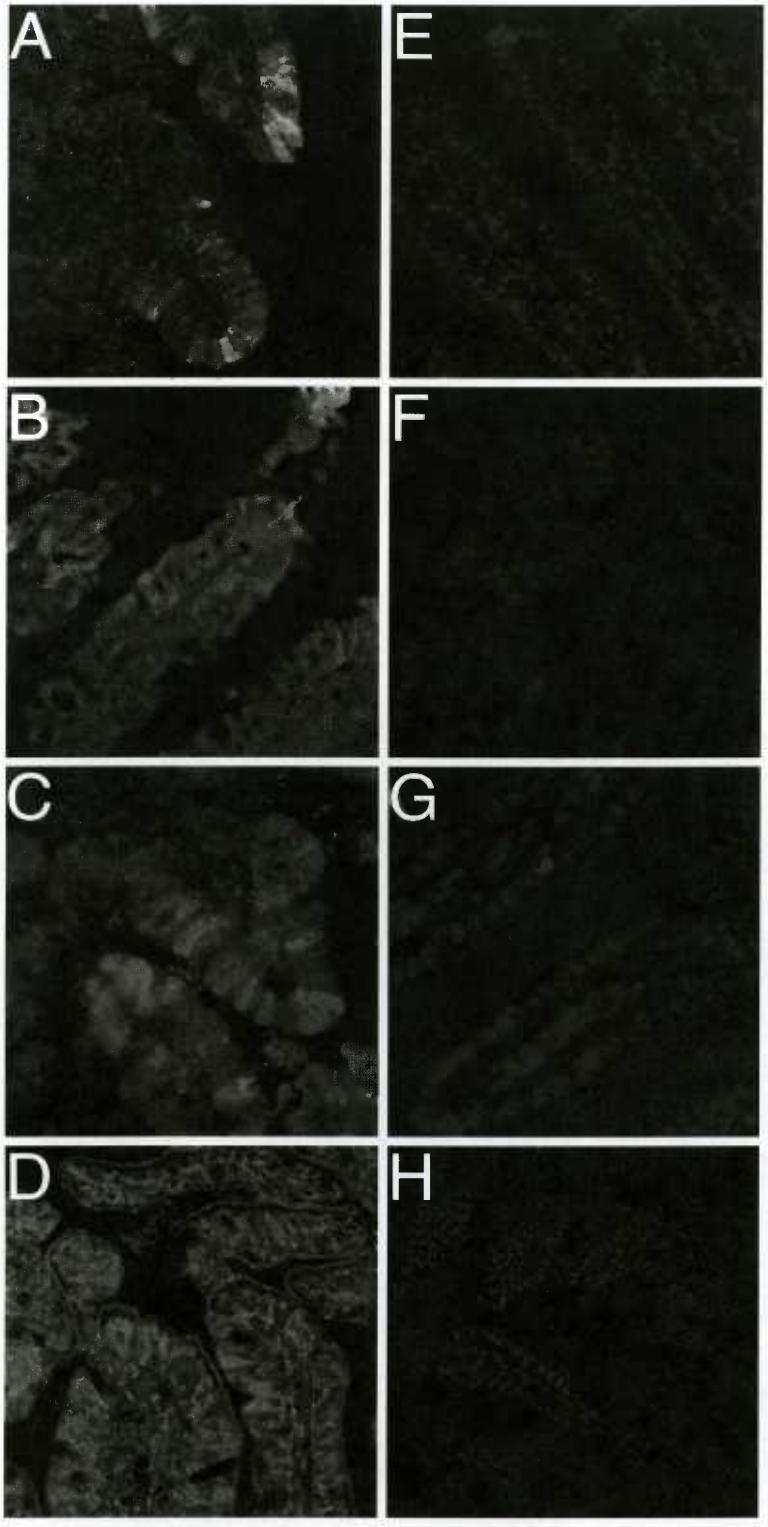

Figure 4.2 Expression of ABCA1 in mouse intestine. Ten micron sections of mouse intestine were stained with an antibody to ABCA1 (green), or with DAPI (blue) or phalloidin (red). (A) Ileum, 20x magnification, (B and C) ileum, 40x magnification from *Abca1*$^{+/+}$ mice. (E, F and G) are

Fasting plasma lipid and apolipoprotein concentrations in *Abca1*$^{+/+}$, *Abca1*$^{+/-i}$ and *Abca1*$^{-i/-i}$ mice are shown in Table 4.1. Total plasma cholesterol was significantly reduced by approximately 30% in *Abca1*$^{-i/-i}$ compared to *Abca1*$^{+/+}$ mice, due primarily to a significant reduction in plasma HDL cholesterol (~30%, $P<0.001$). Plasma levels of apoA-I and apoA-II were significantly decreased by ~25% and 35%, respectively ($P\leq0.05$, Table 4.1). Plasma apoB was also significantly reduced in *Abca1*$^{-i/-i}$ mice compared to controls by approximately 30% ($P<0.01$, Table 4.1). Notably, a gene-dosage effect was evident, with *Abca1*$^{+/-i}$ heterozygotes showing total plasma cholesterol and HDL cholesterol levels intermediate between wildtype and knockout mice and significantly reduced compared to wildtype ($P<0.05$, Table 4.1). Plasma apoA-I concentrations in *Abca1*$^{+/-i}$ heterozygotes tended to be reduced compared to controls, but this difference was not statistically significant.

Table 4.1 Plasma lipid and apolipoprotein values for ABCA1 intestinal specific knock-out mice consuming a standard lab chow diet

Abca1 Genotype	TPC (mg/dl)	HDL-C (mg/dl)	TG (mg/dl)	ApoA-I (mg/dl)	ApoA-II (mg/dl)	ApoCIII (mg/dl)	ApoB (mg/dl)
+/+	116 ± 5 (21)	75 ± 2 (31)	105 ± 12 (9)	81 ± 4 (9)	152 ± 9 (9)	58 ± 7 (9)	21 ± 1 (9)
+/-*i*	96 ± 6 (13)[a]	63 ± 5 (12)[a]	91 ± 9 (8)	76 ± 6 (8)	128 ± 15 (8)	51 ± 7 (8)	18 ± 2 (8)
-*i*/-*i*	81 ± 7 (14)[b]	49 ± 6 (10)[b]	105 ± 10 (6)	61 ± 10 (6)[a]	101 ± 17 (6)[a]	43 ± 4 (6)	15 ± 1 (6)[a]

Blood was obtained from mice after a 4 hour fast. Values are mean ± SEM. Number of mice is indicated in parentheses. TPC, total plasma cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglyceride; ApoA-I, apolipoprotein A-I. [a]$P\leq0.05$, [b]$P<0.001$ compared to +/+.

FPLC analysis confirmed the significant decrease in HDL cholesterol concentration in *Abca1*$^{-i/-i}$ mice (Figure 4.3a). No shift in particle size was evident, indicating that the reduction in HDL cholesterol primarily reflected a reduction in the number of similarly sized particles. Quantification of the cholesterol concentration in the FPLC-separated lipoprotein classes confirmed that HDL cholesterol was reduced by approximately 30% in *Abca1*$^{-i/-i}$ homozygotes (Figure 4.3b, $P<0.05$) and also revealed a significant decrease in LDL cholesterol concentration

(Figure 4.3b, $P<0.05$), similar to that observed in Tangier Disease patients and in ABCA1 total knockout mice (40;41).

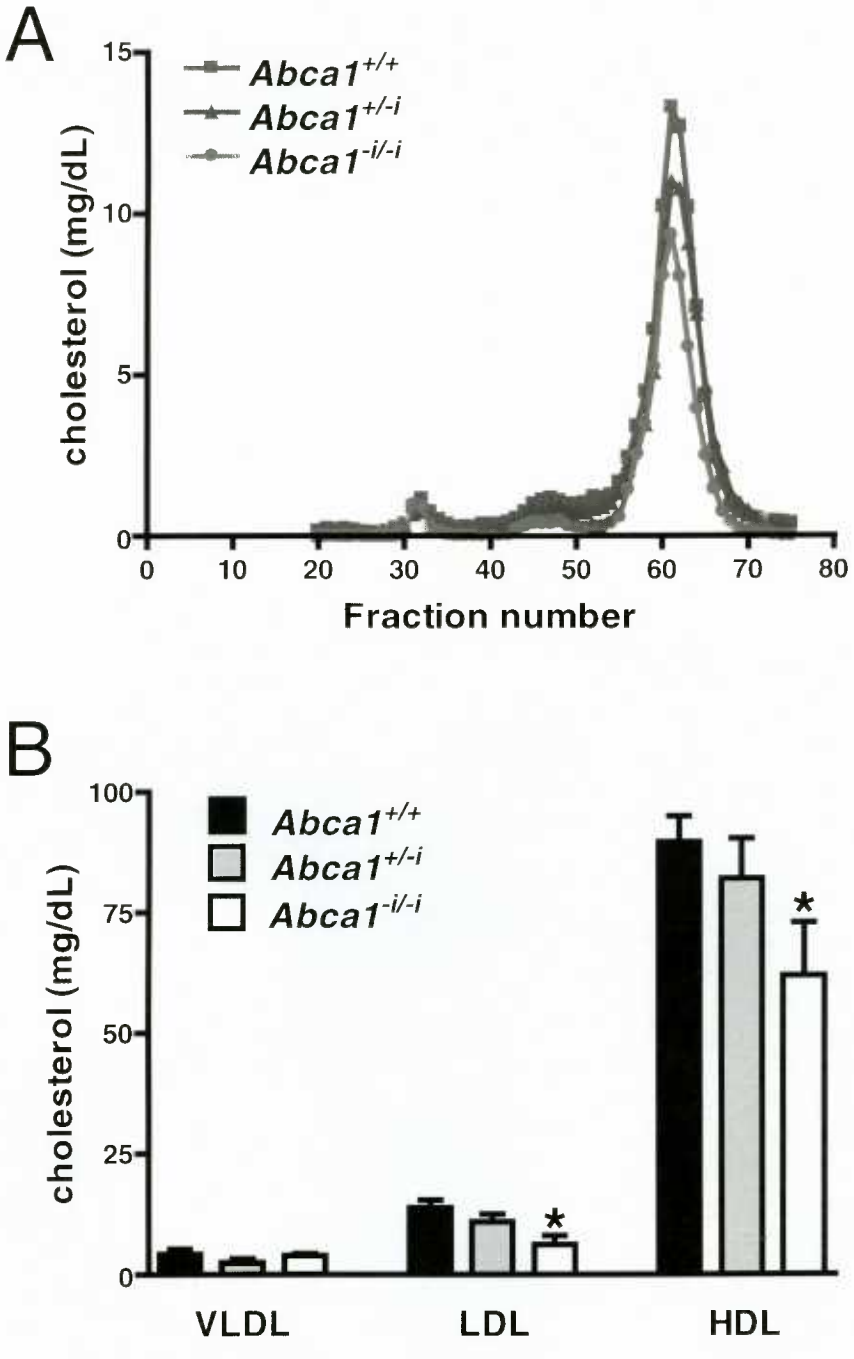

Figure 4.3 Analysis of plasma lipoproteins by fast protein liquid chromatography (FPLC). (A) Equal volumes of plasma from 4-hour fasted *Abca1*$^{+/+}$, *Abca1*$^{+/-i}$ and *Abca1*$^{-i/-i}$ mice were pooled and fractionated by FPLC. Total cholesterol in each fraction was determined by enzymatic assay. (B) Plasma from individual 4-hour fasted mice was fractionated and cholesterol concentration determined on-line by FPLC. n=6-9 mice per group. * $P<0.05$.

To determine the cumulative contribution of intestinal and hepatic ABCA1 to plasma HDL cholesterol levels, we bred mice that carry both the Villin-Cre and Albumin-Cre transgenes, such that ABCA1 is deleted from both liver and intestine (*Abca1*$^{-iL/-iL}$). Figure 4.4a shows plasma HDL cholesterol levels in control mice, and mice lacking intestinal ABCA1, hepatic ABCA1, or both. Compared to mice lacking hepatic ABCA1, mice lacking both intestinal and hepatic ABCA1 display a further significant decrease in plasma HDL cholesterol levels, indicating that the contribution of intestinal ABCA1 to HDL cholesterol levels is independent of hepatic ABCA1. Figure 4.4b shows HDL cholesterol levels as a percentage of respective age- and strain-matched controls. The combined deletion of both hepatic and intestinal ABCA1 results in ~90% decrease in plasma HDL cholesterol levels, similar to that seen in ABCA1 total knockout mice. Therefore, the intestine and liver are the two major sites of initial HDL particle assembly, and ABCA1 in other tissues cannot compensate for the loss of hepatic and intestinal ABCA1.

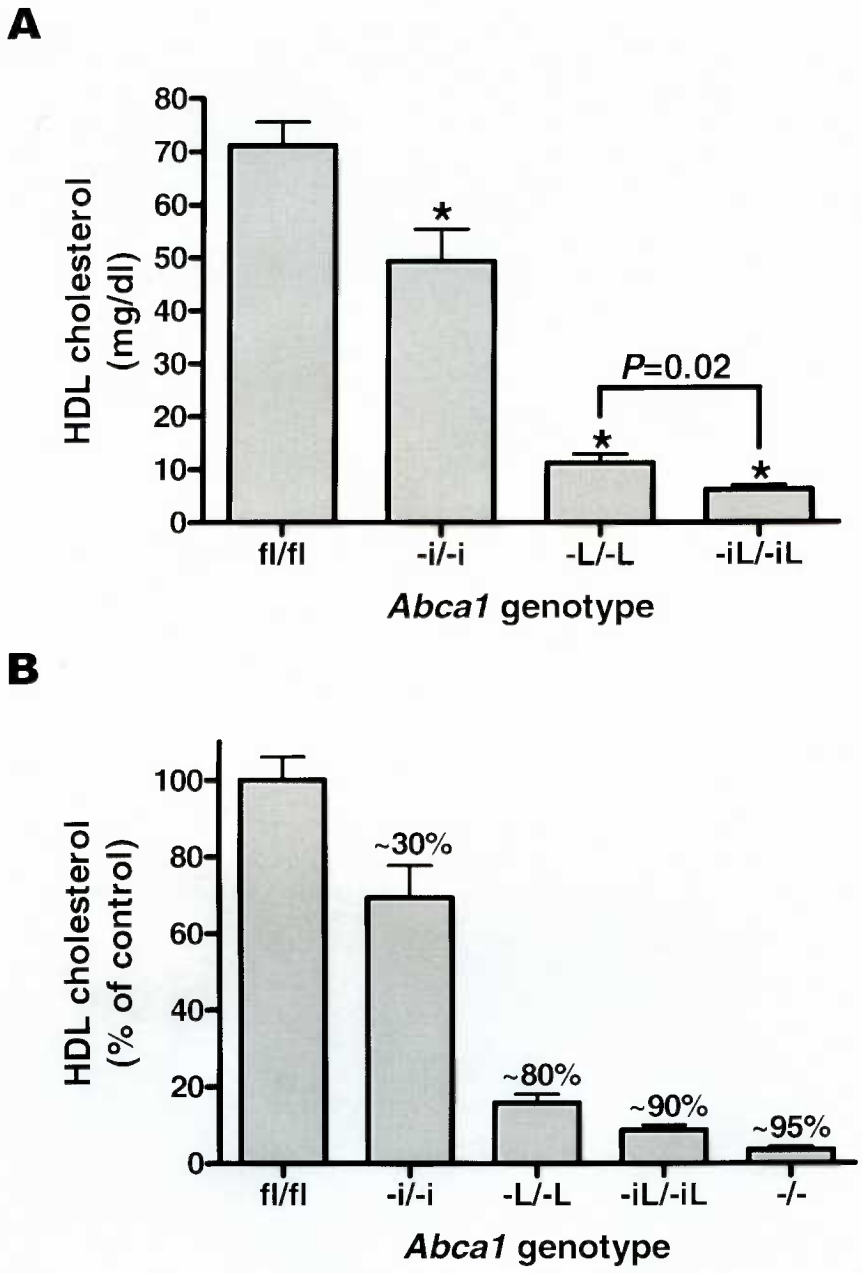

Figure 4.4 Tissue-specific contributions of ABCA1 to plasma HDL cholesterol levels. (A) Plasma HDL cholesterol levels in control mice, and mice lacking intestinal ABCA1 (-i/-i), hepatic ABCA1 (-L/-L), or both (-iL/-iL). Mice lacking both hepatic and intestinal ABCA1 have a further significant decrease in plasma HDL cholesterol compared to mice lacking hepatic ABCA1. (B) Plasma HDL cholesterol levels as a percentage of strain-matched controls. The percentage decrease compared to strain-matched control is indicated over each bar. Deletion of hepatic and intestinal ABCA1 results in a ~90% decrease in plasma HDL cholesterol, similar to that in mice lacking ABCA1 globally (-/-). n≥4 mice per group. *$P<0.01$.

To study the metabolic consequences of loss of intestinal ABCA1 on intestinal cholesterol transport, we fed *Abca1*$^{+/+}$ and *Abca1*$^{-i/-i}$ mice [^{3}H] cholesterol and measured the appearance of the cholesterol tracer in plasma, liver and intestine after 2 hours. Figure 4.5a shows the appearance of [^{3}H] cholesterol in plasma, HDL and non-HDL 2 hours after oral gavage. Mice lacking intestinal ABCA1 had a significant ~35% reduction in the level of [^{3}H] cholesterol in plasma and HDL after 2 hours (P<0.05). [^{3}H] cholesterol appearance in non-HDL was not significantly different between genotypes. Figures 4.5b and 4.5c show the amount of [^{3}H] cholesterol recovered in the liver and in the intestine after a luminal rinse, at 2 hours after the oral gavage. A*bca1*$^{-i/-i}$ mice displayed significantly less [^{3}H] cholesterol in liver (P<0.05, Figure 4.5b), as well as significant retention of [^{3}H] cholesterol in the small intestine compared to controls (P<0.05, Figure 4.5c). These data indicate that cholesterol uptake from the intestinal lumen is not impaired in mice lacking intestinal ABCA1, but that the transport of absorbed cholesterol into plasma is significantly reduced. The reduced appearance of radiolabeled cholesterol in plasma is unlikely to be secondary to the reduced plasma HDL cholesterol pool in these mice, because it has previously been shown that raising plasma HDL levels does not prevent decreased appearance of a gavaged cholesterol tracer in plasma in a chicken model of total ABCA1 deficiency (42).

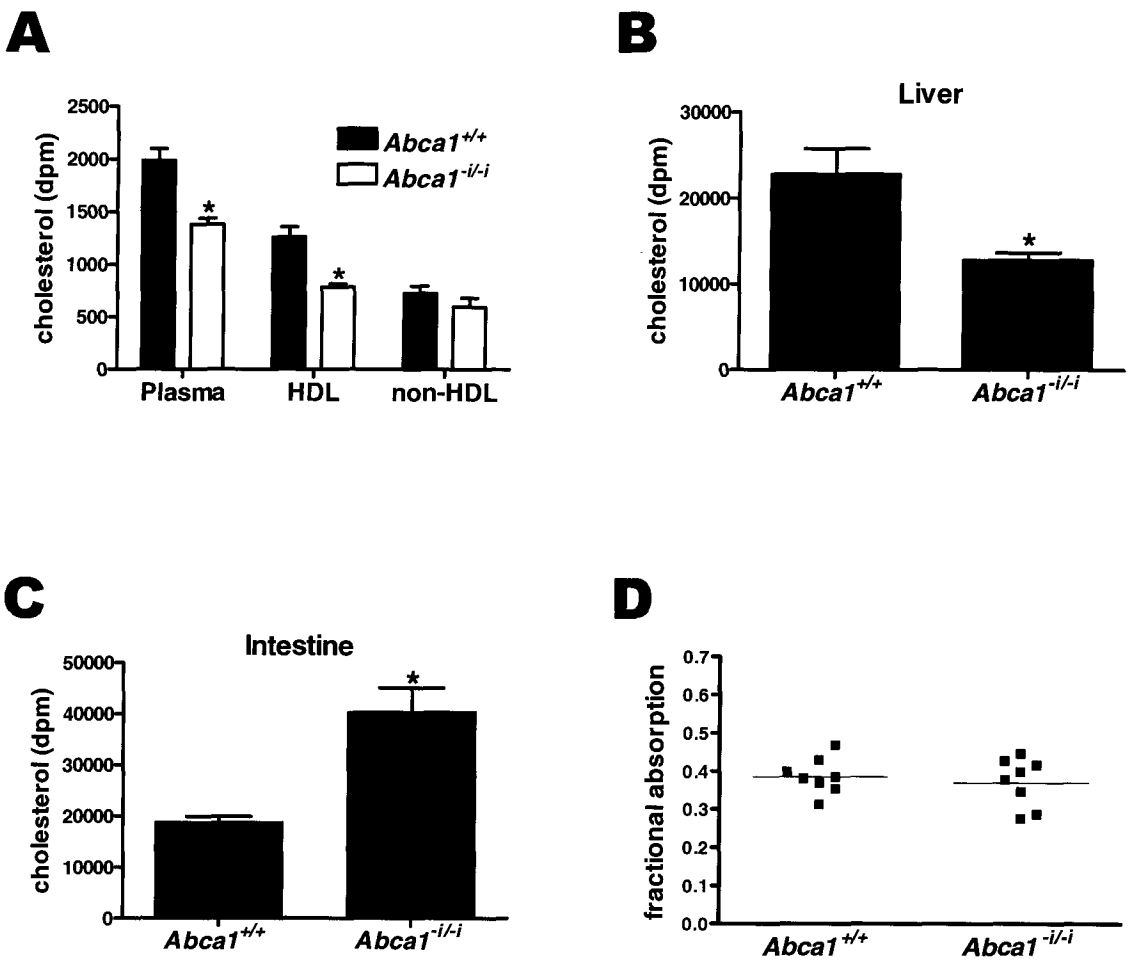

Figure 4.5 Intestinal cholesterol transport in mice lacking intestinal ABCA1. (A) Appearance of [^{3}H] cholesterol in whole plasma, HDL and non-HDL lipoprotein fraction after 2 hours. Mice were gavaged with 0.2 μCi of [^{3}H] cholesterol and the appearance of the tracer was assessed after 2 hours. (B) [^{3}H] cholesterol in liver 2 hours after oral gavage. (C) [^{3}H] cholesterol in small intestine 2 hours after oral gavage. The small intestine was rinsed with PBS to remove luminal contents. (D) Fractional cholesterol absorption determined by fecal dual-isotope method. n=6 mice per group. *P<0.05

To further assess the role of enterocyte ABCA1 in the uptake of luminal cholesterol, we measured fractional cholesterol absorption by the dual-fecal isotope method. Figure 4.5d shows fractional cholesterol absorption in *Abca1*$^{+/+}$ and *Abca1*$^{-i/-i}$ mice. We found no effect on this parameter in the absence of intestinal ABCA1, indicating that enterocyte ABCA1 does not influence luminal cholesterol absorption.

