Atherosclerosis and Lipoproteins

Deficiency of Niemann-Pick C1 Like 1 Prevents Atherosclerosis in ApoE−/− Mice

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Objective—The objective of this study was to determine whether the deficiency of Niemann-Pick C1 Like 1 (Npc1l1) prevents atherosclerosis in apoE null mice.

Methods and Results—Npc1l1+/+apoE null−/− mice were generated and found to have a significant reduction in cholesterol absorption (−77%) compared with wild-type or apoE−/− mice. Npc1l1/apoE−/− mice were fed a chow or Western diet for 24 weeks, then lipoprotein, hepatic, and biliary cholesterol, and atherosclerosis development was compared with apoE−/−, Npc1l1+/+, wild-type, and ezetimibe-treated apoE−/− mice. Chylomicron remnant/ VLDL cholesterol levels were reduced 80% to 90% in both chow and Western diet–fed Npc1l1/apoE−/− mice relative to apoE−/− mice. Male Npc1l1−/− and Npc1l1/apoE−/− mice were completely resistant to diet induced hypercholesterolemia, and both male and female mice were completely resistant to increases in hepatic and biliary cholesterol levels. Atherosclerosis was reduced 99% in aortic lesion surface area, 94% to 97% in innominate artery intimal lesion area, and >90% in aortic root lesion area in both male and female Npc1l1/apoE−/− mice relative to apoE−/− mice.

Conclusions—Lack of Npc1l1, the molecular target of the cholesterol absorption inhibitor ezetimibe, in apoE−/− mice results in a significant reduction in cholesterol absorption and plasma cholesterol levels, and causes a nearly complete protection from the development of atherosclerosis, under both cholesterol-fed and non–cholesterol-fed conditions.

Key Words: NPC1L1 ■ cholesterol absorption ■ atherosclerosis ■ ezetimibe ■ apoE−/− mice

Hypercholesterolemia is a major clinical risk factor for the development of atherosclerosis. Endogenously synthesized cholesterol, absorption of dietary cholesterol, and the reabsorption of biliary cholesterol in the small intestine all contribute to the regulation of plasma cholesterol levels. Ezetimibe, a potent cholesterol absorption inhibitor, has been shown to lower plasma cholesterol by selectively inhibiting dietary and biliary cholesterol uptake at the brush border of the small intestine, inferring that ezetimibe could be interacting with an intestinal cholesterol transporter.1–3 The understanding of the molecular mechanism by which intestinal cholesterol absorption occurs has recently been elucidated.4 The protein, Niemann-Pick C1 Like 1 (NPC1L1), was identified and found to be highly expressed in the proximal intestine and localized to the surface of enterocytes.4 Further investigation demonstrated that mice deficient in Npc1l1 expression had reduced cholesterol absorption and that ezetimibe was ineffective at preventing residual levels of cholesterol absorption in Npc1l1−/− mice.4,5 Furthermore, labeled ezetimibe glucuronide was found to specifically bind to a single site in the brush border membrane and to HEK293 cells expressing NPC1L1, but did not bind to membranes prepared from mice lacking Npc1l1 protein.6 These results established that NPC1L1 was the intestinal cholesterol transporter and that ezetimibe directly interacted with NPC1L1 to prevent cholesterol from being taken up by intestinal enterocytes and absorbed from the intestinal lumen, thereby decreasing plasma cholesterol levels.7

It was previously demonstrated that ezetimibe treatment inhibits cholesterol absorption, reduces plasma cholesterol, and inhibits the development and progression of atherosclerosis in apoE−/− mice fed Western or cholesterol-free diets.8 Consequently, with the discovery of NPC1L1 as the molecular target of ezetimibe,6 studies were initiated to determine the effect(s) that mice lacking Npc1l1 would have on the development and progression of atherosclerosis in apoE−/− mice.9

Materials and Methods

Animals and Diets
Npc1l1−/− mice, generated as previously described,4 were crossed with apoE−/− mice (Jackson Laboratory, Bar Harbor, Me) to generate homozygous Npc1l1/apoE−/− mice (approximately 75% C57Bl/6, 25% 129/OlaHsd). This colony and that of Npc1l1−/− and wild-type
mice were maintained at Schering Plough Research Institute. Age-matched apoE−/− mice (C57Bl/6) were purchased from Jackson Laboratory. At 8 weeks of age, littermate or age-matched male and female Npc1l1/apoE−/−, wild-type, Npc1l1+/+/apoE−/− mice (n=5 to 11 per group) were placed on a Western (40 kcal% butter fat, 0.15% w/w cholesterol) diet (D12079, Research Diets, Inc) and remained on diet for 24 weeks. Matching groups of mice (n=5 to 11 per group) were fed a standard rodent chow (LabDiet, #5053), which contained <0.02% cholesterol, for 24 weeks. A subset of male, age-matched apoE−/− mice (age 8 weeks) were placed on chow or Western diet containing ezetimibe (0.005%, n=10 per group) for 24 weeks. All mice were euthanized at 32 weeks of age. In addition, a group of male Npc1l1/apoE−/− mice (n=8) were fed the chow diet and euthanized at 18 months of age.

