Age and Dietary Polyunsaturated Fat Alter High Density Lipoprotein Subfraction Cholesterol Concentrations in a Pediatric Population of African Green Monkeys

Mary S. Wolfe, John S. Parks, Timothy M. Morgan, and Lawrence L. Rudel

African green monkeys were raised from birth to 60 months of age on diets containing cholesterol (0.8 mg/kcal) and enriched in polyunsaturated (polyunsaturated to saturated fat ratio \([P:S]=2.5\)) or saturated (\([P:S]=0.3\)) fat. Lipoproteins were isolated from plasma of a group of animals (\(N=123\)) and were separated by gel filtration chromatography at 9, 14, 26, 38, and 50 months of age, which covered a period through adolescence into young adulthood. Total plasma cholesterol (TPC) concentrations were 16% lower (\(p=0.01\)) in the polyunsaturated fat-fed group, and high density lipoprotein (HDL) cholesterol concentrations averaged 20% lower (\(p=0.008\)) in this group between 14 and 50 months of age, while plasma apolipoprotein A-I (apo A-I) averaged 7% lower (\(p=0.06\)) over this age interval in the animals. The HDL cholesterol to apo A-I ratio was found to be significantly lower (\(p=0.006\)) in the animals fed the polyunsaturated fat diet. This suggested that the HDL subfraction distribution might differ between groups. In a subset of animals (\(n=105\), 64 male and 41 female), HDL was subfractionated by density gradient ultracentrifugation into six subfractions, HDL-I to HDL-VI, from lowest to highest density. The saturated fat-fed animals had significantly higher cholesterol concentrations in HDL-I and significantly lower cholesterol concentrations in HDL-III, HDL-IV, and HDL-V. These effects held across all ages studied; therefore, these diet effects were not age dependent. In both diet groups, the HDL subfraction pattern changed with age such that the HDL-I and HDL-II cholesterol concentrations decreased, and those of HDL-IV, HDL-V, and HDL-VI increased as the animals matured. The decrease in HDL-I with age appeared to result primarily from a decrease in HDL-I in males, while the HDL-I cholesterol concentration in females did not change with age. We conclude that diet, age, and gender all affect HDL subfraction distribution and therefore can potentially modify the relative atherogenicity of the plasma HDL populations. It remains for future studies to demonstrate the effectiveness of each subfraction in promoting or preventing the cholesterol deposition of atherosclerosis. (Arteriosclerosis and Thrombosis 1991;11:617–628)

Several studies have recognized an inverse relation between high density lipoproteins (HDLs) and both the risk for coronary heart disease (CHD) in human beings and the extent of diet-induced atherosclerosis in experimental animals. The proposed role of HDL in atherogenesis is an ability to prevent the cholesterol accumulation in the artery wall that occurs due to an influx of low density lipoprotein (LDL) cholesterol; however, the actual mechanisms for the effects of HDL on atherosclerosis remain to be determined.

Numerous investigators have studied the relation between dietary fat saturation and HDL levels in an attempt to understand the relation between diet and CHD. The African green monkey has been shown to be a good animal model for studying dietary cholesterol and fat effects on HDL. Consumption of polyunsaturated fat–enriched diets by adult African green monkeys has been associated with lower HDL cholesterol concentrations than when saturated...
fat–fed diets are consumed, and animals fed polyunsaturated fat have also been found to have less coronary artery atherosclerosis (CAA). The paradox of lower HDL cholesterol and less CAA in adult animals consuming polyunsaturated fat–enriched diets is unexplained. This paradox requires special consideration of the influence of dietary polyunsaturated fat on HDL and suggests either that HDL cholesterol concentration by itself may not provide enough information for understanding HDL’s antiatherogenic potential or that the beneficial effects of polyunsaturated fat on LDL are of overriding importance.

HDL of human and nonhuman primate species has been shown by several laboratories10–16 to be a heterogeneous lipoprotein class composed of particles differing in cholesterol and apolipoprotein concentration. A previous study in adult African green monkeys fed saturated or polyunsaturated fat–enriched diets found that overall, the type of HDL heterogeneity was similar to that seen in human beings, but the subtraction distribution was different between the two groups.11 A higher concentration of intermediate density HDL was found in the polyunsaturated fat–fed group despite lower total HDL concentrations, and the polyunsaturated fat–fed animals had less atherosclerosis (L. Rudel et al, unpublished observations).

Atherosclerosis is believed to begin early in life and to progress as an individual ages and matures; therefore, early dietary intervention could be important in modulating lipoprotein metabolism. In cross-sectional studies, HDL cholesterol concentrations have been measured in children from several demographic regions17–20; however, there are limited data on HDL subpopulations,21,22 and we are not aware of data defining the effects of dietary fat type thereon. Given the importance of HDL to atherosclerosis and the similarity between human beings and adult African green monkeys in their response to dietary fat and cholesterol, we undertook this project. We have attempted to determine if dietary polyunsaturated fat could alter HDL and its subtraction heterogeneity during the pediatric-age years. Animals entered this study at birth and consumed the same diet for up to 5 years. This gave us the opportunity to monitor long-term effects of dietary polyunsaturated fat on HDL and HDL heterogeneity from adolescence through periods of growth and development into young adulthood. We have found that aging of the animals was associated with a redistribution of cholesterol from less dense to more dense HDL. In addition, we have identified patterns of HDL response to dietary fat in juvenile animals that were similar to those seen in adults in which polyunsaturated fat actually protected against atherosclerosis. These findings suggest that protection against atherosclerosis induced by diet can begin early in life.

