Dietary Fat–Induced Alterations in Atherosclerosis Are Abolished by ACAT2-Deficiency in ApoB100 Only, LDLr−/− Mice

Thomas A. Bell III, Kathryn Kelley, Martha D. Wilson, Janet K. Sawyer, Lawrence L. Rudel

Objective—The enzyme acyl-coenzyme A (CoA):cholesterol O-acyltransferase 2 (ACAT2) in the liver synthesizes cholesteryl esters (CE) from cholesterol and fatty acyl-CoA, which get incorporated into apoB-containing lipoproteins that are secreted into the bloodstream. Dietary fatty acid composition influences the amount and fatty acid composition of CE within apoB-containing lipoproteins. We hypothesized that when ACAT2 activity is removed by gene deletion, hepatic CE synthesis and secretion would be minimal and, as a result, dietary fat-related differences in atherosclerosis would be eliminated.

Methods and Results—Groups of female apoB100 only, LDLr−/− mice with and without ACAT2 were fed diets enriched in either ω-3 or ω-6 polyunsaturated fat, saturated fat, and cis or trans monounsaturated fat. After 20 weeks on diet, mice fed diets enriched in monounsaturated or saturated fat exhibited significantly higher amounts of plasma cholesterol, larger LDL particles enriched in monounsaturated CE, and more atherosclerosis than mice fed polyunsaturated fat. The dietary fat-induced shifts in plasma cholesterol, LDL size, LDL CE composition, and atherosclerosis were not observed in ACAT2−/− mice. Regardless of the diet fed, the ACAT2−/− mice were protected from atherosclerosis.

Conclusions—The results indicate that in apoB100 only, LDLr−/− mice, ACAT2 plays an essential role in facilitating dietary fat type–specific atherosclerosis through its various effects on plasma lipoprotein concentration and composition.

Key Words: ACAT2 ■ cholesteryl esters ■ lipoproteins ■ liver ■ aortic atherosclerosis

Plasma cholesterol has been established as a predictor of coronary heart disease (CHD) risk. The seminal epidemiological studies conducted in Framingham, Mass found that, beyond total plasma cholesterol measurements, the LDL and HDL cholesterol concentrations provide a more accurate assessment of CHD risk. The incidence of CHD is increased in subjects with elevated amounts of LDL cholesterol or lower quantities of HDL cholesterol. The numerous observations associating elevated LDL with increased CHD risk has prompted studies to unlock the qualities of LDL that give it this proatherogenic behavior.

A growing body of evidence from atherosclerosis studies conducted in animal models suggests the fatty acid composition of the cholesteryl ester of LDL plays a critical role in determining the atherogenic potential of LDL. Studies using nonhuman primates fed diets containing fat and cholesterol found a positive correlation between LDL size and coronary artery atherosclerosis. Analysis of the lipoproteins found the increase in LDL size was attributable to an enrichment of the neutral lipid core with cholesteryl ester (CE), primarily as cholesteryl oleate. Cholesteryl oleate is the primary product of the enzyme acyl CoA: cholesterol acyltransferase (ACAT). ACAT2 has been identified as the isoform of ACAT responsible for the synthesis of cholesteryl esters incorporated into apoB-containing lipoproteins secreted from the liver and intestine.

The relationship between ACAT2 and LDL atherogenicity has been further examined in studies in which African Green monkeys were fed cholesterol-containing diets enriched in either saturated, monounsaturated or polyunsaturated fat. Subsequent studies identified that the majority of hepatic ACAT activity can be attributed to the ACAT2 isoform. The consequence of increased hepatic ACAT2 activity was increased secretion of cholesteryl ester from the liver and subsequent enrichment of LDL with cholesteryl oleate. The enrichment of LDL with cholesteryl oleate increased mean LDL size which was highly correlated with coronary artery atherosclerosis in the monkeys fed saturated and monounsaturated fat, and higher than in the monkeys fed polyunsaturated fat.

Using the apoB100 only, LDLr−/− mouse model, preliminary studies showed similar effects of dietary fat on LDL composition and atherosclerosis. We hypothesized that when ACAT2 activity is negated by gene deletion, relationships...
between LDL composition and atherosclerosis would be lost. ApoB100-only, LDLr<sup>−/−</sup> mice and apoB100 only, LDLr<sup>−/−</sup>, ACAT2<sup>−/−</sup> mice were fed diets enriched in several different fats including polyunsaturated, saturated, and monounsaturated fats. Mice lacking ACAT2 were protected from atherosclerosis regardless of the type of dietary fat fed. Overall, our results show that ACAT2 is a potent mediator of dietary fat-induced atherosclerosis.