All animals were housed, treated, and cared for according to NIH guidelines for the humane treatment of laboratory animals and the Animal Welfare Act in a program accredited by the American Association for Accreditation of Laboratory Animal Care under a protocol approved by the Schering-Plough Research Institute’s Animal Care and Use Committee.

**Cholesterol Absorption and Synthesis**

Cholesterol absorption and synthesis was determined in wild-type, Npc1l1+/+, apoE−/−, and Npc1l1+/+ apoE−/− littermate or age-matched female mice (n=5 to 6 per group) fed a standard chow diet. For cholesterol absorption, the mice were gavaged with 3H-cholesterol, 1 μCi with 0.1 mg of unlabeled cholesterol in 0.1 mL corn oil. Two hours later, plasma, livers, and small intestines were analyzed by liquid scintillation counting as previously described. Lipid extracts were formed as previously described. A uniform liver sample was isolated from the gallbladders and total cholesterol concentrations chromatographically as previously described. Lipid extracts were assayed for cholesteryl ester, free cholesterol, and triglyceride concentrations as previously described. Bile (2 μL, diluted 10X) was isolated from the gallbladders and total cholesterol concentration assayed using the Wako Cholesterol E enzymatic colorimetric method and plasma triglyceride and FPLC fraction triglyceride were determined using the Wako L-Type TG H method (Wako Chemicals).

**Plasma Cholesterol, Triglyceride, and Lipoprotein Profile Determination**

Nonfasting terminal plasma samples were collected at the end of the 24-week Western-fed and standard chow-fed phases. Plasma lipoprotein profiles of individual mice (0.1 mL plasma) were determined by fast protein liquid chromatography (FPLC) with a Pharmacia Superose 6 column as previously described. FPLC fraction cholesterol and total plasma cholesterol levels were determined using the Wako Cholesterol E enzymatic colorimetric method and plasma triglyceride and FPLC fraction triglyceride were determined using the Wako L-Type TG H method (Wako Chemicals).

**Hepatic and Biliary Cholesterol Determination**

Standard samples of liver were collected, and hepatic lipids were extracted and assayed as previously described. Lipid extracts were dried under nitrogen into high-performance liquid chromatography (HPLC) sample vials, resuspended in hexane/isopropanol, and assayed for cholesteryl ester, free cholesterol, and triglyceride concentrations chromatographically as previously described. Bile (2 μL, diluted 10X) was isolated from the gallbladders and total cholesterol concentration assayed using the Wako Cholesterol E enzymatic colorimetric method.

**Real-Time Quantitative Polymerase Chain Reaction Analysis**

Quantitative polymerase chain reaction (qPCR) analysis was performed as previously described. A uniform liver sample was collected from each mouse, rapidly placed in RNAlater (Ambion, cat no. 7021) and kept at 4°C until use. Total RNA was isolated from the liver samples and then reverse transcribed as previously described. qPCR analysis was performed using Platinum qPCR SuperMix-UDG (Invitrogen, cat no. 11730-017) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Two independent reverse transcriptase reactions were each analyzed twice. Each analysis performed in triplicate included both 18S RNA and the gene of interest. Primer/probe pairs for the qPCR analysis of 18S RNA was purchased from ABI (part no. 4308329); and that for 3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) synthase were synthesized by primer/ probe [6FAM]AGGCATTATTTTAGGTTAGTTACATCC[TAMRA]6FAM. Hepatic expression level of HMG CoA synthase mRNA was quantified and normalized against the control reaction for mouse 18S RNA.

**Determination of Atherosclerosis**

Atherosclerotic lesion development and progression was evaluated at 32 weeks of age in mice fed either a Western diet for 24 weeks (started at age 8 weeks) or a standard chow diet. Following exsanguination, the small intestine and liver, with intact gallbladder attached, were removed. The mice were then perfusion fixed (4% paraformaldehyde in phosphate-buffered saline) via a cannula placed in the left ventricle of the heart. The heart and aorta, including the carotid arteries, were excised intact to the iliac bifurcation. The aorta was removed from the heart, opened longitudinally between the intercostal ostia to the iliac bifurcation, pinned open, and the percent of intimal surface occupied by grossly discernable atherosclerotic lesions in the arch, thoracic, and abdominal regions were measured morphometrically (Biorquant Imaging System). Cross sections of the innominate artery and the base of the aorta (serial sections from the aortic valves) were stained (Verhoeff’s van Gieson Elastic Tissue Method) and evaluated for intimal lesion area by image analysis as previously described. Atherosclerotic lesions were quantified based on the size of the cross-sectional areas (mm2) in the aortic intima and innominate artery and by the percent of lesion surface area of the aortic arch, thoracic aorta, and abdominal aorta. The atherosclerotic lesion areas were compared between all genotypes of the Western-fed groups and the appropriate standard chow-fed groups for each genotype.

**Statistical Analysis**

Results are presented as means±SEM. Statistical significance among responses in genotypes and diet groups were assessed using one way analysis of variance (ANOVA), Dunnett multiple comparisons test, and unpaired Student t tests. Probability values less than 0.05 were considered significant.