**Methods**

**Animals**

African green monkeys were raised from birth in the primate facility of our institution. The animals in this study consumed diets enriched in polyunsaturated or saturated fat over their lifetimes. The animals’ mothers consumed the diets during pregnancy and lactation, so that the animals entered their respective experimental group at birth. The animals were weaned from their mothers into social groups at 5 months of age. This report is from a larger project investigating the long-term effects of diets enriched in polyunsaturated fat on CHD risk factors and atherosclerosis. HDL was isolated from blood samples of 123 animals at ages 9, 14, 26, 38, and 50 months. This postweaning age period spanned from adolescence into early adulthood in this species. In a subset of these animals (n=105, 64 male and 41 female), HDL was subfractionated by density gradient ultracentrifugation at the same ages.

**Diet**

Semipurified diets11 were prepared in the diet kitchen of our center, were stored frozen, and then were thawed under refrigeration for feeding to the animals twice daily (approximately 150 kcal/kg body wt). The diets were calorically balanced and differed only in fatty acid composition. Both diets provided approximately 40% kcal from fat, 20% kcal from protein, and 40% kcal from carbohydrate. The fatty acid extract of each diet sample was methylated,23 and the fatty acids beginning with myristate (14:0) were separated on a Hewlett–Packard Model 5890A gas–liquid chromatograph (Palo Alto, Calif.) on a DB-225 column (30 m×0.25 mm, J&W Scientific, Folsom, Calif.) with a flame ionization detector. The fatty acid composition of the diets is shown in Table 1. The diet enriched in saturated fat was designed to mimic the dietary fatty acid consumption pattern of the typical North American and had a polyunsaturated to saturated fat (P:S) ratio of 0.3. The diet enriched in polyunsaturated fat substituted safflower oil for lard and had a P:S ratio of 2.5. To induce atherosclerosis and to achieve total plasma cholesterol (TPC) concentrations equivalent to those in human beings at increased risk for CHD, the animals consumed dietary cholesterol, principally from dried egg yolk (4.1±0.1 mg/g dry wt [n=14] and 4.0±0.1

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Saturated (%)</th>
<th>Polyunsaturated (%)</th>
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<tbody>
<tr>
<td>Myristic acid, 14:0</td>
<td>1.2</td>
<td>0.2</td>
</tr>
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<td>27.1</td>
<td>15.7</td>
</tr>
<tr>
<td>Stearic acid, 18:0</td>
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<td>5.1</td>
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<td>Palmitoleic acid, 16:1 α7</td>
<td>2.8</td>
<td>1.4</td>
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<tr>
<td>Oleic acid, 18:1 α9</td>
<td>40.9</td>
<td>24.2</td>
</tr>
<tr>
<td>Linoleic acid, 18:2 α6</td>
<td>13.1</td>
<td>52.1</td>
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<td>1.3</td>
</tr>
<tr>
<td>P:MS</td>
<td>1/3.3/3.1</td>
<td>1/0.5/0.4</td>
</tr>
<tr>
<td>PS</td>
<td>0.3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

mg/g dry wt \( [n=15] \), mean±SEM for saturated and polyunsaturated fat diets, respectively) at an intake of 0.8 mg/kcal. \( \beta\)-Sitosterol was added to the saturated fat-enriched diet to compensate for the amount found in safflower oil of the polyunsaturated fat-enriched diet.

**High Density Lipoprotein Isolation and Subfractionation**

Blood samples were obtained from the femoral vein by venipuncture after the animals were fasted overnight (16–18 hours). Blood was collected into a cocktail containing 1 mg/ml (final concentration) \( \text{Na}_2 \) EDTA, 1 mg/ml (final concentration) \( \text{NaNO}_3 \), and 0.4 mg/ml (final concentration) 5,5'-dithio-bis(2-nitrobenzoic acid). The samples were placed on wet ice immediately after collection. Plasma was isolated by low-speed centrifugation at 2,700 rpm for 30 minutes, 4°C. The lipoprotein fraction of whole plasma (5–9 ml) was isolated by ultracentrifugation as described previously. The samples were spun at 50,000 rpm for 24 hours, 15°C, in a 70.1 Ti rotor (Beckman Instruments, Palo Alto, Calif.). The lipoprotein fraction at the top of the tube of \( d<1.225 \) g/ml was removed by pipetting after the tube was sliced.