Figure 4.6 shows intestinal and hepatic cholesterol content in control mice and mice lacking intestinal ABCA1. Mice lacking intestinal ABCA1 displayed a trend towards increased total and free cholesterol in the small intestine, and a significant 10-fold increase in intestinal cholesterol ester content ($P<0.05$, Figure 4.6a). There was no difference in hepatic cholesterol content (Figure 4.6b). Figure 4.6c shows the relative amounts of mRNAs involved in sterol metabolism in intestines of control and *Abca1*$^{-i/-i}$ mice. Mice lacking intestinal ABCA1 had significantly increased levels of *Abcg5* and *Abcg8* ($P<0.05$) as well as a trend towards reduced levels of HMG-CoA synthase (*Hmgcs*) mRNA ($P=0.06$). These gene expression changes are consistent with increased sterol content in enterocytes, leading to activation of LXR target genes such as ABCG5 and ABCG8 (43), and suppression of cholesterol synthesis genes. No change was observed in the levels of *Npc1l1* mRNA, the putative cholesterol absorption protein (44;45), consistent with our finding of normal cholesterol absorption in *Abca1*$^{-i/-i}$ mice (Figure 4.5d).

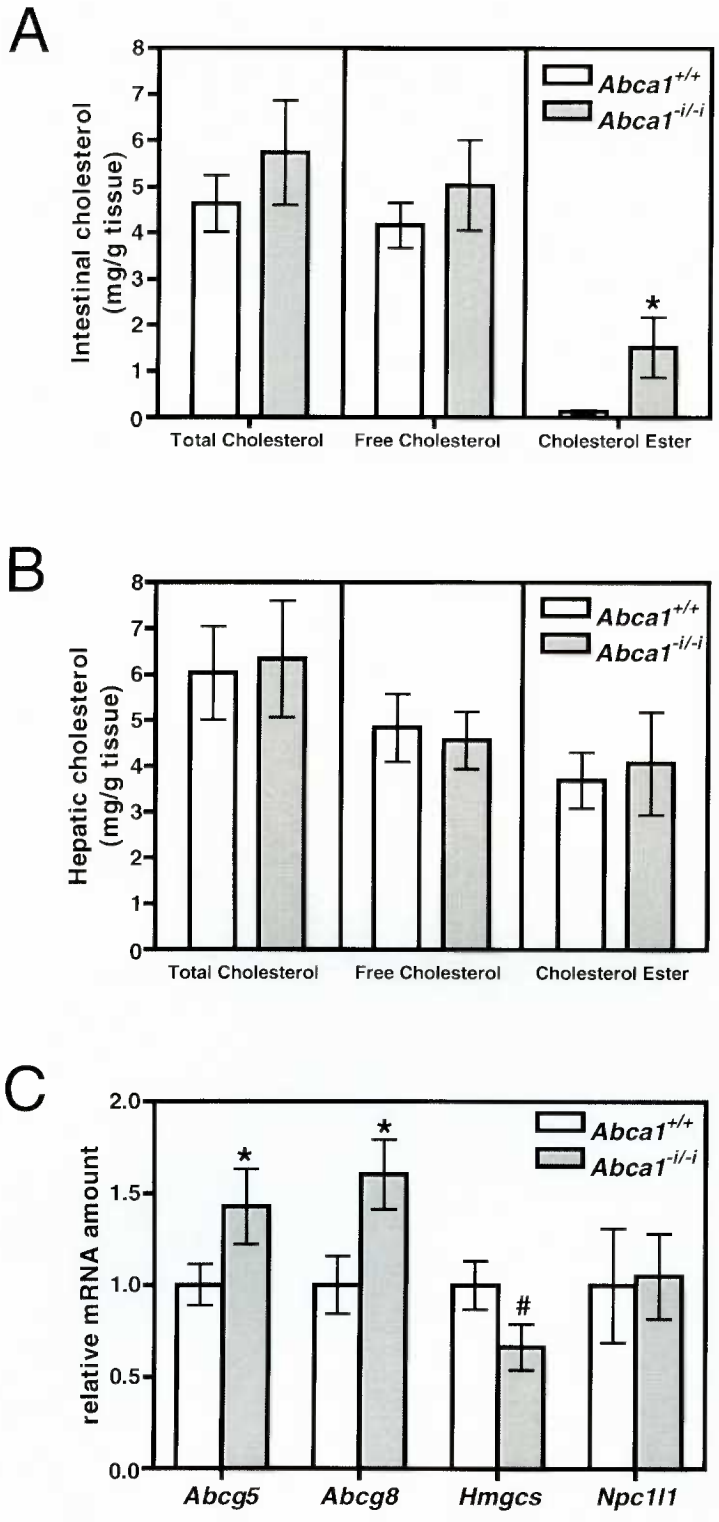

Figure 4.6 Tissue cholesterol levels and gene expression. (A) Intestinal cholesterol levels in control mice and mice lacking intestinal ABCA1. n=3 mice per group. (B) Hepatic cholesterol levels in control mice and mice lacking intestinal ABCA1. n=3 mice per group. (C) Relative amounts of various mRNAs in intestines from control mice and mice lacking intestinal ABCA1. Values are relative to the mRNA amount in control mice which is arbitrarily set as 1. n=4-8 mice per group. *$P<0.05$. #$P=0.06$.

To further characterize the mechanism by which intestinal ABCA1 influences plasma HDL cholesterol levels and intestinal lipid transport, we investigated cholesterol secretion by primary enterocytes isolated from *Abca1*$^{+/+}$ and *Abca1*$^{-i/-i}$ mice. This system has previously been shown to accurately model the secretion of cholesterol into both apoB-containing chylomicron and VLDL-sized particles, as well as non-apoB-containing particles in the size range of HDL (29;30). Primary enterocytes were isolated and incubated with [^{3}H] cholesterol and then chased for 2 hours in the presence of micelles. Figures 4.7a and 4.7b show [^{3}H] cholesterol levels in cells and media, respectively, after 2 hours. Enterocytes from *Abca1*$^{-i/-i}$ mice contained significantly more [^{3}H] cholesterol (Figure 4.7a) and secreted less [^{3}H] cholesterol into the media over 2 hours (Figure 4.7b).

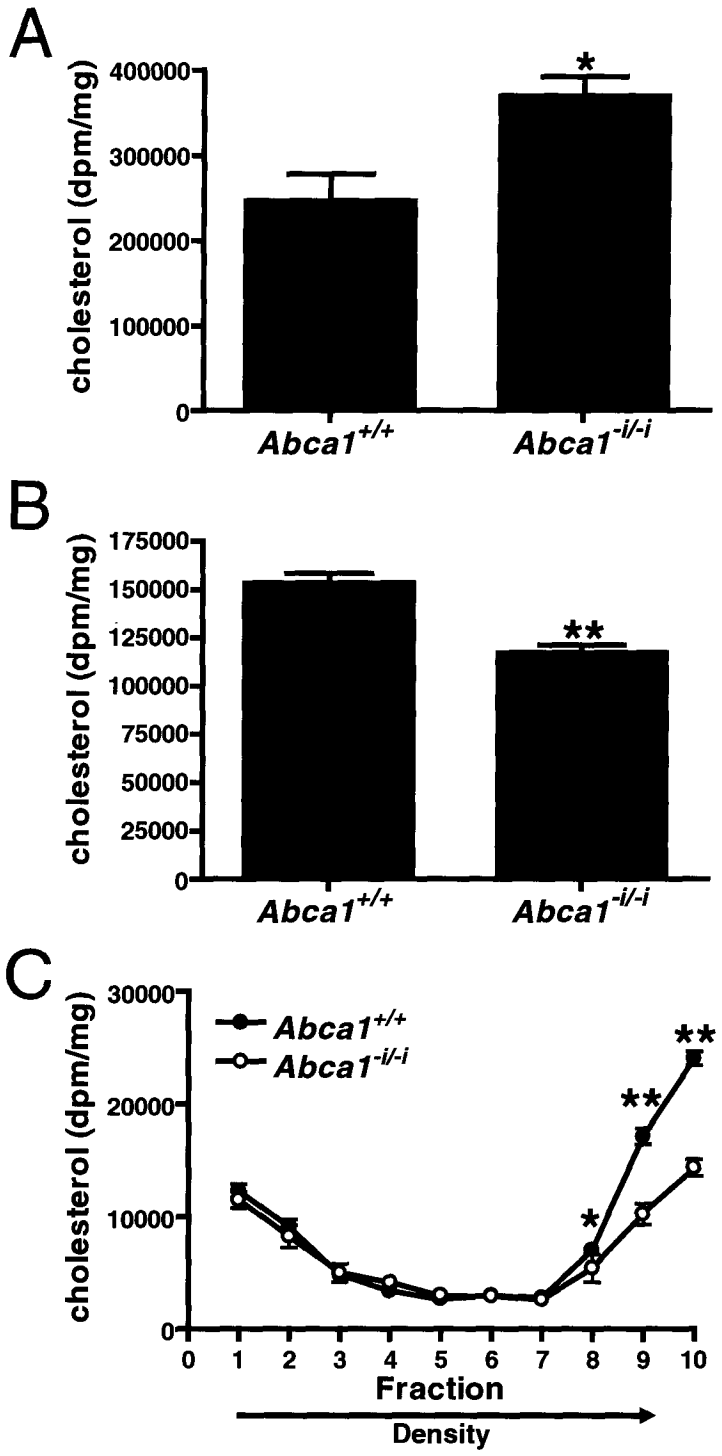

Figure 4.7 Cholesterol secretion from primary enterocytes. Primary enterocytes from *Abca1*$^{+/+}$ and *Abca1*$^{-i/-i}$ mice were isolated and radiolabeled with [^{3}H] cholesterol, then chased in the presence of micelles. (A) [^{3}H] cholesterol remaining in cells after 2 hour chase. (B) [^{3}H] cholesterol in media after 2 hour chase. (C) Media was fractionated by ultracentrifugation and [^{3}H] cholesterol measured in each fraction. n=6 per group. *P<0.05. **P<0.001.

The media were subsequently subjected to density gradient ultracentrifugation and [^{3}H] cholesterol was determined in the isolated fractions. Figure 4.7c shows [^{3}H] cholesterol levels in the isolated fractions. Enterocytes lacking ABCA1 secreted 40% less [^{3}H] cholesterol specifically into the highest density fractions in the density range of HDL and containing most of the secreted apoA-I (29;30) (Figure 4.7c, $p<0.001$). In contrast, there was no difference in cholesterol secretion into the lower density fractions representing chlyomicrons and VLDL (fractions 1-3), and which contain most of the apoB (29;30). These in vitro results therefore recapitulate the findings in vivo that absence of enterocyte ABCA1 results in accumulation of cellular cholesterol, and reduced secretion of cholesterol. These data further indicate that ABCA1 is directly involved in the secretion of cellular cholesterol into HDL from enterocytes.

Cholesterol secretion by the intestine is thought to occur almost exclusively via the lymphatics (23). As *Abca1*$^{-i/-i}$ mice had impaired transport of dietary cholesterol into plasma, we examined whether this was due to decreased transport of cholesterol via lymph. The mesenteric lymph ducts of *Abca1*$^{+/+}$ and *Abca1*$^{-i/-i}$ mice were cannulated and lymph was collected during an intra-duodenal infusion of 4% Intralipid containing [^{14}C] cholesterol and [^{3}H] oleate. Lymph production rates were similar in both groups of mice (approximately 0.10-0.15 ml/h). Figures 4.8a and 4.8b show the rate of transport of cholesterol and triglyceride into mesenteric lymph in *Abca1*$^{+/+}$ and *Abca1*$^{-i/-i}$ mice. There were no significant differences in lipid transport rate between genotypes indicating that total lymphatic lipid transport is not altered by the absence of intestinal ABCA1. Monitoring the appearance of [^{14}C] cholesterol and [^{3}H] oleate in lymph (Figure 4.8c) revealed that the rate of appearance of [^{14}C] cholesterol in lymph as a fraction of [^{3}H] oleate was not significantly different in *Abca1*$^{+/+}$ compared to *Abca1*$^{-i/-i}$ mice (0.53±0.2 vs. 0.65±0.2 after 4 hours, P=0.7), indicating that intestinal ABCA1 does not participate in the transport of dietary cholesterol into lymph.

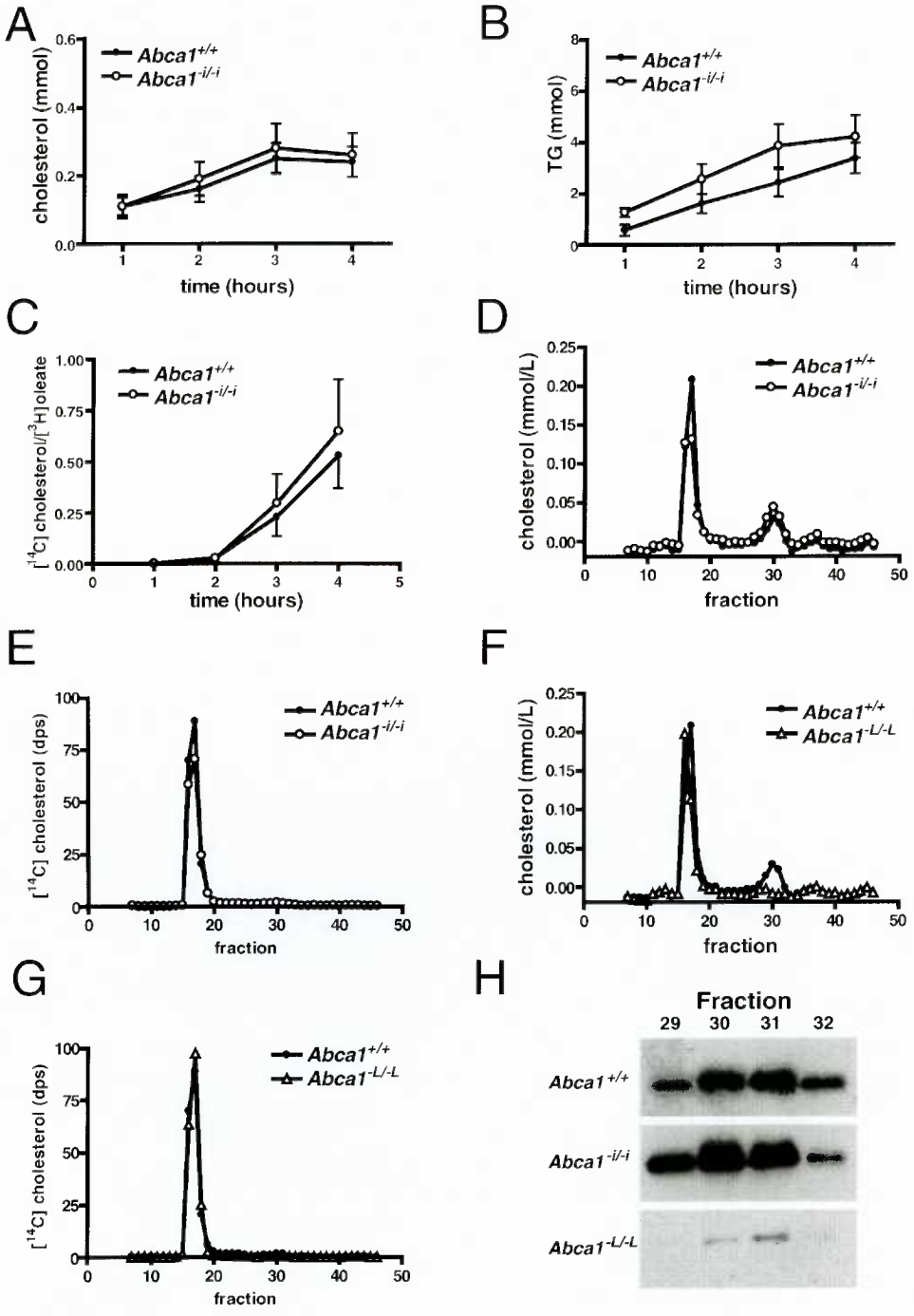

Figure 4.8 Analysis of lymph lipoproteins in *Abca1*$^{+/+}$ and *Abca1*$^{-i/-i}$ mice. (A) Lymph cholesterol transport rate in *Abca1*$^{+/+}$ and *Abca1*$^{-i/-i}$ mice. (B) Lymph triglyceride transport rate in *Abca1*$^{+/+}$ and *Abca1*$^{-i/-i}$ mice. (C) Appearance of [^{14}C] cholesterol in lymph during intra-duodenal infusion as a fraction of [^{3}H] oleate. (D) FPLC analysis of lymph from *Abca1*$^{+/+}$ and *Abca1*$^{-i/-i}$ mice. (E) Distribution of [^{14}C] cholesterol in FPLC fractions of *Abca1*$^{+/+}$ and *Abca1*$^{-i/-i}$ mice. (F) FPLC analysis of lymph from *Abca1*$^{+/+}$ and *Abca1*$^{-L/-L}$ mice. (G) Distribution of [^{14}C] cholesterol in FPLC fractions of *Abca1*$^{+/+}$ and *Abca1*$^{-L/-L}$ mice. (H) Western blot of apoA-I in HDL-sized fractions from lymph of *Abca1*$^{+/+}$, *Abca1*$^{-i/-i}$ and *Abca1*$^{-L/-L}$ mice. n≥6 mice per group.

Lymph collected from mice of each genotype was pooled and subjected to FPLC separation. Lymph from *Abca1*$^{+/+}$ mice showed a major cholesterol peak corresponding to chylomicron/VLDL-sized particles and a minor peak in the size range of HDL, confirming that HDL cholesterol is present in the mesenteric lymph of wildtype mice (Figure 4.8d). Surprisingly, lymph from *Abca1*$^{-i/-i}$ mice showed a similar cholesterol profile to that of wildtype mice. A slight reduction was evident in the amounts of cholesterol in chylomicron/VLDL sized fractions. However, measurement of cholesterol in chylomicrons isolated by density gradient ultracentrifugation revealed that this difference was not statistically significant (0.70±0.07 vs. 0.59±0.06 mM, P=0.3). Notably, *Abca1*$^{-i/-i}$ and *Abca1*$^{+/+}$ mice had equivalent amounts of cholesterol in HDL-sized fractions (Figure 4.8d). This finding indicates that lymph HDL arises independently of intestinal ABCA1. Assessing the distribution of [^{14}C] cholesterol across the FPLC-separated lipoprotein fractions showed that virtually all of the radiolabeled cholesterol secreted into lymph was associated with the chylomicron/VLDL sized fractions in both and *Abca1*$^{+/+}$ and *Abca1*$^{-i/-i}$ mice with essentially no [^{14}C] cholesterol detectable in HDL-sized fractions (Figure 4.8e).

We reasoned that HDL cholesterol present in mesenteric lymph could either arise from the intestine in an ABCA1-independent manner, or could be derived from the plasma, as some reports have suggested (32-36). To determine if lymph HDL originates from plasma, we collected mesenteric lymph from mice in which ABCA1 is deleted in the liver but is present in extrahepatic tissues, including the intestine (*Abca1*$^{-L/-L}$ mice) (16). *Abca1*$^{-L/-L}$ mice have an ~80% reduction in plasma HDL cholesterol levels, and any alteration in lymph lipoprotein concentration detected in *Abca1*$^{-L/-L}$ mice should be directly attributable to the action of hepatic ABCA1. Lymph from *Abca1*$^{-L/-L}$ mice had no detectable HDL cholesterol (Figure 4.8f), whereas the distribution of [^{14}C] cholesterol among FPLC fractions was unchanged (Figure

4.8g). In addition, the HDL-sized fractions from *Abca1*$^{-L/-L}$ lymph contained only trace amounts of apoA-I in comparison to the equivalent fractions in lymph from wildtype and *Abca1*$^{-i/-i}$ mice (Figure 4.8h). These data indicate that HDL present in mesenteric lymph of mice is not secreted by the intestine, and suggest that it originates from the plasma compartment.

4.3. Discussion

Using a novel mouse model in which ABCA1 is specifically inactivated in the intestine, we have shown that intestinal ABCA1 is critically involved in HDL biogenesis, contributing approximately 30% to the steady-state plasma HDL cholesterol pool. Intestinal ABCA1 acts by directly mediating cholesterol transfer towards plasma HDL, and absence of intestinal ABCA1 is associated with decreased transport of intraluminally administered cholesterol into plasma. The absence of intestinal ABCA1 was shown to have no effect on the transfer of luminal cholesterol into lymph and on lymphatic HDL particle content, suggesting that secretion of HDL by the intestine occurs into plasma. Finally, analysis of lymph from liver-specific ABCA1-deficient mice with very low plasma HDL levels provides evidence that HDL present in mouse lymph originates from plasma.

Since the discovery of the highly significant inverse relationship between HDL cholesterol concentration and risk for coronary heart disease (46), the origin of plasma HDL has been a subject of intense study. Successful development of therapeutic strategies to raise HDL levels relies crucially on knowledge of the molecular nature of HDL particle formation as well as the cellular origin of these particles. However, it has not been until recently that we have had the tools necessary to address these questions.

The original conceptualization of the reverse cholesterol transport theory, as described by Glomset (1;2), postulated that HDL cholesterol would be mainly of peripheral origin and be transported to the liver for removal from the body by excretion into bile. The discovery of ABCA1 as the cause of Tangier Disease (6-8), and the finding that the ABCA1 gene product is indispensable for the maintenance of plasma HDL cholesterol levels in mice (39;41;47), has shed new light on the molecular mechanisms of HDL biogenesis. ABCA1 is expressed widely throughout the body (12;13), but only the liver and the intestine are known to synthesize apoA-I (18;48). However, co-expression in the same tissue does not constitute evidence for a functional relationship. Overexpression of ABCA1 in the liver of mice via adenoviral delivery (14;15) or by transgenesis under the control of either its endogenous (49) or an *Apoe* (50) promoter raises HDL levels and protects against atherosclerosis in mice with the $Apoe^{-/-}$ background (51). We have recently described mice with liver-specific deletion of hepatic ABCA1 (16), demonstrating the essential role of hepatic ABCA1 in both the biogenesis and maintenance of plasma HDL. Here we report that deletion of intestinal ABCA1 results in a significant reduction in plasma HDL cholesterol in mice, establishing the intestine as a second crucial player in HDL biogenesis.