**Results**

Phenotypically, Npc1l1/apoE−/− mice were not different from wild-type or Npc1l1+/− mice. Npc1l1/apoE−/− mice appeared healthy throughout the study and were fertile with normal sized litters. Terminal body weights of chow-fed Npc1l1/apoE−/− mice were not significantly different from wild-type or Npc1l1+/− mice and no significant differences in liver weights among the groups were observed (Table). However, the body weights of both male and female apoE−/− mice were significantly less than wild-type, Npc1l1+/−, and Npc1l1/apoE−/− mice. The Western diet caused increases in body weight relative to the chow-fed groups, which reached statistical significance in the male wild-type and ezetimibe treated apoE−/− and female Npc1l1+/− mice (Table).

**Cholesterol Absorption and Synthesis**

Intestinal cholesterol uptake and cholesterol absorption into plasma and liver was determined in wild-type, Npc1l1+/−, apoE−/−, and Npc1l1/apoE−/− female mice after oral admin-
was less dramatic in Npc1l1
Whereas the increase in intestinal cholesterol synthesis
increased to similar levels, relative to wild-type and apoE
wild-type mice, even with their pronounced hypercholes-
Hepatic cholesterol synthesis in-
creased 2-fold increase in hepatic and a 3- to 4-fold increase in
HMG CoA synthase reflected the changes observed with
the cholesterol synthesis findings, with a greater than
2-fold increase in hepatic and a 3- to 4-fold increase in
small intestinal mRNA levels in the Npc1l1−/− and Npc1l1/
apoE−/− mice relative to the wild-type and apoE−/− mice
(Figure 1B, P<0.001). These results demonstrate that mice deficient in
Npc1l1 have a dramatic reduction in the ability to absorb
cholesterol through the intestine and into the plasma and liver.

To determine whether synthesis of cholesterol is af-
fected by the decreased cholesterol absorption observed in
Npc1l1−/− and Npc1l1/apoE−/− mice, hepatic and intestinal
cholesterol synthesis was determined in female mice by
measuring the incorporation of 14C-cholesterol (Figure 1). While cholesterol
absorption into the plasma and liver of apoE−/− mice was
equivalent to wild-type mice, Npc1l1/apoE−/− and Npc1l1−/−
mice absorbed 77% and 83% less cholesterol, respectively,
without type mice (Figure 1A, P<0.001). Cholesterol
uptake into the proximal small intestine was also decreased in
Npc1l1/apoE−/− and Npc1l1−/− mice by 75% and 65%,
respectively, relative to wild-type mice (Figure 1B, P<0.001).
These results demonstrate that mice deficient in
Npc1l1 have a dramatic reduction in the ability to absorb
cholesterol through the intestine and into the plasma and liver.

To determine whether synthesis of cholesterol is af-
fected by the decreased cholesterol absorption observed in
Npc1l1−/− and Npc1l1/apoE−/− mice, hepatic and intestinal
cholesterol synthesis was determined in female mice by
measuring the incorporation of 14C-cholesterol into free and
esterified cholesterol. Hepatic cholesterol synthesis was similar
and not reduced in apoE−/− mice relative to wild-type mice, even with their pronounced hypercholes-
terolemia (see below). Hepatic cholesterol synthesis increased
to similar levels, relative to wild-type and apoE−/−
mice, in Npc1l1−/− mice and in Npc1l1/apoE−/− mice (4.9- and
3.8-fold, respectively for both groups; Figure 1C). Whereas the increase in intestinal cholesterol synthesis
was less dramatic in Npc1l1−/− and Npc1l1/apoE−/− mice,
2.8- and 2.4-fold, respectively, when compared with wild-
type mice (Figure 1D), it is consistent with previously
reported findings.5 Changes in the mRNA expression of
HMG CoA synthase reflected the changes observed with
the cholesterol synthesis findings, with a greater than
2-fold increase in hepatic and a 3- to 4-fold increase in
small intestinal mRNA levels in the Npc1l1−/− and Npc1l1/
apoE−/− mice relative to the wild-type and apoE−/− mice
(Figure 1E and 1F). A compensatory increase in synthesis
of cholesterol in both the liver and small intestine occurred
in response to decreased levels of intestinal cholesterol
uptake and absorption in the Npc1l1−/− and Npc1l1/
apoE−/− mice.

### Plasma Total Cholesterol Determination
Plasma lipid and lipoprotein profiles were determined on all
mice after 24 weeks on either a standard chow or Western diet
(Figure 2). When fed a standard chow diet, plasma cholesterol
values were consistently higher in male and female
apoE−/− mice (Figure 2A). Plasma cholesterol in apoE−/−
mice was 4.3-fold higher than male and 5.8-fold higher than
female wild-type mice (P<0.001), 4.8-fold higher than male
and 7.3-fold higher than female Npc1l1−/− mice (P<0.001)
and 2.5-fold higher than male and 3.5-fold higher than female
Npc1l1/apoE−/− mice (P<0.001). When fed a Western diet
for 24 weeks (Figure 2B), plasma cholesterol levels more

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### Table: Body and Liver Weights, Liver Cholesteryl Ester, and Bile Cholesterol

