**High Dietary n-6/n-3 PUFA Ratio Promotes HDL Cholesterol Level, but does not Suppress Atherogenesis in Apolipoprotein E-Null Mice**

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**Aim:** Dietary fatty acids affect atherogenesis, which was presumed to be partly related to HDL cholesterol (HDL-C) metabolism. The major aim of the work was to analyze various ratios of n-6/n-3 PUFA diets on HDL-C metabolism in apolipoprotein E-null (apoE<sup>-/-</sup>) mice, which have similar symptoms to human type II familial hyperlipoproteinemia.

**Methods:** Two-month-old male apoE<sup>-/-</sup> mice were fed four types of n-6/n-3 PUFA diet (group 1, 1.28; group 2, 5.03; group 3, 9.98 and group 4, 68.26) and control diet, respectively, for 6 weeks. With respect to serum apolipoprotein (apo) A-I concentration, lecithin-cholesterol acyltransferase (LCAT) activity and mRNA abundance of genes involved in HDL-C metabolism in the liver were analyzed.

**Results:** Group 4 diet significantly increased the plasma HDL-C and apoA-I concentrations compared with other groups. LCAT activity in serum increased with decreased ratios of n-6/n-3 PUFA. As the dietary ratio of n-6/n-3 fatty acids increased, so did mRNA levels of hepatic apoA-I, scavenger receptor B class-1 (SR-B1), LCAT, ATP binding cassette transporter A1 (ABCA1), ABCG1 and liver X receptor alpha (LXRα). ApoA-II mRNA level, however, had a tendency to fall. Group 4 diet increased apoA-I and ABCA1 and decreased apoA-II transcriptional levels, whereas group 1 diet decreased mRNA levels of apoA-I, LCAT, SR-B1 and ABCG1.

**Conclusion:** Our data indicated that a high ratio of n-6/n-3 PUFA increased the serum HDL-C level, but did not effectively suppress atherogenesis in apoE<sup>-/-</sup> mice. The elevated HDL-C level is possibly due to up-regulated hepatic apoA-I and ABCA1 with suppression of apoA-II expression.


**Key words:** Liver, Reverse cholesterol transport, Alpha linolenic acid, Linoleic acid

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**Introduction**

Epidemiological and clinical studies have established that the n-6 fatty acid, linoleic acid (LA), the n-3 fatty acids, alpha linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) collectively protect against atherosclerotic cardiovascular diseases (ACVD)<sup>1,2</sup>). Both n-6 and n-3 PUFA have distinct biological cardioprotective effects. Their distinct functions make the ratio of dietary n-6 and n-3 PUFA an important factor influencing cardiovascular health. The optimal beneficial n-6/n-3 ratio is not clear, although certain guidelines in several countries propose a broad ratio of 4:10<sup>3-5</sup>). The specific ratio closely related to atherosclerosis has not been well established, and precise values regarding the most beneficial n-6/n-3 ratio need to be further studied, through both animal and human investigations. Such researches
could be especially important for the population suffering from lipid metabolism disorder and its related syndromes.

In humans and experimental animals, fatty acids modulate plasma HDL cholesterol (HDL-C) levels. It is known that HDL plays a pivotal role in reverse cholesterol transport (RCT). A number of epidemiologic observations and clinical trials have consistently documented a negative relation between HDL-C concentration and the risk of ACVD. The protective effect of HDL can largely be attributed to its role in RCT, a process of HDL-mediated removal of excess cholesterol from peripheral tissues and delivery to the liver for biliary excretion. The mechanism(s) involved in the modulation of plasma HDL-C level by dietary fatty acids is not well understood. The purpose of this study, therefore, was to determine the molecular action mechanism of HDL-C metabolism, and to assess the effects of n-6/n-3 PUFA on atherogenesis by varying dietary n-6/n-3 PUFA ratios in apolipoprotein E-null (apoE−/−) mice, which spontaneously develop atherosclerosis with similar features to those observed in human type III familial hyperlipoproteinemia.