**Methods**

**Mice, Diets, and Study Outline**
The apoB100-only, LDLr<sup>−/−</sup> and apoB100 only, LDLr<sup>−/−</sup>, ACAT2<sup>−/−</sup> female mice were created as described in the expanded Materials and Methods (see online supplement, available at http://atvb.ahajournals.org). These mice were fed 1 of 6 diets consisting of 10% calories from fat and 0.02% cholesterol by weight for 20 weeks. The diets were enriched in specific types of dietary fat, either fish- or plant-derived ω-3 polyunsaturated fat, ω-6 polyunsaturated fat, saturated fat, and cis or trans monounsaturated fat. The complete details of dietary ingredients and fatty acid composition are summarized in supplemental Tables I through III (available online at http://atvb.ahajournals.org). The time course for lipid measurement and the tissue collection techniques used at termination are described in the online supplement.

**Plasma Lipid and Lipoprotein Measurements**
Measurement of plasma lipid concentrations were performed using enzymatic assays according to established methods as described in detail in the online supplement. Plasma VLDL, LDL, and HDL were isolated for compositional analyses as described previously. These techniques are described in detail in the online supplement.

**Quantification of Atherosclerosis**
Atherosclerosis was evaluated by biochemical methods according to techniques previously described. A detailed analysis of lipid measurements from the terminal plasma sample is displayed in Table 1. In the control mice, the concentration of plasma free cholesterol (FC) was not significantly different. Control mice fed fish oil had the lowest concentration of plasma FC, at 76 mg/dL, and control mice fed the trans-Mono diet had the highest concentration of plasma FC, at 209 mg/dL. In the ACAT2<sup>−/−</sup> mice, the different diets did not provide similar shifts in plasma FC and no differences in plasma FC were found.

A detailed analysis of lipid measurements from the terminal plasma sample is displayed in Table 1. In the control mice, the concentration of plasma free cholesterol (FC) increased as the type of dietary fat fed was changed from polyunsaturated to saturated and monounsaturated. Control mice fed fish oil had the lowest concentration of plasma FC, at 76 mg/dL, and control mice fed the trans-Mono diet had the highest concentration of plasma FC, at 209 mg/dL. In the ACAT2<sup>−/−</sup> mice, the different diets did not provide similar shifts in plasma FC and no differences in plasma FC were found.

**Results**
The type of fat fed as well as the presence of ACAT2 had demonstrable effects on total plasma cholesterol (TPC) concentration during the treatment period as shown in the data summarized in Figure 1. As early as 2 weeks on diet, control (ACAT2<sup>−/−</sup>) mice segregated themselves into 3 response groups by cholesterol level (Figure 1A). Control mice fed the diet containing fish-oil consistently had the lowest concentrations of TPC, which remained around 200 mg/dL for the duration of the study. The n-6 polyunsaturated fat fed mice had the next lowest concentrations of TPC, which started around 330 mg/dL and increased to approximately 470 mg/dL after 20 weeks on diet. The saturated and monounsaturated fat fed control mice had the highest concentrations of TPC, which at 2 weeks was slightly less than 500 mg/dL. Control mice fed saturated fat maintained TPC levels around 500 mg/dL for much of the treatment period with an unexpected drop-off to 460 mg/dL at 20 weeks. The TPC concentration for control mice fed cis-monounsaturated fat continued to increase throughout the treatment period and reached 625 mg/dL at the termination of the study, whereas the mice fed trans-Mono achieved the highest average TPC after 2 weeks which increased to approximately 400 mg/dL by the end of the treatment period. The exception was control mice fed the fish oil diet, which started with a lower TPC that had increased to about 400 mg/dL by 20 weeks.

Dietary fat type and ACAT2 activity had a greater impact on plasma cholesteryl ester (CE) concentrations (Table 1). In control mice, the concentration of plasma free cholesterol (FC) increased as the type of dietary fat fed was changed from polyunsaturated to saturated and monounsaturated. Control mice fed fish oil had the lowest concentration of plasma FC, at 76 mg/dL, and control mice fed the trans-Mono diet had the highest concentration of plasma FC, at 209 mg/dL. In the ACAT2<sup>−/−</sup> mice, the different diets did not provide similar shifts in plasma FC and no differences in plasma FC were found.

Dietary fat type and ACAT2 activity had a greater impact on plasma cholesteryl ester (CE) concentrations (Table 1). In control mice, dietary fat type had a significant effect on plasma CE. Control mice fed fish oil had significantly lower concentrations (249 mg/dL) when compared with the other diet groups except for the saturated fat group. The other control diet groups had plasma CE concentrations over 500 mg/dL, with mice fed trans-Mono having the highest concentration of CE at 858 mg/dL. The differences in plasma CE concentrations between dietary fat groups of ACAT2<sup>−/−</sup> mice were not significantly different.

Surprisingly, the most dramatic effect of ACAT2 deletion on plasma lipid values was on triglycerides (TG) as shown in Table 1. With the exception of the fish oil– and n-6 Poly–fed.
mice, ACAT2−/− mice exhibited a significant increase in plasma TG when compared with control mice fed the same diet. No discernable trends could be established between the type of dietary fat fed and plasma TG concentration in either genotype. The only significant difference found between 2 diet groups of the same genotype was between ACAT2−/− mice fed fish oil and trans-Mono fat, which had plasma TG concentrations of 230 and 495 mg/dL, respectively.