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body Weight (g)</th>
<th>Liver Weight (g)</th>
<th>Liver/B.W.</th>
<th>Liver CE (mg/liver)</th>
<th>Bile Cholesterol (μmol/mL)</th>
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<td></td>
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<td></td>
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<tr>
<td>Male</td>
<td>Chow 6</td>
<td>37.6±1.7</td>
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<td>0.043±0.001</td>
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<td>Western 5</td>
<td>49.9±1.9*</td>
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<td>41.2±2.0</td>
<td>1.95±0.14</td>
<td>0.048±0.005</td>
<td>74.60±14.86*</td>
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<td>Male</td>
<td>Chow 6</td>
<td>40.6±1.2</td>
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<td>13.86±0.64</td>
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<td>9.72±1.31</td>
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<tr>
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<td>Chow 6</td>
<td>29.5±1.7</td>
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<td>42.9±1.6*</td>
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<td>0.045±0.003</td>
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<td>Western 6</td>
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<td>0.046±0.002</td>
<td>8.51±0.87</td>
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<tr>
<td>Female</td>
<td>Chow 10</td>
<td>29.9±0.7</td>
<td>1.39±0.07</td>
<td>0.047±0.002</td>
<td>15.01±0.94</td>
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<td>Western 11</td>
<td>35.1±2.0</td>
<td>1.91±0.24</td>
<td>0.055±0.003</td>
<td>15.59±3.07</td>
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<td><strong>ApoE(−/−)</strong></td>
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<tr>
<td>Male</td>
<td>Chow 10</td>
<td>33.3±0.9</td>
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<td>0.049±0.001</td>
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<td>0.049±0.001</td>
<td>14.50±0.55</td>
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<td>Western 10</td>
<td>27.6±1.1</td>
<td>1.48±0.08</td>
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<td>46.76±6.52*</td>
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<tr>
<td>Male</td>
<td>Chow 10</td>
<td>29.5±0.8</td>
<td>1.38±0.05</td>
<td>0.046±0.001</td>
<td>10.52±0.34</td>
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<td>2.01±0.13*</td>
<td>0.050±0.002</td>
<td>13.57±1.64</td>
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</table>

Values are Mean±SEM. *P<0.05 Western-fed compared with appropriate chow-fed group (wild-type bile cholesterol pooled by sex [see text]).
than doubled in male apoE−/− mice relative to chow-fed apoE−/− mice, rising from 460 mg/dL to 1091 mg/dL (P<0.001), and increased 44% in female apoE−/− mice from 481 mg/dL to 854 mg/dL (P<0.001). While plasma cholesterol rose significantly in male Western-fed wild-type mice, from 106 mg/dL to 159 mg/dL (P<0.05), plasma cholesterol did not significantly increase in male Npc1l1−/− or Npc1l1/apoE−/− mice relative to their chow-fed groups (Figure 2A and 2B). Conversely, plasma cholesterol rose 2-fold in all Western-fed female groups relative to chow diet, from 83 mg/dL to 172 mg/dL in wild-type (P<0.05), from 66 mg/dL to 150 mg/dL in Npc1l1−/− (P<0.001) and from 139 mg/dL to 277 mg/dL in Npc1l1/apoE−/− mice (P<0.001). Ezetimibe treatment resulted in a 60% reduction in plasma cholesterol levels in both chow or Western diet treatment groups (P<0.001) relative to male apoE−/− mice fed either a standard chow or Western diet alone (Figure 2A and 2B).

### Lipoprotein Profile Determination

Lipoprotein profile analysis of apoE−/− mice deficient in Npc1l1 protein after 24 weeks on either standard chow or Western diets showed that cholesterol levels were reduced in the chylomicron remnant/VLDL and LDL fractions and raised in the HDL fraction (Figure 2C through 2F). When compared with apoE−/− mice, chylomicron remnant/VLDL-cholesterol was reduced by 80% in chow fed and 90% in Western diet fed male Npc1l1/apoE−/− mice (P<0.001), whereas chylomicron remnant/VLDL-cholesterol was reduced by 80% and 84% in female Npc1l1/apoE−/− mice fed a chow or Western diet, respectively (P<0.001). LDL-cholesterol concentrations in Npc1l1/apoE−/− mice were also lowered in both diet regimens relative to apoE−/− mice, with a 50% reduction in LDL-cholesterol in both chow-fed male and female mice.
Figure 2. Plasma cholesterol and lipoprotein profiles in wild-type (WT, Npc1l1+/+), Npc1l1−/−, Npc1l1/apoE−/−, apoE−/− alone and ezetimibe-treated mice. Nonfasting terminal plasma cholesterol levels were determined after 24 weeks on either a chow (A) or Western (B) diet. Lipoprotein profiles were determined by FPLC (n=3 to 6 per group for wild-type and Npc1l1−/−, n=7 to 9 per group for apoE−/− and Npc1l1/apoE−/− groups). Fractions 14 to 19 were designated as chylomicron remnant/VLDL, 20 to 26 as LDL, and 27 to 34 as HDL. Results represent male mice on chow (C) or Western (D) diets and female mice on chow (E) or Western (F) diets. EZ indicates ezetimibe.

(P<0.001) and a 70% and 50% reduction in LDL-cholesterol in Western-fed male and female mice, respectively (P<0.001). Furthermore, HDL-cholesterol values were consistently low in all apoE−/− mice, relative to all other groups, whether fed standard chow or Western diets, ranging from only 18 mg/dL to 30 mg/dL. However, the elimination of the Npc1l1 protein raised HDL-cholesterol levels in apoE−/− mice substantially. HDL-cholesterol increased 38% in male and 50% in female chow fed Npc1l1/apoE−/− mice (P<0.05) and increased 45% in male and 53% in female (P<0.05) Npc1l1/apoE−/− mice fed a Western diet when compared with apoE−/− mice (Figure 2C through 2F).