Absence of intestinal ABCA1 was shown to result in decreased transport of dietary cholesterol into HDL in vivo and in vitro, along with accumulation of cellular cholesterol. However, our data indicate that in chow-fed mice absence of intestinal ABCA1 does not affect fractional cholesterol absorption as determined by the dual-fecal isotope method. Previous studies assessing intestinal cholesterol absorption in mice lacking ABCA1 globally have yielded conflicting results (41;52;53), suggesting the overall effect of ABCA1 on absorption is at most minor (54). Our findings indicate that the primary function of enterocyte ABCA1 is to mediate the basolateral efflux of cholesterol and, presumably phospholipids, into plasma, a model which is supported by studies in CaCo-2 cells (29;55;56) and chickens lacking ABCA1 globally (42).

Structural and functional polarity is a fundamental feature of enterocytes, and indeed of a variety of epithelial cell types involved in vectorial transport (57). It is therefore perhaps not surprising that while absence of intestinal ABCA1 impairs the basolateral transport of cellular cholesterol into plasma (Figure 4.5a), fractional cholesterol absorption as measured by dual-fecal isotope, which assesses only luminal uptake, is not affected (Figure 4.5d). These results also indicate that the reduction in HDL cholesterol in plasma of *Abca1*$^{-i/-i}$ mice is the direct consequence of a secretory defect in enterocytes.

Results obtained in ABCA1 deficient enterocytes were similar to those observed in primary enterocytes lacking apoA-I (30), indicating that ABCA1 and apoA-I are obligate partners for the secretion of HDL cholesterol from the intestine. While these data suggest that the intestine, along with the liver (16), is essential for the biogenesis of HDL particles, likely by initial lipidation of newly secreted apoA-I, it remains to be determined if the bulk of cholesterol carried by these lipoproteins originates from the intestine and the liver, or, as implicated by the reverse cholesterol transport hypothesis, from peripheral tissues.

Cholesterol that is absorbed or synthesized locally in the intestine is thought to be secreted almost exclusively via the lymphatics, mostly in the form of cholesterol ester in chylomicrons and VLDL (23). Studies of the HDL of mesenteric lymph of rats also suggested that a portion of cellular cholesterol is secreted as HDL into lymph (25-27). Rat mesenteric lymph contains both discoidal and spherical HDL particles which are enriched in phospholipids and protein compared to plasma HDL (25;26), suggesting a distinct origin of these particles. We therefore hypothesized that *Abca1*$^{-i/-i}$ mice would have reduced HDL cholesterol in lymph, underlying the reduction in HDL cholesterol in plasma.

Surprisingly, our lymph cannulation experiments showed that lymph HDL cholesterol and apoA-I contents were essentially unaffected in *Abca1*$^{-i/-i}$ mice, and furthermore that transport of luminal cholesterol into lymph is independent of enterocyte ABCA1. The fact that lymph HDL did not appear to decrease in *Abca1*$^{-i/-i}$ mice in proportion to their reduction in plasma HDL cholesterol may simply reflect an inability to detect small differences in the very small amounts of HDL present in mouse lymph. In addition, plasma lipid values were determined in fasted mice, whereas lymphatic analysis was performed on non-fasted, anesthetized mice, and it cannot be excluded that transudation of HDL from plasma into lymph, or lipolysis of lymph chlymomicrons and VLDL, may be altered under these conditions. Importantly, the finding that absence of intestinal ABCA1 impairs the transport of luminal cholesterol into plasma (Figure 5A), but not into lymph (Figure 8C), suggests the existence of an alternative lipid transport system in enterocytes, by which ABCA1-mediated cholesterol efflux to form HDL occurs selectively into capillaries, while chylomicrons are secreted predominantly into lymphatics. The intestinal epithelium is in close contact with both capillaries and lymphatic vessels of the lamina propria, and indeed other nutrients, such as medium-chain fatty acids, are transported directly into the portal circulation (23). To our knowledge, intestinal transport of cholesterol into the circulation in mammals has not been previously demonstrated.

In contrast to *Abca1*$^{-i/-i}$ mice, lymph HDL cholesterol and apoA-I were virtually absent in *Abca1*$^{-L/-L}$ mice, suggesting that the HDL present in the lymph of wildtype mice is dependent on the activity of hepatic ABCA1 and enters the lymph via the plasma. Consistent with this, orally administered [^{14}C]-labeled cholesterol was recovered only in chylomicron/VLDL-sized fractions in *Abca1*$^{+/+}$, *Abca1*$^{-i/-i}$ and *Abca1*$^{-L/-L}$ mice, indicating that in the time-course of our experiments the intestine did not secrete cholesterol into lymph HDL. The origin of HDL particles in mesenteric lymph has been a subject of considerable controversy. Bearnot et al. demonstrated

that following intravenous injection of [^{125}I] apoA-I labeled HDL, the specific activity of lymph HDL-associated apoA-I did not exceed approximately 30% of that of plasma HDL-associated apoA-I, suggesting intestinal secretion of HDL apoA-I into lymph (28). In addition, Forester et al. reported that after duodenal infusion of [^{3}H] cholesterol the specific activity of lymph HDL was much higher than that of plasma HDL, although the same study also reported significant transfer of free cholesterol between different lipoprotein classes (26). Other studies have suggested that transudation from plasma represents the major origin of lymph HDL (32-36). Because of the rapid exchange of apoproteins and lipid constituents among lipoprotein classes (58;59) radiolabeling experiments may not be able to correctly determine the origin of the radiolabeled particles. The reasons for the discrepancies between our results and those suggesting secretion of intestinal HDL into lymph (25;26;28;60) remain unclear. In most of the studies performed in rats lymph was collected in the presence of an inhibitor of lecithin:cholesterol acyltransferase, which we did not use, and some of these discrepancies may also be due to species differences between mice and rats. In addition, the small amounts of apoA-I in lymph HDL fractions of *Abca1*$^{-L/-L}$ mice may represent intestinally-derived HDL that arises independently of ABCA1. Our data showing near absence of lymph HDL cholesterol and apoA-I in *Abca1*$^{-L/-L}$ mice are most consistent with a model in which lymph HDL is predominantly derived from the plasma.

Patients with Tangier Disease have long been recognized to have reduced LDL cholesterol and altered LDL composition, in addition to near absent HDL cholesterol (61). The mechanisms by which ABCA1 impacts the metabolism of apoB-containing lipoproteins is unclear (62). One mechanism involves an inability of HDL to supply cholesterol esters to LDL via cholesterol ester transfer protein (CETP) (63). However, this is unlikely to be the sole mechanism, since patients with apoA-I deficiency also lack plasma HDL cholesterol but do not exhibit the reduction in

LDL cholesterol observed in Tangier Disease (64), and mice that lack ABCA1 globally or in the liver have reduced LDL cholesterol but do not have CETP (16;41). *Abca1*$^{-i/-i}$ mice have significantly reduced LDL cholesterol and apoB despite their more modest decrease in HDL cholesterol, suggesting that the reduced HDL cholesterol may not entirely underlie the reduction in plasma LDL cholesterol. In addition, lymph chylomicron/VLDL cholesterol was slightly decreased in *Abca1*$^{-i/-i}$ mice, though this difference was not significantly different when lymph chylomicron levels were compared after isolation by density gradient ultracentrifugation. Our studies in primary enterocytes from *Abca1*$^{-i/-i}$ mice suggest that secretion of cholesterol into apoB containing particles is not impaired (Figure 4.7c), similar to enterocytes isolated from mice lacking apoA-I (30), and in contrast to enterocytes in which microsomal transfer protein is inhibited (29). These results suggest that intestinal and hepatic ABCA1 may have direct effects on plasma LDL cholesterol that are not entirely dependent on their effects on HDL cholesterol concentration.

In summary, our results establish that the intestine is crucially involved in the maintenance of plasma HDL levels through the actions of ABCA1 and in addition suggest that intestinally-derived HDL may enter the circulation directly rather than via the lymph. Additional studies are necessary to determine if deletion of intestinal ABCA1 confers increased risk of atherosclerosis and thus whether the intestine plays an atheroprotective role as a component of its normal physiological function.

4.4. Methods

4.4.1. Animals

Abca1 floxed mice were generated by inserting loxP sites into introns 44 and 46 of the murine *Abca1* gene in a 129Sv/Ev embryonic stem cell that was injected into a C57BL/6 blastocyst, as previously described (16). Mice with intestinal-specific deletion of ABCA1 were generated by crossing *Abca1* floxed mice to Villin-Cre transgenic mice, generously provided by Dr. Deborah Gumucio (University of Michigan) (37), to generate $Abca1^{+/-i}$ heterozygotes. Intercrossing led to the $Abca1^{+/+}$, $Abca1^{+/-i}$ and $Abca1^{-i/-i}$ mice used in this study. Control mice, collectively designated $Abca1^{+/+}$, consisted of littermates carrying floxed *Abca1* alleles in the absence of Cre, or wildtype *Abca1* alleles in the presence or absence of Cre, and plasma HDL cholesterol levels between these two groups were not different (data not shown). ABCA1 liver-specific knock-out mice ($Abca1^{-L/-L}$) have been previously described (16). Mice lacking hepatic and intestinal ABCA1 were generated by breeding $Abca1^{-L/-L}$ and $Abca1^{-i/-i}$ mice, and genotypes with regards to Villin-cre or Albumin-cre transgenes determined by promoter-specific PCR. ABCA1 global knock-out mice ($Abca1^{-/-}$) were generously provided by Dr. Omar Francone (Pfizer Global Research and Development) (41). Animals were housed under 12 hour light/dark cycles and received a standard lab chow diet. These studies were approved by the University of British Columbia Animal Care Committee.

4.4.2. Southern Analysis

Southern blotting was performed as previously described (16). Briefly, genomic DNA was isolated from indicated tissues, digested with proteinase K and incubated with EcoRV restriction enzyme (Promega). Digested DNA was separated on a 0.8% agarose gel and transferred to Nytran Super Charge Nylon membrane (Schleicher and Schuell Bioscience). Southern blots

were hybridized with a probe spanning intron 44 to yield the 6 Kb, 7.3 Kb, and 4.2 Kb fragments corresponding to the wild type, floxed, and knockout alleles, respectively.

4.4.3. Western Analysis and Realtime PCR

Western blotting was performed as previously described (12). Briefly, tissues were homogenized and sonicated in 20 mM Hepes, 5 mM KCl, 5 mM $MgCl_2$, 0.5% (v/v) Triton X-100, and complete protease inhibitor (Roche), and protein concentration was determined by the Lowry assay. Equivalent amounts of total protein were separated by SDS-PAGE, transferred to PVDF membranes and probed with anti-ABCA1 (12) or anti-GAPDH (Chemicon) antibodies. For lymph samples, equal volumes of FPLC fractions were separated by SDS-PAGE and probed with an anti-apoA-I antibody (Chemicon).

RNA was isolated using TRIzol® reagent according to manufacturer's instructions (Invitrogen). Three μg of RNA were reverse transcribed with Superscript II (Life Technolgies) to generate cDNA for real time PCR using SYBR® Green PCR Master Mix (Applied Biosystems) in an ABI Prism 7700 Sequence Detection system. The following primer sequences were used: mAbcA1F CGTTTCCGGGAAGTGTCCTA, mAbcA1R CTAGAGATGACAAGGAGGATGGA, mGapdhF TGCACCACCAACTGCTTAG , mGapdhR GATGCAGGGATGATGTTC, mNpc111F TGGACTGGAAGGACCATTTCC, mNpc111R GCGCCCCGTAGTCAGCTAT, mAbcg5F TGGCCCTGCTCAGCATCT, mAbcg5R ATTTTTAAAGGAATGGGCATCTCTT, mAbcg8F CGTCGTCAGATTTCCAATGA, mAbcg8R GGCTTCCGACCCATGAATG, mHmgcsF GCCGTGAACTGGGTCGAA, HmgcsR GCATATATAGCAATGTCTCCTGCAA.

4.4.4. Immunofluoresence

Mouse intestines were fixed in 3% paraformaldehyde for three hours, and unfrozen ten micron sections were cut and mounted on slides. Samples were blocked in 10% normal goat serum for 30 minutes in 0.1% bovine serum albumin/0.1% Tween-20/PBS, incubated with a polyclonal antibody directed to ABCA1 (12) at a concentration of 10μg/ml, followed by a goat anti-rabbit IgG secondary antibody, then washed and counterstained with DAPI or phalloidin prior to coverslipping. Images were captured with a Zeiss Axioplan 2 camera and analyzed using Metamorph software.

4.4.5. Plasma and tissue lipid analysis

Plasma lipid concentrations were determined in 4 hour fasted mice as previously described (51). Briefly, cholesterol and triglyceride concentrations were determined by enzymatic assays using commercially available reagents (BioMerieux). Plasma HDL cholesterol levels were determined after precipitation of apoB-containing lipoproteins with phosphotungstic acid (Roche Diagnostics GmbH). Plasma levels of apoA-I, apoA-II, apoB, and apoC-III were measured by an immunonephelemetric assay using specific mouse polyclonal antibodies as described previously (65). Fast Protein Liquid Chromatography was preformed as previously described (65). This system allows separation of the three major lipoprotein classes, VLDL + IDL, LDL, and HDL in individual animals. Cholesterol concentrations were determined in the eluted fractions. Accumulated data were analyzed by the Millenium 20/0 program (Waters).

Intestinal and hepatic free and total cholesterol was determined by gas-liquid chromatography, as previously described (66).

4.4.6. Enterocyte studies

Mouse primary enterocyte studies were performed according to published protocols (29;30). Briefly, freshly isolated enterocytes were incubated with 1 μCi/mL of [^{3}H] cholesterol in DMEM at 37 °C with constant shaking, and cell suspensions were gassed with 95% O_2, 5% CO_2. After 1 hour, enterocytes were centrifuged at 3,000 rpm for 5 min and the cell pellets were washed twice with DMEM (Gibco), and incubated at 37 °C with micelles containing 0.14 mM sodium cholate, 0.15 mM sodium deoxycholate, 0.17 mM phosphatidylcholine, 1.2 mM oleic acid, and 0.19 mM monopalmitoylglycerol. At the end of the incubation, enterocytes were centrifuged (3,000 rpm, 5 min) and radioactivity in media was counted directly or in fractions following density gradient ultracentrifugation, performed as previously described (29). Total lipids were extracted from cells with isopropanol and counted by liquid scintillation, and proteins were dissolved in 0.1N NaOH and measured by the Bradford method (67). Data represent n = 6 mice per genotype.

4.4.7. Cholesterol absorption

Acute cholesterol absorption was determined as previously described (30). Briefly, overnight fasted mice were gavaged with 0.2 μCi [^{3}H] cholesterol (NEN Life Sciences) and 0.2 mg unlabeled cholesterol in 15 μl olive oil. After 2 hours, plasma, liver and small intestine (first 20 cm from pylorus) were harvested and [^{3}H] content was determined by liquid scintillation counting. Data for liver represent dpm per tissue mass multiplied by total liver mass, and for plasma represent dpm per ml plasma multiplied by total mouse plasma volume (estimated by mass of animal). Data represent n = 3-6 mice per genotype.

For dual fecal isotope studies to quantify fractional cholesterol absorption, mice were gavaged with 0.1 μCi [^{14}C] cholesterol and 0.2 μCi [^{3}H] sitosterol (American Radiolabeled Chemicals) in 0.2 ml soybean oil (Sigma) and feces were collected for 72 hours. Lipids were extracted from

feces and counted by liquid scintillation spectroscopy. Fractional cholesterol absorption was calculated by the formula: (dose [^{14}C]:[^{3}H] - fecal [^{14}C]:[^{3}H]) / dose [^{14}C]:[^{3}H].

4.4.8. Mesenteric lymph collection

Mesenteric lymph cannulation was performed as previously described (68) with minor adjustments. Non-fasted mice were anesthetized by intraperitoneal injection of fentanyl/fluanisone (1 ml/kg) and diazepam (10 mg/kg), and remained anesthetized over the course the experiment. After extra-abdominal displacement of the intestine, the main mesenteric lymph duct was punctured and cannulated with a 0.305 × 0.635 mm (id × od) silicone tubing which was primed with a heparin sodium solution (1000 U/ml). A second cannula was inserted into the duodenum. Both cannulas were secured with tissue adhesive and externalized through the abdominal wall. After repositioning the intestine, the abdominal incision was closed with 8-10 sutures. The animals received an infusion of 4% Intralipid® (0.3 ml/h) through the duodenal cannula during lymph collection. After 1 hour of lymph collection, animals received a lipid bolus via the duodenal cannula containing 1 μCi of [^{14}C] cholesterol and 0.1 μCi of [^{3}H] oleate in 0.1 ml of 4% Intralipid®. Lymph was collected by gravity for 4 hours in total. Body temperature was stabilized using a humidified incubator. Cholesterol and triglyceride concentrations in lymph were determined as described for plasma. Pooled lymph samples of each group were used for lipoprotein separation by fast protein liquid chromatography (FPLC) on a Superose 6B 10/30 column (Amersham Bioscience). The [^{3}H] and [^{14}C] content of lymph was measured by liquid scintillation counting. Data represent n = 6-8 mice per genotype.

4.4.9. Statistical analysis

Differences between groups were compared using Student's t-test or one-way ANOVA using Graphpad Prism software. Data are presented as means ± standard error of the mean.

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Chapter 5 - Intestinal-Specific Induction of ABCA1 Expression with a LXR Agonist Raises Plasma HDL Cholesterol Levels[1]

5.1. Introduction

The ATP-binding cassette transporter, sub-family A, member 1 (ABCA1) mediates the rate-limiting step in the biogenesis of high density lipoprotein (HDL), the assembly of free cholesterol with apolipoprotein A-I. Absence of ABCA1, as occurs in Tangier disease and familial HDL deficiency, results in reduced HDL cholesterol levels and increased risk for coronary artery disease (1). Overexpression of ABCA1 in mice raises HDL levels and protects against atherosclerosis (2;3). In addition, common and rare variants in ABCA1 influence HDL cholesterol levels and risk for coronary artery disease in the general population (4-6), suggesting that modulation of ABCA1 activity may be an effective strategy for raising HDL levels. ABCA1 is expressed in many tissues (7), but it has recently been shown that hepatic (8) and intestinal (9) ABCA1 are the major sites of HDL biogenesis in vivo, establishing these two tissues as the major targets for therapeutic activation of ABCA1 to raise HDL cholesterol levels.

Natural and synthetic agonists of the liver X receptor (LXR) are potent inducers of ABCA1 expression, and significantly raise HDL cholesterol levels and prevent atherosclerosis in murine models (10). However, the therapeutic utility of these compounds is limited by their induction of lipogenic gene expression directly and via activation of hepatic sterol regulatory element binding protein 1c (SREBP1C). Consequently, systemic LXR activation is associated with excessive triglyceride synthesis, hypertriglyceridemia and hepatic steatosis (11;12). The availability of

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synthetic LXR agonists with relative specificity for the intestine (13), together with the identification of intestinal ABCA1 as an important and independent contributor to plasma HDL cholesterol levels (9) raised the possibility that activation of intestinal ABCA1 may increase plasma HDL levels without inducing hepatic steatosis and hypertriglyceridemia. Here we evaluated the ability of a synthetic LXR agonist, GW3965, to raise HDL levels in mouse models of tissue-specific ABCA1 deficiency.

5.2. Results

To investigate the possibility that specific upregulation of intestinal ABCA1 could raise plasma HDL cholesterol levels, we determined the effect of GW3965 on plasma HDL levels in tissue-specific ABCA1 knock-out mice. *Abca1* floxed mice (*Abca1*$^{fl/fl}$) or mice with intestinal (*Abca1*$^{-i/-i}$) or hepatic (*Abca1*$^{-L/-L}$) inactivation of ABCA1 were treated with 0.7 mg of GW3965 by intra-gastric gavage daily for 9 days. Figure 5.1 shows fasting plasma HDL cholesterol levels in *Abca1*$^{fl/fl}$, *Abca1*$^{-L/-L}$ and *Abca1*$^{-i/-i}$ mice at baseline. HDL cholesterol was ~80% lower in *Abca1*$^{-L/-L}$ mice and ~30% lower in *Abca1*$^{-i/-i}$ mice compared to *Abca1*$^{fl/fl}$ mice, consistent with previous reports (8;9).

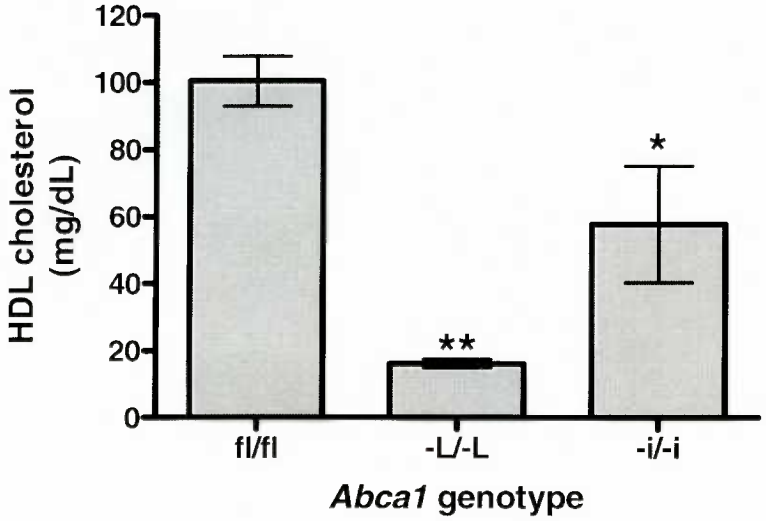

Figure 5.1 Plasma HDL cholesterol levels in control (fl/fl), liver (-L/-L), and intestinal (-i/-i) specific ABCA1 knock-out mice at baseline. * $P<0.01$, ** $P<0.001$. n=5-11 per group.