The lipoproteins of \( d<1.225 \) g/ml were fractionated by agarose column chromatography on Bio-gel A-15 M, 200–400 mesh (Bio-Rad, Richmond, Calif.) in a 1.5×90-cm column (Pharmacia, Piscataway, N.J.) with 0.15 M NaCl containing 0.01% EDTA and 0.01% \( \text{NaNO}_3 \), pH 7.4, as eluent (saline solution). The lipoproteins were pooled into these classes: 1) very low density lipoproteins plus intermediate-sized low density lipoproteins, 2) LDL, and 3) HDL. An aliquot of the isolated HDL was subfractionated by density gradient ultracentrifugation by a modification of the procedure of Rudel et al. A volume of HDL corresponding to approximately 5 mg protein (not <2 mg) was made to a total volume of 12.6 ml with \( d=1.006 \) g/ml saline solution. The HDL sample was raised to \( d=1.10 \) g/ml by the addition of 5.4 ml saturated KBr solution. By use of an underlying procedure in which the solutions were added to the ultracentrifugation tube in order of increasing density to form a discontinuous gradient, the HDL was layered between 12 ml of \( d=1.06 \) g/ml (KBr-saline solution) and 9 ml of \( d=1.16 \) g/ml (KBr-saline solution). The samples were centrifuged in a VTi 50 rotor (Beckman) at 50,000 rpm for 18 hours, 15°C. The tubes were then drained from the top by pumping (Flourinert 3M Co., St. Paul, Minn.) into the bottom of the tube. The optical density of the eluted sample was continuously monitored at 280 nm, and individual fractions of 1.4–1.5 ml were collected with a fraction collector. The density of each fraction was determined by refractometry.

The HDL was pooled into six subfractions that were defined by the following density intervals: HDL-I \( (d<1.09 \) g/ml), HDL-II \( (d=1.09-1.10 \) g/ml), HDL-III \( (d=1.10-1.11 \) g/ml), HDL-IV \( (d=1.11-1.12 \) g/ml), HDL-V \( (d=1.12-1.13 \) g/ml), and HDL-VI \( (d>1.13 \) g/ml). The density fractions were pooled into six subfractions for convenience and because of recognized differences among them in molecular weight, flotation, and composition. The cholesterol concentration of the HDL subfractions was measured, and the plasma cholesterol concentration of each HDL subfraction was calculated by correcting for the recovery of total HDL subfraction cholesterol (86±2%, mean±SEM, \( N=254 \)).

**Chemical Analysis**

The cholesterol concentration of whole plasma and of the \( d>1.225 \) g/ml fraction was quantified by the microassay of Rudel and Morris, and that of individual lipoprotein fractions was measured enzymatically (Guilford Diagnostics, Cleveland, Ohio). Whole-plasma apolipoprotein A-I (apo A-I) concentration was measured by enzyme-linked immunosorbent assay (ELISA) as described previously.

**Statistical Analysis**

All statistical analyses of the longitudinal data in this study were analyzed with the SAS software package (Statistical Analysis System, Raleigh, N.C.). Longitudinal measurements of HDL cholesterol and apo A-I were analyzed by univariate repeated-measures analysis of variance. The statistical model considered various outcome variables measured at several ages as the repeated measures and dietary fat as the treatment effect. All interaction terms were considered in the statistical model and were included in the model only if they were significant at \( p<0.05 \).

The statistical analysis was a hierarchical procedure in which the full model containing main effects (dietary fat and age and, in some cases, gender) and all interactions were tested, and then sequentially nonsignificant interaction terms were removed from the model. When all possible comparisons by age and dietary fat were made, the significance level for the multiple comparisons was corrected for multiple comparisons with the Bonferroni technique. When a significant effect by age was found, the differences in the variable between different ages were tested by Duncan’s multiple range test.

Repeated-measures analysis of covariance was used for analyzing cholesterol concentrations of HDL subfractions. HDL cholesterol was added to the statistical model as the covariate. Repeated-measures analysis of covariance was also used in correlation analysis for determining relations between HDL cholesterol and HDL density subfractions by age and dietary fat treatment. The correlation between variables was estimated by modeling polynomial relations separately across time. The consistency of the model across ages and between dietary fat groups was examined. All interaction terms of age, dietary fat, and HDL cholesterol were found to be nonsignificant, and the slope of the line for each correlation within a dietary fat group did not change significantly over time.
Results

Lipid and Apolipoprotein A-I Concentrations

Lipoprotein measurements for studying HDL were made at ages 9, 14, 26, 38, and 50 months. The measurements of cholesterol and apolipoproteins presented in this article covered a period of adolescence into young adulthood. The ages of 9, 14, and 26 months were before sexual maturation, 38 months was peripubertal, and 50 months of age was after sexual maturity.

Mean TPC concentrations from the two dietary fat groups for the five ages are shown in Figure 1. There was a significant effect of age (p=0.0001) on TPC concentrations in the two groups. TPC concentrations rose significantly in both groups between 9 and 26 months of age (p<0.05). As the animals matured and aged beyond 26 months, TPC declined in both groups; however, the TPC concentrations at the older ages were not significantly different from the values at 26 months of age (p>0.05).