Materials and Methods

Animals and Diets

Homozygous apoE−/− mice were obtained from Jackson Laboratory (Bar Harbor, Maine, USA), and were housed in sterile, filter-top cages (3−4 mice/cage) at 22±1°C with a 12-h light/12-h dark cycle. All mice were first fed the control diet (3% fat and 7.1% total energy) from 22 days old before further experiments. The mice were then randomly assigned to four experimental groups (experimental diets) and one control group (control diet) at 8 weeks old (n=12, respectively). The experimental diets contained 5% (w/w) fat (11.5% total energy) (Table 1). The n-6/n-3 PUFA ratios of the experimental diets, for feeding groups 1−4 mice, were 1.28, 0.65, 0.65, and 0.65, respectively, for 6 weeks with food and water ad libitum. In order to avoid lipid oxidation in the diets, all food was prepared in tablets and packed in zip-locked bags, flushed with nitrogen, sealed, and stored at −20°C before use. Fresh food was provided daily.

Experimental Design

The health status of mice was monitored daily. Body weight was recorded once every 3 days. At the end of the 6-week feeding period, all mice were sacrificed by suffocation with CO2. The animal studies have been reviewed by the Ethics Committee of Shandong Normal University (Jinan, China).

Table 1. Composition of the diets

<table>
<thead>
<tr>
<th></th>
<th>Experimental diet</th>
<th>Control diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Corn starch</td>
<td>640</td>
<td>660</td>
</tr>
<tr>
<td>Fat</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>Vitamin mix¹</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mineral mix²</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

¹Vitamin mix provided the following amounts (mg/kg dry diet): retinol, 12; thiamine, 40; cholecalciferol, 0.125; riboflavin, 30; pantothenic acid, 140; pyridoxine, 20; inositol, 300; cyanocobalamin, 0.1; ascorbic acid, 1,600; dl-a-tocopherol, 340; menadione, 80; nicotinic acid, 200; para-aminobenzoic acid, 100; folic acid, 10; biotin, 0.6.

²Mineral mix provided the following amounts (mg/kg dry diet): CaCO3, 12,000; K2HPO4, 10,750; MgSO4·7H2O, 5,000; NaCl, 3,000; MgO, 2,000; FeSO4·7H2O, 400; ZnSO4·7H2O, 350; MnSO4·H2O, 100; CuSO4·5H2O, 50; Na2SiO3·3H2O, 25; AlK(SO4)2·12H2O, 10; K2Cr2O7, 7.5; NaF, 5; NiSO4·6H2O, 5; H3BO3, 5; CoSO4·7H2O, 2.5; KIO3, 2; LiCl, 0.75; Na2SeO3, 0.75; (NH4)2CO3, 0.5.

Table 2. Fatty acid composition of the five diets

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>SFA</td>
<td>10.25</td>
<td>11.83</td>
<td>12.24</td>
<td>12.64</td>
<td>16.77</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.44</td>
<td>0.44</td>
<td>0.44</td>
<td>0.44</td>
<td>0.73</td>
</tr>
<tr>
<td>C16:0</td>
<td>8.22</td>
<td>9.01</td>
<td>9.22</td>
<td>9.42</td>
<td>13.69</td>
</tr>
<tr>
<td>C18:0</td>
<td>1.27</td>
<td>1.55</td>
<td>1.63</td>
<td>1.70</td>
<td>2.10</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.25</td>
</tr>
<tr>
<td>MUFA¹</td>
<td>12.95</td>
<td>14.91</td>
<td>15.42</td>
<td>15.92</td>
<td>19.70</td>
</tr>
<tr>
<td>C16:1</td>
<td>0.39</td>
<td>0.39</td>
<td>0.39</td>
<td>0.39</td>
<td>0.66</td>
</tr>
<tr>
<td>C18:1</td>
<td>12.39</td>
<td>14.34</td>
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<td>15.36</td>
<td>18.76</td>
</tr>
<tr>
<td>C20:1</td>
<td>0.18</td>
<td>0.18</td>
<td>0.18</td>
<td>0.18</td>
<td>0.30</td>
</tr>
<tr>
<td>MUFA¹</td>
<td>77.05</td>
<td>73.36</td>
<td>72.41</td>
<td>71.46</td>
<td>63.53</td>
</tr>
<tr>
<td>C18:2</td>
<td>43.29</td>
<td>61.20</td>
<td>65.81</td>
<td>70.43</td>
<td>61.81</td>
</tr>
<tr>
<td>C18:3</td>
<td>32.73</td>
<td>11.13</td>
<td>5.57</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C20:5</td>
<td>0.65</td>
<td>0.65</td>
<td>0.65</td>
<td>0.65</td>
<td>1.08</td>
</tr>
<tr>
<td>C22:6</td>
<td>0.39</td>
<td>0.39</td>
<td>0.39</td>
<td>0.39</td>
<td>0.64</td>
</tr>
<tr>
<td>Ratio n-6 to n-3</td>
<td>1.28</td>
<td>5.03</td>
<td>9.98</td>
<td>68.26</td>
<td>35.98</td>
</tr>
</tbody>
</table>