Lipoprotein profiles of mice fed a standard chow or Western diet containing ezetimibe (0.005%) were similar to Npc1l1/apoE−/− mice. Ezetimibe treatment reduced chylomicron remnant/VLDL-cholesterol in male apoE−/− mice 76% in chow-fed and 70% in Western-fed mice (P<0.001), reduced LDL-cholesterol 40% in both diets (P<0.001) and increased HDL-cholesterol 30% in chow-fed and 50% in Western-fed (P<0.001) mice relative to untreated apoE−/− mice (Figure 2C and 2D). Chylomicron remnant/VLDL-triglyceride levels were also reduced by ezetimibe treatment or in Npc1l1/apoE−/− mice relative to apoE−/− (supplemental Figure I, available online at http://atvb.ahajournals.org). Lipoprotein profiles of the apoE−/− mice were improved by ezetimibe or in Npc1l1/apoE−/− mice, but were not completely normalized to the lipoprotein-cholesterol levels found in wild-type or Npc1l1−/− alone mice (Figure 2).
Hepatic and Biliary Cholesterol Determination
Hepatic cholesteryl ester (CE) accumulation, previously reported as an indicator of chronic intestinal cholesterol absorption status,13 was measured in the livers of mice fed either a standard chow or Western diet for 24 weeks. While hepatic CE values remained consistent among all female chow-fed groups (13.05 to 15.01 mg/liver) (Table), there was a slightly higher level of CE seen in the chow-fed male Npc1l1/apoE−/− group (16.48 mg/liver) relative to wild-type (12.72 mg/liver), Npc1l1+/− (13.86 mg/liver), and apoE−/− (13.79 mg/liver) groups. When fed a Western diet, hepatic CE values in female mice rose significantly relative to chow fed mice, in wild-type mice to 74.6 mg/liver, and in apoE−/− mice to 46.8 mg/liver (P<0.001). Whereas liver CE increased significantly in male Western fed apoE−/− mice, relative to chow fed mice, to 39.18 mg/liver (P<0.05), liver CE also rose from 12.72 mg/liver to 39.27 mg/liver in wild-type but was not statistically significant because of considerable variability within the group. However, hepatic CE did not rise significantly in either male or female Western fed Npc1l1+/− or Npc1l1/apoE−/− mice. Furthermore, male apoE−/− mice fed a Western diet containing ezetimibe (0.005%) for 24 weeks did not exhibit a rise in hepatic CE. Compared with male apoE−/− mice fed a Western diet alone, hepatic CE accumulation was significantly reduced by ezetimibe treatment (Table, P<0.05). Hepatic triglyceride levels generally increased in all groups of mice fed the Western diet compared with the chow diet, while the non-fasted plasma triglyceride levels were variable with no consistent trends (supplemental Table I). Reducing cholesterol absorption by ezetimibe or through lack of Npc1l1 prevents cholesterol diet-induced accumulation of hepatic cholesteryl esters.

Because decreased hepatic cholesterol stores may alter bile cholesterol levels, bile was collected from isolated gallbladders and evaluated for cholesterol content (Table). Results were analogous to those seen with hepatic accumulation of cholesteryl esters. There were no significant differences, within genders, in biliary cholesterol content when comparing chow fed groups (means between 1.66 to 1.88 μmol cholesterol/mL of bile in males; 1.97 to 3.10 μmol cholesterol/mL of bile in females). However, after 24 weeks on Western diet, biliary cholesterol in male and female wild-type mice (pooled) increased from 1.88±0.17 to 4.44±1.55 μmol cholesterol/mL (P<0.05). In apoE−/− mice a more moderate increase in biliary cholesterol, relative to chow-fed mice, was found when fed a Western diet (35% increase in males, 37% increase in females, P<0.05). However, biliary cholesterol remained at or below chow-fed levels in Npc1l1+/− and Npc1l1/apoE−/− mice fed the Western diet. Biliary cholesterol levels were also significantly reduced in male apoE−/− mice fed a Western diet containing ezetimibe relative to male apoE−/− mice fed a Western diet alone (Table, P<0.05). Lack of Npc1l1 or treatment with ezetimibe prevents diet-induced increases in biliary cholesterol levels.