The effect of age on TPC was accompanied by a significant effect of dietary fat (p=0.01) (Figure 1). The mean differences in TPC between the two groups were largest at the younger ages. Animals fed dietary polyunsaturated fat had mean TPC concentrations that averaged 16% lower overall than their saturated fat-fed counterparts.

Lipoproteins were subfractionated by agarose column chromatography at 9, 14, 26, 38, and 50 months of age. Mean HDL cholesterol concentrations are shown for the two dietary fat groups in Figure 2A. The effect of dietary fat on HDL cholesterol was not equivalent for all ages, as shown by the significant interaction term of diet x age (p=0.008). As shown in Figure 2, HDL cholesterol concentrations were similar at 9 months of age in the two groups. At 14 months of age, the average HDL cholesterol concentration was higher in the saturated-fat group, and it remained relatively constant at this higher level thereafter. In contrast, in the animals fed polyunsaturated fat, mean HDL cholesterol concentrations were progressively lower between 9 and 38 months of age and somewhat higher at 50 months. Between 14 and 50 months of age, HDL cholesterol concentrations averaged 126 mg/dl in the saturated-fat group and 96 mg/dl in the polyunsaturated-fat group. A significant difference in mean HDL cholesterol concentrations at individual ages between the two dietary fat groups was only found at 26 months (126±12 versus 93±8 mg/dl for saturated and polyunsaturated, respectively, p=0.03). The 18% (p=0.2) and 32% (p=0.09) lower HDL cholesterol levels in the polyunsaturated-fat group at 14 and 38 months of age, respectively, failed to achieve statistical significance at the 95% confidence interval after correction of the significance level for multiple comparisons by the Bonferroni technique, although these differences appeared reproducible.

Whole-plasma apo A-I concentrations were also measured at the individual ages by ELISA, and the mean concentrations for the two groups are shown in Figure 2B. Pilot studies have shown that essentially all of the plasma apo A-I is associated with the HDL fraction. The types of differences in apo A-I concentrations were similar to those of HDL cholesterol, although differences due to diet were less marked. There was an overall effect of age on apo A-I concentration (p=0.02); however, differences in apo A-I concentration between individual ages did not achieve statistical significance at the 95% confidence interval. Apo A-I concentrations were similar between the two groups at 9 months of age and afterward averaged 7% lower in the polyunsaturated-fat group (p=0.06).

Because larger average differences in concentrations of HDL cholesterol than apo A-I were observed between the animals fed saturated and polyunsaturated fat-enriched diets, the HDL cholesterol to plasma apo A-I ratio was calculated and examined for effects of age and dietary fat. The average ratios for the two groups at each age are shown in Figure 2C. At 9 months of age, the ratio appeared higher in the polyunsaturated-fat group, which reflected the patterns of HDL cholesterol and apo A-I (panels A and B) at this age. The statistically significant effect of the type of dietary fat on the ratio (p=0.006) was most evident at 14–50 months of age, when the average concentration of HDL cholesterol relative to apo A-I was higher in the saturated fat–fed group. After correction for multiple comparisons, a statistically significant difference in the ratio at individual ages was
only found at 26 months of age (0.48±0.03 versus 0.38±0.02, p=0.03 for saturated- versus polyunsaturated-fat groups, respectively). The ratio also averaged approximately 20% lower in the polyunsaturated-fat group at 38 and 50 months, but these differences failed to achieve statistical significance at the 95% confidence interval (p=0.09 and p=0.6, respectively).

High Density Lipoprotein Density Subfractions

Since HDL cholesterol was lowered more than plasma apo A-I by dietary polyunsaturated fat at most ages, it seemed likely that HDL subfraction distribution was affected. To investigate HDL heterogeneity, HDL isolated by agarose column chromatography was subfractionated by density gradient ultracentrifugation. Figure 3 shows density gradient profiles of HDL measured at 280 nm from two representative animals at 26 months of age. Although the total amount of HDL protein placed into the gradient was the same for the two animals, the HDL gradient profiles were obviously different. Panel A shows data from a saturated fat-fed animal, and panel B shows data from a polyunsaturated fat-fed animal. The saturated fat-fed animal possessed more lower-density HDL subfractions than the polyunsaturated fat-fed animal, whose major HDL subfraction was of a higher density.