¹MUFA: monounsaturated fatty acids.
Splems were drawn from the heart, and allowed to clot for a minimum of 1 h at room temperature. Serum was obtained by centrifugation at 1,100 $\times$ $g$ for 15 min at 4$^\circ$C. Liver and serum samples were stored in aliquots at $-80$°C until later analysis. The heart was perfused in situ with cold 10% phosphate-buffered formalin for 2 min, removed from the body with the aortic root, and fixed in 10% phosphate-buffered formalin for 48 h.

**Serum Lipids Analysis**

Total cholesterol (TC), free cholesterol, and HDL-C concentrations in serum were determined using commercial kits (Kyowa Medix, Japan) adapted for 96-well microtiter plates. Cholesterol ester was computed as the difference between TC and FC. Non-HDL-C (nHDL-C) was computed as the difference between total and HDL-C.

**Serum Apolipoprotein Analysis**

Serum apoA-I was measured using an immunoturbidimetric method (Auto ApoA-I; Bacton Assay Systems Inc, San Marcos, USA) according to the manufacturer’s instructions.

**LCAT Activity Analysis (Endogenous Assay)**

Endogenous LCAT activity was measured as the utilization rate of FC in native serum according to the method of Ly H. et al. They measured the FC content of serum was measured colorimetrically in pentuplicate by an autoanalyser at zero and after 40 min at 37°C. LCAT activity was expressed as nanomoles of FC consumed/hour per milliliter serum.

**Quantitative RT-PCR Analysis**

Total RNA was extracted from the liver using TRIzol reagent (Invitrogen, USA) in accordance with the manufacturer’s instructions and treated with DNase I (Takara, Japan). RNA quality and integrity were checked by absorbance at 260 and 280 nm and gel electrophoresis to confirm the presence of strong and intact ribosomal 28 S and 18 S bands. Reverse transcription of RNA into cDNA was performed using Superscript reverse transcriptase (Invitrogen, USA) primed with Oligo (dT)$_{18}$ according to the manufacturer’s instructions. The quality and integrity of cDNAs were tested by initially amplifying the housekeeping gene $\beta$-actin. Quantitative real-time PCR was performed using the Rotor-Gene 3000 Real-time PCR System (Corbett Research, Sydney, Australia). The sequences of forward and reverse primers are listed in Table 3. Amplification was detected using the Syber-green (Molecular Probes, USA) method. Specificity of the amplified PCR products was examined after the final cycle by generating a melting curve with a heating rate of 1°C/s between 72 and 99°C. The data were analyzed using Rotor-Gene 3000 software with the 2$^{-\Delta\Delta Ct}$ method. The relative expression values of target gene mRNAs were normalized to that of $\beta$-actin mRNA in each sample. Amplification of the target

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Annealing temperature (°C)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>apo A-I</td>
<td>S$^1$</td>
<td>GCCACGGTATGGCACAGCAAGAT</td>
<td>59</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CCCAGAGGTCGCGGAGTCGTAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>apo A-II</td>
<td>S</td>
<td>CCAGGCATACCTTTGAGAACAC</td>
<td>59</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>GGAGAAACAGCCAGAAGTGAGG</td>
<td></td>
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</tr>
<tr>
<td>LCAT</td>
<td>S</td>
<td>CCAAGGCAGGATGAATTACCTAG</td>
<td>59</td>
<td>40</td>
</tr>
<tr>
<td>SR-B1</td>
<td>AS</td>
<td>AGGCTATGCGCAAATGAGGAAGA</td>
<td>59</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>TGGACAAATGGAACGGACTG</td>
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<td></td>
</tr>
<tr>
<td>ABCA1</td>
<td>AS</td>
<td>GCTTCCATCCCTCCTGTTCATCAT</td>
<td>59</td>
<td>40</td>
</tr>
<tr>
<td>ABCG1</td>
<td>S</td>
<td>CCCATACATAGCCGGAGG</td>
<td>59</td>
<td>40</td>
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<tr>
<td>LXRα</td>
<td>AS</td>
<td>ATCAGCCAAAGGAGGAGG</td>
<td></td>
<td></td>
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<tr>
<td>β-actin</td>
<td>S</td>
<td>AGGAGAACATCAGGCGAT</td>
<td>59</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>GTACTCCTGCTTTGCTGATTC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$S, sense primer; AS, antisense primer.
gene and β-actin was run in separate tubes. All reactions were performed in triplicate. The mRNA expression levels were reported as fold changes compared with control mice.