Development and Progression of Atherosclerosis
To determine the effect of eliminating the cholesterol transporter, Npc1l1, on the development and progression of atherosclerosis in apoE−/− mice, atherosclerotic lesion area was measured in the aorta and innominate artery of mice deficient in Npc1l1 protein (Figure 3). Sparse, scattered atherosclerotic lesions were grossly detectable on the intimal surface of the entire aorta in female and male chow-fed apoE−/− mice, with 13.2% and 7.4% of the aortic surface, respectively, consisting of lesions. However, lesions were essentially undetectable in chow-fed Npc1l1/apoE−/− mice with <0.1% of the surface of the entire aorta containing grossly detectable lesions in both female (P<0.001) and male (P<0.05) mice. Aortic lesion involvement was more extensive in apoE−/− mice when fed a Western diet, with 39.2% of female and 30.0% of male aortas consisting of grossly detectable lesions. Lesions on the surface of the entire aorta in Npc1l1/apoE−/− mice fed a Western diet were still essentially undetectable, with lesion surface area reduced by 99% in both female (0.04%, P<0.001) and male (0.1%, P<0.001) mice relative to female and male Western fed apoE−/− mice. While lesion involvement was most extensive and severe on the surface of the aortic arch in apoE−/− mice fed either a chow (20.6% in males and 35.0% in females, Figure 3A) or Western (50.8% in males and 56.4% in females, Figure 3B) diet, gross surface lesions were barely discernible in the aortic arch of Npc1l1/apoE−/− mice fed either chow (0.03%, females; 0.08%, males) or Western (0.07%, females; 0.1%, males) diets (P<0.001). All male and female wild-type and Npc1l1−/− mice had no detectable lesions on the surface of the aorta whether fed a chow or Western diet (results not shown).

Because gross surface atherosclerotic lesions were essentially undetectable in Npc1l1/apoE−/− mice, microscopic cross sections of the innominate artery and aortic sinus were evaluated. The intimal lesion area of the innominate artery in chow-fed female and male Npc1l1/apoE−/− mice were reduced 96% and 94%, respectively, when compared with apoE−/− mice fed a standard chow (Figure 3C, P<0.01). When fed a Western diet, reductions in the intimal lesion size of Npc1l1/apoE−/− mice appeared even more pronounced given the increased lesion area in the Western fed apoE−/− mice (Figure 3D), with reductions of 96% and 97% in female and male mice, respectively (P<0.001). All male and female wild-type and Npc1l1−/− mice had no detectable lesions in the innominate artery whether fed a chow or Western diet (results not shown).

To further assess the protective effect that the elimination of the Npc1l1 protein provides the apoE−/− mouse, lesion areas of the aortic sinus were evaluated. Serial cross sections from the base of the aorta into the heart were prepared and sections 100 μm distal to the valves were quantified. Atherosclerotic lesion area in both chow and Western-fed Npc1l1/apoE−/− mice was significantly reduced in the aortic sinus when compared with apoE−/− mice (Figure 3E and 3F). Lesion area was reduced >90% in male and female chow-fed Npc1l1/apoE−/− mice relative to apoE−/− mice (Figure 3E, P<0.01). When fed a Western diet, lesion area was reduced >95% in both female and male Npc1l1/apoE−/− mice compared with the apoE−/− groups (Figure 3F, P<0.001). These results indicate that by eliminating the cholesterol transporter, Npc1l1, and
Deficiency of NPC1L1 Prevents Atherosclerosis

Figure 3. Morphometric lesion analysis of aortic arch lesion surface area (LSA) (A and B), innominate arteries (C and D), and aortic sinuses (E and F) from Npc1l1/apoE−/− mice and apoE−/− mice with or without ezetimibe treatment on either chow (A, C, E) or Western (B, D, F) diets. The atherosclerotic LSA is expressed as percent of total aortic arch surface and the innominate artery and aortic sinus is expressed as cross-sectional area of the intima. (Wild-type and Npc1l1−/− mice had no detectable lesions whether fed chow or Western diet, data not shown). EZ indicates ezetimibe. *P<0.05 vs apoE−/−.

Ezetimibe treatment of male apoE−/− mice placed on either a standard chow diet or Western diet for 24 weeks also demonstrated significant reductions in atherosclerosis (Figure 3). Intimal lesion area of the innominate artery and aortic sinus, and the aortic lesion surface area were reduced 83%, 75%, and 87%, respectively (P<0.001), in ezetimibe-treated, chow-fed apoE−/− mice relative to untreated apoE−/− mice (Figure 3A, 3C, and 3E). Western-fed, ezetimibe-treated apoE−/− mice displayed more dramatic reductions in lesion area, because of the increased lesion size in apoE−/− mice fed a Western diet alone. The aortic lesion surface area was reduced from 50% to 6.8% in the arch of ezetimibe treated apoE−/− mice (Figure 3B), whereas the intimal lesion area of the innominate artery and the aortic sinus was reduced from 0.118 mm² to 0.017 mm² and from 0.450 mm² to 0.098 mm², respectively (Figure 3D and 3F, P<0.001). Furthermore, lesions of chow or Western-fed ezetimibe-treated male apoE−/− mice were not significantly different from those of male Npc1l1/apoE−/− mice fed either a standard chow or Western diet. These results confirm that treatment with ezetimibe or the elimination of its molecular target, the cholesterol transporter Npc1l1, both of which prevent the intestinal absorption, inhibits the development and progression of atherosclerosis in apoE−/− mice.

