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

Objectives—The enzyme acyl-coenzymeA (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. ACAT activity can be attributed to the ACAT2 isoform. 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. 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 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

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. (Arterioscler Thromb Vasc Biol. 2007;27:1396-1402.)

Key Words: ACAT2 • cholesteryl esters • lipoproteins • liver • aortic atherosclerosis
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. The data points represent averages±SEM for 9 to 14 mice for each diet/genotype group.

**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 near 725 mg/dL at 20 weeks. These dietary fat effects on TPC were lost in ACAT2<sup>−/−</sup> mice (Figure 1B). All but 1 of the ACAT2<sup>−/−</sup> diet groups had TPC values around 200 mg/dL after 2 weeks which increased to approximately 400 mg/dL by the end of the treatment period. The exception was ACAT2<sup>−/−</sup> mice fed the fish oil diet, which started with a lower TPC that had increased to about 400 mg/dL by 20 weeks.

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.

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...
In the ACAT2 diets were fed. Except for ACAT2 

tions were observed when the saturated and monounsaturated

VLDL-CE concentration although not in a statistically sig-

Table 2: Dietary Fat and ACAT2 Activity Effects on Lipoprotein CE Distribution

<table>
<thead>
<tr>
<th>Diet</th>
<th>Cholesteryl Ester (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VLDL</td>
</tr>
<tr>
<td></td>
<td>Control ACAT2 /−</td>
</tr>
<tr>
<td>Fish</td>
<td>2±11 a</td>
</tr>
<tr>
<td>Poly (n=6)</td>
<td>45±18 a</td>
</tr>
<tr>
<td>Flax</td>
<td>76±5 a</td>
</tr>
<tr>
<td>Sat</td>
<td>140±70 a</td>
</tr>
<tr>
<td>trans-Mono</td>
<td>162±93 a</td>
</tr>
<tr>
<td>cis-Mono</td>
<td>188±58 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 diet/genotype class if they had the same letter; numbers that had only different letters were significantly different. All data are mean±SEM.
curred, 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 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).

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.
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 the diet shifted from polyunsaturated to saturated and monounsaturated. 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 concentrations 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 parameters 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 atherosclerosis was seen, similar to what had been found in studies of coronary artery atherosclerosis in monkeys\(^6\) 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 differences 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 differential 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 monounsaturated CE, primarily cholesteryl oleate.\(^3\) A similar relationship 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 monounsaturated 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 monounsaturated 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\(^{-/-}\) 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.\(^{13,15}\) LCAT in the plasma compartment produces polyunsaturated CE by using phospholipid sn-2 fatty acids as substrates. LCAT in the ACAT2\(^{-/-}\) 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\(^{-/-}\) diet groups (Table 2). Because the control and ACAT2\(^{-/-}\) 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.\(^{16,17}\)

The apoB100 only mice were originally developed to compare the atherogenic potential of LDL-sized particles to compare the atherogenic potential of LDL-sized particles to the apoB100 only mice. Because the control and ACAT2\(^{-/-}\) 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.\(^{16,17}\)

The apoB100 only mice were originally developed to compare the atherogenic potential of LDL-sized particles to the apoB100 only mice. Because the control and ACAT2\(^{-/-}\) 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.\(^{16,17}\)

The apoB100 only mice were originally developed to compare the atherogenic potential of LDL-sized particles to the apoB100 only mice. Because the control and ACAT2\(^{-/-}\) 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.\(^{16,17}\)

The apoB100 only mice were originally developed to compare the atherogenic potential of LDL-sized particles to the apoB100 only mice. Because the control and ACAT2\(^{-/-}\) 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.\(^{16,17}\)

The apoB100 only mice were originally developed to compare the atherogenic potential of LDL-sized particles to the apoB100 only mice. Because the control and ACAT2\(^{-/-}\) 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.\(^{16,17}\)

The apoB100 only mice were originally developed to compare the atherogenic potential of LDL-sized particles to the apoB100 only mice. Because the control and ACAT2\(^{-/-}\) 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.\(^{16,17}\)
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 α-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 accurately predict atherosclerosis.24 The results from our studies in the apoB100 mice show that ACAT2 derived CE are detrimental.25 mice 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.