For individual animals, the HDL was pooled into six subfractions as described previously10,11: HDL-I, HDL-II, HDL-III, HDL-IV, HDL-V, and HDL-VI. In a previous report by Babiak et al,11 HDL heterogeneity within individual density fractions has been analyzed by analytical ultracentrifugation and nonnondenaturing gradient gel electrophoresis. HDL density subfractions I and II contained predominantly HDL_{2a} material (94% and 64%, respectively, by analytical ultracentrifugation), and HDL_{2b} was the predominant species in subfraction III. By analytical ultracentrifugation, HDL_{3a} was the only constituent of HDL-VI and was the major constituent of HDL-IV and HDL-V. Further characterization of fractions IV-VI by nondenaturing gradient gel electrophoresis found peaks in HDL-IV and HDL-V corresponding to HDL_{2a} and HDL_{3a}, respectively, while HDL-VI contained a peak of HDL_{3a} size along with some larger HDL_{3b}-size material.
The measurements of HDL cholesterol concentrations in the young animals had shown a lack of equivalence between the two groups and an age effect within each dietary fat group (Figure 2A). Therefore, differences in HDL subfraction cholesterol concentrations between the diet groups were harder to interpret. To determine if the dietary fat effect on HDL density subfraction cholesterol concentration was dependent on the HDL cholesterol level within an animal, the relation between HDL cholesterol and concentrations of cholesterol in individual subfractions was modeled for the two dietary fat groups at each age interval. The cholesterol concentration of each HDL density subfraction (except HDL-VI) was found to be significantly correlated with HDL cholesterol. The statistical model showed a constancy by age and dietary fat in the relation between HDL cholesterol and the cholesterol concentration of density subfractions I–V.

Increases in HDL cholesterol concentrations were reflected in linear increases in the cholesterol concentration of each density subfraction.

**Effects of Dietary Polyunsaturated Fat on High Density Lipoprotein Density Subfraction Cholesterol Concentrations**

The age period studied in these animals represented a time of rapid growth and development in these animals. Feeding polyunsaturated fat to the animals during this age interval induced an effect on HDL subfraction cholesterol concentrations similar to that observed in adult animals. Overall mean differences showed significantly lower (37%, \( p=0.0001 \)) HDL-I cholesterol concentrations and significantly higher cholesterol concentrations of the intermediate density subfractions HDL-IV (14%, \( p=0.01 \)) and HDL-V (10%, \( p=0.02 \)) in animals fed the polyunsaturated fat–enriched diet compared with the saturated fat–enriched diet.

Because of differences in HDL cholesterol concentrations between groups, the effects of age and dietary fat type on subfraction cholesterol concentrations were evaluated after covarying for HDL cholesterol concentration in the statistical model. Table 2 shows the mean cholesterol concentrations of HDL density subfractions I–VI at five ages. Of the six subfractions, the highest cholesterol concentration was found within the HDL-I subfraction, and the least cholesterol was found in the most dense HDL subfraction, HDL-VI.

HDL density subfraction cholesterol concentrations were affected both by age and dietary fat. As shown in Table 2, at each age HDL-I cholesterol was consistently higher in the saturated-fat group (\( p=0.0008 \)). This difference in HDL-I cholesterol for individual HDL-I cholesterol levels was translated into higher mean HDL-I cholesterol concentrations in the saturated-fat group across all ages. From 9–38 months of age, mean HDL-I cholesterol concentrations in the polyunsaturated fat–fed animals averaged 20–30% lower than in the saturated fat–fed animals. The difference between the two dietary fat groups in HDL-I cholesterol was diminished at 50 months. Unlike HDL-I cholesterol, HDL-II cholesterol concentrations were not affected significantly by the dietary fat group of the animals (\( p=0.3 \)).

For intermediate HDL density subfractions III–V, an opposite effect of the type of dietary fat on cholesterol concentration than for HDL-I was found. Significantly higher mean concentrations of HDL subfractions III–V (HDL-III, \( p=0.0004 \); HDL-IV, \( p=0.0001 \); HDL-V, \( p=0.004 \)) were present in the polyunsaturated fat–fed animals. The statistically significant effect of dietary fat on HDL-III cholesterol was found only after controlling for HDL cholesterol levels within the animals by covariate analysis. Animals fed dietary polyunsaturated fat had mean HDL-III cholesterol concentrations that averaged 12–20% higher than the saturated fat–fed animals across all five ages. Likewise, dietary polyunsaturated-fat feeding was associated with HDL-IV cholesterol concentrations that averaged 10–25% higher than in the saturated fat–fed group.

**Figure 3. Density gradient (g/ml) absorbance profiles as a function of tube volume (ml) of high density lipoprotein (HDL) from representative monkeys at 26 months of age in the saturated fat–fed (panel A) and polyunsaturated fat–fed (panel B) groups. HDL was isolated by a combination of ultracentrifugation and gel filtration chromatography. Density gradient ultracentrifugation was performed on HDL as described in "Methods." Sample was eluted by pumping a dense solution into the bottom of the centrifuge tube, and absorbance at 280 nm was continuously monitored and recorded.**
TABLE 2. Effect of Dietary Fat on Cholesterol Concentrations of High Density Lipoprotein Density Subfractions