To determine whether the absence of Npc1l1 just delays the eventual development of atherosclerosis in apoE−/− mice or if lesion development was delayed because of the mixed genetic background (approximately 75% C57Bl/6, 25% 129/OlaHsd) in the Npc1l1/apoE−/− mice, a group of chow-fed male Npc1l1/apoE−/− mice were evaluated at 18 months of age. The plasma lipoprotein cholesterol profiles (not shown) were essentially identical to the 8-month-old male chow-fed Npc1l1/apoE−/− mice (Figure 2C). Innominate artery atherosclerosis in this 18-month-old Npc1l1/
apoE<sup>−/−</sup> group was reduced by 88% relative to the 8-month-old apoE<sup>−/−</sup> chow-fed male mice from 0.072 mm<sup>2</sup> to 0.009 mm<sup>2</sup> (P>0.001). Aortic sinus lesions were reduced from 0.171 mm<sup>2</sup> in the 8-month-old apoE<sup>−/−</sup> chow-fed male mice to 0.042 mm<sup>2</sup> in 18-month-old Npc1l1/apoE<sup>−/−</sup> mice (P<0.001). The lesions of the 18-month male Npc1l1/apoE<sup>−/−</sup> mice were not significantly different from those of 8-month-old male Npc1l1/apoE<sup>−/−</sup> mice or ezetimibe-treated male apoE<sup>−/−</sup> mice fed a standard chow diet. These results indicate that the absence of Npc1l1 stops any continued development of atherosclerosis over an additional 10-month period in apoE<sup>−/−</sup> mice relative to lesions seen at 8 months of age.

**Discussion**

The purpose of this study was to determine the consequence of disrupting the expression of the Niemann-Pick C1 Like 1 (Npc1l1) protein in the apoE null mouse model of atherosclerosis. NPC1L1 was recently established as the elusive intestinal cholesterol transporter which ezetimibe, a cholesterol absorption inhibitor, selectively binds to inhibit the uptake of dietary and biliary cholesterol into the small intestine. It was reported that mice deficient in Npc1l1 lack uptake of dietary and biliary cholesterol into the small intestine. It was reported that mice deficient in Npc1l1 lack uptake of dietary and biliary cholesterol into the small intestine. It was reported that mice deficient in Npc1l1 lack uptake of dietary and biliary cholesterol into the small intestine. It was reported that mice deficient in Npc1l1 lack uptake of dietary and biliary cholesterol into the small intestine. 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Whereas apoE<sup>−/−</sup> mice absorb cholesterol at levels equivalent to wild-type mice, our acute cholesterol absorption studies demonstrate that Npc1l1 deficiency in the apoE<sup>−/−</sup> background significantly reduced the ability of the mice to absorb intestinal cholesterol. Cholesterol absorption experiments performed using female Npc1l1/apoE<sup>−/−</sup> mice demonstrate that the reduction in cholesterol absorption into the plasma and liver in Npc1l1/apoE<sup>−/−</sup> mice is comparable to that of Npc1l1<sup>−/−</sup> mice and ezetimibe-treated apoE<sup>−/−</sup> mice. In contrast, liver and intestinal cholesterol synthesis was increased in both the Npc1l1/apoE<sup>−/−</sup> and Npc1l1<sup>−/−</sup> relative to wild-type and apoE<sup>−/−</sup> mice. This suggests that reduced cholesterol influx, caused in this case by an intestinal cholesterol transport deficiency, results in increased de novo synthesis of cholesterol in an effort to maintain whole body cholesterol homeostasis. An increased HMG CoA reductase activity has also been found in ezetimibe treated animals, whereby hepatic stores of cholesterol were reduced due to the inhibition of biliary cholesterol absorption and delivery back to the liver.

Whereas plasma and hepatic cholesterol levels increased significantly in wild-type and apoE<sup>−/−</sup> mice after 24 weeks on Western diet, plasma and hepatic cholesterol in male Npc1l1/apoE<sup>−/−</sup> and Npc1l1<sup>−/−</sup> mice remained at levels comparable to chow-fed groups. Plasma cholesterol values doubled in female Npc1l1/apoE<sup>−/−</sup> and Npc1l1<sup>−/−</sup> mice suggesting that they are not completely resistant to the effects of the high saturated fat, 0.15% cholesterol-containing Western diet as male mice. However, plasma cholesterol values in Western diet–fed female Npc1l1/apoE<sup>−/−</sup> and Npc1l1<sup>−/−</sup> mice were still significantly less than apoE<sup>−/−</sup> mice. Moreover, the present cholesterol absorption study demonstrates that male and female Npc1l1/apoE<sup>−/−</sup> and Npc1l1<sup>−/−</sup> mice had equivalent reductions in cholesterol absorption relative to wild-type mice and the degree of reduced cholesterol absorption is also equivalent to those reported previously in dual isotope fecal cholesterol absorption experiments employing both male and female Npc1l1<sup>−/−</sup> mice. Previous experiments, in which only male mice were used, demonstrated that the rise in plasma cholesterol is completely prevented in Npc1l1<sup>−/−</sup> mice when challenged with a very high cholesterol/cholate diet for 7 days. Other investigators have found that male and female Npc1l1<sup>−/−</sup> mice fed a cholesterol/cholate/high fat Paigen diet were completely resistant to diet-induced hypercholesterolemia. The present results confirm that mice deficient in Npc1l1 protein, in the apoE<sup>−/−</sup> mouse model, have decreased dietary and biliary cholesterol absorption which attenuates the rise in plasma cholesterol associated with the apoE<sup>−/−</sup> mouse when fed chow and cholesterol containing diets.

