Influence of LDL apheresis on LDL subtypes in patients with coronary heart disease and severe hyperlipoproteinemia

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Abstract Epidemiologic studies and in vitro experiments indicate that low density lipoprotein (LDL) subtypes differ concerning their atherogenic potential. Small, dense LDL are more atherogenic than large, buoyant LDL. LDL apheresis is a potent therapeutic modality to lower elevated LDL-cholesterol. It is unknown whether such therapy induces a shift in the LDL subtype distribution. In this study we evaluated the influence of LDL apheresis on the LDL subtype distribution in patients with CHD and familial hypercholesterolemia (FH, n = 22), combined hyperlipidemia (CHLP, n = 6), or Lp[a]-hyperlipoproteinemia (Lp[a]-HLP, n = 4) regularly treated by LDL apheresis (immunoadsorption (n = 14), HELP apheresis (n = 8), dextran sulfate adsorption (n = 7), cascade filtration (n = 3)). On the basis of 6 LDL subfractions (d 1.020–1.057 g/mL) isolated by density gradient ultracentrifugation the LDL-density profile was determined in each patient before and after apheresis. There was a relative increase of LDL-subfractions 1, 2, and 3 (P < 0.01, P < 0.05, and P < 0.01, respectively) and a concomitant decrease of LDL subfractions 5 and 6 (P < 0.05) after apheresis. Subgroup analysis indicates that the degree of the small, dense LDL reduction was much more prominent in patients with CHLP compared to patients with FH or Lp[a]-HLP, whereas the type of apheresis technique had no effect. The extent of small, dense LDL reduction correlated with the preapheresis concentrations of small, dense LDL and triglycerides but not with the extent of triglyceride reduction. We conclude that LDL apheresis not only decreases LDL mass, but also improves LDL-density profile, particularly in patients with CHLP. —Schamberger, B. M., H. C. Geiss, M. M. Ritter, P. Schwandt, and K. G. Parhofer.


Supplementary key words LDL subfractions • LDL subtypes • LDL apheresis • density gradient ultracentrifugation • small dense LDL

An increased low density lipoprotein (LDL) cholesterol concentration is a well-known risk factor for the development and progression of coronary heart disease (CHD) (1). LDL can be separated into subtypes of different size and density by density gradient ultracentrifugation (2) and gradient gel electrophoresis (3). In vitro experiments (4–6) and epidemiologic studies (7–10) have shown that small, dense LDL are more atherogenic than large, buoyant LDL. These findings can be partly explained by the association of small, dense LDL with elevated levels of plasma triglycerides and decreased levels of HDL-cholesterol (11), but there is evidence that additional mechanisms contribute to the atherogeneity of small, dense LDL (12, 13). Small, dense LDL are more susceptible to oxidation compared to large, buoyant LDL (4, 14) and have a higher capacity to bind to intimal proteoglycan (5). It is unknown whether small, dense LDL are derived from large, buoyant LDL or produced independently; however, once in plasma these particles are characterized by a lower affinity for the LDL receptor and a longer half-life in the plasma compared to large, buoyant LDL (6).

For patients with CHD the National Cholesterol Education Program recommends an aggressive quantitative reduction of LDL-cholesterol (<100 mg/dL) to avoid progression of CHD. Patients with CHD and hyperlipoproteinemia, which is so severe that dietary and drug therapy fail to reach this goal, are considered candidates for LDL apheresis, a powerful and invasive therapeutic modality to lower LDL-cholesterol. These patients could additionally benefit if apheresis does not only decrease LDL concentrations, but also induces a shift in the subtype distribution. However, it is unknown whether or not LDL apheresis induces a shift in the LDL-subtype distribution, and whether such a shift depends on the apheresis system used or the underlying hyperlipoproteinemia. Furthermore, such changes could also provide information on the metabolism of LDL subtypes.

We investigated the influence of a single apheresis on the distribution of LDL subtypes in patients treated by regular LDL apheresis. Furthermore, we examined whether

Abbreviations: CHLP, combined hyperlipidemia; FH, familial hypercholesterolemia; HDL, high density lipoprotein; HELP, heparin-induced extracorporeal LDL precipitation; LDL, low density lipoprotein; Lp[a]-HLP, lipoprotein[a]-hyperlipoproteinemia; VLDL, very low density lipoprotein.

