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NPC1L1: Evolution From Pharmacological Target to Physiological Sterol Transporter

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Abstract—Niemann-Pick C1-like 1 protein (NPC1L1) was recently shown to be the molecular target of the cholesterol absorption inhibitor class of drugs, of which ezetimibe is the first widely used member. Since its discovery, NPC1L1 has also been shown to play a focal physiological role in intestinal absorption of sterols, including plant sterols and cholesterol. Evidence in support of this new metabolic pathway has been garnered not only through human, animal, and cell studies of function but also through the use of human genetics as an approach to study the association of *NPC1L1* sequence variation with metabolic and drug-response phenotypes. The example of NPC1L1 shows how the elucidation of a pharmacological target can serve as a means to gain understanding of a key physiological pathway. **(*Arterioscler Thromb Vasc Biol*. 2006;26:2433-2438.)**

Key Words: cholesterol ■ enterocyte ■ intestine ■ lipoproteins ■ low-density lipoprotein ■ pharmacogenetics ■ sterol

Pharmacological inhibition of intestinal cholesterol absorption is proving to be a useful strategy for treating patients with dyslipidemia, especially those with elevated plasma low-density lipoprotein (LDL) cholesterol. Ezetimibe is the first and so far only cholesterol absorption inhibitor to achieve widespread clinical use. Interestingly, ezetimibe was initially identified in an acyl-coenzyme A (CoA): cholesterol acyltransferase (ACAT) inhibitor discovery program with the hypothesis that disruption of intracellular cholesterol esterification within macrophages would have a beneficial influence on the development of arterial wall plaques. However, ezetimibe's activity on that enzymatic target was underwhelming compared with some other members of the class, but proved to be a potent inhibitor of cholesterol absorption.[1] Subsequent animal and later human studies showed that it could effectively and safely lower plasma LDL cholesterol.[1] Also, the combination of ezetimibe with statin drugs had an additive effect on LDL-lowering compared with statins alone, suggesting that ezetimibe possessed a distinctive mechanism of action.[1] Although ezetimibe and its metabolites were known to localize to the brush border of the upper small intestine,[2] the molecular target was unknown at the time that it was made available for prescription use in North America, almost 4 years ago.

Identification of NPC1L1 Through Genomics and Bioinformatics

A major advance in the understanding of ezetimibe's mechanism of action occurred in 2004 when Altmann, Davis, and colleagues in the research laboratories of Schering-Plough evaluated sequence data from human, rat, and mouse gastrointestinal cDNA libraries to identify proteins with features, such as transmembrane domains and known cholesterol sensing motifs, that would be expected to be seen in a putative cholesterol transporter.[3] One credible candidate emerged from this search, namely Niemann-Pick C1-like 1 protein, whose gene is designated as *Npc1l1* in mouse or *NPC1L1* in human. *NPC1L1* was first described in 2000;[4,5] its name was derived from the fact that it shared 42% amino acid identity with Niemann-Pick type C1 protein (NPC1), which encodes a protein involved in intracellular cholesterol transport and is also the causative gene for Niemann-Pick disease type C1.[6]

In mouse, rat, and human, the small intestine showed the highest level of *NPC1L1* mRNA expression.[2,3] With the exception of human liver, which showed similar levels of expression as the intestine, *NPC1L1* expression in all other tissues was <10% of intestinal expression and was barely detectable in many tissues, which contrasted starkly with the fairly ubiquitous tissue expression of NPC1. Davies et al,[7] however, found human *NPC1L1* mRNA to be predominantly expressed in the liver, with small intestine detection levels at <5% of the liver expression levels. The role of this protein in human liver biology is still unclear. Further analysis of the duodenal–ileal axis of rat small intestine demonstrated that peak expression of *Npc1l1* mRNA and Npc1l1 protein occurred in the proximal jejunum, which was also the predominant site for sterol absorption.[3] Furthermore, the protein appeared to be discretely localized to the epithelial layer of the rat jejunum, specifically within those enterocytes closest to the luminal space.[3]

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Evidence for an Absorptive Role for NPC1L1 From Animal Models