Figure 5.2a shows fasting total plasma cholesterol levels after 9 days of treatment with GW3965. Total plasma cholesterol levels were significantly increased by GW3965 treatment in $Abca1^{fl/fl}$ and $Abca^{-L/-L}$ mice, but not in $Abca1^{-i/-i}$ mice or $Abca1^{fl/fl}$ mice treated with a vehicle control. These changes in total plasma cholesterol were accounted for by a 48% increase in HDL cholesterol in $Abca1^{fl/fl}$ mice ($P<0.01$) (Figure 5.2b). $Abca1^{-L/-L}$ mice, with very low baseline levels of plasma HDL cholesterol, also displayed a significant 42% increase in plasma HDL cholesterol following GW3965 treatment ($P<0.05$) (Figure 5.2b), indicating that the effect of GW3965 on HDL levels is independent of hepatic ABCA1. Notably, this effect was completely abrogated in $Abca1^{-i/-i}$ mice, which displayed no significant difference in HDL levels at baseline compared to after treatment. This finding indicates that the increase in HDL observed in $Abca1^{fl/fl}$ mice is dependent on intestinal ABCA1. Plasma triglycerides were not altered by GW3965 in any group of mice (Figure 5.2c). Separation of plasma lipoprotein classes by FPLC further indicated that the increase in plasma cholesterol in $Abca1^{fl/fl}$ mice treated with GW3965

was due to a large increase in the HDL fraction (Figure 5.2d). A slight shift towards smaller HDL particles could be observed from the FPLC profile.

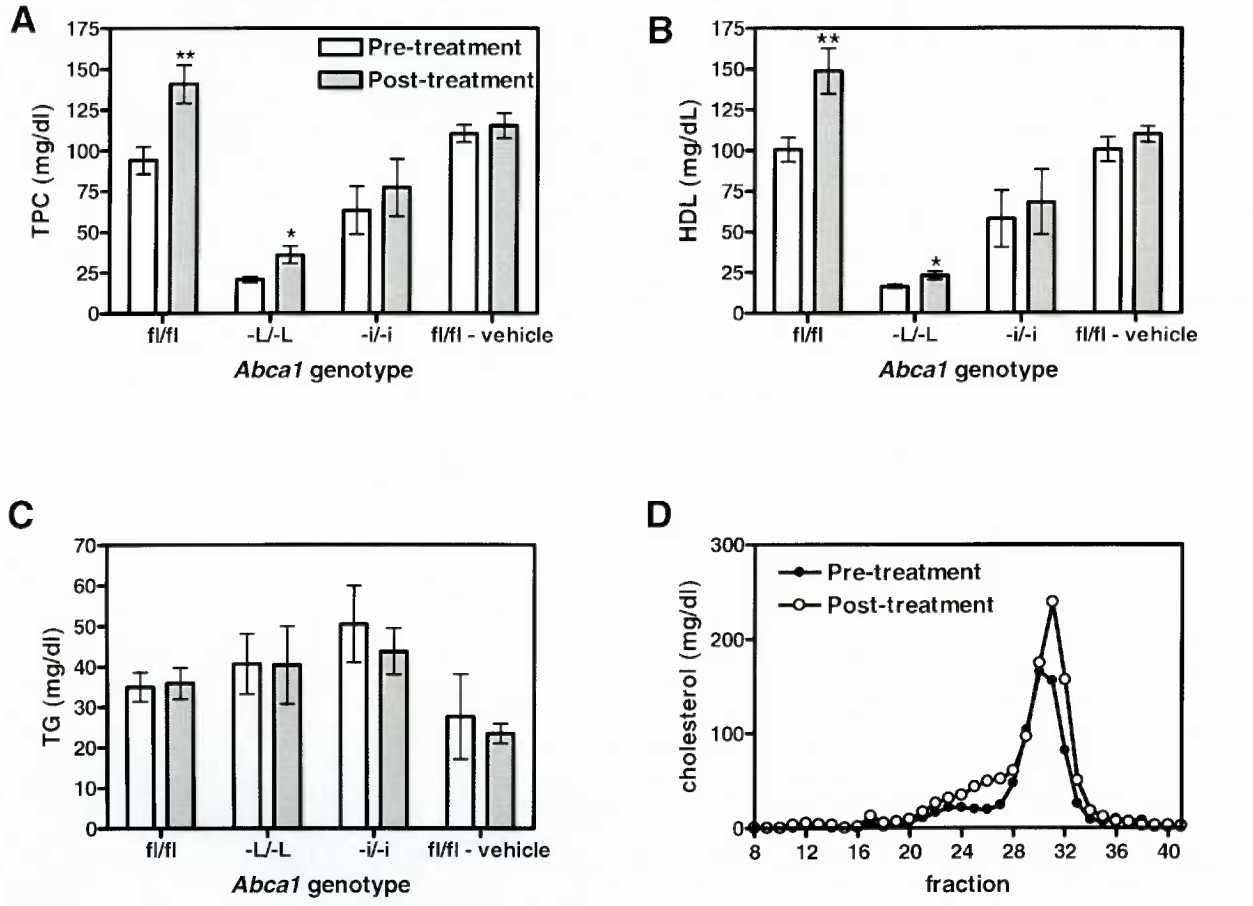

Figure 5.2 Plasma lipid levels before and after treatment with GW3965. A, total plasma cholesterol levels in control (fl/fl), liver (-L/-L) or intestinal (-i/-i) specific knock-out mice before and after treatment with GW3965 or vehicle control. B, plasma HDL cholesterol levels in control (fl/fl), liver (-L/-L) or intestinal (-i/-i) specific knock-out mice before and after treatment with GW3965 or vehicle control. C, plasma triglyceride (TG) levels in control (fl/fl), liver (-L/-L) or intestinal (-i/-i) specific knock-out mice before and after treatment with GW3965 or vehicle control. D, FPLC analysis of plasma from control mice before and after treatment with GW3965. *P<0.05, **P<0.01. n=5-11 per group.

Figures 5.3a and 5.3b show intestinal and hepatic mRNA levels of LXR target genes in $Abca1^{fl/fl}$ mice treated with GW3965 compared to $Abca1^{fl/fl}$ mice treated with a vehicle control. Treatment with GW3965 significantly increased expression of intestinal *Abca1* by ~6-fold (P=0.004) (Figure 5.3a) but had no effect on the expression of hepatic *Abca1* (Figure 5.3b), confirming the selectively of this compound for intestinal rather than hepatic LXR targets in mice. Other LXR

target genes, such as *Abcg8*, *Abcg1* and *Srebp1c* were also significantly upregulated in the intestines of GW3965-treated mice, whereas none of these genes were significantly up-regulated in the livers of GW3965-treated mice. The concurrent raise in intestinal *Abca1* mRNA levels and HDL levels in $Abca1^{fl/fl}$ mice, together with the lack of this effect in $Abca1^{-i/-i}$ mice, further indicate that the effect of GW3965 on plasma HDL levels is predominantly due to its effect on intestinal ABCA1. ABCA1 protein levels were highly induced in the intestines but not livers of mice treated with GW3965, as predicted based on the *Abca1* mRNA levels in these mice (Figure 5.3c).

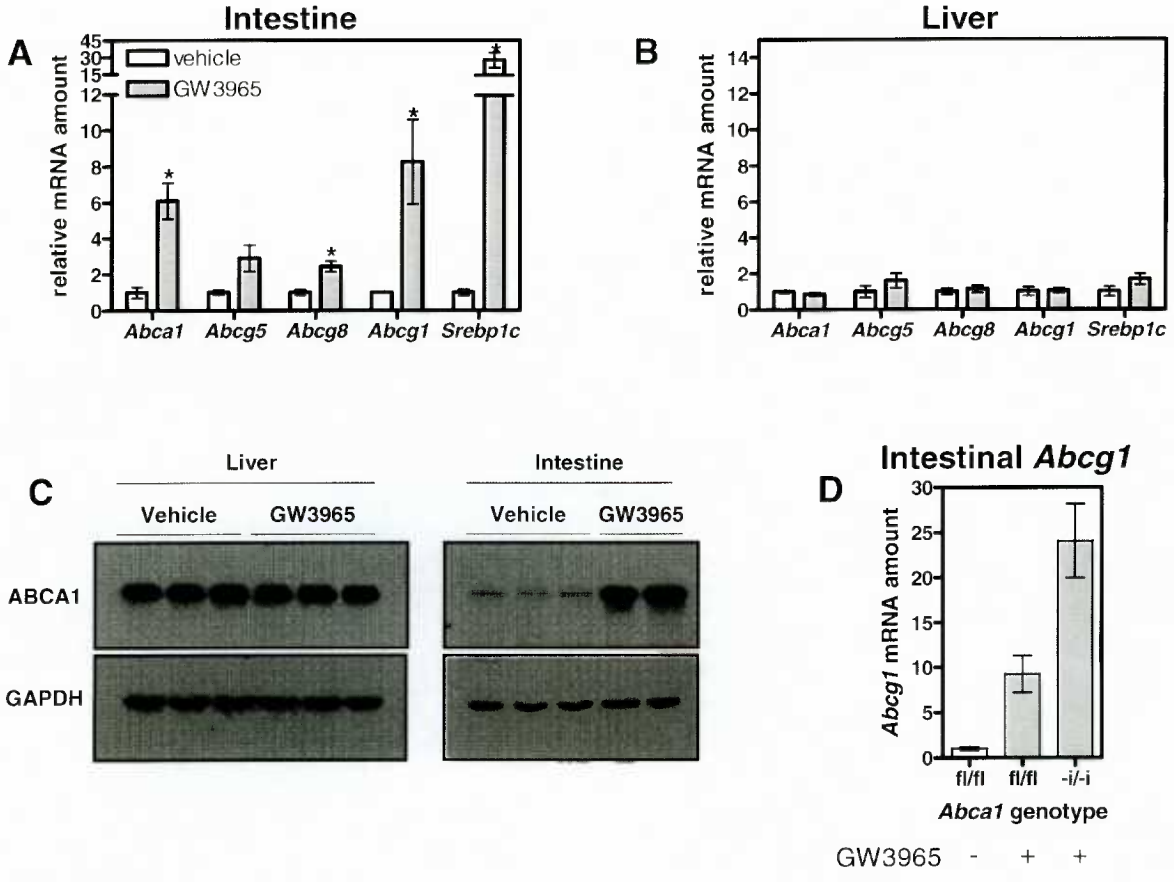

Figure 5.3 Intestinal and hepatic mRNA and protein levels in mice treated with GW3965 (grey bars) or vehicle (white bars). A, intestinal mRNA levels of LXR target genes. B, hepatic mRNA levels of LXR target genes. C, hepatic and intestinal ABCA1 protein expression in $Abca1^{fl/fl}$ mice treated with vehicle or GW3965. D, intestinal *Abcg1* expression level. The level of each gene in the vehicle-treated mice is arbitrarily set as 1. n=4 vehicle-treated mice and 7-GW3965 treated mice. *, $P<0.01$.

Because ABCG1, which mediates the efflux of cholesterol to HDL (14), was also significantly upregulated upon GW3965 treatment, we measured intestinal *Abcg1* mRNA levels in $Abca1^{fl/fl}$ and $Abca1^{-i/-i}$ to determine if this change could contribute to the increase in HDL cholesterol levels observed in wildtype mice. *Abcg1* expression was increased in both $Abca1^{fl/fl}$ and $Abca1^{-i/-i}$ mice (Figure 5.3d). Indeed, *Abcg1* expression was induced to an even greater extent in $Abca1^{-i/-i}$ compared to $Abca1^{fl/fl}$ mice, yet resulted in no change in plasma HDL cholesterol levels, suggesting that the induction of this gene was unlikely to have contributed to the rise in HDL cholesterol observed in wildtype mice. The higher expression of *Abcg1* in the intestines of $Abca1^{-i/-i}$ compared to $Abca1^{fl/fl}$ mice suggests that ABCG1 may be induced in the absence of ABCA1, as has been previously reported (15).

5.3. Discussion

We have recently shown that intestinal-specific deficiency of *Abca1* in mice results in a 30% reduction in plasma HDL cholesterol (9), indicating that intestinal ABCA1, in addition to hepatic ABCA1, is crucial for the maintenance of plasma HDL cholesterol levels. These data suggested that selectively increasing intestinal ABCA1 may lead to elevated plasma HDL cholesterol levels.

To test this hypothesis, we treated mice with the synthetic LXR agonist GW3965. GW3965 significantly raised plasma HDL cholesterol levels in wildtype mice and mice lacking hepatic ABCA1, and this effect was completely abrogated in mice lacking intestinal ABCA1, thereby providing proof-of-principle that activation of intestinal ABCA1 can lead to an increase in HDL levels. Epidemiological data suggest that even small increases in plasma HDL cholesterol are predicted to yield significant reductions in risk for coronary artery disease (16;17), indicating

that the changes in plasma HDL cholesterol caused by inducing intestinal ABCA1 expression may be clinical highly significant.

GW3965 is a non-steroidal carboxylic acid derivative of a tertiary amine identified to possess LXR activity (18) and has previously been shown to effectively raise HDL levels and inhibit the development of atherosclerosis (10) without causing significant induction of lipogenic genes in the liver (13). Different degrees of tissue-selectivity of GW3965 have been reported (13;19;20), indicating that the model system used and the mode and length of administration may influence the selectivity of this compound. Under our experimental conditions, this compound was highly selective for intestinal rather than hepatic LXR targets. GW3965 has previously been demonstrated to exhibit different patterns of co-activator recruitment compared to non-selective LXR agonists (13) which may account for its tissue-selectivity (21). The current study establishes intestinal ABCA1 as the molecular target for this compound's effect on HDL levels. These data further provide evidence for the importance of the intestine in HDL biogenesis and reverse cholesterol transport.

Our data provide the first direct evidence that plasma HDL levels can be raised by raising intestinal ABCA1. The recent identification of partial agonists of LXRα with greater tissue-specificity (22) and LXR ligands with target specificity (23) indicates that it may be possible to design LXR agonists that activate specific genes in a tissue-specific manner. Our data suggest that intestinal ABCA1 may represent a promising therapeutic target for raising HDL levels by transcriptional mechanisms while avoiding the hepatic steatosis and hypertriglyceridemia normally associated with systemic LXR activation.

5.4. Methods

Abca1 intestinal and liver-specific knock-out mice have been previously described (8;9). . GW3965 was kindly provided by GlaxoSmithKline, Stevenage, UK. Fourteen week-old female mice were fed 0.7 mg of GW3965 (corresponding to ~34 mg/kg/day) or vehicle control by intragastric gavage. On each day of the study, GW3965 was dissolved in DMSO under sterile conditions to a concentration of to 70 mg/L, then dissolved in one volume of Cremaphor followed by 9 volumes of 5% mannitol. Two-hundred microliters of this solution, corresponding to 0.7 mg of GW3965, were delivered to each mouse by intragastric gavage using feeding needles (Popper and Sons).

Blood was drawn from 4 hour-fasted mice at baseline and after 9 days of feeding. Liver and small intestine (proximal jejunum to distal ileum) were harvested, snap-frozen in liquid nitrogen and stored at -80°C. All experiments were performed under the approval by the University of British Columbia Animal Care Committee.

Plasma cholesterol, HDL and TG assays were performed as previously described (9).

Total RNA was extracted from livers and intestines using the RNeasy kit (Life Technologies). Two μg of DNase treated RNA were reverse transcribed using Superscript II (Life Technologies). Rnase treated cDNA was used for real time PCR using SYBR® Green PCR Master Mix (Applied Biosystems) in an ABI Prism 7700 Sequence Detection. Gapdh was used as the invariant control. mRNA levels in control mice were arbitrarily set as 1.

For western blots, liver or intestine tissues from *Abca1*$^{fl/fl}$ mice treated with vehicle or GW3965 were homogenized in 20 mM Hepes, 5 mM KCl, 5 mM MgCl2, 0.5% (v/v) Triton X-100, and complete protease inhibitor (Roche), and protein concentration was determined by the Lowry assay. Eighty milligrams of total protein were separated by SDS-PAGE, transferred to PVDF membranes and probed with anti-ABCA1 or anti-GAPDH (Chemicon) antibodies.

Statistical analysis was performed using Graphpad software, using a 2-tailed Student's t-test to compare two groups, and a oneway ANOVA with a Newmal-Keuls post-test to compare three groups. Data are expressed as mean ± standard error.

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Chapter 6 - Contribution of Hepatic ABCA1 to Susceptibility to Atherosclerosis[1]

6.1. Introduction

Plasma HDL cholesterol concentrations are inversely proportional to cardiovascular disease risk (1). Although the precise mechanisms underlying this relationship are incompletely understood, one major atheroprotective function of ABCA1 is thought to be its participation in reverse cholesterol transport (2). The ATP-binding cassette transporter, sub-family A, member 1 (ABCA1) regulates the rate-limiting step in the generation of HDL particles by mediating the efflux of cellular cholesterol and phospholipids to apolipoprotein A-I (3), and therefore plays a critical role in reverse cholesterol transport.

ABCA1 is expressed widely throughout the body (4). We have recently demonstrated using tissue-specific gene targeting that the liver (5) and the intestine (6) are the two major sites of HDL biogenesis in mice. However, the contribution of ABCA1 in specific tissues to susceptibility to atherosclerosis is less clear.

Humans carrying mutations in ABCA1 exhibit an increased risk of coronary artery disease (7-9), though the prevalence of coronary artery disease in homozygous Tangier Disease is not as great as might be expected based on their extremely low HDL cholesterol levels (10). This has been postulated to be due to the reduction in LDL cholesterol levels observed in TD patients (11), which may mitigate to some extent the pro-atherogenic effect of low HDL cholesterol. Mice lacking ABCA1 globally exhibit no change in atherosclerosis susceptibility (12). In contrast, deletion (12) or overexpression (13) of ABCA1 in hematopoetic cells modulates susceptibility to

[1] A version of this chapter will be submitted for publication.

atherosclerosis, indicating that ABCA1 plays an important role in atherogenesis in specific tissues.

Hepatic ABCA1 is the primary contributor to plasma HDL cholesterol levels in vivo (5;14), and is therefore a key site for therapeutic interventions to raise HDL levels. This raises the question of what role hepatic ABCA1 plays in susceptibility to atherosclerosis. Based on studies in transgenic mice overepexpressing a murine *Abca1* cDNA in liver and macrophages it has been postulated that hepatic ABCA1 may actually play a pro-atherogenic role because of an effect on the metabolism of apoB-containing lipoproteins (15;16). In addition, ABCA1 has been shown to impact atherogenesis independently of effects on HDL cholesterol (3), suggesting that the relationship between ABCA1 and atherosclerosis may be complex. In order to determine the contribution of hepatic ABCA1 to atherosclerosis susceptibility we generated mice deficient for hepatic ABCA1 on the atherogenic apoE-deficient background and determined the extent of atherosclerosis in these mice. The *Apoe*$^{-/-}$ mice develop spontaneous lesions throughout the arterial tree on standard chow diet by 12 weeks of age and are therefore an ideal model to study genetic factors that modulate atherosclerosis (17).

6.2. Results

6.2.1. Generation of Apoe$^{-/-}$*;Abca1*$^{-L/-L}$ *mice*

Mice deficient for hepatic ABCA1 (*Abca1*$^{-L/-L}$) (5) on a mixed 129SvEv/C57BL6 genetic background were bred to congenicity on the C57BL6 background by crossing them to wildtype C57BL6 mice. At two separate generations male progeny were genotyped for ~150 polymorphic markers and the most congenic male used for breeding in the following generation. By 3 generations, a male mouse with >90% C57BL6 background was identified and crossed to female

Apoe$^{-/-}$ mice on a pure C57BL6 background, to generate double heterozygotes on a "pure" (~95%) genetic background. Double heterozygotes were intercrossed to generate the *Apoe*$^{-/-}$;*Abca1*$^{+/+}$ and *Apoe*$^{-/-}$:*Abca1*$^{-L/-L}$ mice used in this study.

Apoe$^{-/-}$;*Abca1*$^{-L/-L}$ mice displayed a marked reduction in hepatic ABCA1 protein expression (Figure 6.1a). ABCA1 expression in thioglycollate-elicited peritoneal macrophages was not altered between *Apoe*$^{-/-}$;*Abca1*$^{-L/-L}$ and *Apoe*$^{-/-}$;*Abca1*$^{+/+}$ mice (Figure 6.1b), indicating that ABCA1 was selectively inactivated in hepatocytes.

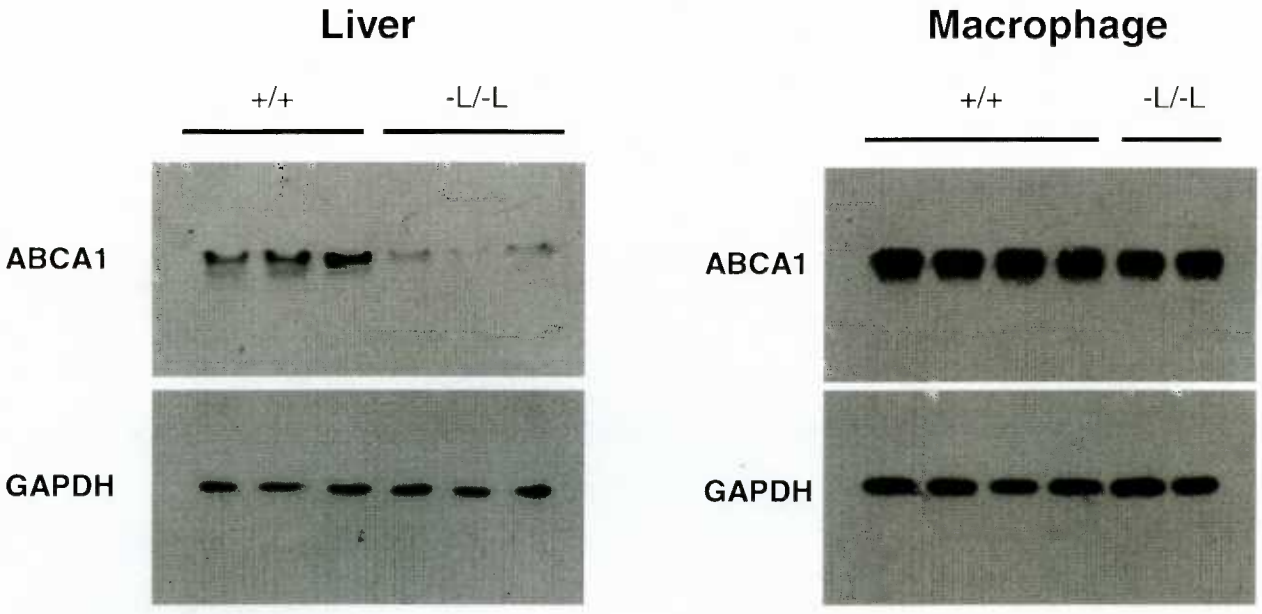

Figure 6.1 ABCA1 protein expression in liver and macrophages. Western blot of (A) liver lysates and (B) thioglycollate-elicited peritoneal macrophages from *Apoe*$^{-/-}$;*Abca1*$^{+/+}$ and *Apoe*$^{-/-}$;*Abca1*$^{-L/-L}$ mice.