Sources of Funding
This work was supported by NIH Grants AT-002782 and HL-49373.

Disclosures
None.

References
3. Tall AR, Small DM, Atkinson D, Williford K. LDLr
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 and Lawrence L. Rudel

Arterioscler Thromb Vasc Biol. 2007;27:1396-1402; originally published online April 12, 2007; doi: 10.1161/ATVBAHA.107.142802

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/27/6/1396

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2007/04/17/ATVBAHA.107.142802.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Expanded Materials and Methods Section

Mice, Diets, and Study Outline

The control mice used in this study were female apoB100-only, LDLr\(^{-/+}\) 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\(^{-/-}\) used in this study, the apoB100-only, LDLr\(^{-/-}\) mice were crossed with ACAT2\(^{-/-}\), LDLr\(^{-/-}\) 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\(^{-/-}\) 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 \(\omega-3\) polyunsaturated fat, \(\omega-6\) polyunsaturated fat, saturated fat, and \emph{cis} or \emph{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>Fatty Acid (% w/w)</th>
<th>Diet</th>
<th>Sat</th>
<th>Mono</th>
<th>n-6 Poly</th>
<th>n-3 Poly</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>α-LA</td>
</tr>
<tr>
<td>Fish</td>
<td>35</td>
<td>27</td>
<td>9</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>n-6 Poly</td>
<td>22</td>
<td>21</td>
<td>54</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Flax</td>
<td>36</td>
<td>29</td>
<td>15</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Sat</td>
<td>55</td>
<td>24</td>
<td>17</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cis-Mono</td>
<td>26</td>
<td>55</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Trans-Mono</td>
<td>24</td>
<td>50</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*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>Diet</th>
<th>Sat</th>
<th>Mono</th>
<th>n-6 Poly</th>
<th>α-LA</th>
<th>EPA</th>
<th>DHA</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-3 Poly</td>
<td>3.5</td>
<td>2.7</td>
<td>0.9</td>
<td>0.2</td>
<td>1.0</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>n-6 Poly</td>
<td>2.2</td>
<td>2.1</td>
<td>5.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>Flax</td>
<td>3.6</td>
<td>2.9</td>
<td>1.5</td>
<td>1.9</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>Sat</td>
<td>5.5</td>
<td>2.4</td>
<td>1.7</td>
<td>0.1</td>
<td>0.1</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>Cis-Mono</td>
<td>2.6</td>
<td>5.5</td>
<td>1.6</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td>Trans-Mono</td>
<td>2.4</td>
<td>5.0</td>
<td>1.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Table 3: Experimental Diet Ingredients

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Diet Group</th>
<th>Sat Blend</th>
<th>Oleic Fatty Acid Blend</th>
<th>Poly Blend</th>
<th>Fish Oil</th>
<th>trans Blend</th>
<th>Flaxseed Oil</th>
<th>Palm Oil</th>
<th>Casein, USP</th>
<th>Lactalbumin</th>
<th>Dextrin</th>
<th>Sucrose</th>
<th>Wheat Flour</th>
<th>Alphacel</th>
<th>Cholesterol, Crystalline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.0</td>
<td>4.0</td>
<td>1.6</td>
<td>2.4</td>
<td>8</td>
<td>4</td>
<td>17</td>
<td>17</td>
<td>35</td>
<td>7</td>
<td>0.019*</td>
</tr>
<tr>
<td>Same for All Diets</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein, USP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>Vitamin Mixture</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactalbumin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>Salt Mixture</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextrin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17</td>
<td>B-sitosterol</td>
<td>0.011</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17</td>
<td>Tenox 20A</td>
<td>0.004</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat Flour</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>35</td>
<td>MTS-50</td>
<td>0.018</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alphacel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>Vit E 5-67</td>
<td>0.002</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol, Crystalline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.019</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>* No additional cholesterol added to n-3 Poly Diet</td>
</tr>
</tbody>
</table>
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)