An in vivo role was characterized by the creation of mice in which the *Npc1l1* gene had been deleted by homologous recombination.[3] Intestinal expression of *Npc1l1* in the homozygous knockout mice was completely absent. Plasma cholesterol and triglycerides were not different among wild-type *Npc1l1*$^{+/+}$, heterozygous *Npc1l1*$^{+/-}$, and homozygous *Npc1l1*$^{-/-}$ knockout mice. After oral administration of radiolabeled cholesterol, the *Npc1l1*$^{+/+}$ and *Npc1l1*$^{+/-}$ mice absorbed ≈50% of the administered dose of cholesterol, whereas cholesterol absorption was decreased by ≈70% in the *Npc1l1*$^{-/-}$ mice. In *Npc1l1*$^{+/+}$ mice, ezetimibe inhibited cholesterol absorption to the same extent (≈70%) as gene deletion in *Npc1l1*$^{-/-}$ mice. Ezetimibe caused no further reduction in cholesterol absorption in *Npc1l1*$^{-/-}$ mice, indicating an essential role for Npc1l1 in the ezetimibe-sensitive cholesterol absorption pathway.[3]

Plant sterol absorption, specifically of radiolabeled sitosterol, was also significantly reduced in *Npc1l1*$^{-/-}$ mice compared with *Npc1l1*$^{+/+}$ mice. Liver sitosterol was decreased by ≈70% and sitosterol uptake into the proximal third of the small intestine was decreased by ≈50% in *Npc1l1*$^{-/-}$ mice. Ezetimibe treatment of *Npc1l1*$^{+/+}$ mice substantially decreased sitosterol uptake into the liver and the proximal small intestine to levels comparable to those in *Npc1l1*$^{-/-}$ mice. In ezetimibe treated chow-fed mice, plasma sitosterol and campesterol were decreased by >80%. These findings supported the concept that Npc1l1 plays an essential role in the ezetimibe-sensitive pathway of plant sterol absorption.[8]

Administration of a diet enriched in cholesterol and cholate to *Npc1l1*$^{+/+}$ mice increased plasma cholesterol 2-fold and liver cholesteryl ester 6-fold. These increases were reduced by 90% in *Npc1l1*$^{+/-}$ mice and were completely attenuated in *Npc1l1*$^{-/-}$ mice. Ezetimibe treatment of cholesterol-fed *Npc1l1*$^{+/+}$ mice decreased plasma and liver cholesterol to levels similar to those observed in *Npc1l1*$^{-/-}$ mice. Thus, the absence of *Npc1l1* prevented the elevation in plasma and liver cholesterol seen with increasing dietary cholesterol.[8]

Npc1l1 expression was apparent in the proximal jejunum of *Npc1l1*$^{+/+}$ and *Npc1l1*$^{+/-}$ mice, but was undetectable in *Npc1l1*$^{-/-}$ mice. Furthermore, a diet enriched in cholesterol and cholate reduced *Npc1l1* expression by 75% compared with chow-fed animals of the same genotype, demonstrating that its expression was sensitive to intestinal cholesterol uptake, although this result has not been conclusively established.[9,10] Studies of other sterol-sensitive genes had comparable results. For instance, *Hmgcs1* encodes 3-hydroxy-3-methylglutaryl (HMG) CoA-synthase, a rate-limiting enzyme in cholesterol biosynthesis. Decreased intestinal cholesterol uptake in chow-fed *Npc1l1*$^{-/-}$ mice resulted in a 3.5-fold increase in expression of *Hmgcs1* and this remained upregulated in cholesterol-fed *Npc1l1*$^{-/-}$ mice, indicating that absence of absorbed cholesterol failed to downregulate this sterol-sensitive gene. In addition, *Abcg5* encodes a transporter that participates in the efflux of plant sterols and cholesterol from the enterocyte back into the intestinal lumen, and is sensitive to cholesterol through activation of the liver X receptor (LXR). *Abcg5* expression increased with cholesterol feeding in wild-type mice and this was attenuated in *Npc1l1*$^{-/-}$ mice. Therefore, in the absence of *Npc1l1*, levels of genes regulated by sterol-sensitive transcription factors (SREBPs and LXRs) suggested that intra-enterocyte sterol levels were diminished.[8]