<table>
<thead>
<tr>
<th>Age group</th>
<th>HDL-I</th>
<th>HDL-II</th>
<th>HDL-III</th>
<th>HDL-IV</th>
<th>HDL-V</th>
<th>HDL-VI</th>
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<tbody>
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<td>9 mo</td>
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<tr>
<td>Sat'd</td>
<td>56±4</td>
<td>29±2</td>
<td>12±1*</td>
<td>7±1</td>
<td>4±1</td>
<td>3±1</td>
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<tr>
<td>Sat'd</td>
<td>48±5</td>
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<td>4±1</td>
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<tr>
<td>Sat'd</td>
<td>48±4</td>
<td>26±2</td>
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<tr>
<td>Sat'd</td>
<td>33±8</td>
<td>17±3</td>
<td>17±2</td>
<td>17±2</td>
<td>15±1</td>
<td>7±1</td>
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<tr>
<td>fat effect</td>
<td>p=0.0008</td>
<td>p=0.3</td>
<td>p=0.0004</td>
<td>p=0.0001</td>
<td>p=0.004</td>
<td>p=0.9</td>
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</table>

Values are mean±SEM. Data were analyzed as described in "Methods" by repeated-measures analysis of covariance, where HDL cholesterol was the covariate. Significance level is given for main effects of dietary fat across all ages. Within individual HDL density subfractions for specific ages, cholesterol concentrations with the same symbol are significantly different.

HDL, high density lipoprotein; Sat'd, saturated fat-fed group; Poly, polyunsaturated fat-fed group.

At 9 months, •p=0.05; ‡p=0.02; §p=0.03.
At 26 months, †p=0.05; ‡p=0.02; †‡p=0.03.
At 38 months, †p=0.04; ‡p=0.03.

Urated fat similar to that for HDL-III cholesterol and HDL-IV cholesterol were found for HDL-V cholesterol; however, the dietary fat effect was diminished at 50 months of age.

As was found for HDL-II cholesterol, mean HDL-VI cholesterol concentrations were not significantly different (p=0.9) between the two dietary fat groups.

Effects of Age on High Density Lipoprotein Density Subfraction Cholesterol Concentrations

The age effects on HDL subfraction cholesterol concentrations were equivalent for the two dietary fat groups; therefore, for simplicity in illustrating the effect of age, the data were combined for the two dietary fat groups, and the mean concentrations by age for the individual subfractions are shown in Figure 4. The effect of age on HDL density subfraction cholesterol concentrations could be divided into subfractions where cholesterol decreased, did not change, or increased as the animals aged. In the fraction of least density, HDL-I, there was a significant (p=0.03) downward trend in mean cholesterol concentration with age (panel A), and as a result, more cholesterol was present in this fraction at 9 than at 50 months of age (p<0.05). HDL-II cholesterol showed a similar significant (p=0.0001) downward trend in concentration. In contrast to HDL-I cholesterol and HDL-II cholesterol, changes in HDL-III with age were not significant (p=0.1); therefore, cholesterol concentrations were comparable at all ages for HDL-III cholesterol.

Figure 4. Bar graphs of effect of age (months, mo) on high density lipoprotein (HDL) subfraction cholesterol concentrations (mg/dl) in animals combined from the two diet groups. Data were analyzed as described in Figure 4 and "Methods." Age effect for HDL-I, p=0.03; for HDL-II, p=0.0001; for HDL-III, p=0.1; for HDL-IV, p=0.0001; for HDL-V, p=0.0001; and for HDL-VI, p=0.003.
The effects of age on mean cholesterol concentrations of HDL-IV, HDL-V, and HDL-VI are shown in panel B. In contrast to the decreases in cholesterol in subfractions I and II as the animals aged, HDL-IV, HDL-V, and HDL-VI all showed significant increases in cholesterol concentration with age (HDL-IV, p<0.0001; HDL-V, p=0.0001; HDL-VI, p=0.003).

**Effects of Type of Dietary Fat and Age on Cholesterol Distribution Among High Density Lipoprotein Density Subfractions**

The previous data presented evidence for changes in HDL density subfraction cholesterol concentrations as the animals aged. The data also showed that the type of dietary fat can modify the concentration of cholesterol among the six subfractions independent of changes in total HDL cholesterol. These changes in absolute concentration with age and dietary fat group resulted in a redistribution of cholesterol among the six subfractions. The mean percentage distribution for dietary fat groups by age are shown in Figures 5A and 5B. There were significant effects (p<0.0002) of dietary fat saturation on the percentage distribution of cholesterol for HDL subfractions I, III, IV, and V. These are the same subfractions that showed significant effects of dietary fat saturation on mean cholesterol concentrations.