6.2.2. Plasma lipid levels

Table 1 shows plasma lipid and apolipoprotein concentrations in 4-hour fasted 12-week old *Apoe*$^{-/-}$;*Abca1*$^{+/+}$ and *Apoe*$^{-/-}$:*Abca1*$^{-L/-L}$ mice. Plasma total cholesterol and phospholipid levels were reduced by ~30% in mice lacking hepatic ABCA1 (Table 1). Plasma HDL and apoA-I levels were reduced by ~50% in *Apoe*$^{-/-}$;*Abca1*$^{-L/-L}$ mice. Plasma LDL and VLDL cholesterol levels were reduced by ~25% and ~35%, respectively.

Table 6.1 Plasma Lipid and Apolipoprotein Concentrations

Abca1 genotype	TPC (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	PL (mg/dl)	TG (mg/dl)	ApoA-I (mg/dl)
+/+	380 ± 19	29 ± 6	169.6 ± 6	180.2 ± 12	258.4 ± 16	68.9 ± 18	40.7 ± 4
-L/-L	263 ± 20	14 ± 1	124.1 ± 10	116.5 ± 11	204.2 ± 16	44.9 ± 8	24.0 ± 3
P value	0.0006	0.04	0.0009	0.002	0.03	0.3	0.009

Figure 6.2 shows the distribution of cholesterol in plasma lipoproteins as determined by fast protein liquid chromatography (FPLC). Although the amounts of HDL cholesterol in *Apoe*$^{-/-}$ mice are small, a reduction in the cholesterol content of the HDL fractions could be observed in *Apoe*$^{-/-}$;*Abca1*$^{-L/-L}$ mice. Cholesterol content was also reduced in the LDL fractions (Figure 6.2).

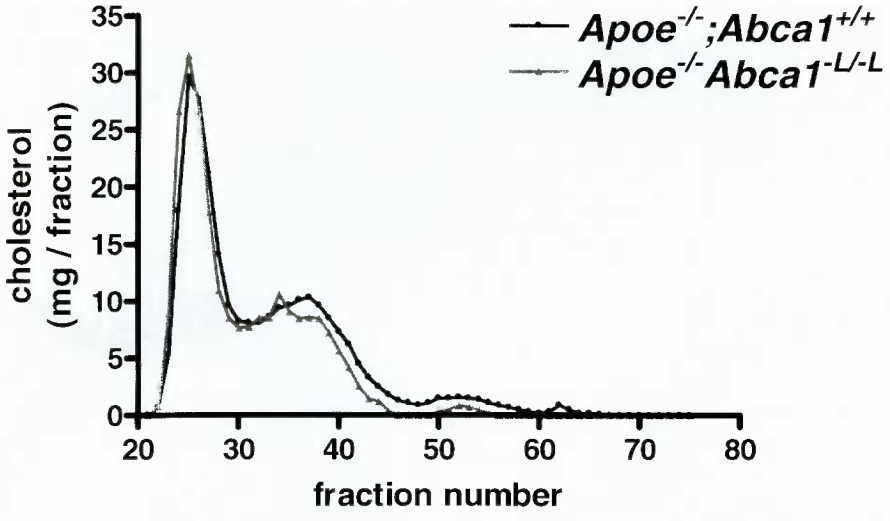

Figure 6.2 Fast Protein Liquid Chromatography profile of plasma cholesterol. Equal volumes of plasma from n=10-14 mice per group were pooled.

6.2.3. Susceptibility to Atherosclerosis

Twelve-week old *Apoe*$^{-/-}$;*Abca1*$^{-l/-L}$ and littermate *Apoe*$^{-/-}$;*Abca1*$^{+/+}$ mice fed a laboratory chow diet were sacrificed to determine the extent of aortic atherosclerosis. Aortic lesion area is shown in Figure 6.3a. Mice lacking hepatic ABCA1 displayed a ~75% increase in aortic lesion area compared to *Apoe*$^{-/-}$;*Abca1*$^{+/+}$ mice (P<0.01). The increase in atherosclerotic lesion area was observed in both male and female mice. Percent of aortic root covered by lesions was also significantly increased in the absence of hepatic ABCA1 (3.2±0.4% vs. 6.5±0.7%, P<0.0001) (Figure 6.3b).

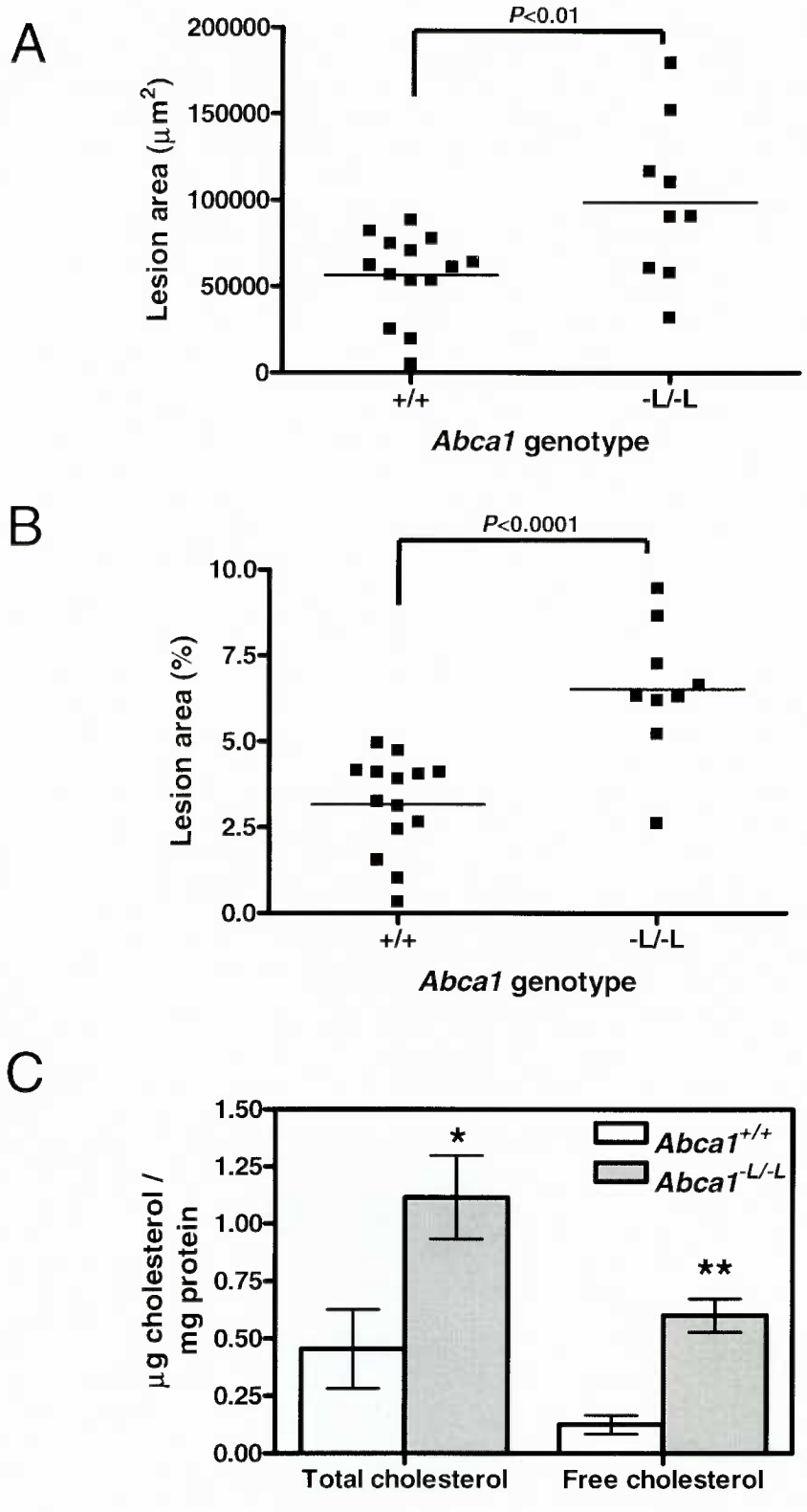

Figure 6.3 Susceptibility to atherosclerosis in mice lacking hepatic ABCA1. A, aortic lesion area in *Apoe*$^{-/-}$;*Abca1*$^{+/+}$ and *Apoe*$^{-/-}$;*Abca1*$^{-L/-L}$ mice. B, percent of aortic root covered by lesions in *Apoe*$^{-/-}$;*Abca1*$^{+/+}$ and *Apoe*$^{-/-}$;*Abca1*$^{-L/-L}$ mice. C, aortic total and free cholesterol levels. **P*<0.05, ***P*<0.001. n=4-5 per group.

Figure 6.3c shows the concentrations of total and free cholesterol in the aortas of *Apoe*$^{-/-}$;*Abca1*$^{+/+}$ and *Apoe*$^{-/-}$;*Abca1*$^{-L/-L}$ mice, as determined by gas-liquid chromatography. *Apoe*$^{-/-}$;*Abca1*$^{-L/-L}$ mice displayed a significant increase in both aortic total and free cholesterol levels indicating that *Apoe*$^{-/-}$;*Abca1*$^{-L/-L}$ mice have an impaired ability to clear cholesterol from aortic cells.

6.3. Discussion

To determine the contribution of hepatic ABCA1 to atherosclerosis susceptibility, we bred mice that lack hepatic ABCA1 on the *Apoe*$^{-/-}$ background. *Apoe*$^{-/-}$;*Abca1*$^{-L/-L}$ mice had reduced plasma total and HDL cholesterol levels, and increased aortic atherosclerotic lesion area. In addition, aortic cholesterol concentrations were significantly greater in *Apoe*$^{-/-}$;*Abca1*$^{-L/-L}$ mice.

Hepatic ABCA1 is a major determinant of plasma HDL cholesterol levels (5); however, the role of hepatic ABCA1 in atherogenesis has not been previously assessed. Based on studies in overexpression models of ABCA1 it has been hypothesized that hepatic ABCA1 may actually play a pro-atherogenic role by impacting the metabolism of apoB-containing lipoproteins (15;16). Because of the interest in ABCA1 as a potential therapeutic target to raise HDL cholesterol levels (18), it is important to determine the role of hepatic ABCA1 in atherogenesis.

The ability of HDL particles to return excess tissue cholesterol to the liver for excretion in bile is thought to be one of the primary mechanisms by which HDL exerts its anti-atherogenic effect. Reverse cholesterol transport begins with the production by ABCA1 in the liver (5) and intestine (6) of HDL particles that can subsequently acquire cholesterol from peripheral tissues by

ABCA1-dependent and -independent mechanisms. In support of this concept, overexpression of apoA-I has previously been shown to increase macrophage-to-feces RCT in mice (19) and deletion of apoA-I reduces macrophage-to-feces RCT (20). Our data suggest that inhibition of one of the initial steps in the RCT pathway, the production of HDL particles by hepatic ABCA1, results in a diminished capacity to eliminate excess cholesterol from aortic cells and increased atherosclerotic lesion development. These results are therefore consistent with the important role of the RCT pathway in limiting the accumulation of tissue cholesterol and preventing atherosclerosis.

Previous studies of the role of ABCA1 in atherogenesis have examined either overexpression or deletion of ABCA1 in 12-week old *Apoe*$^{-/-}$ mice (12;21;22). We therefore chose to study *Apoe*$^{-/-}$;*Abca1*$^{L/L}$ at a similar time-point to allow comparison with these studies. Deletion of ABCA1 in macrophages by bone marrow transplantation results in increased atherosclerotic lesion formation on the *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ genetic backgrounds (12;23). Conversely, bone marrow transplantation of ABCA1 overexpressing cells reduces atherosclerosis susceptibility with no change in plasma lipid levels (13). In contrast, mice lacking ABCA1 globally have been reported to display no change in atherosclerosis susceptibility on either a wildtype background or the A*poe*$^{-/-}$ or *Ldlr*$^{-/-}$ backgrounds (12). This suggests that absence of ABCA1 in specific cell-types such as macrophages increases susceptibility to atherosclerosis while global deletion of ABCA1 may be associated with anti-atherogenic effects (such as reduced LDL cholesterol) that offset the otherwise pro-atherogenic effects of ABCA1 deletion. In addition, overexpression of ABCA1 under the control of its endogenous promoter reduced atherosclerosis lesion development (21), while overexpression of ABCA1 in liver and macrophages under the control of an apoE promoter has been shown to have contrasting effects on atherosclerosis depending on the diet and genetic background (22). Our findings of increased atherosclerosis in mice lacking hepatic

ABCA1 indicate that both hepatic and macrophage ABCA1 play anti-atherogenic functions, though by different mechanisms, likely involving HDL particle formation in the case of the liver and inhibition of foam cell formation in the case of macrophages.

In summary, we have demonstrated that tissue-specific deletion of ABCA1 increases susceptibility to atherosclerosis in mice deficient for apoE. These data suggest an important role for hepatic HDL production in RCT and inhibiting atherogenesis, and suggest that upregulation of hepatic ABCA1 may be a useful therapeutic strategy to raise HDL levels and inhibit atherosclerosis.

6.4. Methods

6.4.1. Animals

ABI PRISM© Mapping Primers (Applied Biosystems (ABI), Foster City, CA), informative between the C57BL/6 and 129Sv/Ev strains, and spaced at an average distance of 20 cM across the genome (excluding the X and Y chromosomes) were used for the speed backcrossing assay. Primers were fluorescently labeled with one of 6-FAM, VIC, or NED (AB standard dye set DS-31). Each assay was prepared in a total volume of 15 μl using 10-50 ng of genomic template, and the following reagents from Invitrogen (Burlington, Ontario): 1.5 μl 10X PCR buffer, 0.75 μl 50 mM Mg2+, 1.5 μl 2.5 mM dNTPs, 1.0 μl 5 μM primer mix and 0.12 μl Taq (5 U/ul) polymerase. Thermal cycling, post-amplification dilution and pooling, and ABI3100 plate setup was performed as per manufacturer's instructions (ABI, Foster City, CA). PCR fragments were analyzed using Gene Mapper 3.0 (ABI, Foster City, CA).

6.4.2. Western Analysis and Realtime PCR

Western blotting was performed as previously described (4). Briefly, tissues were homogenized and sonicated in 20 mM Hepes, 5 mM KCl, 5 mM $MgCl_2$, 0.5% (v/v) Triton X-100, and complete protease inhibitor (Roche), and protein concentration was determined by the Lowry assay. Equivalent amounts of total protein were separated by SDS-PAGE, transferred to PVDF membranes and probed with anti-ABCA1 (4) or anti-GAPDH (Chemicon) antibodies.

Real-time quantitative PCR was performed according to previously described methods and primers (6). RNA was isolated using TRIzol® reagent according to manufacturer's instructions (Invitrogen). Three μg of RNA were reverse transcribed with Superscript II (Life Technolgies) to generate cDNA for real time PCR using SYBR® Green PCR Master Mix (Applied Biosystems) in an ABI Prism 7700 Sequence Detection system.

6.4.3. Plasma lipid levels

Plasma lipid levels were determined as previously described (6). Briefly, cholesterol and triglyceride concentrations were determined by enzymatic assays using commercially available reagents (BioMerieux). Plasma levels of apoA-I by an immunonephelemetric assay using specific mouse polyclonal antibodies as described previously (24). Fast Protein Liquid Chromatography was preformed as previously described (24) and accumulated data were analyzed by the Millenium 2010 program (Waters).

6.4.4. Atherosclerosis quantification

Twelve-week-old *Apoe*$^{-/-}$;*Abca1*$^{L/L}$ and *Apoe*$^{-/-}$ littermate controls maintained on a chow diet were fasted for 4 hours prior to sacrifice. Tissues were harvested for RNA and protein analysis. Mice were perfused transcardially with 4% paraformaldehyde (Fisher Scientific Co., Nepean, Ontario, Canada), and the hearts with attached aortas were isolated. Aortas were dissected below

the levels of aortic arch for gas chromatography analysis. Hearts were embedded in Tissue-Tek OCT (Sakura Finetek USA Inc., Torrance, California, USA) media in a plastic mold, frozen, and sixteen 10-μm sections were cut. For oil red O (ORO) staining, the sections were rinsed in water and isopropanol and stained in 0.25% ORO for 20 minutes, followed by an isopropanol rinse. Sections were counterstained in Gill's hematoxylin for 1 minute and mounted. Transverse sections were obtained from the apex of the heart moving toward the aortic region, with sections beginning at the point where all three aortic valve cusps became clearly visible. Every fourth section was placed on a slide for ORO staining of neutral lipid and counterstained with hematoxylin, such that each slide had four sections 40 μm apart. Sections adjacent to those stained with ORO were stained with Movat's pentachrome for the identification of elastin, collagen, glycosaminoglycans, smooth muscle cells, and foam cells.

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Chapter 7 - The Role of ß-cell ABCA1 in Glucose Homeostasis, Insulin Secretion and Response to Thiazolidinedione Treatment[1]

7.1. Introduction

Type 2 diabetes (T2D) is caused by a relative inability of the endocrine pancreas to secrete sufficient insulin to meet the increasing metabolic demands of insulin resistance associated with obesity and ageing. Loss of first-phase glucose-stimulated insulin secretion is an invariant finding amongst individuals with T2D(1). However, the pathogenesis of ß-cell dysfunction in T2D remains obscure. One proposed mechanism is lipotoxicity, in which toxic lipids accumulate leading to ß-cell apoptosis and loss of insulin secretion(2). Triglycerides (TG) and saturated free fatty acids (FFAs) such as palmitate, which increase production of ceramide, can induce ß-cell dysfunction and death(3;4), but the role of cholesterol in modulating ß-cell function and survival has received little attention.

The ATP-binding cassette transporter, sub-family A, member 1 (ABCA1) is a plasma membrane protein that mediates the rate-limiting step in HDL biogenesis by effluxing cellular cholesterol and phospholipids to an apolipoprotein acceptor. Since non-hepatic cells are unable to degrade cholesterol, ABCA1 plays a critical role in regulating intracellular cholesterol homeostasis(5). Mice lacking Abca1 globally display impaired glucose tolerance but have normal insulin sensitivity, suggesting that these mice have a defect in ß-cell function.

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The finding of impaired glucose tolerance in mice lacking Abca1 globally led us to hypothesize that ABCA1 is important for ß-cell cholesterol homeostasis and function. Mice lacking Abca1 globally have very low levels of total plasma cholesterol(6), and may therefore not be a suitable model in which to assess the effect of altered islet cholesterol homeostasis on ß-cell function. In addition, it is not possible to study the function of ß-cell Abca1 independent of its role in other tissues in mice with global deletion of Abca1. To test the hypothesis that ABCA1 plays a functional role in ß-cells in vivo, we generated mice that specifically lack ß-cell Abca1 (*Abca1*$^{-P/-P}$). Our results indicate that ß-cell ABCA1 plays a critical role in ß-cell cholesterol homeostasis, and is required for insulin-secretion and the response to thiazolidinedione treatment, establishing ABCA1 as a novel gene involved in ß-cell cholesterol metabolism and glucose homeostasis.

7.2. Results

7.2.1. Abca1 expression and function in murine islets

Mice that lack Abca1 globally (*Abca1*$^{-/-}$) have normal fasting blood glucose levels but display impaired glucose tolerance by 4 months of age (Figure 7.1a). Insulin sensitivity in these mice is indistinguishable from wildtype controls (Figure 7.1b), suggesting that this impairment reflects islet dysfunction. Consistent with previous reports(7), we found that Abca1 protein is almost undetectable in whole pancreatic lysates (Figure 7.1c). In contrast, Abca1 is highly expressed in isolated murine islets (Figure 7.1c), which make up ~1% of the adult pancreas. Abca1 protein is also present in the transformed ß-cell line INS-1 (Figure 7.1c) where it mediates apolipoprotein A-I dependent cholesterol

efflux from cells (Figure 7.1d), indicating that Abca1 is expressed and functions as a cholesterol efflux transporter in ß-cells.