An Early Model for NPC1L1 Complex Function

Using alternative approaches, Smart et al[11] pursued the identification of the ezetimibe target by disrupting putative cholesterol processing genes in zebrafish larvae. They determined that annexin-2 (Anx2) complexed with caveolin-1 (Cav1) in the zebrafish and mouse intestine. Antisense RNA directed against either *Anx2* or *Cav1* prevented formation of this complex and antisense RNA directed against *Anx2* resulted in reduced intracellular sterol mass. Also, ezetimibe administered to hypercholesterolemic mice seemed to disrupt the heterocomplex, through interaction of ezetimibe with Cav1. Together, the data suggested that Anx2 and Cav1 were components of an intestinal sterol transport complex.[11] However, Valasek et al[12] subsequently showed that *Cav1*$^{-/-}$ mice displayed normal mRNA and protein levels of Anx2 and Npc1l1 in proximal intestinal mucosa, and ezetimibe was equally effective in attenuating cholesterol absorption in *Cav1*$^{-/-}$ and wild-type mice. This implies that a heterocomplex containing Anx2 and Cav1 is not essential for Npc1l1 function.

Evidence for Direct Interaction of NPC1L1 With Ezetimibe

Additional evidence has come from in vitro binding assays that tested directly the interaction between the drug and NPC1L1. Garcia-Calvo, with Davis, Thornbury, and colleagues,[13] developed a binding assay and showed that labeled ezetimibe glucuronide bound specifically to a single site in intestinal epithelial brush border membranes and in embryonic kidney cells engineered to express NPC1L1. Furthermore, the binding affinities of ezetimibe and its analogs to recombinant NPC1L1 were indistinguishable from those observed for native enterocyte membranes. Values for the dissociation constant of ezetimibe glucuronide for NPC1L1 were evaluated in intestinal enterocyte membranes and human embryonic kidney cells engineered to express NPC1L1 from various species. Binding affinities were highest for rhesus monkey compared with more moderate values for rat and human, with the lowest values reported for mouse, which also correlates well with the in vivo potency observed for ezetimibe across species. Also, ezetimibe failed to bind to membranes collected from *Npc1l1*$^{-/-}$ null mice compared with wild-type mice. These results directly established NPC1L1 as ezetimibe's target.

Regulation of *NPC1L1* Expression and Subcellular Location of NPC1L1

The regulation of *NPC1L1* expression has not been completely defined. Davis et al reported that intestinal *Npc1l1* mRNA expression was downregulated in wild-type and *Npc1l1*$^{+/-}$ mice fed a cholesterol/cholate diet.[8] This is consistent with sterol-regulated elements (SRE) within the