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the elimination of LDL subtypes depends on the type of underlying hyperlipoproteinemia or the apheresis system used for LDL elimination.

**METHODS**

LDL density profiles were determined in 32 patients undergoing regular apheresis treatment immediately before and after a single apheresis to examine the effect of LDL apheresis on the distribution of LDL subtypes.

**Patients**

The entire study population consisted of 32 patients (19 male, 13 female; mean age: 52 years, range 34–75 years) treated by regular LDL apheresis because of severe, heterozygous familial hypercholesterolemia (FH, n = 22), combined hyperlipidemia (CHLP, n = 6), or Lp[a]-hyperlipoproteinemia (Lp[a]-HLP, n = 4). Four patients had no lipid-lowering medication due to side effects; in 28 patients statins were given at the maximal tolerable dose for a minimal treatment time of 6 months (simvastatin in 3, lovastatin in 2, and atorvastatin in 23 patients).

**Apheresis techniques**

In 24 patients LDL apheresis was performed at weekly intervals, in 8 patients at biweekly intervals. As described elsewhere (15) plasma was separated from whole blood by a plasma filter or by centrifugation. LDL elimination was done from plasma using immunoadsorption (n = 14 patients; column with polyclonal anti-human apoB antibodies coupled to Sepharose, Therasorb, Unterschleissheim, Germany), HELP apheresis (n = 8; LDL precipitation by acetic acid buffer and heparin, Braun, Melsungen, Germany), dextran sulfate adsorption (n = 7; columns with cellulose-bound dextran sulfate, Kaneka, Osaka, Japan) and cascade-filtration (n = 3; LDL removal by a filter with a pore size of 15 nm DIAMED, Cologne, Germany (16)).

Anticoagulation was performed with heparin (1000–5500 IU as a bolus and up to 3000 IU/h continuously) and, in the case of plasma separation by centrifugation, additionally with citrate dextrose solution (15). The duration of LDL apheresis was standardized such that a postapheresis LDL concentration of 50–60 mg/dL was reached corresponding to a treatment time between 2.15–4.15 h and a mean plasma volume of 3185 mL (Table 1).

Five patients were studied at two occasions while treated with different apheresis systems (2 on immunoadsorption and dextran sulfate adsorption, 3 on HELP apheresis and dextran sulfate adsorption). Thus, 37 index aphereses could be analyzed.

**Preparative and analytical methods**

Fasting blood was taken in EDTA-containing tubes immediately before and after apheresis treatment. After centrifugation at 3000 rpm for 10 min, plasma was stored at 4°C. Lipid analyses and the determination of LDL subtypes were performed within 48 h.

Plasma lipids. Total plasma cholesterol and triglycerides were determined by enzymatic methods using an autoanalyzer (EPOS; Eppendorf, Hamburg, Germany). HDL cholesterol was measured after precipitation of apolipoprotein B (apoB)-containing particles by dextran sulfate and magnesium acetate. LDL-cholesterol was calculated by the formula of Friedewald, Levy, and Fredrickson (17) unless plasma triglycerides exceeded 400 mg/dL. Otherwise preparative ultracentrifugation was performed (18 h, d = 1.006 g/mL, 270,000 g, 4°C; Beckman Ti 50.4 rotor, Palo Alto, CA) to measure VLDL cholesterol and triglycerides in the supernatant and total cholesterol in the infranatant (containing HDL and LDL). After precipitation of apoB-containing lipoproteins by dextran sulfate and magnesium acetate, HDL-cholesterol was determined in the infranatant. LDL-cholesterol was calculated by subtraction of HDL-cholesterol from total cholesterol in the infranatant. ApoB, apoA-I, and Lp[a] were determined by nephelometry (Behring, Marburg, Germany) using antibodies against human apoB, apoA-I, and Lp[a] (anti-ApoB, anti-apolipoprotein A-I, anti-Lp[a] from rabbit, Dade Behring, Marburg, Germany).