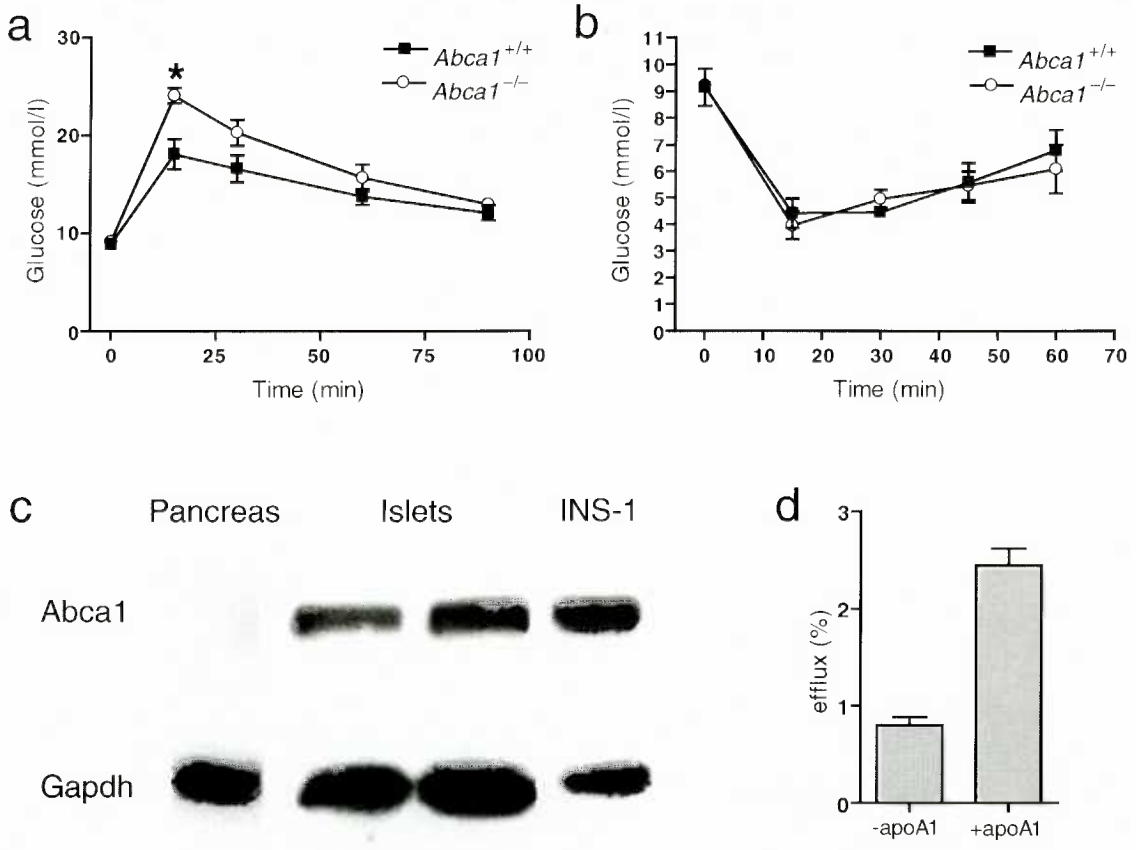

Figure 7.1 Glucose homeostasis in Abca1 global knock-out mice (*Abca1-/-*) and Abca1 expression in islets. (a) glucose tolerance testing in mice lacking Abca1 globally (*Abca1-/-*) and littermate controls (*Abca1*$^{+/+}$). n=8 per group. (b) insulin tolerance testing in mice lacking Abca1 globally (*Abca1*$^{-/-}$) and littermate controls (*Abca1*$^{+/+}$). n=4 per group. (c) Abca1 protein expression in whole pancreas, isolated islets and INS-1 cells. (d) cholesterol efflux in INS-1 cells in the presence or absence of apolipoprotein A-I. * *P*=0.004, 2-tailed Student's t-test.

To investigate the role of Abca1 in ß-cells, we generated mice lacking ß-cell Abca1 (*Abca1*$^{-P/-P}$) by crossing *Abca1* floxed mice(8) to mice expressing Cre under the control of the ß-cell specific rat insulin promoter(9). The expression of *Abca1* mRNA in isolated islets of *Abca1*$^{-P/-P}$ mice was markedly reduced (Figure 7.2a) as was Abca1 protein expression in islets (Figure 7.2b). The residual Abca1 expression evident in islets from

these mice (Figure 7.2b) suggests the expression of Abca1 in non-ß-cells. *Abca1* mRNA levels were not significantly different in other tissues (Figure 7.2a), indicating that Abca1 had been selectively deleted from ß-cells.

Robust Abca1 expression was observed by immunoflourescence in pancreas sections from wildtype mice and co-localized with insulin (Figure 7.2c). In contrast, pancreas sections from *Abca1*$^{-P/-P}$ mice displayed scant islet Abca1 immunoreactivity restricted to non-insulin expressing islet cells (Figure 7.2c).

Abca1$^{-P/-P}$ mice displayed no significant differences in body weight over time (Figure 7.2d) and had normal food intake (Figure 7.2e). In addition, plasma cortisol levels were not significantly different between genotypes (Table 1) suggesting that hypothalamic function is not altered in *Abca1*$^{-P/-P}$ mice.

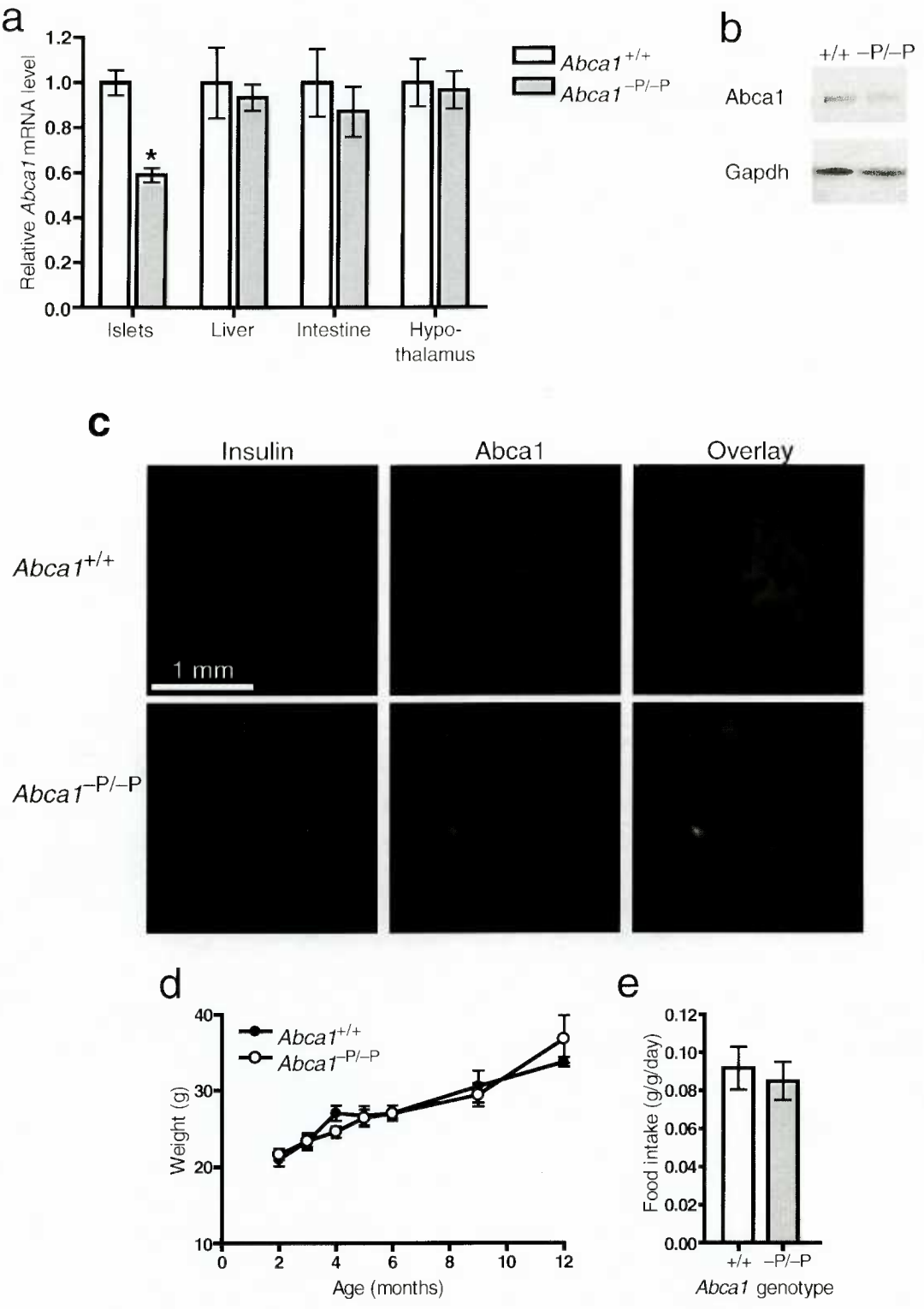

Figure 7.2 Generation of mice lacking Abca1 in ß-cells (Abca1–P/–P). (a) Abca1 mRNA expression in tissues from Abca1+/+ and Abca1–P/–P mice. n=4-7 per group. (b) Western blot for Abca1 on islets isolated from Abca1+/+ and Abca1–P/–P mice. (c) immunoflouresence for insulin (red) and Abca1 (green) and overlay in pancreas sections from 3 month-old Abca1+/+ and Abca1–P/–P mice. (d) weight curves of Abca1+/+ and Abca1–P/–P mice (n≥5 mice per time point). (e) average daily food intake (n=6). * P=0.0002, 2-tailed Student's t-test.

7.2.2. Glucose intolerance, defective insulin secretion, and altered ß-cell cholesterol homeostasis in Abca1$^{-P/-P}$ mice

Heterozygous *Abca1*$^{+/-P}$ mice had normal glucose tolerance at 8 weeks of age (Figure 7.3a) but by 16 weeks displayed impaired glucose tolerance (Figure 7.3b). Homozygous *Abca1*$^{-P/-P}$ displayed a more severe phenotype, with glucose intolerance detectable at 8 weeks (Figure 7.3c), and markedly impaired glucose tolerance by 16 weeks of age (Figure 7.3d). We observed no difference in glucose tolerance between littermates with wildtype Abca1 alleles in the presence or absence of the RIP-Cre transgene or mice with floxed Abca1 alleles (Figure 7.3d), and mice of all 3 genotypes were therefore combined as controls in subsequent experiments, denoted collectively herein as "*Abca1*$^{+/+}$". Plasma insulin levels during the glucose tolerance test were significantly lower in *Abca1*$^{-P/-P}$ mice 15 minutes following the i.p. glucose challenge (Figure 7.3e). Insulin sensitivity was unaltered in *Abca1*$^{-P/-P}$ mice compared to controls (Figure 7.3f).

To further examine insulin secretion in vivo, we measured first-phase insulin response 2 minutes following a glucose (3 mg/g) challenge. Plasma insulin levels were lower in *Abca1*$^{-P/-P}$ mice compared to controls at 2 minutes following the glucose challenge (Figure 7.3g) (P<0.01), indicating that Abca1 is essential for first-phase glucose stimulated insulin secretion in vivo. Similarly, insulin secretion in response to L-arginine (0.3 mg/g) was significantly lower in *Abca1*$^{-P/-P}$ mice compared to controls (Figure 7.3h) (P<0.05). The reduction in insulin secretion in response to both glucose and non-glucose secretagogues suggests that absence of ß-cell Abca1 results in a generalized insulin secretory defect.

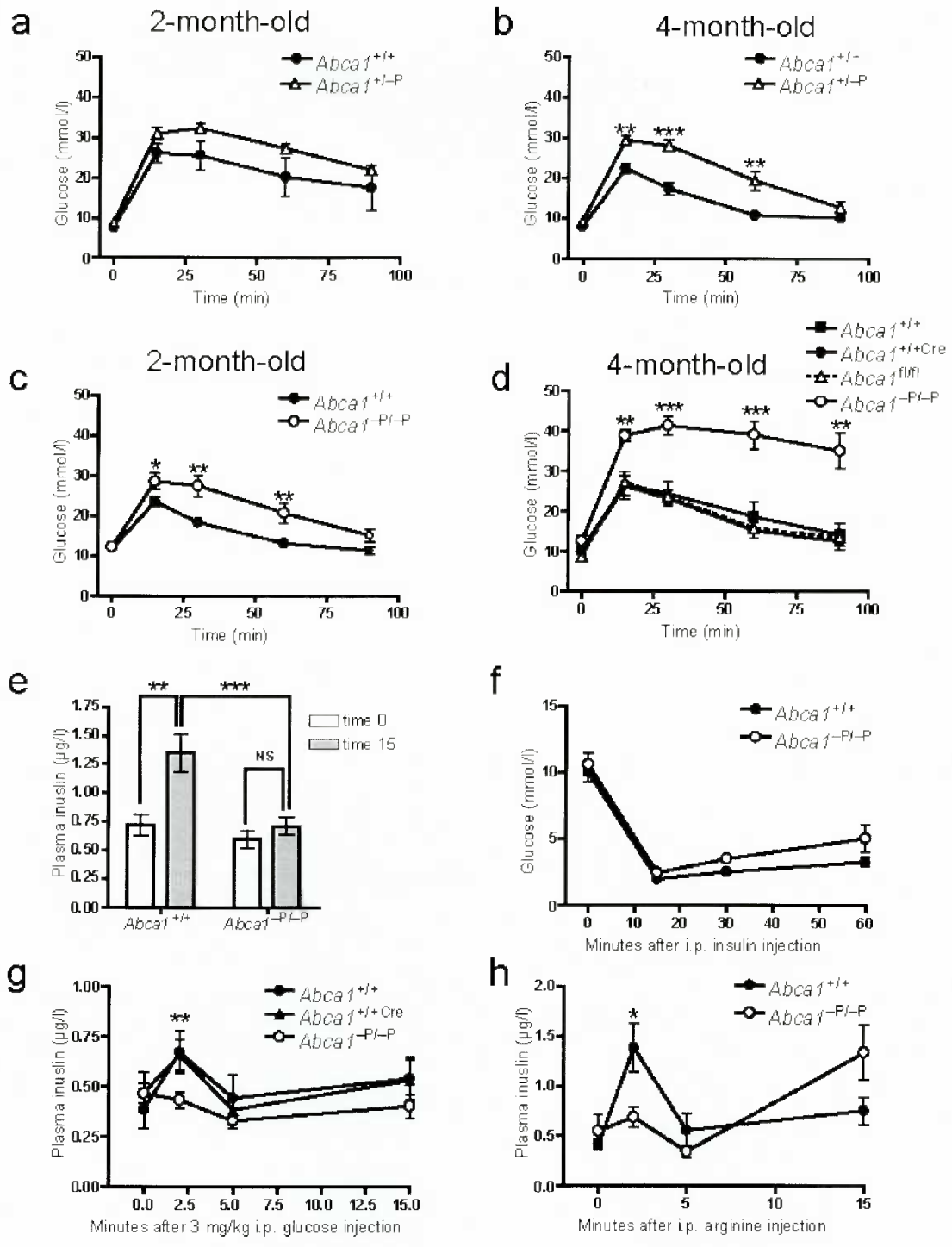

Figure 7.3 Glucose homeostasis in Abca1 ß-cell specific knock-out mice. (a, b, c, d) Glucose tolerance testing in 2 month old Abca1$^{+/-P}$ heterozygotes (a) (n=4 per group), 4 month-old Abca1$^{+/-P}$ heterozygotes (b) (n=8 per group), 2 month-old Abca1$^{-P/-P}$ homozygotes (c) (n=5 per group) or 4 month-old Abca1$^{-P/-P}$ homozygotes (d) (n=8 per group). (e) plasma insulin levels during the IPGTT in 4-month old Abca1$^{-P/-P}$ homozygotes and controls (n=18 per group). (f) I.P. insulin tolerance test in Abca1$^{-P/-P}$ mice (n=4 per group). (g, h) acute phase insulin secretion after an i.p. injection of glucose (g) (n=9 per group) or L-arginine (h) (n=5 per group). * P<0.05, ** P<0.01, *** P<0.001, 2-tailed Student's t-test. NS, not significant.

The reduction in insulin secretion observed in *Abca1*$^{-P/-P}$ mice could be due to a reduction in ß-cell mass, an impairment of ß-cell function, or a combination of both. We performed quantitative histo-morphometrical analysis on evenly-spaced pancreas sections from *Abca1*$^{-P/-P}$ mice and controls, and observed no difference in ß-cell mass among genotypes (Figure 7.4a). Similarly, gross morphology of islets was not altered (Figure 7.4b), nor was the overall abundance or size of islets (Figure 7.4c). The reduction in insulin secretion observed in vivo could therefore not be explained by a reduction in ß-cell mass.

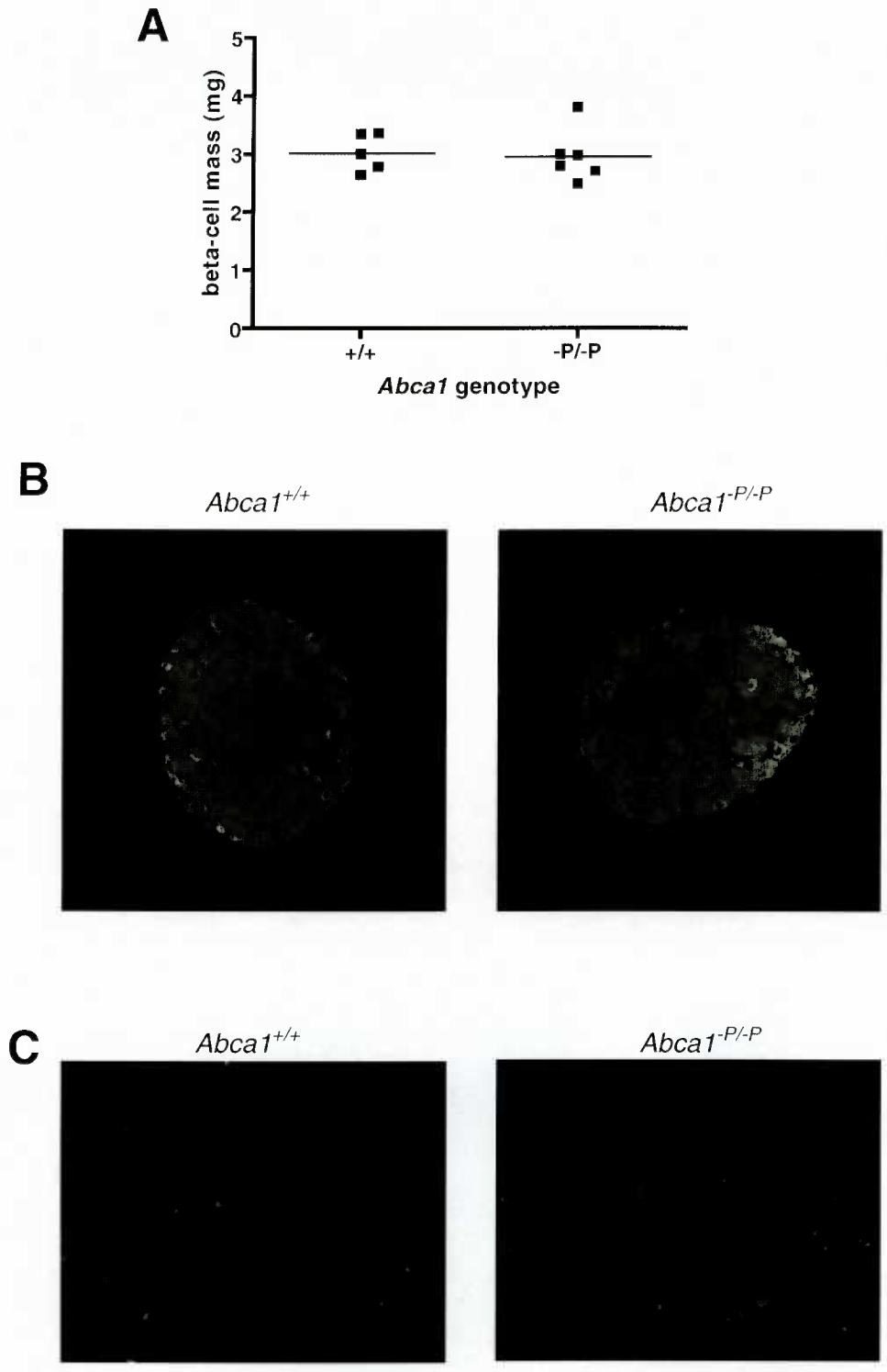

Figure 7.4 ß-cell mass in mice lacking ß-cell Abca1. (a) ß-cell mass was determined by histomorphometry from 10 evenly spaced pancreatic sections. (b) immunoflourescence for insulin (green) and glucagon (red) from ***Abca1***$^{+/+}$ and ***Abca1***$^{-P/-P}$ mice. (c) low power view of pancreata from ***Abca1***$^{+/+}$ and ***Abca1***$^{-P/-P}$ mice showing immunoflourescence for insulin (green).

Plasma total and HDL cholesterol levels were not significantly different between *Abca1*$^{+/+}$ and *Abca1*$^{-P/-P}$ mice (Table 1), indicating that ß-cell Abca1 does not contribute significantly to steady-state plasma HDL levels, in contrast to the function of Abca1 in the liver(8) and intestine(10). However, absence of ß-cell Abca1 resulted in a significant increase in islet total and free cholesterol levels and cholesterol esters (Figure 7.5a) (P<0.05). Islet TG levels were not significantly altered (Figure 7.5a). Measurement of islet cholesterol content in mice with global deletion of Abca1 revealed no increase in islet total or free cholesterol or cholesterol ester levels (Figure 7.5a). This finding suggests that the higher circulating cholesterol levels to which islets from *Abca1*$^{-P/-P}$ mice are exposed (92.9±4 vs 9.7±3 mg/dl, P<0.0001) results in a greater degree of cholesterol accumulation and more severe impairment in glucose tolerance compared to mice with global deletion of Abca1.

Table 7.1 Plasma metabolic parameters in *Abca1*$^{+/+}$ and *Abca1*$^{-P/-P}$ mice.

Abca1 genotype	Plasma cholesterol (mg/dL)	HDL cholesterol (mg/dL)	FFA (mM)	ß-hydroxy-butyrate (mM)	Cortisol (µg/dl)
+/+	88.0 ± 4 (11)	66.4 ± 2 (11)	1.6 ± 0.1 (10)	0.20 ± 0.04 (7)	0.61 ± 0.05 (7)
–P/–P	92.9 ± 4 (4)	69.2 ± 5 (4)	1.9 ± 0.3 (7)	0.20 ± 0.07 (5)	0.56 ± 0.08 (5)
P value	0.5	0.6	0.2	0.9	0.6

Number of mice is indicated in parentheses. Mice were fasted for 4 hours prior to sample collection. Plasma for cortisol was collected at 2:00 pm.

To further study the cellular sterol content of islets lacking ß-cell Abca1, we examined the expression of various sterol-sensitive mRNAs in isolated islets (Figure 7.5b). The expression of HMG-CoA reducatase (*Hmgcr*) and LDL receptor (*Ldlr*) mRNA, both of

which are downregulated by cholesterol(11), was significantly reduced in islets lacking Abca1 (Figure 7.5b). The expression of the *Srebp-1c* transcript of sterol regulatory element binding factor 1 (*Srebf1*) was not significantly different in *Abca1*$^{-P/-P}$ islets. *Abcg1* mRNA was readily detectable in isolated mouse islets, and the expression of *Abcg1* was increased in islets lacking ß-cell Abca1 (Figure 7.5b). This suggests that ABCG1, a cholesterol transporter essential for preventing excess cellular cholesterol accumulation(12), may increase to compensate for the loss of ß-cell Abca1 and the resultant increased intracellular cholesterol content.