In Table 2, the effects of dietary fat and ACAT2 activity on plasma lipoprotein CE distribution in subsets of mice at 20 weeks from each diet group are shown. In control mice, the different dietary fat–enriched diets apparently altered VLDL-CE concentration although not in a statistically significant way, and higher averages for VLDL-CE concentrations were observed when the saturated and monounsaturated diets were fed. Except for ACAT2−/− mice fed fish oil, a lower average VLDL-CE concentration was seen in all ACAT2−/− diet groups when compared with control mice fed the same diet. In the ACAT2−/− mice, the different diets did not appear to have a similar effect on mean VLDL-CE concentration to what was observed in the control mice.

In control animals, the average LDL-CE concentration was lowest in the fish oil group and was highest in the cis-Mono group; for these small subsets of animals, the value in the latter group was only significantly higher than for the fish oil group. With the exception of the fish oil diet groups, LDL-CE values tended to be lower in the ACAT2−/− mice when compared with the control groups. The largest difference among the ACAT2−/− animals was between the LDL-CE of fish oil and flaxseed oil groups, averaging 208 and 494 mg/dL, respectively.

Among diet groups of control animals, the lowest HDL-CE concentrations were seen in the fish oil and the trans-Mono groups, whereas the highest value was seen in the cis-Mono group. With the exception of the cis-Mono diet groups, HDL-CE values tended to higher in ACAT2−/− mice when compared with the control mice. In the fish oil and the trans-Mono groups, HDL-CE was almost 300% higher in ACAT2−/− mice, a finding suggesting that the absence of ACAT2 can markedly increase HDL concentrations, at least in some dietary fat groups.

Although dietary fats and presence or absence of ACAT2 activity did not produce large shifts in LDL-CE concentrations, a significant alteration in LDL-CE fatty acid composition occurred, as shown in Figure 2. In control mice, the percentage of monounsaturated CE was higher in mice fed flaxseed oil, saturated, or monounsaturated fat when compared with mice fed fish oil or the ω-6 polyunsaturated diet. The higher percentages

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**TABLE 1. Dietary Fat and ACAT2 Activity Effects on Plasma Lipids**

<table>
<thead>
<tr>
<th>Diet</th>
<th>FC Control</th>
<th>ACAT2−/−</th>
<th>CE Control</th>
<th>ACAT2−/−</th>
<th>TG Control</th>
<th>ACAT2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish</td>
<td>76±4 a</td>
<td>114±25 ab</td>
<td>249±20 a</td>
<td>434±90 abc</td>
<td>68±6 a</td>
<td>230±82 abcd</td>
</tr>
<tr>
<td>Poly (n-6)</td>
<td>140±10 abc</td>
<td>131±17 ab</td>
<td>630±61 bcd</td>
<td>428±50 ab</td>
<td>152±32 abcd</td>
<td>303±77 bcd</td>
</tr>
<tr>
<td>Flax</td>
<td>146±9 bc</td>
<td>184±18 bc</td>
<td>533±49 bc</td>
<td>576±66 bcd</td>
<td>83±17 a</td>
<td>430±75 ef</td>
</tr>
<tr>
<td>Sat</td>
<td>155±8 bc</td>
<td>160±16 bc</td>
<td>520±96 abc</td>
<td>416±56 ab</td>
<td>87±16 ab</td>
<td>314±62 def</td>
</tr>
<tr>
<td>trans-Mono</td>
<td>209±14 c</td>
<td>187±18 bc</td>
<td>858±73 days</td>
<td>498±47 ab</td>
<td>125±25 abc</td>
<td>495±71 f</td>
</tr>
<tr>
<td>cis-Mono</td>
<td>190±28 bc</td>
<td>140±11 ab</td>
<td>730±79 cd</td>
<td>386±32 ab</td>
<td>71±12 a</td>
<td>351±50 def</td>
</tr>
</tbody>
</table>

Plasma FC, CE, and TG measurements were made on the terminal plasma sample of 9–14 mice per diet/genotype group. For each plasma lipid class (FC, CE, TG), all 12 diet/genotype groups were compared using a Tukey Honestly Significant Difference Test with P<0.05. Any No. with one or more letters was not different from another No. within that diet/genotype class if it had the same letter; numbers with only different letters were significantly different. All data are mean±SEM.