Effects of the type of dietary fat did not necessarily reflect similar effects of age on cholesterol distribution. Significant effects (p<0.01) of age on percentage cholesterol distribution affected all HDL subfractions, with the mean percentage decreasing in HDL-I-II and increasing in HDL-III-VI as the animals grew older. At 9 months of age, the greatest percentage of cholesterol for both dietary fat groups was found in HDL-I, and there was a stepwise decrease to HDL-VI. As young adolescents, these animals had the majority of their HDL cholesterol localized within the least-dense HDL subfractions I and II (76% saturated- and 69% polyunsaturated-fat groups, panels A and B). As the animals entered a period of maturation and development (26–38 months), the pattern of declining cholesterol in HDL-I and HDL-II continued. By the time the animals reached young adulthood at 50 months of age, HDL-I cholesterol and HDL-II cholesterol had decreased 30–50%, such that these subfractions together contained no more than one half of all HDL cholesterol (50% saturated- and 39% polyunsaturated-fat groups). Aging and maturing of the animals was associated with increasing concentrations of the remaining four subfractions (HDL-III–VI, Figure 5), resulting in increases in the percentage of cholesterol within these subfractions. At 50 months of age, HDL-I still had the highest mean percentage of HDL cholesterol, while the percentages found in HDL-III-V were similar within both dietary fat groups.

**Effects of Gender on High Density Lipoprotein Density Subfractions**

Since the animals were studied during an interval before puberty and were followed into young adulthood, we wanted to determine if sexual maturation might influence HDL subfraction cholesterol concentrations. There was no significant overall effect of gender on total HDL cholesterol (p=0.9), plasma apo
FIGURE 6. Line plots of effect of type of dietary fat and age (months, mo) on mean ± SEM high density lipoprotein (HDL) subfraction cholesterol concentrations (mg/dl) for HDL-I (panel A), HDL-IV (panel B), and HDL-V (panel C) for male (■) and female (□) animals. Data were analyzed as described in Figure 1 and “Methods.” Age×sex effect: for HDL-I, p=0.0008; for HDL-IV, p=0.02; and for HDL-V, p=0.002.

A-I (p=0.7), or the HDL cholesterol to apo A-I ratio (p=0.4); however, both HDL cholesterol and apo A-I mean concentrations were consistently 10–20% lower in male animals at 26 months of age and older. There was a pattern of age-related change by gender in HDL density subfraction cholesterol concentrations that was not different for the two diet groups. The cholesterol concentrations within HDL subfractions I, IV, and V changed significantly with age, depending on the sex of the animals (age×sex: HDL-I, p=0.0008; HDL-IV, p=0.02; HDL-V, p=0.002). This effect of gender was not different for the two diet groups and therefore is illustrated for combined groups in Figures 6A, 6B, and 6C. As the male animals matured and aged, cholesterol concentration of less-dense HDL-I (panel A) was found to decrease, while the cholesterol concentrations of intermediate density, HDL-IV and HDL-V, increased (panels B and C, respectively). In females, mean concentrations of these subfractions showed much smaller changes with age, and the sum of cholesterol in the intermediate fractions IV and V (panels B and C, respectively) was the same at each of five ages. The male and female animals showed a crossover of higher mean HDL-I cholesterol beginning at 26 months of age in females (panel A) due to the continued decreases in cholesterol concentrations of this subfraction between 9 and 50 months in the male animals.

**Discussion**

In the present study, animals were subjected to lifelong consumption of atherogenic diets enriched in either saturated fat or polyunsaturated fat to study the long-term effects of such diets on lipoprotein metabolism and atherogenesis. In this article, we
report modestly lower plasma concentrations of TPC, HDL cholesterol, and apo A-I in the animals fed polyunsaturated fat throughout their life. Although similar effects have been reported for adult animals and for human adults, children, and infants, when the degree of polyunsaturation was high, most of the studies in human populations have been evaluated after short-term consumption of the polyunsaturated fat diets. In addition, the data in this report, which highlighted five ages from adolescence into young adulthood, showed that age in addition to the type of dietary fat affected lipid concentrations in the animals. While more extensive longitudinal data will be presented in a separate publication, it is of note that TPC and HDL cholesterol levels were found to be reasonably consistent within animals of both dietary fat groups. The mean correlations for values 4–36 months apart were $r=0.7$ and $r=0.6$ for TPC and HDL cholesterol, respectively (L. Rudel et al., unpublished observation). This study provides evidence that lipoprotein alterations that are associated with consumption of dietary polyunsaturated fat can be initiated early in life and can be maintained long-term as the individual grows and develops.

In addition, this study showed that the distribution of cholesterol among HDL subfractions was significantly different, with higher concentrations of cholesterol in intermediate density HDL subfractions despite lower total HDL cholesterol concentrations in animals consuming polyunsaturated fat. This diet-induced shift in the pattern of HDL subfraction distribution occurred together with an age-related shift toward decreased concentrations of HDL-I and HDL-II cholesterol and an increase in the cholesterol concentrations of HDL-IV, HDL-V, and HDL-VI. Furthermore, the decrease in HDL-I cholesterol concentration that occurred with age occurred only in the male animals, resulting in higher HDL-II in females as young adults. These changes in HDL subfraction distribution indicate that the factors regulating HDL metabolism are sensitive to each of the three main variables of this study, that is, dietary fat type, age, and gender. To our knowledge, this is the first study to indicate this degree of complexity in HDL metabolism. It is not possible to explain the metabolic basis of these effects with our current information about the factors controlling HDL metabolism. However, with what has been documented in studies of humans showing that polyunsaturated fat lowers HDL cholesterol concentrations and that HDL cholesterol concentrations decrease during maturation in young boys along with changes in HDL subfraction patterns, we believe that these studies in African green monkeys document a valuable animal model with which to further investigate mechanisms for such effects.

At the same time, we must point out that the African green monkey is an animal that maintains very low plasma triglyceride concentrations, even though diets with 40% kcal as fat were fed. This is important since others have noted a close relation between HDL subfraction concentration and the metabolism of the triglyceride-rich lipoproteins. We did not document the various aspects of lipoprotein lipase and hepatic lipase levels and of postprandial lipemia in the animals of these studies. However, the uniformly low plasma triglyceride concentrations have been documented (L. Rudel et al., unpublished observation), and studies of many of these variables of triglyceride metabolism have been done in the past in adult monkeys, demonstrating little correlation with HDL subfraction distribution. It seems fair to say that other factors are contributing to the pattern of HDL subfraction distribution seen in these animals. While we can only speculate on what the nature of the factors involved might be, we have indications that apo A-I production is high in African green monkeys and that polyunsaturated fat affects apo A-I production, and we know that levels of cholesteryl ester transfer activity are high (Reference 36 and L. Rudel et al., unpublished observation). Each of these is potential factors that could influence the concentration and composition of HDL subfractions although as yet in an undefined manner.

The animals were raised on semipurified diets with a nutrient composition similar to that of Western society. The fatty acid composition of the saturated fat diet was also representative of that of Western society, while the fatty acid composition of the polyunsaturated-fat diet essentially represents an extreme that could be achieved by humans. Since the purpose of this study was to demonstrate differences where they occur, the selection of fatty acid compositions that were markedly different was an advantage. The cholesterol level of the diets was chosen to induce a modest hypercholesterolemia in the young animals, which was necessary for atherosclerosis induction. The animals achieved TPC levels during their pediatric-age years similar to those of human beings at increased risk for CHD, which allowed us, in separate studies, to monitor long-term effects of hypercholesterolemia on atherosclerosis development in a pediatric population in addition to its effects on plasma lipids and lipoproteins.

While the data on atherosclerosis progression will be presented in a separate publication, it is of note that less atherosclerosis developed in the polyunsaturated-fat group and less atherosclerosis developed in the pediatric-age animals compared with fully adult animals fed the same diets for the same lengths of time. Therefore, the changes in plasma lipids and lipoproteins observed in the pediatric-age animals are likely associated with decreased atherogenicity in the African green monkey.

The specific lipid and lipoprotein factors associated with the decreased rate of atherosclerosis progression remain unknown. It should be of note that a decreased amount of atherosclerosis occurred in the polyunsaturated-fat group despite consistently lower HDL cholesterol and apo A-I concentrations. In this case, it seems useful to consider the possibility that the modification in HDL subfractions toward increased con-
centrations of intermediate density HDL subfractions may actually be beneficial for protection against atherosclerosis. The pathway of reverse cholesterol transport postulates the movement of cholesterol from peripheral tissues to HDL with direct removal from plasma or with transfer of the cholesteryl ester to triglyceride-rich lipoproteins for uptake and excretion by the liver. The higher concentrations of intermediate subfractions may be reflective of more efficient transport of cholesterol than for larger HDLs. Possibly, intermediate-density HDLs are better than are larger HDLs in promoting cholesteryl efflux from arteries due to their lower cholesterol to phospholipid ratio. In this way, intermediate-density HDL would aid in reducing buildup of cholesterol in arteries, thereby decreasing atherosclerosis.

In addition, the modification of HDL subfraction pattern by dietary polyunsaturated fat may be an indication of a difference in plasma cholesteryl ester transfer. Smaller HDL subfractions may promote a higher rate of cholesteryl ester transfer than larger HDL since the smaller particle would provide more surface area per unit mass. The decreased concentration of HDL-I cholesterol may also indicate an increased transfer via cholesteryl ester transfer protein from intermediate-density HDL into apo B-containing lipoprotein instead of into HDL. Under conditions in vivo where cholesteryl ester transfer protein activity has been inhibited in rabbits, cholesterol esterification was maintained; however, HDL cholesteryl ester concentration was increased and the initial rates of HDL and plasma cholesterol ester clearance were reduced. The buildup of larger, cholesterol-enriched HDL in the circulation of the saturated fat-fed group may in fact represent an impairment of reverse cholesterol transport, possibly from inefficient cholesteryl ester transfer into triglyceride-rich particles.

Acknowledgments

The authors acknowledge the technical assistance of Beverly Sonbert, Joy Martin, and Susan Pelkey as well as the skilful manuscript preparation of Linda Odham.

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**KEY WORDS** • high density lipoproteins • cholesterol • age • polyunsaturated fats • nonhuman primates
Age and dietary polyunsaturated fat alter high density lipoprotein subfraction cholesterol concentrations in a pediatric population of African green monkeys.

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doi: 10.1161/01.ATV.11.3.617

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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