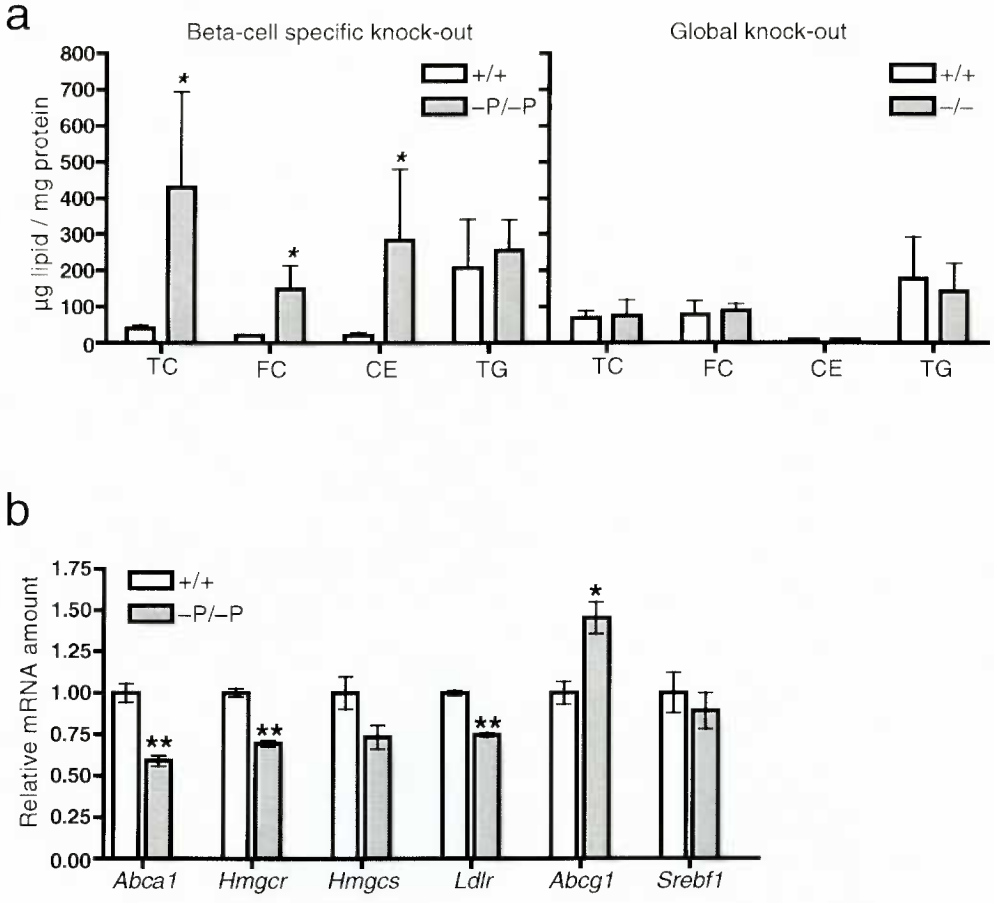

Figure 7.5 Absence of ß-cell Abca1 results in altered cholesterol homeostasis in isolated islets. (a) Total (TC), free (FC) and esterified (CE) cholesterol and triglyceride (TG) levels in islets isolated from ß-cell specific Abca1 knock-out mice and controls (+/+ and –P/–P) and Abca1 global knock-out mice and controls (+/+ and –/–) determined by gas-liquid chromatography and enzymatic assay. n=3-6 per group. (b) relative amount of various mRNAs in isolated islets from Abca1$^{+/+}$ and Abca1$^{-P/-P}$ mice. The Srebf1 gene was amplified with primers specific for the Srebp-1c gene product. Each value represents the amount of mRNA relative to Abca1$^{+/+}$ mice, which is arbitrarily set as 1 for each transcript. Values represent pooled data from 2-5 separate experiments in which equal amount of total RNA was pooled from 3 animals. * P<0.05, ** P<0.01

We next investigated insulin secretion in isolated islets from *Abca1*$^{-P/-P}$ mice and controls. Insulin secretion in response to 24 mM glucose was significantly blunted in islets lacking Abca1 (Figure 7.6a). Incubation of islets with KCl, which directly depolarizes the cell membrane, led to significant stimulation of insulin secretion in wildtype but not *Abca1*$^{-P/-P}$ islets (Figure 7.6a). This finding suggests that the impairment in insulin secretion in Abca1-deficient islets lies downstream of nutrient sensing and secondary signal generation by the ß-cell. Insulin content was increased in islets lacking Abca1 (Figure 7. 6b), as well as in whole pancreata from *Abca1*$^{-P/-P}$ mice (Figure 7. 6c), while static insulin mRNA levels were not significantly altered (Figure 7. 6d), consistent with a defect in the exocytosis of insulin-containing secretory granules. Examination of the dynamics of glucose-stimulated insulin secretion by perifusion of isolated islets demonstrated a defect in first-phase glucose-stimulated insulin release in mice lacking ß-cell Abca1 (Figure 7. 6e,f).

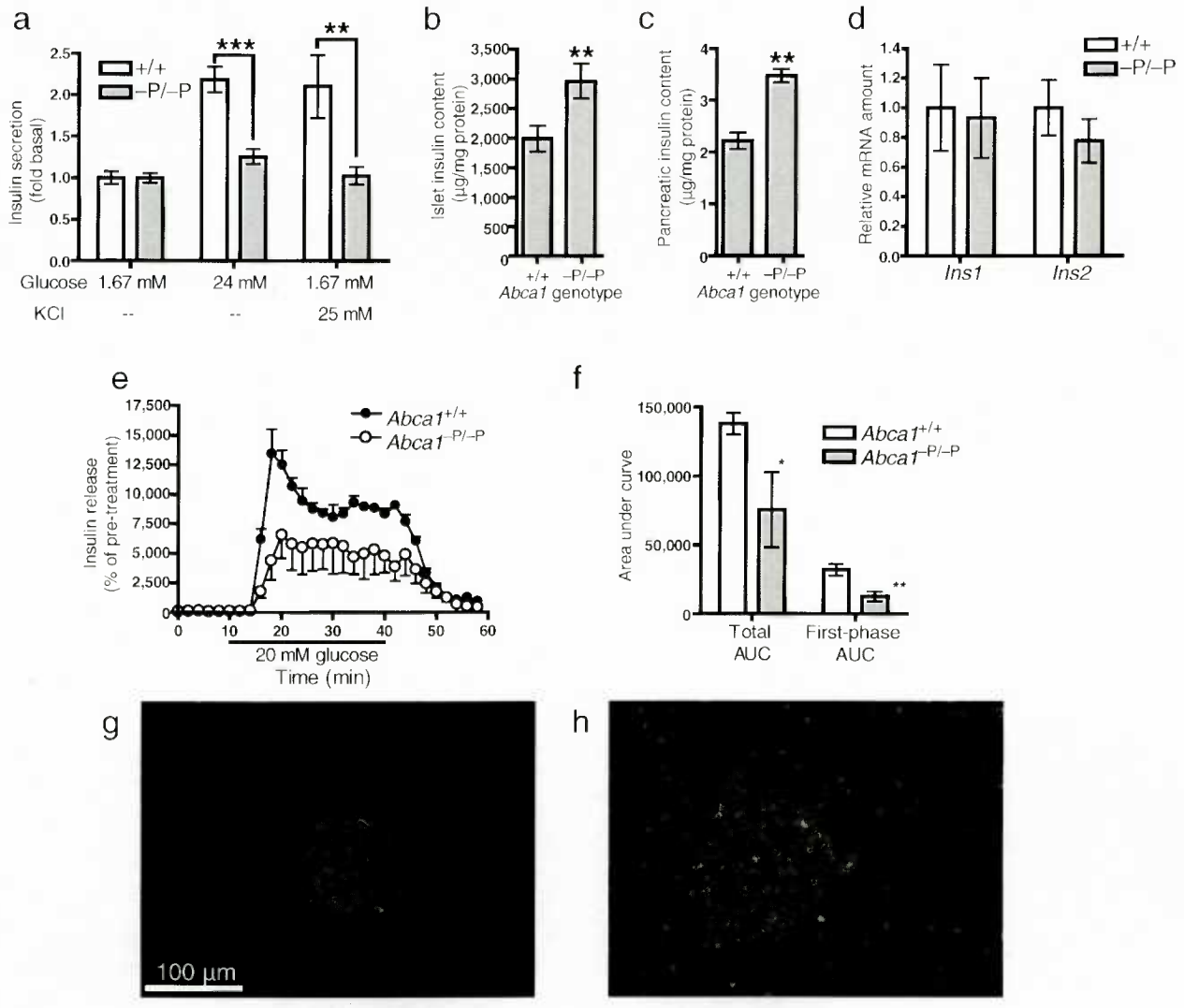

Figure 7.6 Insulin secretion is impaired in isolated islets lacking ß-cell Abca1. (a) insulin secretion from isolated islets. Islets were cultured overnight prior to stimulation in the conditions indicated for 1 hour. Data represents pooled data from 3 separate experiments each consisting of pooled islets from 3 animals per genotype, and values are expressed as percent of islet content relative to basal secretion which is arbitrarily set as 1. (b) islet insulin content. (c) pancreatic insulin content. (d) mRNA expression of *Ins1* and *Ins2* genes in islets isolated from *Abca1*$^{-P/-P}$ mice and controls. (e) Islet perifusion. (f) area under the curve from islet perifusion experiment. (g, h) Filipin (blue) and propidium iodide (red) staining of pancreas sections from *Abca1*$^{+/+}$ (g) and *Abca1*$^{-P/-P}$ (h) mice. n=18 per group for (a), (b) and (d). n=3 per group for (c), (e) and (f). * *P*<0.05, ** *P*<0.01, * *P*<0.001, 2-tailed Student's t-test.**

Exocytosis of insulin-containing granules is thought to occur at specific lipid microdomains of the plasma membrane, and depletion of plasma membrane cholesterol has been reported to enhance glucose-stimulated insulin secretion(13). We examined cellular cholesterol levels in sections of pancreas from *Abca1*$^{-P/-P}$ mice using the cholesterol probe filipin. Islets from *Abca1*$^{-P/-P}$ mice displayed a marked increase in the

intensity of filipin staining (Figure 7. 6g,h), consistent with our quantitative analysis of islet cholesterol content. Filipin staining appeared to be specifically enhanced at the plasma membrane of islet cells from *Abca1*$^{-P/-P}$ mice. These data raise the possibility that defective Abca1-mediated efflux from ß-cells leads to cholesterol accumulation at the plasma membrane which is associated with impaired insulin secretion.

7.2.3. ß-cell ABCA1 in the Response to Thiazolidinedione Treatment

Thiazolidinediones (TZDs) such as rosiglitazone are agonists of the nuclear hormone receptor PPARγ and are widely used in the treatment of T2D. PPARγ is known to activate ABCA1 in macrophages via the liver X receptor (LXR)(14). We found that rosiglitazone treatment significantly increased Abca1 expression in the transformed rat ß-cell line, INS-1 (Figure 7.7a). We therefore hypothesized that rosiglitazone-mediated induction of ß-cell ABCA1 could in part contribute to the beneficial effect of these compounds on ß-cell function and glucose homeostasis.

To test this hypothesis we fed *Abca1*$^{-P/-P}$ and control mice a high-fat diet (30% fat by weight) alone or in combination with 30 mg/kg/day rosiglitazone . Both diet regimens resulted in significant weight gain after 2 weeks, but weight did not differ significantly between genotypes in any group (22.4±4 g vs 22.5±4 g on chow, 27.0±4 g vs 25.2±4 g on high-fat, 29.2±7 g vs 27.1±3 g on high-fat plus rosiglitazone, for *Abca1*$^{+/+}$ vs *Abca1*$^{-P/-P}$, respectively). Glucose tolerance tests were performed prior to and 2 weeks after initiation of high-fat feeding. High-fat feeding resulted in significantly impaired glucose tolerance in wildtype mice, and rosiglitazone restored glucose tolerance (Figure 7.7b). High-fat feeding also worsened glucose tolerance in *Abca1*$^{-P/-P}$ mice, but rosiglitazone had no effect on glucose tolerance in these animals (Figure 7.7c). The total area under

the glucose tolerance curve (AUC_{ipgtt}) was increased in *Abca1*$^{+/+}$ mice fed the high-fat diet, and returned to levels not significantly different from that of chow-fed mice following rosiglitazone (Figure 7.7d). In contrast, the AUC_{ipgtt} for *Abca1*$^{-P/-P}$ mice was significantly increased by high-fat feeding, and did not improve in response to rosiglitazone (Figure 7.7d).

Fasting insulin levels were significantly increased in *Abca1*$^{+/+}$ mice fed a high-fat diet and were returned to baseline levels by rosiglitazone (Figure 7.7e). *Abca1*$^{-P/-P}$ mice showed no change in fasting insulin levels in response to either high-fat diet or rosiglitazone (Figure 7.7e). Insulin sensitivity was improved to a similar extent in both genotypes in response to rosiglitazone (Figure 7.7f), indicating that the difference in glucose tolerance observed between *Abca1*$^{+/+}$ and *Abca1*$^{-P/-P}$ mice could not be explained by different effects of rosiglitazone on insulin sensitivity.

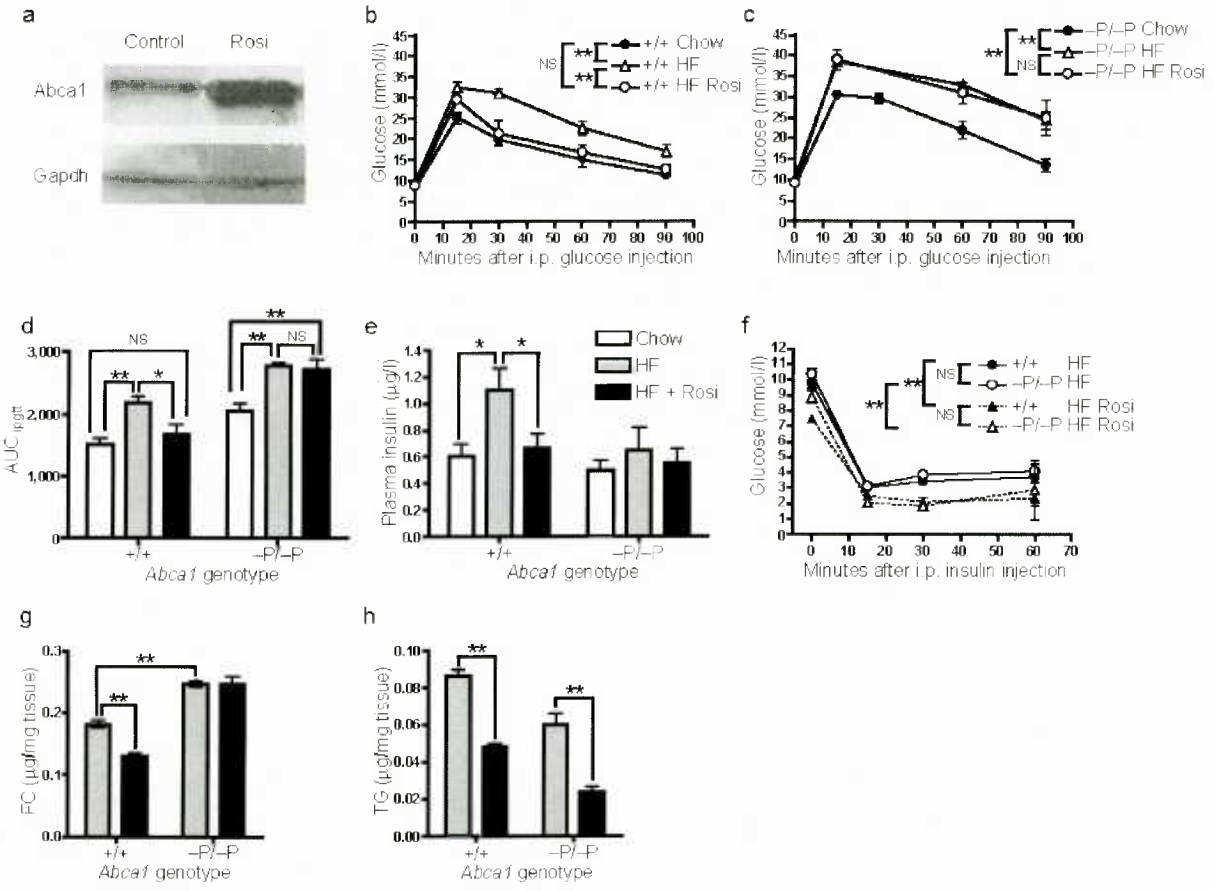

Figure 7.7 ß-cell Abca1 is essential for the response to rosiglitazone treatment. (a) Western blot for Abca1 in INS-1 cells following overnight culture with 1 µM rosiglitazone or control. (b) glucose tolerance testing in *Abca1*$^{+/+}$ receiving a chow diet, and after 2 weeks of high-fat or high-fat+rosiglitazone feeding. n=12 per group. (c) glucose tolerance testing in *Abca1*$^{-P/-P}$ receiving a chow diet, and after 2 weeks of high-fat or high-fat+rosiglitazone feeding. n=12 per group. Statistical comparison of IPGTT curves in (b) and (c) is by repeated-measures ANOVA with Neuman-Keuls post-test. (d) Area under the i.p. glucose tolerance curve (AUCipgtt) for the mice described in (b) and (c). (e) fasting plasma insulin concentrations for the mice described in (b) and (c). n=6 per group. (f) insulin tolerance testing in *Abca1*$^{+/+}$ and *Abca1*$^{-P/-P}$ mice receiving high-fat or high-fat+rosiglitazone diets. n=6 per group. (h, i) islet free cholesterol levels (h) and triglyceride levels (i) in islets isolated from *Abca1*$^{+/+}$ and *Abca1*$^{-P/-P}$ mice after 2 weeks of high-fat or high-fat+rosiglitazone feeding. Lipid levels were determined by gas-liquid chromatography and enzymatic assay on pooled islets from n=3 mice per group, and error bars represent standard deviation of inter-assay variation. * *P*<0.05, ** *P*<0.01, NS not significant.

Rosiglitazone lowered the free cholesterol concentration in islets isolated from wildtype mice (Figure 7.7g). Free cholesterol levels were higher in islets isolated from *Abca1*$^{-P/-P}$ mice and were unchanged by rosiglitazone (Figure 7.7g), suggesting that an inability to lower islet cholesterol content may contribute to the failure to respond to rosiglitazone in these mice. Notably, rosiglitazone lowered islet TG concentration equivalently in both genotypes of mice (Figure 7.7h).

7.3. Discussion

We have documented a novel role for ABCA1 in mediating cholesterol homeostasis and insulin secretion in pancreatic ß-cells. Abca1 is highly expressed in ß-cells and absence of ß-cell Abca1 results in accumulation of cellular cholesterol, marked reduction in insulin secretion in vivo and progressive impairment in glucose tolerance. The defect in insulin secretion is not due to a reduction in ß-cell mass, suggesting that ABCA1 is not involved in islet development or in maintenance of ß-cell mass. Insulin secretion is significantly reduced in islets isolated from mice lacking ß-cell Abca1, showing that cellular cholesterol homeostasis plays a crucial functional role in islets. Finally, we have shown that ß-cell ABCA1 contributes to the beneficial effect of thiazolidinediones on glucose tolerance, suggesting that specific upregulation of ß-cell ABCA1 may be a useful therapeutic approach to improve ß-cell function in type 2 diabetes.

Low levels of HDL cholesterol frequently co-occur with type 2 diabetes in the context of the metabolic syndrome(15). However, a role for cholesterol in lipotoxic disease of islets has not been clearly established. Lipoprotein receptors, in particular the low density lipoprotein (LDL) receptor and LDL-related protein are expressed in mouse islets(16), and atherogenic lipoproteins such as LDL and VLDL induce apoptotic death in isolated islets and transformed ß-cell lines(16-18), an effect blocked by high density lipoproteins (HDL)(16). The current studies are the first to our knowledge to show a specific defect in ß-cell cholesterol homeostasis associated with impaired ß-cell function.

Rare and common variation at the *ABCA1* locus is an important determinant of HDL cholesterol levels in the general population(19;20). This raises the possibility that alterations in ABCA1 may be an important unifying mechanism underlying both the low HDL cholesterol and impaired ß-cell function in individuals with T2D and the metabolic syndrome. In support of this concept, ABCA1 loss of function polymorphisms have been shown to be over-represented in a T2D compared to normo-glycemic population(21). Human islets contain lipid storage vesicles which are absent in those of mice(22), and it will therefore be important to determine if cholesterol accumulation induces dysfunction in human ß-cells.

Loss of ß-cell Abca1 results in gene-dose dependent and age-related progressive impairment in glucose tolerance. Notably, even heterozygous $Abca1^{+/-P}$ mice display significant glucose intolerance by 4 months of age, indicating that appropriate levels of ß-cell Abca1 are critical for normal ß-cell function. Homozygous $Abca1^{-P/-P}$ mice display a more severe impairment in glucose tolerance at 4 months of age. By six months of age fasting blood sugars were significantly higher in $Abca1^{-P/-P}$ than their littermate controls (12.0 ± 1 vs. 9.2 ± 0.4, P=0.01), although fasting hyperglycemia was not a uniform feature of older $Abca1^{-P/-P}$ mice. These findings point to a progressive decline in ß-cell function in the absence of ABCA1, consistent with age-related accumulation of cholesterol.

Absence of ß-cell Abca1 resulted in no overt alteration in islet morphology or ß-cell mass in chow-fed mice. In contrast, glucose-stimulated insulin secretion was dramatically reduced in islets isolated from $Abca1^{-P/-P}$ mice, indicating a specific defect in ß-cell function. The stimulus-secretion pathway by which insulin is released from ß-

cells begins with the uptake of glucose via GLUT2 glucose transporters followed by its metabolism within the ß-cell. The subsequent rise in the cellular ATP:ADP ratio causes closing of ATP-sensitive K^+ channels, leading to depolarization of the plasma membrane and Ca^{2+} influx triggering exocytosis of insulin containing granules(23). The ß-cell abnormality in T2D is thought to occur either at the level of glucose metabolism or exocytosis(24). We observed impaired insulin secretion in response to both glucose and the non-glucose secretagogue L-arginine in vivo, suggesting a more generalized defect in insulin release. In support of this idea, in vitro insulin secretion in response to KCl, which directly depolarizes the cell membrane and leads to insulin release independently of the upstream glucose-sensing pathway, was markedly decreased in islets isolated from mice lacking Abca1. This finding suggests that the defect in *Abca1*$^{-P/-P}$ islets resides at the level of insulin exocytosis, downstream of glucose sensing or metabolism.