**TABLE 2. Dietary Fat and ACAT2 Activity Effects on Lipoprotein CE Distribution**

<table>
<thead>
<tr>
<th>Diet</th>
<th>VLDL Control</th>
<th>ACAT2−/−</th>
<th>LDL Control</th>
<th>ACAT2−/−</th>
<th>HDL Control</th>
<th>ACAT2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish</td>
<td>2±11 a</td>
<td>9±2 a</td>
<td>170±63 a</td>
<td>208±25 a</td>
<td>15±11 a</td>
<td>50±4 a</td>
</tr>
<tr>
<td>Poly (n-6)</td>
<td>45±18 a</td>
<td>19±2 a</td>
<td>415±39 a,b</td>
<td>372±17 a,b</td>
<td>40±1 a,b</td>
<td>73±16 a</td>
</tr>
<tr>
<td>Flax</td>
<td>76±5 a</td>
<td>61±30 a</td>
<td>545±26 a,b</td>
<td>494±86 b</td>
<td>28±8 a,b</td>
<td>33±8 a</td>
</tr>
<tr>
<td>Sat</td>
<td>140±70 a</td>
<td>23±8 a</td>
<td>494±29 a,b</td>
<td>400±66 a</td>
<td>36±8 a,b</td>
<td>56±6 a</td>
</tr>
<tr>
<td>trans-Mono</td>
<td>162±93 a</td>
<td>71±32 a</td>
<td>472±79 a,b</td>
<td>418±75 a,b</td>
<td>16±3 a</td>
<td>46±9 a</td>
</tr>
<tr>
<td>cis-Mono</td>
<td>188±58 a</td>
<td>26±2 a</td>
<td>629±169 b</td>
<td>264±28 a,b</td>
<td>60±14 b</td>
<td>52±11 a</td>
</tr>
</tbody>
</table>

Lipoprotein CE concentrations were determined in the terminal plasma sample of 3 mice for each of the 12 diet/genotype groups. Within each lipoprotein subclass (VLDL, LDL, HDL), all 12 diet/genotype groups were compared by a Tukey Honestly Significant Difference Test with P<0.05. Any No. with one or more letters was not different from another No. within that lipoprotein class if they had the same letter; numbers with only different letters were significantly different. Lipoprotein subfractions were collected from whole plasma after separation by HPLC. Lipoprotein CE values were calculated from TC and FC measurements determined by enzymatic assays as described in Materials and Methods. All data are mean±SEM for the 3 plasma samples analyzed. The trends seen for VLDL CE to increase from poly to sat to monos, and to be lower in ACAT2−/− mice are believed to represent true diet and genotype effects even though the apparent differences were not found to be statistically significant in the 3 plasma samples analyzed.
of saturated and monounsaturated CE were no longer present in the ACAT2−/− mice, and Figure 2A and 2B show the inverse effects on polyunsaturated fatty acid CE percentages when compared with the saturated plus monounsaturated CE percentages.

Figure 3 shows the data on effects of dietary fat and genotype on LDL size and atherosclerosis. In control mice the different dietary fats were able to alter mean LDL size, becoming larger as the fatty acid composition of the diet shifted from polyunsaturated to saturated and monounsaturated (Figure 3A). Fish oil-fed mice had the smallest mean LDL at 4.1 g/μmole, and the largest LDL particles were found in control mice fed cis-Mono at 5.9 g/μmole. Any effects of dietary fat on LDL size were lost when ACAT2 activity was absent and LDL size was equivalent in all dietary fat groups with means near 4.0 g/μmole for each group (Figure 3B).

A parallel relationship occurred in the extent of atherosclerosis quantified by chemical measurement of aortic CE and FC accumulation (Figure 3C and 3E). In control mice, aortic CE accumulation increased as the fatty acid composition of

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**Figure 2.** Fatty acid methyl ester (FAME) analyses were performed on LDL-CE of control (A) and ACAT2−/− (B) mice (n=3). The bars represent the mean (±SEM) percentages of saturated, monounsaturated, and polyunsaturated CE in LDL for the control and ACAT2−/− mice; the inset indicates which pattern/color corresponds to which type of CE.

**Figure 3.** Quantification of LDL size and atherosclerosis extent in diet fed control and ACAT2−/− mice. The mean size of LDL from (A) control and (B) ACAT2−/− mice was determined from 4 mice per diet/genotype group by the high-performance liquid chromatography (HPLC) technique described in the Materials and Methods section and LDL size is expressed as g/μmole. The extent of atherosclerosis was assessed by aortic CE and FC accumulation in (C, E) control and (D, F) ACAT2−/− mice with 9 to 14 mice per diet/genotype group as measured by GLC; results are expressed as mg of CE or FC/g of aortic protein. When comparing each measurement across both genotypes, different letters represent a significant difference with P<0.05 as indicated by a post hoc Tukey Honestly Significant Difference test.
the diet shifted from polyunsaturated to saturated and mono-
unsaturated. Mice fed fish oil had the lowest average aortic
CE at 9.0 mg/g protein, and mice fed trans-Mono had the
highest average at 38.3 mg/g protein. In the control mice,
with exception of the mice fed cis-Mono, the concentration of
aortic FC also increased with the type of dietary fat fed
although not to the same extent as CE. Control mice fed the
n-6 Poly diet had the lowest concentration of aortic FC at 19.5
mg/g protein, and mice fed trans-Mono had the highest
concentration at 47.1 mg/g protein. The ACAT2−/− mice did
not have a similar trend of diet-induced atherosclerosis
among dietary fat groups; markedly lower aortic CE concen-
trations were found for all ACAT2−/− diet groups averaging
6.9 mg/g protein, a value lower than in any of the control diet
groups. The ACAT2−/− mice also did not show an effect of
dietary fat on aortic FC concentration, and the bulk of the FC in
these groups likely represents membrane cholesterol. In Figure
4, LDL size and atherosclerosis extent measured as aortic CE
were correlated. A strong relationship was found between these
2 variables with a correlation coefficient of \( r = 0.87 \). Significant
correlations of atherosclerosis with other plasma lipid parame-
ters were also found (for example, a correlation of \( r = 0.78 \) with
TPC as shown in supplemental Figure I) but none were as strong
as the correlation between LDL size and atherosclerosis.