Atherosclerotic Lesions Analysis

The upper half of the heart was dissected, soaked overnight in 30% sucrose, and embedded in OCT. Sequential 10 μm frozen sections were cut from the apex towards the base of the heart until the aortic valve leaflets appeared. From this point, 20 sections representing every second serial section over a distance of 200 μm were collected and stained with Sudan IV. Images were captured of each Sudan IV-stained cross-section using an Olympus DP71 camera mounted on a microscope (Olympus IX-71, Japan). From these sections, atherosclerotic lesions were determined using Image Pro-Plus 6.0 software (Media Cybernetics, USA). The results are reported as the average of all 20 aortic sections analyzed. In order to minimize artificial error, all analyses were performed by the same person.

Statistical Analysis

Data are presented as the means ± SD. Continuous variables were compared among groups by one-way ANOVA with post-hoc LSD t test. A p value < 0.05 was considered significant. A standard software package (SPSS for Windows 15.0) was used.

Results

Diets and Serum Lipids Analysis

The ratio of n-6/n-3 fatty acids of the formulated diets ranged from 1.28 to 68.26 (Table 2). No significant difference was observed in the growth of mice fed with different experimental diets (data not shown).

After feeding the mice with the diets for 6 weeks, the serum HDL-C and apoA-1 protein levels of group 4 mice were significantly higher than those of other groups (p < 0.05, Table 4). The ratio of TC/HDL-C in group 4 mice was significantly lower than that of mice in other groups (p < 0.05). On the other hand, no significant differences were found in the serum concentrations of FC, TC and CE among groups fed different diets. The extent of atherosclerotic lesions area in aortic sinus was limited in all mice, and did not differ significantly among experimental groups (p > 0.05, Table 4).

LCAT Activity Analysis

In order to evaluate the effect of different ratios of n-6/n-3 fatty acids on the endogenous activity of LCAT, the FC utilization rate was assessed in native serum at the end of each feeding period. Group 1 mice have the highest endogenous LCAT activity (Fig. 1). As the ratio of n-6/n-3 increased, endogenous LCAT activity revealed a tendency to decrease. LCAT activity in mice of group 3 was significantly higher than in group 4 and the control group, but no significant difference was observed between groups. These data suggest that endogenous LCAT activity increased with the increase of n-3 PUFA content in diets.

The Effect of Dietary n-6/n-3 PUFA Ratios on Gene Expression

In an attempt to elucidate the mechanisms by which the fatty acid profile of the diet altered HDL-C metabolism, the mRNA abundance of hepatic genes involved in HDL-C metabolism was assessed by real-time RT-PCR. As the dietary ratio of n-6/n-3 fatty acids increased, the expressions of hepatic apoA-1, SR-B1, LCAT, ABCA1, ABCG1 and LXRs were increased; however, the apoA-II mRNA level decreased.

Table 4. Effect of different diets on serum lipids and lipoproteins, serum apoA-I concentrations, and aortic lesion formation in apoE-/- mice1,2

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC (mmol/L)</td>
<td>5.65 ± 0.96</td>
<td>5.75 ± 1.02</td>
<td>5.11 ± 0.68</td>
<td>6.37 ± 1.36</td>
<td>6.03 ± 1.55</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>15.06 ± 1.75</td>
<td>16.07 ± 2.61</td>
<td>13.59 ± 1.18</td>
<td>15.0 ± 2.51</td>
<td>14.64 ± 2.86</td>
</tr>
<tr>
<td>CE (mmol/L)</td>
<td>9.29 ± 1.27</td>
<td>10.21 ± 1.85</td>
<td>8.89 ± 1.36</td>
<td>8.87 ± 1.26</td>
<td>8.61 ± 1.50</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.56 ± 0.34b</td>
<td>1.34 ± 0.19b</td>
<td>1.24 ± 0.16b</td>
<td>2.03 ± 0.50a</td>
<td>1.42 ± 0.37b</td>
</tr>
<tr>
<td>nHDL-C (mmol/L)</td>
<td>13.10 ± 2.17ab</td>
<td>14.30 ± 2.69a</td>
<td>12.77 ± 1.57ab</td>
<td>12.97 ± 1.79b</td>
<td>13.24 ± 2.85ab</td>
</tr>
<tr>
<td>apo A-I (g/L)</td>
<td>5.01 ± 1.29a</td>
<td>5.91 ± 0.89a</td>
<td>5.19 ± 1.68a</td>
<td>3.71 ± 0.47b</td>
<td>5.46 ± 1.66a</td>
</tr>
<tr>
<td>aortic lesions area (μm²)</td>
<td>51,625 ± 20,118</td>
<td>50,220 ± 12,775</td>
<td>43,942 ± 19,105</td>
<td>61,328 ± 25,724</td>
<td>51,541 ± 36,676</td>
</tr>
</tbody>
</table>

1Values are the means ± SD, n = 12. Means in a row with superscripts without a common letter differ significantly, p < 0.05.
2To convert values for FC, TC, CE, HDL-C, and nHDL-C to mg/dL, multiply by 38.67.
as the dietary n-6/n-3 ratio increased (Fig. 2).

The data from real-time RT-PCR revealed that group 1 to 3 diets markedly decreased the expression of LCAT and SR-B1 in the liver compared with the control diet. Hepatic LCAT and SR-B1 mRNA levels were reduced by 32% in mice fed the group 4 diet compared with those fed the group 1 diet ($p < 0.05$); however, there were no significant differences among groups 1, 2 and 3. This difference in hepatic LCAT mRNA levels was not reflected in serum LCAT activity, suggesting that post-transcriptional regulation plays an important role in LCAT function. The group 1 diet significantly attenuated the expression of SR-B1 mRNA compared with the group 4 diet, as determined by real-time RT-PCR ($0.36 \pm 0.05$ vs. $0.81 \pm 0.08$ arbitrary units, $p < 0.05$). Compared with the control diet, the group 1 diet decreased the apoA-I mRNA level ($0.62 \pm 0.10$ vs. $1.0 \pm 0.12$ arbitrary units, $p < 0.05$), but the group 4 diet up-regulated apoA-I expression ($1.30 \pm 0.14$ vs. $1.0 \pm 0.12$ arbitrary units, $p < 0.05$). These results suggest that n-3 PUFA down-regulated apoA-I expression, but n-6 PUFA had an opposite affect on apoA-I expression. The mRNA levels of ABCA1 and ABCG1, the two factors involved in cellular cholesterol efflux, were increased by 1.2-fold

Fig. 1. Effect of dietary fatty acid levels on endogenous LCAT activity in serum.

LCAT activity was measured as the utilization rate of FC in native serum. Endogenous LCAT activity was expressed as nanomoles FC consumed/hour per milliliter serum. Data are presented as the means $\pm$ SD ($n = 12$). Means without a common letter differ significantly, $p < 0.05$.

Fig. 2. Effect of dietary fatty acid levels on the mRNA expression of genes involved in HDL-C metabolism in the liver by real-time RT-PCR.

Expression levels of mRNA are indicated as fold differences compared with mice fed the control diet. The values of the control diet are set as 1.0. Bars represent the means $\pm$ SD ($n = 12$). Means without a common letter differ significantly, $p < 0.05$. Abbreviations: apoA-I, apolipoprotein A-1; apoA-II, apolipoprotein A-II; LCAT, lecithin-cholesterol acyltransferase; SR-B1, scavenger receptor B class 1; ABCA1, ATP binding cassette transporter A1; ABCG1, ATP binding cassette transporter G1; LXRa, liver X receptor alpha.
(p<0.05) in group 4, and reduced by 44% (p<0.05) in group 1 compared with the control group, respectively. The group 4 diet significantly decreased the mRNA level of apoA-II compared with the control group (0.71±0.05 vs. 1.0±0.14 arbitrary units, p<0.05); however, the group 1 diet did not increase the apoA-II mRNA level significantly compared with the control group. The expression of LXRα in the liver decreased by 78% in group 1 and increased by 17% in group 4 compared with the control group. These results suggest that n-3 PUFA inhibited LXRα expression, while n-6 PUFA moderately up-regulated LXRα expression.

Discussion

The present study was designed to assess the antiatherogenic effects and investigate potential mechanisms responsible for the characteristics of varying ratios of n-6/n-3 PUFA representing a wide range of PUFA profiles on HDL-C metabolism in apoE<sup>-/-</sup> mice.

ApoE<sup>-/-</sup> mice spontaneously develop atherosclerosis with features similar to those observed in human type III familial hyperlipoproteinemia. A previous study showed that apoE plays scarcely any role in HDL metabolism, and the absence of apoE did not influence the effect of fatty acids on HDL<sup>10</sup>. Several studies using apoE<sup>-/-</sup> mice have observed a relationship between HDL metabolism and atherosclerosis recent years<sup>11-13</sup>; therefore, we studied the antiatherogenic effect and HDL-C metabolism by varying the n-6/n-3 PUFA using apoE<sup>-/-</sup> mice. Our results and suggestions may be useful for dietary guidance in human type III familial hyperlipoproteinemia or those suffering from hyperlipidemia. In our knowledge, this study was the first report to analyze the mechanism(s) of varying n-6/n-3 PUFA ratios on the HDL-C metabolism in lipid metabolism disorder mice at the molecular level.

Our results indicate that the highest dietary n-6/n-3 PUFA ratio (68.26) elevated HDL-C and apoA-I levels significantly. These results are consistent with several studies<sup>14,17</sup>, but are not in agreement with others<sup>18-20</sup>

Epidemiological investigations<sup>15,21-23</sup> and animal studies<sup>14,16-19</sup> showed a discrepancy between HDL-C levels and the n-6 and n-3 PUFA. The present studies showed that n-6 PUFA moderately promoted the HDL-C level in the apoE<sup>-/-</sup> mice, which is consistent with other animal studies<sup>14,16,17</sup>, but different from epidemiological investigations. This may be due to different responses to HDL-C by PUFA between mice and humans. Moreover, differences in the experimental design of the studies, including the population, the profiles of fatty acids, the dietary amounts, the period of administration, as well as the animal model and control, could explain some of the heterogeneous responses or separate phenotypes. Although the present findings supported that a high ratio of n-6/n-3 PUFA increased HDL-C concentration, the atherosclerotic lesions in group 4 mice seem to progress, suggesting that a high n-6/n-3 PUFA ratio diet is detrimental to atherogenesis in apoE<sup>-/-</sup> mice. This is consistent with the views that a high ratio of n-6/n-3 PUFA is not beneficial for cardiovascular protection in humans<sup>20</sup>; hence, a high ratio of n-6/n-3 PUFA has not been recommended. An elevated HDL-C level does not suppress atherosclerosis in apoE<sup>-/-</sup> mice. It is possible that the major risk factors for atherogenesis, such as eicosanoids and inflammatory mediators produced by LA and ALA, influence the development of atherosclerotic lesions more seriously than the HDL-C level. Dietary LA can be metabolized to arachidonic acid (AA), which can be metabolized further to prostaglandins and leukotrienes that are relatively prothrombotic, proinflammatory, and vasoconstricting. It is well known that the counteracting effects of two such eicosanoids, thromboxane A2 (TXA2) and prostacyclin (PGI2), are pivotal in the regulation of vascular hemostasis. TXA2 induces platelet aggregation and acts as a vasoconstrictor, whereas PGI2 inhibits platelet aggregation and acts as a vasodilator. A high dietary n-6/n-3 PUFA ratio is beneficial to thrombotic conditions via the detrimental balance of TXA2/PGI2. Numerous data indicate that inflammatory processes are involved in all stages of atherosclerotic lesion development<sup>25,26</sup>. Increased LA intake may result in increased inflammatory responses. Also, n-6 PUFAs may be reduced by competition for cyclooxygenase in the formation of antiinflammatory mediators from n-3 PUFAs. Another possible explanation is that HDL particles are a heterogeneous class of lipoproteins with subtypes identified on the basis of their density, electrophoretic mobility, particle size, and apolipoprotein composition. Not all HDL particles share the same biological properties. Thus, the HDL-C concentration may not adequately reflect antiatherogenic effects.