Cellular cholesterol levels have been shown to modulate exocytosis in a variety of neuroendocrine cell types(25-27). Our results implicate ABCA1 and cellular cholesterol homeostasis in insulin exocytosis in vivo. Specifically, we show with filipin staining that islets lacking ß-cell Abca1 have accumulation of cholesterol, likely at the plasma membrane, and that this is associated with impaired insulin secretion. It remains to be determined if deletion of Abca1 impairs other homeostatic systems in the ß-cell that contribute to islet dysfunction and impaired glucose tolerance in these mice.

Thiazolidinediones (TZDs) such as rosiglitazone are widely used in the treatment of diabetes. In addition to their insulin-sensitizing actions, TZDs are recognized to have beneficial effects on ß-cell function in humans(28) and ß-cell mass and function in animal models(29). Culture of isolated pancreatic islets in the presence of the TZD

troglitazone has been shown to lower cellular lipid content(30) and enhance ß-cell function. The molecular target of TZDs is PPARγ, a nuclear hormone receptor involved in glucose and lipid metabolism and cell differentiation. PPARγ induces ABCA1 expression and cholesterol efflux via LXR in macrophages(14;31). PPARγ-induced activation of ABCA1 enhances the removal of cellular cholesterol and prevents lipid accumulation in macrophages(14) and mesangial cells(32). We therefore hypothesized that activation of PPARγ would also lead to activation of ABCA1 in ß-cells, and that this effect could contribute to the beneficial effect of TZDs on ß-cell function and glucose homeostasis in T2D.

Rosiglitazone treatment reduced islet TG content in both *Abca1*$^{+/+}$ and *Abca1*$^{-P/-P}$ mice, and lowered islet free cholesterol levels in wildtype but not *Abca1*$^{-P/-P}$ mice. This was associated with significantly improved glucose tolerance in wildtype mice with no effect of rosilglitazone on glucose tolerance in mice deficient for ß-cell Abca1. Rosiglitazone improved insulin tolerance equally in both genotypes. The failure of rosiglitazone to improve glucose tolerance in *Abca1*$^{-P/-P}$ mice suggests that specific activation of ß-cell ABCA1 and subsequent reduction of islet cholesterol content is an important mechanism by which rosiglitazone improves glucose tolerance. It remains to be determined whether the effect of rosiglitazone on ABCA1 requires LXR.

Recently it was reported that mice deficient for LXR-ß, a major transcriptional activator of ABCA1, display impaired glucose tolerance and reduced ß-cell function, together with accumulation of neutral lipids in islets(33). The metabolic defect in these mice is similar to what we observed in *Abca1*$^{-P/-P}$ mice, and indeed a reduction of islet *Abca1* mRNA

was observed in *LXR-ß*$^{-/-}$ mice, suggesting that modulation of Abca1 levels may in part explain the phenotype in these mice.

In summary, we demonstrate that ABCA1 plays a critical functional role in mediating cholesterol homeostasis in ß-cells, and that absence of ß-cell ABCA1 leads to defective insulin secretion and impairment of glucose tolerance, suggesting that cholesterol toxicity may be an important component of lipotoxic disease of islets. Rosiglitazone, which activates ABCA1, improves glucose tolerance in the presence but not in the absence of ß-cell ABCA1, suggesting that strategies which specifically target ß-cell ABCA1 may be beneficial for improving ß-cell function in T2D while avoiding the undesirable effects of TZDs on adiopogenesis.

7.4. Methods

7.4.1. Animals

Abca1 global knock-out mice were generously provided by Dr. Omar Francone, Pfizer Global Research. *Abca1* floxed mice have been previously described(8). *Abca1* ß-cell specific knock-out mice and controls were generated by crossing *Abca1* floxed mice on a mixed C57Bl6/129SvEv background with B6.Cg-Tg(Ins2-cre)25Mgn/J mice (Jackson laboratory)(9). Animals received a standard laboratory chow, or high-fat diet (Harlan Teklad TD 93075) with or without 0.36 % rosiglitazone (to yield a dose of 30 mg/kg/day based on measured food intake), as indicated. All studies were approved by the University of British Columbia Animal Care Committee

7.4.2. Physiological and metabolic studies

Intraperitoneal glucose tolerance tests were performed on 4-hour fasted mice injected with 2 mg/g glucose. Blood glucose was measured with a glucometer (Lifescan). Insulin

tolerance tests were performed on 4-hour fasted mice injected with 1 U/kg human recombinant insulin (Novo Nordisk). Insulin secretion in vivo was determined in 4-hour fasted mice injected with 3 mg/g glucose or 0.3 mg/g L-arginine. Plasma insulin was determined by ELISA (Crystal Chem). Insulin secretion in vitro was performed on hand-picked islets isolated following intra-ductal collagenase injection as previously described(34). Islets were cultured overnight in Ham's F10 media, plated at 40 islets per well in Kreb's Ringer Bicarbonate Buffer (KRB-BSA) containing 1.67 mM glucose for 2 hours, then incubated with buffer containing 1.67 mM glucose, 20 mM glucose or 1.67 mM glucose plus 25 mM KCl. After one hour media was removed and islets lysed in 1 M glacial acetic acid, and insulin levels determined by ELISA (Alpco). Insulin secretion is expressed as percent of islet insulin content and is normalized to basal levels to allow comparison between multiple experiments. Insulin secretion in isolated mouse islets was also studied using the perifusion technique, essentially as described(35). Temperature- and CO_2-controlled perifusion columns were loaded with ~150 sized-matched islets from a given mouse and allowed to equilibrate to a 0.3 ml/minute perifusion flow for 1 hour after which islets were subjected to a step-wise increase from 3mM to 20mM glucose. Insulin was assayed in the samples using Linco's Rat Insulin RIA Kit. Data are normalized to the pre-treatment values (first 7 time-points) to control for uneven loading of columns. Area-under-the-curve values were calculated as the cumulative percent pretreatment.

Plasma cholesterol and HDL cholesterol (on polyethylene glycol-precipitated plasma) were determined by enzymatic assay according to the manufacturer's instructions (Thermo Electron Corporation). Tissue cholesterol levels were determined by gas-liquid chromatography(36) and tissue triglyceride content was determined by enzymatic

assay(37). Plasma free fatty acids were determined by NEFA C kit (WAKO), plasma ß-hydroxybutyrate and cortisol levels were determined by enzymatic assay (Stanbio and Alpco).

7.4.3. Real-time PCR, Western blotting and immunoflourescence

Real-time PCR was performed as described(10). Briefly, total RNA was extracted from isolated islets using the RNeasy kit (Life Technologies), and DNase treated RNA was reverse transcribed using Superscript II (Life Technologies). Rnase treated cDNA was used for real time PCR using SYBR® Green PCR Master Mix (Applied Biosystems) in an ABI Prism 7700 Sequence Detection. GAPDH was used as the invariant control. mRNA levels in control mice were arbitrarily set as 1.

Western blotting was performed as previously described(38). Briefly, tissues were homogenized and sonicated in 20 mM Hepes, 5 mM KCl, 5 mM $MgCl_2$, 0.5% (v/v) Triton X-100, and complete protease inhibitor (Roche), and protein concentration was determined by the Lowry assay. Equivalent amounts of total protein were separated by SDS-PAGE, transferred to PVDF membranes and probed with anti-Abca1(38) or anti-GAPDH (Chemicon) antibodies.

Immunoflourescence was performed with antibodies to insulin and glucagon (DAKO) on paraffin-embedded sections. Immunoflourescence for Abca1 was performed as previously described(10) on 3-month old mice. For ß-cell mass measurements, the percentage insulin-positive surface area was determined from 10 evenly-spaced slides per pancreas. The mean insulin-positive area of the 10 slides was then multiplied by

pancreatic weight to estimate ß-cell mass. Filipin (Sigma) staining was performed on cryostat sections as previously described(39) and counterstained with propidium iodide (Sigma).

7.4.4. Statistical analysis

Data are presented as means plus or minus standard error. Differences between groups were calculated by Student's t-test (for 2 groups) or One-way ANOVA with Neuman-Keuls post-test (for 3 groups) with a *P* value of 0.05 considered significant.

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Chapter 8 - Conclusion

The studies described herein were designed to investigate the role of ABCA1 in HDL biogenesis, susceptibility to atherosclerosis and cellular cholesterol homeostasis. Specifically, I have examined genotype-phenotype interactions for the ABCA1 gene, and have examined the specific roles of ABCA1 in the intestine, liver and pancreatic islet in HDL biogenesis, atherogenesis and glucose homeostasis.

8.1. ABCA1 genetics

More than 100 coding variants have been described in the ABCA1 gene, resulting in a broad spectrum of clinical and biochemical phenotypes. We have provided a catalogue of these variants, and by analyzing them in relation to their in vivo phenotypes have arrived at an initial grouping of mutation by severity. This information should be useful for future, more detailed biochemical analysis of these variants.

A major question in human genetics is how to predict which specific DNA variants will have functional effects on the encoded protein. For rare diseases like TD and FHA these genotype-phenotype correlations are a particular challenge because of the small number of patients with any given mutation. We employed a recently described method for predicting the consequence of DNA variants in ABCA1 using an evolutionary approach. By comparing these predictions with functional assessment of the variants we showed that it is possible to accurately predict which specific variants will impair the function of the ABCA1 protein. These results clarify the impact of genetic variation in ABCA1, and more broadly represent a new approach for identifying functionally significant DNA variants.

8.2. HDL biogenesis

The major advance contributed by this study to the field of HDL metabolism is the identification of intestinal ABCA1 as an important contributor to plasma HDL levels in vivo. We have shown that intestinal ABCA1 contributes to ~30% of the fasting plasma HDL cholesterol pool. An updated figure of HDL biogenesis and metabolism is shown in Figure 8.1.

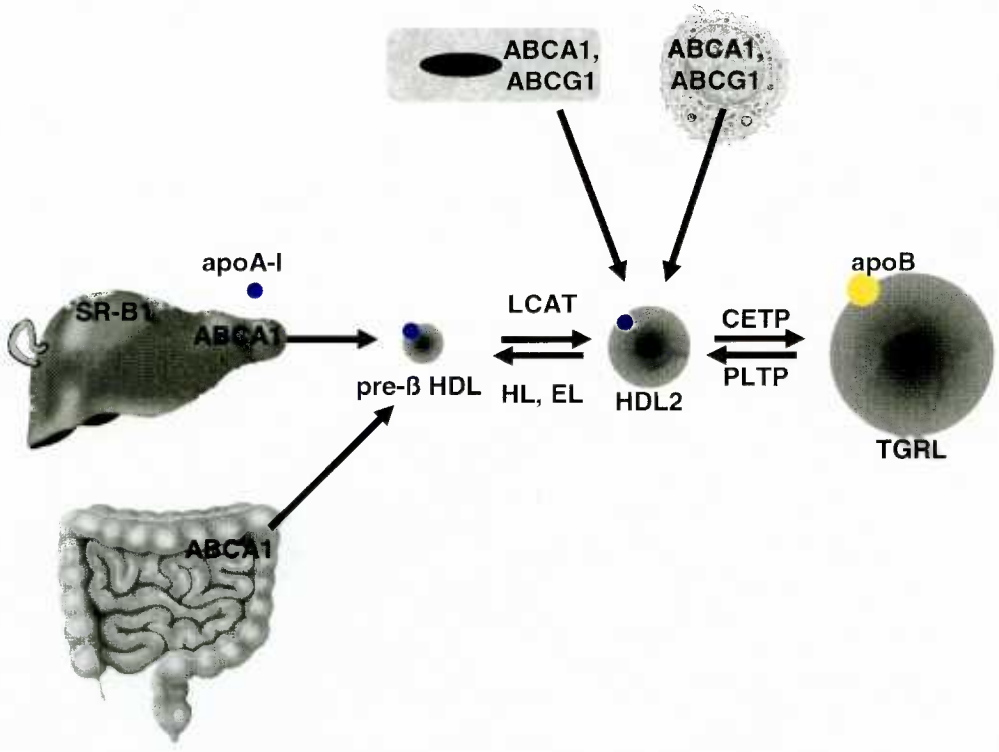

Figure 8.1 Updated pathways of HDL metabolism. ABCA1 in the liver and intestine are primarily responsible for the biogenesis of HDL particles. Nascent HDL particles secreted into the circulation from the liver or intestine can act as acceptors for cholesterol efflux by ABCA1 from peripheral tissues. More mature HDL particles can act as acceptors for efflux by ABCG1. The return of HDL to the liver is omitted for clarity.

A surprising aspect of these studies was that HDL produced by intestinal ABCA1 appears to be secreted directly into the portal circulation, rather than into the lymph. Cholesterol transport by the intestine was thought to occur exclusively via the lymphatics (1), and studies in rats

suggested that nascent HDL particles in lymph were secreted by the intestine (2-5). However, the origin of mesenteric lymph HDL has been a subject of debate (6-10). Our data suggest the existence of a high-affinity transport system that specifically transports newly synthesized HDL into the circulation.

The importance of intestinal ABCA1 to HDL biogenesis was further demonstrated by the finding that selective activation of intestinal ABCA1 using a novel agonist of the Liver X receptor raises plasma HDL cholesterol levels. LXR agonists are attractive compounds for the treatment of cardiovascular disease, and prevent atherosclerosis in animal models (11). However, these compounds also have the undesirable effect of inducing fatty acid synthesis in the liver (12;13). The identification of intestinal ABCA1 as a target for raising HDL levels by LXR activation therefore suggests that intestinal specific LXR agonists may hold promise as therapeutic compounds to raise HDL without causing hypertriglyceridemia.

8.3. ABCA1 and atherosclerosis

Humans with mutations in ABCA1 have increased incidence of CAD. However, the relative increase in CAD risk, about 5.85-fold, is less than would be expected based on the absence of HDL in these patients. This paradox was thought to be due to the reduction in LDL cholesterol in Tangier disease patients, which would mask to some extent the pro-atherogenic effects of low HDL cholesterol. Studies in mouse models of ABCA1 deficiency or overexpression confirmed the complex relationship between ABCA1 and atherosclerosis. Overexpression of ABCA1 globally (14) or in macrophages (15) reduces atherosclerosis, while deletion of ABCA1 in macrophages increases susceptibility to atherosclerosis without changing plasma lipid levels (16). In contrast, mice with global inactivation of ABCA1 have no increased susceptibility to

atherosclerosis even on atherogenic genetic backgrounds (16), reminiscent of the less than expected increase in atherosclerosis in humans.

One explanation for these results is that ABCA1 in macrophages plays an important anti-atherogenic role by preventing lipid accumulation and inhibiting foam cell formation, but does not significantly contribute to plasma HDL cholesterol levels. In contrast, mice with global deletion of ABCA1, because of their reduction in non-HDL cholesterol, have on balance no change in susceptibility to atherosclerosis compared to controls. However, these results also suggested that study of the role of ABCA1 in atherosclerosis in specific tissues may yield insights that are not obtainable by studying global knock-out models. Hepatic ABCA1 is the major contributor to plasma HDL cholesterol levels, and a natural question was therefore the contribution of hepatic ABCA1 to atheroslceorsis susceptibility.

We demonstrated that mice with liver-specific deletion of ABCA1 on the apoE-deficient background have increased susceptibility to atherosclerosis, and accumulate cholesterol in their aortas. This result has implications for our conception of reverse cholesterol transport. By failing to generate HDL particles in the liver, plasma HDL cholesterol levels are reduced. Even in the presence of extra-hepatic ABCA1, this results in a diminished capacity to remove excess cholesterol from peripheral tissues and a subsequent increase in atherosclerosis.

These results are informative from a therapeutics perspective. Macrophage ABCA1 is widely considered an anti-atherogenic pharmacological target, but is clearly technically difficult to specifically target. Hepatic ABCA1 in contrast is relatively easy to target, such as by using viral gene transfer, but it has been controversial whether the effect on atherosclerosis would be

beneficial. Our results suggest that hepatic ABCA1, by virtue of its role on HDL biogenesis, does play an anti-atherogenic role and could therefore represent an attractive therapeutic target.

8.4. ABCA1 and glucose metabolism

ABCA1 is expressed in many tissues in the body (17) but only the liver and intestine appear to be important for the production of HDL particles. What is the function of ABCA1 in other tissues? We discovered serendipitously that mice lacking ABCA1 have impaired glucose tolerance, and that ABCA1 protein is expressed in the islets of Langerhans, suggesting that ABCA1 may play a previously unrecognized role in islet function. The finding that mice with specific inactivation of ß-cell ABCA1 have profound glucose intolerance and defective insulin secretion in vivo and in vitro in association with altered ß-cell cholesterol homeostasis established a new role for ABCA1 in ß-cell function. More broadly, these studies suggest that cholesterol homeostasis may be an important determinant of ß-cell function.

We further demonstrated that ß-cell ABCA1 is required for the beneficial effects of rosiglitazone on glucose tolerance. Rosiglitazone is one of the most commonly used drugs in the treatment of type 2 diabetes with over 10 million prescriptions filed in the United States in 2005. While rosiglitazone is recognized to have beneficial effects on ß-cell function, the mechanisms underlying these effects were not well established. Our data showing a requirement for ß-cell ABCA1 in the response to rosiglitazone suggests that part of the beneficial effect of this compound is by regulating cellular cholesterol homeostasis via ABCA1. This also suggests that therapeutic strategies that specifically target ß-cell ABCA1 may have beneficial effects on ß-cell function while circumventing the negative effects of TZDs on adipogenesis.

8.5. Future Directions

Several lines of future investigation are suggested by these studies. Firstly, it will be of interest to extend our results of the predicition of the functional consequences of ABCA1 variants to other genes with established functional assays. If our results with ABCA1 prove generalizable to other genes it would suggest that the evolutionary-based approach we employed could be a useful method in general for making genotype-phenotype predictions.

Our results indicating that intestinal ABCA1 is an important source of plasma HDL cholesterol raise the question of what role intestinal ABCA1 may play in atherogenesis. By crossing mice with intestinal specific deletion of ABCA1 to one of the established strains of atherosclerosis-suceptible mice it should be possible to answer this question. In addition, it will be of interest to study whether susceptibility to atherosclerosis is modified by intestinal specific activation of ABCA1.

The increased atherosclerosis in mice lacking hepatic ABCA1 suggests that blockage of this specific step in the RCT pathway increases atherogenesis. It will be of interest in future studies to measure rates of reverse cholesterol transport in these mice, such as using labeled macrophages (18), or by assessing the efflux potential of sera from these mice to more accurately determine the impact of hepatic HDL biogenesis on RCT rates.

Finally, it will be informative to extend our studies of ABCA1 function in islets. Two lines of inquiry are immediately suggested. First, study of the precise mechanism by which ABCA1 and cholesterol homeostasis influences insulin secretion. It should be possible to gain insight into this question using cell biology models of the effect of cellular cholesterol accumulation on specific aspects of insulin exocytosis. Secondly, is ß-cell impairment in the absence of ABCA1 worsened

by hypercholesterolemia? By crossing *Abca1*$^{-P/-P}$ mice to hypercholesterolemic strains or using various high-fat/high-cholesterol feeding regimens it should be possible to determine whether the phenotype in these mice can be exacerbated by worsening the degree of cellular cholesterol accumulation.

8.6. Summary

We employed both naturally occurring and engineered variants of ABCA1 in a variety of model systems in order to study the function of this transporter in physiological processes and disease pathogenesis. This has allowed us to identify new roles of ABCA1 in specific tissues in HDL biogenesis, cellular cholesterol homeostasis, atherosclerosis and diabetes. The results of these studies lend support to the notion of studying rare diseases to gain fundamental insights into biological processes as well as the use of tissue-specific gene targeting in order to dissect the discrete function of genes. This work also demonstrates how a single gene can evolve multiple and myriad functions in discrete tissues, adding complexity and wonder to the mysterious phenomenon of life.

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Appendix 1 Copy of UBC Animal Care Committee Certificates

The University of British Columbia

ANIMAL CARE CERTIFICATE
Breeding Programs

PROTOCOL NUMBER: **A03-0247**

INVESTIGATOR OR COURSE DIRECTOR: **Hayden, M.R.**

DEPARTMENT: **Medical Genetics**

PROJECT TITLE: **Breeding: The Contribution of Hepatic ABCA1 to HDL Levels and Composition and Atherosclerosis**

ANIMALS: **Mice 270**

APPROVAL DATE: **04-01-12**

The Animal Care Committee has examined and approved the use of animals for the above breeding program, and have been given an assurance that the animals involved will be cared for in accordance with the principles contained in Care of Experimental Animals - A Guide for Canada, published by the Canadian Council on Animal Care.

Amanda L. Fidler for Dr J. Love

Approval of the UBC Committee on Animal Care by one of:
Dr. W.K. Milsom, Chair
Dr. J. Love, Director, Animal Care Centre
M. L. Macdonald, Manager

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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110, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 FAX: 604-822-5093

The University of British Columbia

ANIMAL CARE CERTIFICATE

PROTOCOL NUMBER: **A04-0143**

INVESTIGATOR OR COURSE DIRECTOR: **Hayden, M.R.**

DEPARTMENT: **CMMT**

PROJECT OR COURSE TITLE: **Contribution of Hepatic ABCA1 to HDL cholesterol levels and composition and susceptibility to atherosclerosis**

ANIMALS: **Mice 300**

START DATE: **04-07-01** APPROVAL DATE: **04-07-15**

FUNDING AGENCY: **Heart and Stroke Foundation of B.C. & Yukon**

The Animal Care Committee has examined and approved the use of animals for the above experimental project or teaching course, and have been given an assurance that the animals involved will be cared for in accordance with the principles contained in Care of Experimental Animals - A Guide for Canada, published by the Canadian Council on Animal Care.

Approval of the UBC Committee on Animal Care by one of:
Dr. W.K. Milsom, Chair
Dr. J. Love, Director, Animal Care Centre
Ms. L. Macdonald, Manager, Animal Care Committee

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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