**Discussion**

In pilot studies in the apoB100 only, LDLr−/− mouse a
relationship between dietary fat type, LDL size, and athero-
sclerosis was seen, similar to what had been found in studies
of coronary artery atherosclerosis in monkeys\(^6,7,9\) where
ACAT2 activity was implicated in the diet-induced difference
in atherosclerosis. In the present study, ACAT2 activity was
removed by gene deletion so that the role of this enzyme
could be examined. Remarkably, dietary fat–related differ-
ences in atherosclerosis were all lost and atherosclerosis was
minimal in ACAT2−/− mice (Figure 3). This is the first
demonstration of a liver enzyme that can control the differ-
ential atherosclerosis responses to individual dietary fats. The
striking association with LDL size (Figure 4) suggests that
the prevention of dietary fat–related shifts in types and
amounts of CE in LDL is a major contributor to the effect.
The association with LDL size was higher even than with
TPC (see supplemental Figure I) and although it was only
determined for the blood samples collected at 20 weeks, there
is little room for the strength of the association to increase
with more data such as from additional time points.

Earlier results from studies in nonhuman primates showed
that the increase in LDL size that occurred when saturated and
monounsaturated fat were fed was attributable to an
enrichment of the lipoprotein particle core with monounsatur-
ated CE, primarily cholesteryl oleate.\(^3\) A similar relation-
ship between dietary fat and LDL-CE fatty acid composition
occurred in the apoB100 only, LDLr−/− mice as seen in the
data of Figure 2. An increase in the percentage of monounsatur-
ated CE in LDL occurred as the type of fat shifted from
polyunsaturated to saturated and monounsaturated fat (Figure
2A). When this shift in LDL-CE composition is coupled with
the change in LDL size shown in Figure 3A, the net effect is
a marked increase in the amount of monounsaturated CE
within the LDL particles of mice fed saturated and monounsatur-
ated fat enriched diets. In the monkey studies and in
our current work, enrichment of the LDL core with
monounsaturated CE is highly associated with an increase
in atherosclerosis.
The ACAT2\textsuperscript{−/−} mice in these studies allowed for an assessment of the role of ACAT2 derived CE in the pathogenesis of atherosclerosis. Figure 2B shows that LDL-CE fatty acid composition is dramatically altered when ACAT2 activity is removed, resulting in a shift in LDL-CE fatty acid composition to be predominantly polyunsaturated with a lower CE content per particle. The change in LDL-CE fatty acid composition is believed to be attributable to a compensatory action of lecithin:cholesterol acyltransferase (LCAT) to provide CE for the LDL particle core.\textsuperscript{13,15} LCAT in the plasma compartment produces polyunsaturated CE by using phospholipid sn-2 fatty acids as substrates. LCAT in the ACAT2\textsuperscript{−/−} mice provided LDL-CE concentrations nearly comparable to those observed in the control mice. Further, LCAT can potentially contribute to the increased HDL-CE found in most of the ACAT2\textsuperscript{−/−} diet groups (Table 2). Because the control and ACAT2\textsuperscript{−/−} mice had similar concentrations of LDL-CE, the differences in atherosclerosis observed between these two genotypes (Figure 3C and 3D) appear to be importantly related to the shift in LDL-CE fatty acid content and composition. This result strengthens the conclusion that ACAT2-derived CE determines the atherogenicity of LDL. The higher the percentage of saturated and monounsaturated CE in LDL particles, especially as they become larger in size, will result in an increase in the number of these higher melting cholesteryl esters per LDL particle. This shift is thought to result in cholesteryl ester accumulation in atherosclerotic lesions, possibly as a result of a decreased rate of CE clearance from foam cells.\textsuperscript{16,17}

The apoB100 only mice were originally developed to compare the atherogenic potential of LDL-sized particles to larger VLDL remnant-sized particles that were free of apoB48.\textsuperscript{18} To do so, the apoB100 only mice were crossed onto both the LDLr\textsuperscript{−/−} and apoE\textsuperscript{−/−} mouse models of atherosclerosis. Despite having similar levels of plasma cholesterol, the apoB100 only, LDLr\textsuperscript{−/−} mice had significantly higher amounts of atherosclerosis when compared with the apoB100 only, apoE\textsuperscript{−/−} mice. The difference in atherosclerosis between the 2 groups of mice was suggested to be attributable to the significantly increased particle number in the apoB100 only, LDLr\textsuperscript{−/−} mice. Our current studies in the apoB100 only, LDLr\textsuperscript{−/−} mice, allow a further refinement to the assessment of particle size/atherosclerosis associations. By using different dietary fats, we have shifted mean apoB100-LDL sizes while keeping the majority of the particles within the LDL size range. The results from our studies indicate in the apoB100 only, LDLr\textsuperscript{−/−} mice, an increase in LDL size is attributable to an increase in ACAT2-derived cholesteryl oleate in LDL; the effect to increase LDL size enhances atherosclerosis.

LDL size as an indicator of CHD risk has been examined in detail in clinical studies.\textsuperscript{19,20} Both large and small LDL have been positively associated with CHD in humans. The data indicate that the utility of LDL size as a predictor of atherosclerosis in the absence of other measurements such as LDL particle concentration and composition is limited. In earlier studies in nonhuman primates\textsuperscript{7,9} and in our current work in mice, an increase in LDL size is associated with an enrichment of LDL with ACAT2-derived monounsaturated CE. Additionally, a recent study in humans found a positive relationship between monounsaturated CE in plasma and carotid artery intimal thickness.\textsuperscript{21} The results from these studies suggest that assessing LDL size alone is not sufficient and that beyond LDL size, measurements of LDL-CE fatty acid composition including the content of cholesteryl oleate in LDL, may provide a more accurate indication of CHD risk.

When ACAT2 was present, the effects the different dietary fats had on TPC were rapid and specific to the type of fat fed (Figure 1A). In nonhuman primates, dietary saturated and monounsaturated fat enhanced CE production and secretion from the liver when compared with polyunsaturated fat.\textsuperscript{5,9} The current results suggest that the effects the different fatty acid enriched diets had on hepatic ACAT2 activity and hepatic CE secretion may have paralleled the earlier observations from nonhuman primates and the absence of ACAT2 blocked this response. Using liver perfusion experiments in mice with and without ACAT2, Lee et al directly assessed the function of ACAT2 in lipoprotein CE secretion from the liver.\textsuperscript{22} Liver perfusate from ACAT2\textsuperscript{−/−}, LDLr\textsuperscript{−/−} mice had an 87% decrease in VLDL-CE accumulation when compared with LDLr\textsuperscript{−/−} mice, indicating ACAT2 was vital in supplying newly synthesized apoB-lipoproteins with CE.

Regardless of the diet fed, the apoB100 only, LDLr\textsuperscript{−/−}, ACAT2\textsuperscript{−/−} mice had elevated plasma TG (Table 1). This observation is typical of those made in other studies conducted in ACAT2 knockout mice.\textsuperscript{15,22} Based on the results from isolated liver perfusion, ACAT2\textsuperscript{−/−}, LDLr\textsuperscript{−/−} mice showed an increased hepatic secretion of VLDL measured as a higher accumulation rate of TG in perfusate when compared with LDLr\textsuperscript{−/−} mice.\textsuperscript{22} The increased incorporation of TG into newly formed lipoproteins in the liver of ACAT2 knockout mice apparently compensates in some way for the decreased CE production in the hepatocyte. Exactly why this phenomenon occurs is not clear and needs further study. Nevertheless, the hypertriglyceridemia identified in ACAT2 knockout mice appears to be benign because atherosclerosis was much attenuated at the same time that the elevation in triglyceride concentrations occurred.

Three different polyunsaturated fats were compared in these studies, including \( \omega-6 \) fatty acid enriched diet and 2 \( \omega-3 \) polyunsaturated fat–containing diets. One diet used fish oil and another used flaxseed oil as the \( \omega-3 \) fatty acid source. The key difference between the flax and fish oil diets is that the primary \( \omega-3 \) fatty acid in the fish diet is \( \alpha \)-linolenic acid, a precursor to the eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids of the fish oil diet. The fish diet did not decrease plasma cholesterol, VLDL-CE, LDL size, or the percentage of monounsaturated CE in LDL in an analogous fashion to what was observed in the fish oil fed mice. The lack of equivalent effects on plasma lipoprotein measurements apparently led to higher amounts of atherosclerosis in mice fed the flax versus fish oil diets (Figure 3C). The bulk of the \( \omega-3 \) fatty acids found in plasma and liver lipids in mice fed the flax diet were EPA and DHA, not \( \alpha \)-linolenic acid (data not shown). However, the percentage of EPA and DHA in plasma and liver cholesteryl esters and triglycerides of mice fed the flax diet was only about half as much as was found in mice fed the fish oil diet (data not shown). The results suggest that \( \alpha \)-linolenic acid is not as effective as EPA.
and DHA in atheroprotection. Others have shown that the Δ6-desaturase enzyme required for the conversion of α-linolenic acid to EPA can be limiting, resulting in poor conversion of α-linolenic acid to more potent ω-3 fatty acids.23

Interestingly, mice fed the ω-6 polyunsaturated fat and fish oil diets presented with a similar reduction in aortic CE (Figure 3C), despite the ω-6 fatty acid fed mice having plasma CE and LDL-CE concentrations more similar to what was found in mice fed saturated or monounsaturated fat. The smaller LDL size (Figure 3A) and reduced percentage of monounsaturated CE in LDL (Figure 2A) appears to have protected the ω-6 fatty acid fed mice from atherosclerosis. These data in mice fed ω-6 fatty acids highlight how measurements of plasma lipoprotein cholesterol distribution are not always sufficient in and of themselves to accurately predict atherosclerosis.

The relative impact that different dietary fats have on CHD risk has been studied and debated for quite some time, and yet a consensus as to which types of fatty acids promote or prevent atherosclerosis has not been reached. The data from the present study are consistent with other observations in showing that monounsaturated fatty acids do not protect against atherosclerosis.7,24,25 In humans, the LDL to HDL ratio is the biomarker commonly used to support beneficial effects of monounsaturated fat.26 However, usefulness of this ratio is believed to be confounded by the effects of monounsaturated fat to promote LDL cholesteryl oleate enrichment.27 Several studies in humans have indicated that a decrease in the proportion of cholesteryl linoleate together with an increase in cholesteryl oleate in plasma can be detrimental.28 The results from our studies in the apoB100 only, LDLr-/- mice show that ACAT2 derived CE are atherogenic and when ACAT2 activity is removed, less atherosclerosis occurs and dietary fat differences are eliminated. The implication is that ACAT2 might also be important in humans, although this has yet to be directly tested.

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Disclosures
None.

References
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Expanded Materials and Methods Section

Mice, Diets, and Study Outline

The control mice used in this study were female apoB100-only, LDLr<sup>−/−</sup> mice. The original breeders were acquired from Dr. Steven Young and had a mixed background (~75% C57BL/6 and ~25% 129Sv/Jae) (1). To generate the ACAT2<sup>−/−</sup> used in this study, the apoB100-only, LDLr<sup>−/−</sup> mice were crossed with ACAT2<sup>−/−</sup>, LDLr<sup>−/−</sup> mice on a mixed background (50% C57BL/6 and 50% 129Sv/Jae) (2) that were originally obtained from Dr. Robert Farese, Jr. This cross generated the apoB100-only, LDLr<sup>−/−</sup> mice lacking functional ACAT2. Since the mice used in this study are not from a pure background strain, sibling controls were used to minimize genetic heterogeneity. At seven to eight weeks of age, the mice were switched from a diet of rodent chow to one of six prepared diets consisting of 10% calories from fat and 0.02% cholesterol by weight. The diets were also enriched in specific types of dietary fat, either fish or plant derived ω-3 polyunsaturated fat, ω-6 polyunsaturated fat, saturated fat, and cis or trans monounsaturated fat. The complete details of the dietary ingredients and fatty acid composition are summarized in Supplemental Tables 1, 2 and 3. The diet groups consisted of 9-14 mice of each genotype and the diets were administered for 20 weeks. All protocols involving mice had prior approval from the institutional animal care and use committee. The mice were maintained in a facility fully approved by the American Association for the Accreditation of Laboratory Animal Care.
At 2, 8, and 16 weeks during the study, the mice were restrained by the administration of ketamine (50 mg/kg) and xylazine (10 mg/kg) and a blood sample was collected from the retro-orbital plexus. At the end of the 20 week diet intervention period the mice were fasted for 4 hours and overdosed with anesthetic. Plasma was isolated after centrifugation at 12,500 RPM at 4°C from the blood collected by heart puncture into EDTA; aliquots of plasma were stored in 10% sucrose at -20°C. Liver samples were collected at the time of sacrifice, snap-frozen in liquid N₂, and then stored in an ultralow freezer at -80°C. The aortas beginning at the aortic sinus and ending at the iliac bifurcation were removed from the mice and fixed in a 10% neutral buffered formalin solution.

At 2, 8, 16 and 20 weeks, body weight measurements were taken for each mouse in the study with no significant differences in body weight identified due to the type of dietary fat. However, the body weights of ACAT2⁻/⁻ mice were significantly heavier, averaging 30 grams, than the control mice which averaged 25 grams. Separate studies with additional crossbreeding indicated that the difference in body weight is not due to ACAT2, per se, but more likely due to a subtle difference in background strain.

Plasma Lipid and Lipoprotein Measurements

Plasma cholesterol (TPC), free cholesterol (FC), and triglyceride (TG) concentrations were measured using enzymatic assays as previously described (3,4). Cholesteryl ester (CE) was determined by subtracting FC from TPC, then
multiplying the difference by 1.67. The lipoprotein subclasses were isolated from fresh aliquots of plasma from individual mice according to methods previously described (5). Whole plasma was injected onto a Superose 6 chromatography column, which was subsequently run at 0.5 mL/min with 0.9% NaCl containing 0.05% EDTA (pH 7.4), and 0.05% NaN₃. The average LDL particle size, estimated as LDL molecular weight in g/µmol, was measured from a standard curve constructed from LDL standards of known size plotted against elution time. Fractions containing VLDL, LDL, and HDL were collected and pooled according to elution time. These aliquots of isolated lipoproteins were then measured for TC and FC via enzymatic assays and CE was determined by the equation described previously. The fatty acid composition of CE from isolated LDL was determined by fatty acid methyl ester analysis as previously described (5,6). Briefly, the lipid of the LDL samples was extracted in chloroform and methanol as described by Bligh and Dyer (7), and lipid classes were separated by thin layer chromatography (TLC) on silica gel G plates. The band corresponding to CE was removed, saponified, and the fatty acids were then methylated. Fatty acid composition was determined after separation of methyl esters by gas-liquid chromatography.