LDL subfractionation. LDL subfractions were separated by isopycnic density gradient ultracentrifugation as described elsewhere (2) with some modifications. In brief, dry solid KBr was added to the plasma to increase its density to 1.21 g/mL. A discontinuous density gradient was constructed by 2 mL of a NaCl/KBr solution (d 1.26 g/mL), 3 mL plasma (d 1.21 g/mL), 2 mL of a NaCl/KBr solution (d 1.063 g/mL), 2.5 mL of another NaCl/KBr solution (d 1.019 g/mL), and 2 mL of a NaCl solution (d 1.006 g/mL). All solutions contained NaN3 (0.1%) and EDTA (0.04%). Densities were measured by a precision density meter (Anton Paar DMA 38, Graz, Austria). Ultracentrifugation was performed in a Beckmann SW 40 Ti rotor at 40,000 rpm for 48 h at 15°C. Fifteen fractions were collected successively by aspiration of 0.5 mL with an Eppendorf pipette beginning at the top of each gradient. Seven LDL subfractions were isolated corresponding to fractions 5–11. They refer to following density intervals: LDL-1, 1.020–1.024 g/mL; LDL-2, 1.025–1.029 g/mL; LDL-3, 1.030–1.034 g/mL; LDL-4, 1.035–1.040 g/mL; LDL-5, 1.041–1.047 g/mL; LDL-6, 1.048–1.057 g/mL; LDL-7, 1.058–1.066 g/mL.

In 6 patients with elevated Lp[a] levels (45–120 mg/dL), apoA-I and apoB concentrations were measured in fractions 5–13, and Lp[a] levels in fractions 5–11 (LDL subfractions 1–7). As LDL-7 contained considerable amounts of apoA-I and Lp[a], indicating contamination with HDL and Lp[a], this subfraction was excluded from further analysis. LDL subfractions 1–4 con-

**Table 1**. Plasma lipid concentrations before and after LDL apheresis, plasma volume treated and heparin dose applied during apheresis in 32 patients

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<tr>
<td><strong>LDL-cholesterol</strong></td>
<td>158 ± 40</td>
<td>61 ± 16</td>
<td>166 ± 34</td>
<td>62 ± 11</td>
<td>147 ± 61</td>
<td>65 ± 32</td>
<td>132 ± 25</td>
<td>51 ± 7</td>
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<td></td>
<td>46 ± 9</td>
<td>38 ± 7</td>
<td>48 ± 10</td>
<td>39 ± 8</td>
<td>40 ± 7</td>
<td>36 ± 5</td>
<td>44 ± 8</td>
<td>35 ± 8</td>
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<td><strong>Triglycerides</strong></td>
<td>166 ± 135</td>
<td>89 ± 88</td>
<td>117 ± 35</td>
<td>62 ± 29</td>
<td>366 ± 217</td>
<td>209 ± 153</td>
<td>139 ± 31</td>
<td>55 ± 3</td>
</tr>
<tr>
<td><strong>Lp[a]</strong></td>
<td>3185 ± 1010</td>
<td>3346 ± 920</td>
<td>3033 ± 1480</td>
<td>2527 ± 360</td>
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<td><strong>Heparin dose (I.U.)</strong></td>
<td>6187 ± 3480</td>
<td>6700 ± 3340</td>
<td>5267 ± 4000</td>
<td>4750 ± 3750</td>
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Values given as mean ± SD.

* Median (range).
tained no detectable Lp[a] and LDL-5 was nearly free of Lp[a] (Lp[a]-conc. <3 mg/dL), whereas LDL subfraction 6 showed some amount of Lp[a] depending on the total Lp[a] concentration. As LDL-6 showed Lp[a] concentrations up to 35 mg/dL in patients with preapheresis Lp[a] levels >60 mg/dL, all statistical analyses were repeated after exclusion of patients with high Lp[a]-levels (>60 mg/dL).

On the basis of 6 LDL subfractions, LDL-1 and 2 were defined as large, buoyant LDL (d 1.020–1.029 g/mL), LDL-3 and 4 as intermediate dense LDL (d 1.030–1.040 g/mL), and LDL-5 and 6 as small, dense LDL (d 1.041–1.057 g/mL). Density limits were determined by a standard curve derived from control gradients constructed with a NaCl/KBr solution (d 1.21 g/mL) instead of plasma and fractionated in 1-mL aliquots. Each run contained one control gradient. Intra-assay and inter-assay variability was <5%.