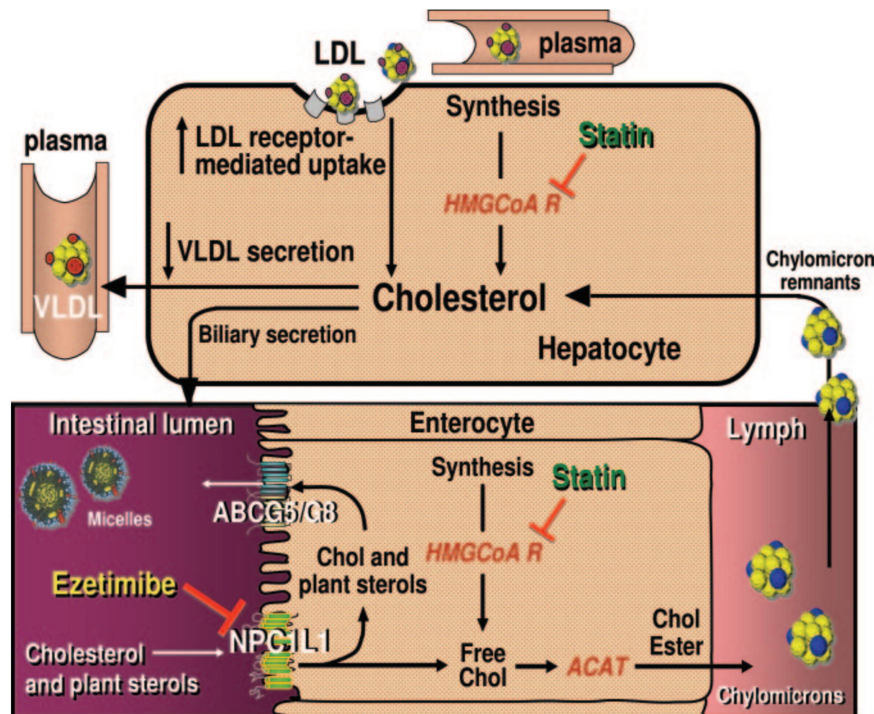

Figure 1. NPC1L1 function. Schematic showing NPC1L1 localized to the brush border of the enterocyte, acting as the gate-keeper for absorption of cholesterol and plant sterols from the lumen of the small intestine and also as the molecular target of ezetimibe. Cholesterol (chol) and plant sterols that have entered the enterocyte can be resecreted into the lumen via the ABCG5/G8 heterodimer. Absorbed cholesterol contributes to the free cholesterol pool, as does the cholesterol from the endogenous biosynthetic pathway for which hydroxymethyl glutaryl-CoA reductase (HMGCoA R) is the rate-limiting step; this activity is pharmacologically inhibited by statin drugs. AcylCoA:cholesterol acyl transferase (ACAT) then esterifies the free cholesterol, which is packaged into chylomicrons and secreted into the lymphatics. After processing in the peripheral circulation, chylomicron remnants are taken up by hepatocytes, contributing to the hepatic pool of cholesterol. Depletion of this pool results in decreased secretion of very low-density lipoproteins (VLDL) and increased catabolism of low-density lipoprotein (LDL) by LDL receptors. Kinetic studies of ezetimibe- treated patients and animals indicated that reduction of plasma apolipoprotein (apo) B was a consequence of both a moderate decrease in the secretion of apoB-containing lipoproteins (LDL and VLDL), and a markedly increased catabolic rate, probably via the LDL receptor. Thus, just as statin drugs reduce the hepatic cholesterol pool by inhibiting cholesterol biosynthesis, ezetimibe ultimately contributes to a decreased hepatic cholesterol pool, albeit via a different mechanism, namely a decreased supply of chylomicron remnant-derived cholesterol to the liver. The diagram does not show the possibility that ezetimibe might also interact with NPC1L1 expressed in human liver, as discussed in the text.

Npc1l1 promoter, suggesting that its expression is regulated like many other genes coding for proteins involved in cholesterol metabolism.[5] Repa et al reported that *Npc1l1* expression was lower in the intestine of wild-type mice fed a cholesterol-enriched diet compared with those on chow alone and fell even further in ACAT2 null mice fed a cholesterol-enriched diet compared with those on chow alone.[10] In contrast, intestinal *Npc1l1* expression in hamsters was much less sensitive to a cholesterol-enriched diet.[9] In preliminary studies, *Npc1l1* expression was increased in both the jejunum and liver in pigs treated with ezetimibe. The enhanced expression correlated with cholesterol depletion in both tissues, consistent with transcriptional regulation by sterols via an SRE.[14]

The subcellular location of NPC1L1 within enterocytes was primarily in the plasma membrane.[3,15] Using rat hepatoma cells over-expressing human NPC1L1, Yu et al[16] recently reported that NPC1L1 was present simultaneously in both intracellular compartments and the cell membrane. Furthermore, its subcellular distribution was regulated by cholesterol availability. Cholesterol depletion induced translocation of NPC1L1 to the cell surface, preferentially to an apical domain, resulting in an increased uptake of free cholesterol through NPC1L1.[16] Whether a similar pattern of cholesterol-regulated translocation of endogenous NPC1L1 occurs in human hepatocytes remains to be determined.

In contrast to mice and rats, human liver also expresses NPC1L1.[3,7] The apical location of NPC1L1 in hepatoma cells predicted a canalicular distribution of NPC1L1 in vivo, which has been confirmed in monkey liver.[16] The physiological significance of NPC1L1 in hepatocytes and their canalicular location remains to be elucidated. Both enterocytes and hepatocytes are polarized cells with their apical surface exposed to micelles containing cholesterol. NPC1L1 mediates intestinal cholesterol absorption from micelles in the intestinal lumen (Figure 1), and it is tempting to speculate that it may also promote cholesterol re-uptake from micelles in the canalicular bile. Furthermore, it is possible that in humans and species that express NPC1L1 in liver, the pharmacological efficacy of ezetimibe may be partially attributed to the blocking of this canalicular re-uptake mechanism.[16] Consequently, ezetimibe might also predispose some individuals to gallstone formation by increasing cholesterol saturation of

bile, although extensive clinical trial experience would indicate that this is not a major concern.

Additional Evidence of the Centrality of NPC1L1 in Sterol Absorption

LXRs function as nuclear cholesterol sensors that become activated in response to elevated intracellular cholesterol and then induce the expression of numerous genes involved in cholesterol absorption, efflux transport, and excretion.[17] Duval et al[18] showed that LXR activation with a synthetic agonist downregulated intestinal expression of *NPC1L1*, supporting a crucial role for both LXR and NPC1L1 in intestinal cholesterol homeostasis. *Npc1l1* repression was also associated with protection against a Western diet in another transgenic model, perhaps mediated by bile acid responsive genes.[19] These findings further emphasized the sterol-sensitivity of *Npc1l1* and its central position in intestinal cholesterol absorption. The final word on the regulation of NPC1L1 expression is still, however, largely unknown, with mixed results from several labs for the regulation of NPC1L1 by LXR agonists.

Inhibition of NPC1L1 by Ezetimibe Decreases LDL Cholesterol

Apolipoprotein B100 (apoB) kinetic studies in men with primary hypercholesterolemia revealed that ezetimibe decreased LDL cholesterol, primarily through enhanced catabolic rates of very-low-density lipoprotein (VLDL), intermediate-density lipoprotein, and LDL, which is consistent with an upregulation of hepatic LDL receptor activity.[20] In the pig model, apoB kinetic analyses demonstrated that the combination of ezetimibe plus simvastatin decreased VLDL and LDL apoB100 concentrations; ezetimibe inhibited cholesterol absorption and simvastatin blocked the compensatory increase in cholesterol synthesis observed with ezetimibe monotherapy, resulting in a significant reduction in hepatic cholesterol and a marked synergistic increase in hepatic LDL receptor expression.[14] Plasma apoB was significantly decreased by a modest reduction in VLDL apoB production and a greatly enhanced LDL receptor-mediated LDL apoB clearance, both of which are attributed to the reduction in hepatic cholesterol (Figure 1). The efficacy of ezetimibe for lowering plasma LDL cholesterol both in patients with homozygous familial hypercholesterolemia[21] and in *Ldlr*-deficient mice[22] further support a role for this agent in reducing hepatic VLDL production. Therefore, inhibition of NPC1L1 by ezetimibe enhances LDL cholesterol-lowering compared with statins alone, through a distinct yet complementary mechanism of action.

Human Genetic Studies

Human genetic studies from 2004 provided some of the earliest evidence in favor of NPC1L1 as the target of ezetimibe. The rationale for these studies was as follows: if NPC1L1 is ezetimibe's target, then naturally occurring coding mutations in the *NPC1L1* gene might affect response to the drug among individuals who carried such mutations. Thus, Wang et al selected 8 individuals from a lipid clinic who exhibited no plasma LDL cholesterol response to ezetimibe treatment and determined the genomic sequence of the coding regions and intron–exon boundaries of *NPC1L1* in these patients.[23] This strategy found a compound heterozygous subject among the ezetimibe nonresponders: an elderly European man who had 2 rare nonsynonymous mutations of the *NPC1L1* gene, namely V55L and I1233N (Figure 2) on different chromosomes.[23] This provided indirect evidence that NPC1L1 was the ezetimibe target, albeit in the absence of direct functional data.

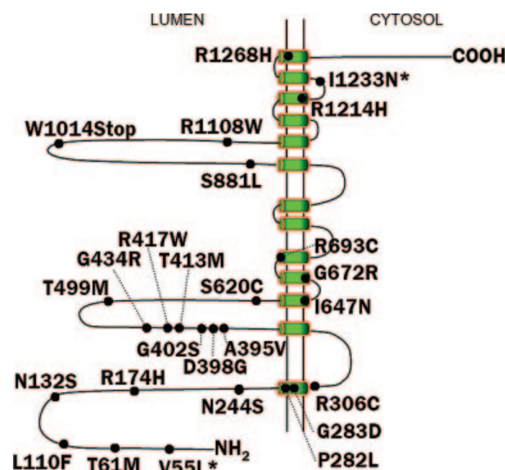

Figure 2. NPC1L1 protein variants. Schematic protein structure of NPC1L1 showing orientation of extracellular and intracellular domains. Positions of rare amino acid variants are indicated. The majority were reported by Cohen et al[26] and are associated with inter-individual variations in sterol absorption. The 2 human *NPC1L1* genomic variants indicated by asterisks were first described in a nonresponder to ezetimibe,[23] and the I1233N variant was among the variants reported by Cohen et al.[26]

After examining the extreme situation of ezetimibe nonresponders, the next hypothesis tested was whether common genetic variation in *NPC1L1* would underlie more subtle interindividual differences in plasma LDL cholesterol response to ezetimibe. Three informative common single nucleotide polymorphisms (SNPs) in *NPC1L1,* namely 1735C>G [L272L], 25342A>C, and 27677T>C [V1296V] (Figure 3), were used to build a "3-site haplotype" which was in turn used to genotype 101 subjects who were treated with ezetimibe for primary hypercholesterolemia.[24] For subjects carrying the most common 1735C-25342A-27677T haplotype (termed "haplotype 2"), plasma LDL cholesterol concentration decreased by ≈24% on ezetimibe treatment. However, ≈12% of individuals did not carry the common *NPC1L1* haplotype 2, and among these individuals plasma LDL cholesterol concentration decreased by ≈35% in response to ezetimibe, representing a highly significant between-genotype difference.[24] Whereas the study was limited because of small sample size, lack of a replication sample and no direct functional consequence for the SNPs, the statistical association again strongly linked NPC1L1 with ezetimibe in humans.

Simon et al[25] similarly evaluated the hypothesis that genetic variation in *NPC1L1* would influence the LDL cholesterol response to ezetimibe in 2 large independent clinical trial cohorts. The most significant individual association with drug response was found for a promoter poly-

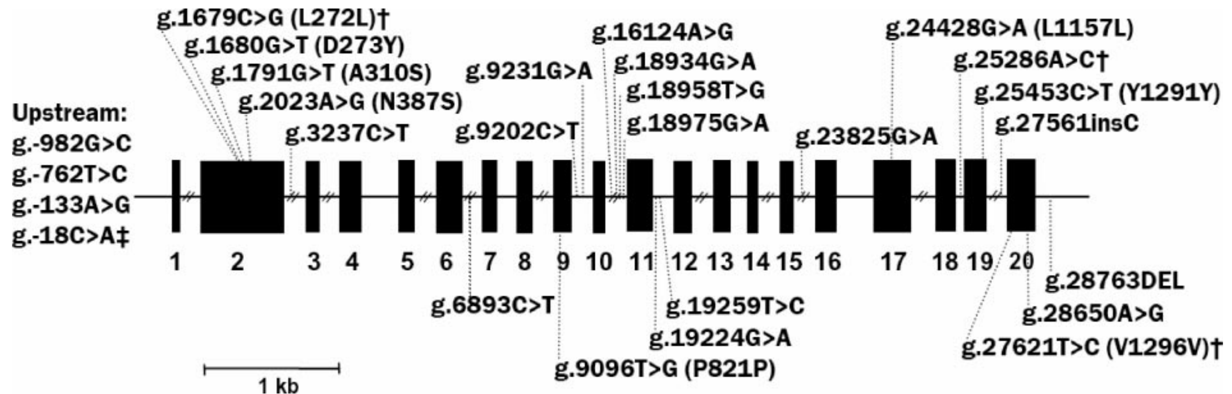

Figure 3. *NPC1L1* genomic variants. Schematic genomic coding map of *NPC1L1* showing positions of relatively common synonymous and nonsynonymous noncoding genomic variants of *NPC1L1*. Intron sizes are not to scale. The numbering is based on the GenBank reference sequence NT_007819, with the numbers harmonized to match those used by Simon et al.[25] Most SNP alleles shown were present at a frequency >5%. The SNPs indicated by daggers formed a haplotype used by Hegele et al[24] that was found to be associated with variation in plasma LDL cholesterol response to ezetimibe. The SNP with the double-dagger (g.-18C>A) was focal to the detection of an association signal, again regarding LDL cholesterol response to ezetimibe, as reported by Simon et al.[25]

morphism, g.−18C>A (Figure 3), for which carriers of the minor allele had a 15% greater LDL cholesterol decrement from baseline after 6 weeks of ezetimibe treatment.[25] Haplotypes composed of the variant and surrounding markers were also significantly associated with variation in LDL cholesterol response. These results added significant weight in favor of the hypothesis that the effect of NPC1L1 genetic variation is on intestinal cholesterol transporter activity.

Finally, additional genetic proof of a physiological role for NPC1L1 was provided by the recent report of a significant relationship between sterol absorption and the presence of multiple rare *NPC1L1* sequence variants.[26] In this population-based study, intestinal sterol absorption was estimated by the ratio of plasma campesterol to lathosterol (Ca: L); campesterol is a plant sterol absorbed only from the diet whereas lathosterol is a cholesterol precursor that correlates with rates of endogenous cholesterol synthesis. Screening of the genomic DNA sequences of *NPC1L1* coding regions in individuals at each extreme of the Ca:L ratio distribution curve showed an excess of sequence variations among the low absorbers (low Ca:L), with ≈4 times as many nonsynonymous sequence variants, tending to involve highly conserved residues (Figure 2), identified in the low absorbers compared with the high absorbers.[26] Interestingly, the majority of the sequence changes (≈75%) were found among blacks. The variants also appeared to be associated with lower baseline plasma concentrations of LDL cholesterol.[26] The I1233N mutation found in 1 subject of the 128 in the high absorber group (Figure 2) coincidentally had been initially found in the original ezetimibe non-responder reported by Wang et al.[23] It is unknown whether the I1233N heterozygous nonresponder to ezetimibe reported by Wang et al was also a hyperabsorber of sterols. Nor is it known whether the I1233N heterozygote hyperabsorber of sterols reported by Cohen et al[26] also responded abnormally to ezetimibe. Further studies might show an overlap between genetic determinants of sterol absorption and ezetimibe response phenotypes.

Until direct functional assessment of any of these common or rare variants has been performed, no definitive conclusions can be drawn about the gene mutations and NPC1L1 function. However, the evidence taken together, including their significant: (1) associations with response to ezetimibe; (2) significant association with measures of sterol absorption; and (3) associations with concentrations of plasma LDL cholesterol implicate the *NPC1L1* gene product as the target for ezetimibe and physiological determinant of intestinal sterol absorption in humans.

Conclusion

Thus, in <3 years, NPC1L1 has catapulted from virtually unknown status to being a key player in normal physiology and as a determinant of response to a useful cholesterol-lowering medication, ezetimibe. Ezetimibe has, in effect, served as a probe to expose a new pathway, to work "backward" from protein function to protein identification. Evidence in support of this new pathway has been garnered not only through traditional reductionist models and in vitro binding assays, but also through the use of human genetics as a tool to study the impact of *NPC1L1* sequence variation in patients and healthy subjects. It is fortuitous that ezetimibe, which was identified in an ACAT inhibitor discovery program, was a much better inhibitor of cholesterol absorption than of ACAT[1] and perhaps ironic that other members of the ACAT inhibitor class of drugs have lately been shown to have minimal efficacy in reducing coronary arterial lesions.[27,28]

The example of ezetimibe and NPC1L1 shows how a widely used pharmaceutical agent can serve as a means to gain understanding of a key physiological pathway. Other examples include the elucidation of peroxisomal proliferator-activated receptors that was afforded by study of fibric acid derivatives and of nicotinic acid receptors by study of niacin. With the ability to develop higher-throughput functional assays, it is inevitable that this approach to discovery of physiological pathways and genes will grow in importance.

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Disclosures

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