Even when fed the hypercholesterolemic Western diet, in which cholesterol levels of male apoE<sup>−/−</sup> mice increased to more than 1000 mg/dL, the lipoprotein profile in the Npc1l1/apoE<sup>−/−</sup> mice remained dramatically improved. In male Npc1l1/apoE<sup>−/−</sup> mice, chylomicron remnant/VLDL and LDL cholesterol were maintained at chow-fed levels and HDL cholesterol was doubled to equal that of chow-fed mice in both sexes. Female chylomicron remnant/VLDL and LDL cholesterol profiles also improved but, once again, not to the extent of males. Ezetimibe treatment also dramatically improved the lipoprotein profile in male apoE<sup>−/−</sup> mice by lowering chylomicron remnant/VLDL and LDL cholesterol and raising HDL. A similar improvement of apoE<sup>−/−</sup> mouse lipoprotein profile has been reported for mice deficient in ACAT2 and apoE. ACAT2 is also in the intestinal cholesterol absorption pathway and its deficiency in chow-fed apoE<sup>−/−</sup> mice decreases chylomicron remnant/VLDL cholesterol levels and increases HDL cholesterol. Although VLDL triglyceride levels were reduced in the Npc1l1/apoE<sup>−/−</sup> mice (supplemental Figure I), it was reported that VLDL triglyceride levels were significantly increased in ACAT2/apoE<sup>−/−</sup> mice. Overall, the dramatic improvement in the lipoprotein profile of Npc1l1/apoE<sup>−/−</sup> mice indicates that Npc1l1 deficiency can reverse the deleterious effects of apoE protein deficiency.

While the improvement in plasma and hepatic cholesterol and lipoprotein profiles were dramatic, the most remarkable effects of Npc1l1 deficiency was observed while evaluating atherosclerotic lesion development in the apoE<sup>−/−</sup> mouse model. There was a distinct difference in lesion involvement between genders of apoE<sup>−/−</sup> mice, with female mice having larger lesions than the males. It has been documented that, despite tremendous in-breeding of...
the apoE−/− mouse, variability is apparent between mice on normal or high-fat diets and between gender. However, male and female Npc1l1/apoE−/− mice fed chow or Western diets consistently had barely discernible atherosclerotic lesions in all areas evaluated, which were reduced >90% relative to apoE−/− mice. The reduction in atherosclerosis was not just attributable to a delay in development of lesions in the mixed genetic background Npc1l1/apoE−/− mice, because lesions in 18-month-old mice were still dramatically reduced similar to 8-month-old mice in all arterial sites evaluated. This level of atherosclerosis inhibition has also been reported in ACAT2/apoE−/− female mice fed a chow diet for 27 weeks, which demonstrated a similar reduction in VLDL/LDL cholesterol and HDL cholesterol as the Npc1l1/apoE−/− mice fed chow or Western diets consistently had barely discernible atherosclerosis in all arterial sites evaluated. This level of atherosclerosis deficiency and prevented the development of atherosclerosis in the apoE−/− mouse. Decreasing dietary cholesterol absorption and preventing the reuptake of biliary cholesterol into the intestine in apoE−/− mice, either by disrupting the cholesterol transporter, Npc1l1, or by drug intervention with ezetimibe, plasma and hepatic cholesterol concentrations were decreased resulting in the amelioration or prevention of atherosclerosis in the apoE−/− mouse model. Therefore, drugs that interact with NPC1L1 may be useful for treating individuals with hypercholesterolemia and potentially reduce their risk of coronary heart disease.

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Galya Vassileva and Andrei Golovko provided excellent technical assistance in mouse genotyping, breeding, and husbandry.

Disclosures
All authors are employed at Schering-Plough Corporation.

References
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Supplemental Material:

**Deficiency of Niemann-Pick C1 Like 1 (NPC1L1) Prevents Atherosclerosis in apoE -/- Mice**

Harry R. Davis Jr.¹, Lizbeth M. Hoos¹, Glen Tetzloff ¹, Maureen Maguire², Li-ji Zhu¹, Michael P. Graziano¹ and Scott W. Altmann¹

**Plasma, Hepatic, and Lipoprotein Triglyceride Levels**

Total non-fasting plasma triglyceride and hepatic triglyceride levels were not consistently reduced (Table S1), while chylomicron remnant/VLDL triglyceride levels were reduced by ezetimibe treatment of apoE(-/-) mice and in Npc1l1/apoE(-/-) mice (Figure S1).

Figure S1. Plasma triglyceride lipoprotein profiles in wild type (Npcl1l+/+), Npcl1l(-/-), Npc1l1/apoE(-/-), apoE(-/-) alone and ezetimibe treated mice. Lipoprotein profiles were determined by FPLC (n=3-6/group for wild type and Npc1l1(-/-), n=7-9/group for apoE(-/-) and Npc1l1/apoE(-/-) groups). Fractions 14-19 were designated as chylomicron remnant/VLDL, 20-26 as LDL, and 27-34 as HDL. Results represent male mice on chow (A) or western (B) diets and female mice on chow (C) or western (D) diets. EZ, ezetimibe.
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Values are mean ± SEM
Figure S1.

A. Chow-fed, Male

B. Western-fed, Male

C. Chow-fed, Female

D. Western-fed, Female