Statistical analysis. For statistical analyses, LDL subfractions were expressed in relative terms. Therefore, the cholesterol concentration of each LDL subfraction was divided by the total cholesterol concentration of all 6 subfractions. In each patient, corresponding LDL subfractions (before vs. after apheresis) were compared with non-parametric tests (Wilcoxon test). The mean relative reduction of total LDL-cholesterol achieved by the index apheresis was compared between the four different apheresis systems using the Mann-Whitney U test. Spearman-rho correlation coefficients (double-sided test for significance) were calculated to examine possible influences of the preapheresis triglyceride, small, dense LDL, and Lp[a] concentrations as well as the extent of triglyceride and Lp[a] reduction during apheresis on the degree of small, dense LDL reduction observed after apheresis.

Similarly, the effect of statin therapy (high dose vs. low dose vs. no statin therapy) was evaluated using the Kruskal-Wallis analysis and the Mann-Whitney U test. To evaluate the effect of heparin application on the change of the LDL subtypes during apheresis, we compared patients who were given only a bolus heparin application on the change of the LDL subtypes during apheresis and the Mann-Whitney U test. To evaluate the effect of heparin on the change of the LDL subtypes during apheresis, we compared patients who were given only a bolus versus those additionally receiving a continuous heparin infusion. We also tested whether the total heparin dose was correlated with the degree of small, dense LDL reduction with apheresis (Spearman-rho).

RESULTS

In the entire group (n = 32 patients, Table 1) the index apheresis reduced LDL-cholesterol from 158 ± 40 mg/dL to 61 ± 16 mg/dL (−61%), Lp[a] from 44 ± 35 mg/dL to 19 ± 15 mg/dL (−53%), triglycerides from 166 ± 135 mg/dL to 89 ± 88 mg/dL (−49%), and HDL-cholesterol from 46 ± 9 mg/dL to 38 ± 7 mg/dL (−16%). The relative reduction of LDL-cholesterol, Lp[a] and triglycerides did not differ among patients suffering from FH (−62%, −51%, −47%), CHLP (−55%, −52%, −46%), and Lp[a]-HLP (−61%, −63%, −59%, respectively, Kruskal-Wallis analysis, P > 0.12).

Distribution of LDL subfractions

Table 2 and Fig. 1 show the distribution of LDL subfractions 1–6 (d 1.020–1.057 g/mL) before and after apheresis in all patients (n = 32). After apheresis there was a relative increase of LDL subfractions 1, 2, and 3 (P < 0.01, P < 0.05, and P < 0.01, respectively) and a concomitant decrease of LDL subfractions 5 and 6 (P < 0.05). Exclusion of patients with isolated Lp[a]-HLP (n = 4) and of pa-
The preapheresis triglyceride concentration was positively correlated with the proportion of small, dense LDL (r = 0.43, P < 0.01). The extent of the small, dense LDL reduction during apheresis was positively correlated with the pretreatment amount of LDL-5, LDL-6, and small, dense LDL (r = 0.52, r = 0.44, r = 0.49; P < 0.01, P < 0.05, P < 0.01, respectively) and with the preapheresis triglyceride level (r = 0.37, P < 0.05). The extent of triglyceride reduction during apheresis did not correlate with the degree of small, dense reduction (P > 0.6).

The degree of small, dense LDL reduction did not correlate with the preapheresis Lp[a] concentration (P > 0.27) and the extent of Lp[a] elimination (P > 0.15) during apheresis in patients with Lp[a] concentrations ≤60 mg/dL (maximal Lp[a] conc. in LDL-6: 8 mg/dL).

The comparison of patients with versus without lipid-lowering medication as well as patients treated at high versus low statin doses did not show any difference in the proportion of small, dense LDL subtypes before apheresis (P > 0.7, P > 1.0, respectively) and in the extent of their reduction during apheresis (P > 0.3, P > 0.7, respectively, