**Abstract**
Plasma remnant-like particle-cholesterol (RLP-C) and the RBC (n-3) index are novel risk factors for cardiovascular disease. Effects of docosahexaenoic acid (DHA) supplementation on these risk factors in hypertriglyceridemic men have not been studied. We determined effects of DHA supplementation on concentrations of plasma RLP-C, the RBC (n-3) index, and associations between concentrations of plasma RLP-C with those of plasma lipids and fatty acids. Hypertriglyceridemic men aged 39–66 y, participated in a randomized, placebo-controlled, parallel study. They received no supplements for 8 d and then received either 7.5 g/d DHA oil (3 g DHA/d) or olive oil (placebo) for the last 90 d. Fasting blood samples were collected on study d –7, 0 (baseline), 45 (mid-intervention), 84, and 91 (end-intervention). DHA supplementation for 45 d decreased (P < 0.05) fasting RLP-C (36%) and increased plasma eicosapentaenoic acid (EPA):arachidonic acid (AA) (100%) and the RBC (n-3) index (109%). Continued supplementation with DHA between d 45 and 91 further increased the RBC (n-3) index (162%) and plasma EPA:AA (137%) compared with baseline values. RLP-C concentration was positively associated (P < 0.01) with the plasma concentrations of triacylglycerols (Kendall’s correlation coefficient or r = 0.46), triacylglycerol: HDL cholesterol (HDL-C) (r = 0.44), total cholesterol:HDL-C (r = 0.26), Apo B (r = 0.22), C III (r = 0.41), and E (r = 0.17), and 18:1(n-9) (r = 0.32); it was negatively associated (P < 0.05) with plasma concentrations of DHA (r = −0.32), EPA (r = −0.25), HDL-C (r = −0.21), LDL cholesterol:Apo B (r = −0.30), and HDL-C:Apo A (r = −0.25). Supplementation with placebo oil did not alter any of the response variables tested. Decreased atherogenic RLP-C and increased cardio-protective (n-3) index may improve cardio-vascular health. J. Nutr. 138: 30–35, 2008.

**Introduction**
Cardiovascular disease (CVD) and stroke are the leading causes of mortality in the United States, accounting for >38% of all deaths (1). Elevated total cholesterol (total-C) and LDL cholesterol (LDL-C), total and small dense LDL particles, and triacylglycerols and low HDL cholesterol (HDL-C) are established independent risk factors for the development of CVD (1–4). Additional novel blood lipid markers used as risk factors for CVD include increased plasma concentration of remnant-like particle-cholesterol (RLP-C) or remnant lipoprotein cholesterol (5–8), decreased ratio between plasma eicosapentaenoic acid (EPA) and arachidonic acid (AA) (9,10), and decreased (n-3) index [sum of EPA and docosahexaenoic acid (DHA) as a percentage of total fatty acid content] of the RBC (11–14). RLP that are produced from VLDL are the major atherogenic lipoproteins that can be taken up by macrophages to produce foam cells without oxidative modification (15). An increase in the ratio between EPA and AA reduces the inflammatory response (9). An (n-3) index of <4% was associated with a 10-fold greater risk of sudden cardiac death compared with an (n-3) index of >7–8% (12). Thus, plasma RLP-C, the ratio between EPA and AA, and the RBC (n-3) index are important risk factors for evaluating the risk for CVD.
Diets rich in (n-3) fatty acids have been shown to be cardioprotective; these diets decreased inflammation, platelet aggregation, cardiac arrhythmias, triacylglycerols, number of total LDL, and small dense LDL particles and increased the (n-3) index, endothelial relaxation, and atherosclerotic plaque stability (12,16,17). Most of the earlier studies regarding the effects of long chain (n-3) PUFA on blood lipids were conducted with fish oils that contain a mixture of EPA and DHA. Recently a number of studies have been conducted with EPA and DHA individually (18–34). Results from studies with individual fatty acids show that EPA and DHA have similar effects on some of the lipid variables, but they are assimilated to different concentrations in tissues and have different effects on lipoprotein particle size, heart rate, and blood pressure (27–33). To the best of our knowledge, the effects of DHA supplementation on the plasma concentration of RLP-C and the ratio between EPA and AA, and the RBC (n-3) index in hypertriglyceridemic men (who are at increased risk for CVD) have not been previously published. Therefore, the main aim of this study was to examine the effects of DHA supplementation on the above 3 risk factors. We further determined the associations between concentrations of plasma RLP-C and those of plasma lipids and individual fatty acids.

**Subjects and Methods**

**Study design and subjects.** The study protocol was approved by the Institutional Review Boards of the University of California Davis and the Veterans Administration Medical Center, Mather, CA. It was a double blind, placebo-controlled, parallel study with 2 metabolic periods: baseline (first 8 d) and intervention (last 90 d). Group codes were revealed to the primary investigator and the statistician after sample collection from all subjects was completed, but the laboratory staff was unaware of group assignments until all analyses were completed. During the baseline period, subjects did not receive supplements, whereas during the intervention period, subjects’ diets were supplemented with either placebo or DHA capsules. The DHA group received 7.5 g/d DHA oil (DHA 3.0 g/d and no EPA), which is produced in the microalga *Crypthecodinium cohnii* (Martek Biosciences). The placebo group received 7.5 g/d olive oil. Subjects continued to consume their regular diets and were instructed not to change their usual diets and activity levels throughout the study. Presudy physical characteristics, dietary intake, and fasting blood lipids for men who participated in the study have been reported (35). All selected subjects had fasting serum triacylglycerol concentrations of 150–400 mg/dL (1.70–4.53 mmol/L), total-C < 300 mg/dL (7.78 mmol/L), LDL-C < 220 mg/dL (5.69 mmol/L), and BMI between 22 and 35 kg/m². Thirty-four men (17 in each group) completed the study, but for the analyses reported here, we had samples from only 14 subjects in each group. For the DHA group, all response variables were tested in all 14 subjects; in the placebo group, RLP-C concentrations were analyzed in 14 subjects, but the plasma and RBC fatty acids were analyzed only in 10 and 6 subjects, respectively, because these variables did not change. For the same reason, we did not analyze the RBC fatty acids composition in the placebo group at the middle of the study.

**Analysis of plasma lipids and plasma and RBC fatty acids.** Blood samples were drawn from subjects after they had fasted for 12 h on d 7 and 0 (baseline), d 45 (mid-intervention), and d 84 and d 91 (end of intervention) into EDTA-containing tubes. Plasma and RBC samples were prepared, flushed with nitrogen, and stored at −70°C until lipid extraction. Total RBC lipids were extracted using the methods of Bligh and Dyer (36) and were methylated with 14% BF₃/methanol at 100°C for 30 min (37). Butylated hydroxytoluene was added before saponification and all samples were purged with N₂ throughout the process to minimize oxidation. Fatty acid methyl esters were analyzed by GLC using a Hewlett Packard 6890 equipped with a flame ionization detector. Plasma total lipids were extracted, transmethylated, and their fatty acids analyzed on an Agilent 6890 gas chromatograph as previously reported (20,38). We measured fatty acid concentrations for only the plasma and RBC samples obtained on study d 0, 45, and 91, which are expressed as a percentage of the total mg of fatty acids (wt%). Concentrations of lipids and lipoproteins were determined in plasma samples prepared on each of the 5 blood draw days as previously reported (35). Fasting plasma RLP-C concentrations were evaluated using the RLP-C Assay kit distributed by Polymedco (Corlandt Manor). The RLP-C assay is a quantitative determination of cholesterol contained in remnant lipoproteins in the plasma after removal of the apoB-100 and apo-A1 lipoproteins.

**Results**

**Fatty acid composition of plasma and RBC lipids.** At baseline, the plasma levels (wt%) of none of the fatty acids except 18:2(n-6), 20:3(n-6), and the sum of monounsaturated fatty acids (MUFA) differed between the 2 groups (Table 1). The plasma 18:2(n-6) wt% was significantly higher and those of 20:3(n-6) and the sum of MUFA were significantly lower in the DHA group compared with the placebo group. DHA supplementation significantly decreased the levels of 20:4(n-6), 22:4(n-6), and total (n-6) PUFA and significantly increased those of 18:0, 20:5(n-3), 22:6(n-3), and total (n-3) PUFA. The plasma DHA level was 255% greater than at baseline on both d 45 and 91, whereas that of EPA was 60 and 81% greater on those 2 d, respectively. Plasma concentrations of 18:1(n-9) and the sum of MUFA decreased (P = 0.002) with DHA supplementation, but the interaction between time and treatment was not significant (P = 0.056). DHA supplementation did not alter the wt% of 14:0, 16:0, 18:1(n-7), 18:2(n-6), 18:3(n-3), 20:3(n-6), 22:5(n-6), or total SFA. Continued supplementation between d 45 and 91 did not cause any further changes in the levels of any of the fatty acids other than a significant decrease in total (n-6) PUFA. Supplementation with the placebo oil did not alter plasma fatty acid composition at either time point (Table 1).

Presupplementation concentrations of the RBC fatty acids did not differ between the 2 groups except those of 18:2(n-6), and 20:4(n-6) (Table 2). Concentration of 18:2(n-6) was significantly higher and that of 20:4(n-6) was significantly lower in the DHA group compared with the corresponding values in the placebo group. DHA supplementation significantly decreased the wt% of 20:3(n-6), 20:4(n-6), 22:4(n-6), 22:5(n-3), and total (n-6) PUFA; it significantly increased concentrations of 16:0, 20:5(n-3), 22:6(n-3), total (n-3) PUFA, and total SFA. DHA concentrations of RBC lipids were 75% greater than at baseline on d 45 and 179% greater on d 91 and those of EPA were 47 and 120% greater on those 2 d, respectively. Continued supplementation between d 45 and 91 further decreased levels of (n-6) PUFA, 20:4(n-6), 22:4(n-6), 22:5(n-3), and 22:6(n-3) and increased those of 20:5(n-3) and 22:6(n-3). Plasma concentrations of 14:0, 18:0, 18:1(n-9), 18:1(n-7), 18:2(n-6), 18:3(n-3), and the sum of MUFA in the
TABLE 1  Effect of DHA supplementation on plasma fatty acid composition in hypertriglyceridemic men

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Treatment</th>
<th>0</th>
<th>45</th>
<th>91</th>
<th>P-value, time × treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0 DHA</td>
<td>1.24 ± 0.10</td>
<td>1.20 ± 0.10</td>
<td>1.34 ± 0.08</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>1.37 ± 0.08</td>
<td>1.38 ± 0.14</td>
<td>1.27 ± 0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:0 DHA</td>
<td>21.43 ± 0.68</td>
<td>21.45 ± 0.64</td>
<td>21.99 ± 0.50</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>23.17 ± 0.72</td>
<td>22.73 ± 0.56</td>
<td>22.86 ± 0.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1(n-9) DHA</td>
<td>6.19 ± 0.18b</td>
<td>6.59 ± 0.15a</td>
<td>6.56 ± 0.17b</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>6.26 ± 0.18</td>
<td>6.44 ± 0.22</td>
<td>6.20 ± 0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1(n-7) DHA</td>
<td>20.11 ± 1.61</td>
<td>18.96 ± 0.58</td>
<td>19.88 ± 0.54</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>23.59 ± 0.64</td>
<td>22.90 ± 0.86</td>
<td>23.36 ± 0.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2(n-6) DHA</td>
<td>28.34 ± 0.78</td>
<td>30.17 ± 0.91</td>
<td>27.98 ± 0.96</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>25.53 ± 0.62</td>
<td>26.10 ± 0.50</td>
<td>26.33 ± 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3(n-3) DHA</td>
<td>0.81 ± 0.04</td>
<td>0.98 ± 0.07</td>
<td>0.78 ± 0.06</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.73 ± 0.06</td>
<td>0.71 ± 0.06</td>
<td>0.64 ± 0.08</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion

DHA supplementation significantly decreased plasma concentrations of RLP-C and increased the RBC (n-3) index and the ratio between plasma EPA and AA concentrations in hypertriglyceridemic men. The decreased plasma RLP-C was mediated

TABLE 2  Effect of DHA supplementation on RBC fatty acid composition in hypertriglyceridemic men

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Treatment</th>
<th>0</th>
<th>45</th>
<th>91</th>
<th>P-value, time × treatment</th>
</tr>
</thead>
</table>

Data are means ± SEM, n = 14 (DHA) or 10 (placebo). Means in a row with superscripts without a common letter differ, P < 0.05.

DHA group and concentrations of all fatty acids in the placebo group did not change following fatty acid supplementation.

18:1(n-9), total-C:HDL-C, Apo B, and Apo E (Table 3). The greatest negative association of RLP-C concentration was with the plasma concentration of 22:6(n-3), followed by those of LDL-C: Apo B, 20:5(n-3), HDL-C: Apo A, and HDL-C. Plasma RLP-C concentration was not correlated with the concentrations of other plasma lipids and apo proteins (total-C, LDL-C, LDL-C: HDL-C, Apo A1, lipoprotein a) and fatty acids 18:0, 18:2(n-6), 18:3(n-6), 20:3(n-6), 20:4(n-6), 22:4(n-6), and 22:5(n-6) (not shown).

Associations between plasma RLP-C, lipids, and fatty acids. Plasma concentration of RLP-C showed the highest positive association with the plasma concentration of triacylglycerols, followed by the ratio of triacylglycerol: HDL-C; Apo CIII,
through changes in both plasma lipids and fatty acid composition; some of the lipids and fatty acids were positively associated with RLP-C, whereas others were negatively associated (Table 3). We previously reported that DHA supplementation lowered fasting and postprandial triacylglycerols by 25–30% in these subjects (35). Because the RLP-C are produced from the triacylglycerol-rich chylomicrons and VLDL, the DHA-induced decreases in plasma RLP-C and triacylglycerols is in good agreement. Furthermore, blood triacylglycerol concentration showed the strongest positive association with RLP-C. Negative associations of RLP-C with the plasma concentrations of EPA and DHA were anticipated, because both these fatty acids lowered the plasma triacylglycerols, but the positive association between plasma concentrations of RLP-C and 18:1(n-9) (r = 0.32; P = 0.003; Table 3) was quite unexpected. DHA supplementation provided 1.7 g/d and the placebo supplement provided 5.7 g/d of 18:1(n-9). Despite the small increase in the intake of 18:1(n-9) from the DHA oil, plasma concentrations of this fatty acid decreased following DHA supplementation (P = 0.002), although the day × treatment interaction was not significant (P = 0.058). The positive association between plasma concentrations of RLP-C and 18:1(n-9) may be due to the reduction of both these variables by DHA and not by the dietary intake of 18:1(n-9). It is generally believed that dietary 18:1(n-9) improves the lipid profile (41); this positive association between plasma 18:1(n-9) and RLP-C may be viewed as an adverse effect. The decreased plasma 18:1(n-9) in this case resulted from the altered tissue metabolism of this fatty acid and not from increased dietary intake.

Presupplement fatty acid concentrations of the RBC lipids (Table 2) were quite distinct from those of the plasma lipids. Concentration of 18:2(n-6) in plasma lipids was twice that in RBC lipids, whereas concentrations of 18:0, 20:4(n-6), 22:4(n-6), 22:5(n-3), and 22:6(n-3) in RBC lipids were 2 or more times those of the corresponding concentrations in plasma lipids (Tables 1 and 2). DHA supplementation significantly decreased plasma concentrations of 22:5(n-3) (DPA, an intermediate in the biosynthesis of DHA), but it did not change DPA concentration in plasma lipids (Table 1). The decreased RBC DPA was most likely due to inhibition of the elongase/desaturase enzymes involved in the synthesis of DHA by the end product (DHA) (42). An increase in EPA concentrations of both plasma and RBC lipids may be due to retro conversion of DHA to EPA (42). The maximum change in plasma fatty acid composition and RLP-C concentration was attained within the first 45 d of DHA supplementation, whereas changes in RBC fatty acid continued for the next 45 d. These associations suggest that plasma and not RBC fatty acid composition is a better predictor of plasma RLP-C.

Decreased plasma and RBC (n-6) PUFA and increased (n-3) PUFA after DHA supplementation in the hypertriglyceridemic subjects are similar to changes previously reported in other subject populations (20,23,32,42–44). The increase in the RBC (n-3) index by 162% in our study is much greater than the increase of 35% observed in another recent study with hypertriglyceridemic men and women who consumed a mixture of EPA and DHA (1 g/d for 3 mo) from foods (45). This discrepancy is most likely due to the differences in the (n-3) fatty acids used, their dose, and source. The effect of DHA on plasma RLP-C concentration observed in our study is consistent with that reported with EPA in diabetic patients (46); our results differ from those of a study with patients

TABLE 3 Kendall’s correlation coefficients between plasma RLP-C and lipids or fatty acids in hypertriglyceridemic men taking DHA supplements

<table>
<thead>
<tr>
<th>Lipid or fatty acid</th>
<th>R</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacylglycerols</td>
<td>0.46</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Triacylglycerols:HDL-C</td>
<td>0.44</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>18:1(n-9)</td>
<td>0.32</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total-C:HDL-C</td>
<td>0.26</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Apo B</td>
<td>0.22</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>22:6(n-3)</td>
<td>−0.32</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LDL:C:Apo B</td>
<td>−0.30</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>20:5(n-3)</td>
<td>−0.25</td>
<td>0.02</td>
</tr>
<tr>
<td>HDL-C</td>
<td>−0.21</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

1 Correlation coefficients were calculated between RLP-C and plasma lipids using data for the DHA group from d −7, 0, 45, 84, and 91, and between RLP-C and plasma fatty acids using the data for d 0, 45, and 91. Only those variables are listed that showed significant associations.
having metabolic syndrome, in which fish oil supplementation did not alter the clearance of the stable isotope-labeled remnant-like emulsions in subjects with visceral obesity (47). This discrepancy may be due to the differences in the characteristics of the study subjects or the use of different methods (isotope ratio vs. immunological methods).

The decreased atherogenic RLP-C and increased cardioprotective (n-3) index caused by DHA may be clinically important in reducing the risk for CVD. Results previously published from this study showed that DHA decreased plasma triacylglycerols and number of total and small dense LDL particles and increased the concentration of HDL-C and the number of large LDL and HDL particles (35). Thus, the overall effect of DHA supplementation to improve cardiovascular health can be quite significant. Further studies are necessary to determine the minimum dose of DHA needed and its effectiveness in human subjects with other risk factors of CVD.

Acknowledgments
We thank Drs. Ellen Bonnel, Leslie Woodhouse, and their staffs in the coordination of the study and analysis of blood samples; we are grateful to Dr. Edward Nelson and Eileen Bailey, Martek Biosciences, for donating the DHA capsules and for RBC fatty acid analysis.

Literature Cited