ABCA1 and ABCG1 are members of the ABC family of transporters. Previous studies support that hepatic ABCA1 and ABCG1 exert a generalized antiatherogenic effect via its contribution to HDL formation and remodeling<sup>27,30</sup>. Our results showed that hepatic ABCA1 mRNA was increased by a high ratio of n-6/n-3 PUFA, whereas aortic ABCA1 mRNA was increased by a low ratio of n-6/n-3 PUFA (data not
n-3 PUFA decreases the expression of genes involved in HDL-C metabolism, such as apoA-I, ABCG1, SR-B1 and LCAT; however, it increases endogenous LCAT activity in serum, although n-6 PUFA has no influence on transcriptional levels of ABCG1, LCAT and SR-B1, and endogenous LCAT activity. It moderately increases the expression of hepatic genes apo A-I and ABCA1, but down-regulates apoA-II transcription, which may increase HDL-C concentration in serum. Abbreviations: ABCA1, ATP binding cassette transporter A1; ABCG1, ATP binding cassette transporter G1; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; HDL-C, HDL cholesterol; LCAT, lecithin-cholesterol acyltransferase; NE, no effect; SR-B1, scavenger receptor B class 1.

Fig. 3. An estimated paradigm of the mechanisms of n-3 and n-6 PUFA on HDL-C metabolism based on the present studies.

ApoA-I and LCAT, two essential components of RCT, are shown to modulate HDL-C concentration, which is highly correlated with the amount of apoA-I. Thus, apoA-I expression may be an important determinant of HDL-C levels. Consistent with previous reports, the present findings showed that HDL-C concentration was highly correlated with the amount of serum apoA-I. With the dietary fatty acid profiles used in this study, differences in hepatic apoA-I expression were not completely reflected in serum apoA-I concentrations. ApoA-I was possibly regulated in a post-transcriptional phase, as reported by Azrolan et al. LCAT is critical for the maintenance of normal HDL metabolism in humans and mice. At present, the molecular mechanism(s) that regulates serum LCAT concentrations is not well understood. LCAT activity depends in part on the amount of the enzyme in serum, and in part on the substrate and cofactors available to the enzyme, such as apoA-I, an activator of LCAT, and apoA-II, an inhibitor of LCAT activity. Our results showed that a low ratio of n-6/n-3 PUFA did not influence the expression of apoA-II, but a high ratio of n-6/n-3 PUFA downregulated its expression. The effects of n-3 PUFA on LCAT activity, however, are not consistent. A low ratio of n-6/n-3 PUFA diet increased LCAT activity significantly, consistent with reports that ALA increased LCAT activity in humans, but contrary to a previous report that fish oil inhibited LCAT activity. The reason for this discrepancy may be due to the different n-3 fatty acids in the sn-2 position of phosphatidylcholine.

The best-understood mechanism of direct selective uptake of HDL-C by the liver is that mediated by SR-B1. Abundant data have indicated that, in rodents, SR-B1 is a critical regulator of HDL metabolism. Presently, the roles of dietary fat in modulating the expression of SR-B1 are not clear. It was reported...
that some n-6 PUFA increased the hepatic expression of SR-B1. In our study, however, the expression of SR-B1 in apoE-/- mice fed a high ratio of n-6/n-3 PUFA diet did not differ significantly compared with other groups.

Taken together, an estimated paradigm of the mechanisms of n-3 and n-6 PUFA in HDL-C metabolism in apoE-/- mice, mostly based on the present study, has been summarized in Fig. 3.

In summary, our data suggested that a high dietary ratio of n-6/n-3 PUFA (68.26) exerted a positive impact on the metabolism of serum HDL-C compared with low ratio groups, and did not effectively suppress atherosclerosis in apoE-/- mice. Furthermore, a high dietary ratio of n-6/n-3 PUFA significantly increased the expression of genes (apoA-1, ABCA1) essential for the metabolism of HDL and RCT. Further studies on the effects of dietary ratios of n-6/n-3 PUFA on HDL-C metabolism need to be conducted.

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