Quantification of Atherosclerosis

The extent of aortic atherosclerosis was measured by quantifying the accumulation of CE in the entire aorta (extending from the heart to the iliac
bifurcation) according to published methods (8). Briefly, the formalin preserved aortas were cleaned by removing all adherent adipose and connective tissue, and lipids were extracted in 2:1 chloroform: methanol with 5α-cholestane added as an internal standard, and FC and total cholesterol (after saponification) was measured by gas-liquid chromatography. Aortic CE was determined using the aforementioned equation (TC-FC)*1.67. The correlation between aortic CE and TPC measured at 20 weeks of the study is shown in supplement Figure 1. While the correlation was relatively strong, the association with LDL particle size was even stronger, as shown in Figure 4 of the manuscript. We also measured the area under the time curve for the TPC data gathered at 2, 8, 16, and 20 weeks of diet treatment for each diet group, and the association with aortic atherosclerosis was equivalent to that for TPC at 20 weeks (data not shown).

Reference List


**Supplemental Tables**

**Table 1: Fatty Acid Composition of Diets**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Sat</th>
<th>Mono</th>
<th>n-6 Poly</th>
<th>n-3 Poly</th>
<th>(\alpha)-LA</th>
<th>EPA</th>
<th>DHA</th>
<th>Other</th>
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<tr>
<td>Fish</td>
<td>35</td>
<td>27</td>
<td>9</td>
<td>2</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td></td>
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<tr>
<td>n-6 Poly</td>
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<td>54</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td></td>
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<tr>
<td>Flax</td>
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<td>15</td>
<td>19</td>
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<tr>
<td>Sat</td>
<td>55</td>
<td>24</td>
<td>17</td>
<td>1</td>
<td>1</td>
<td>0</td>
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<tr>
<td>Cis-Mono</td>
<td>26</td>
<td>55</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
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<tr>
<td>Trans-Mono</td>
<td>24</td>
<td>50 (25)*</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
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*Denotes the percentage of total dietary fatty acid that is trans-monounsaturated fat.
Table 2: Fatty Acid Energy Distribution of Diets

<table>
<thead>
<tr>
<th>Fatty Acid (Energy %)</th>
<th>n-3 Poly</th>
<th>n-6 Poly</th>
<th>α-LA</th>
<th>EPA</th>
<th>DHA</th>
<th>Other</th>
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<td>n-6 Poly</td>
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<td>5.4</td>
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<tr>
<td>Flax</td>
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<td>0.1</td>
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<tr>
<td>Sat</td>
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<td>1.7</td>
<td>0.1</td>
<td>0.1</td>
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Table 3: Experimental Diet Ingredients

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<th>Ingredient</th>
<th>Diet Group</th>
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<tr>
<td></td>
<td></td>
<td>Fish</td>
<td>n-6 Poly</td>
<td>Flax (g/100g Dry Weight)</td>
<td>Sat</td>
<td>Cis-Mono</td>
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<td>Oleic Fatty Acid Blend</td>
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<td>4.0</td>
<td>0.2</td>
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<td>0.2</td>
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<tr>
<td>trans Blend</td>
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<td>4.0</td>
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<td>0.2</td>
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<td>1.6</td>
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<tr>
<td>Palm Oil</td>
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Same for All Diets

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<thead>
<tr>
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<td>Casein, USP</td>
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<td>Vitamin Mixture</td>
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<td>Salt Mixture</td>
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<tr>
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<td>B-sitosterol</td>
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<tr>
<td>Sucrose</td>
<td>17</td>
<td>Tenox 20A</td>
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<td></td>
</tr>
<tr>
<td>Wheat Flour</td>
<td>35</td>
<td>MTS-50</td>
<td>0.018</td>
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<tr>
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<td>Vit E 5-67</td>
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<td>Cholesterol, Crystalline</td>
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</table>

* No additional cholesterol added to n-3 Poly Diet
Figure 1.

Legend

Plasma total plasma cholesterol concentration was correlated with aortic atherosclerosis extent measured chemically by cholesteryl ester concentration when the average values for the diet/genotype groups were compared. The correlation coefficient, as shown for the best fit regression line, was $r=0.78$.  

Correlation of Aortic Atherosclerosis and Plasma Cholesterol

![Graph showing correlation between plasma cholesterol and aortic CE concentration](image)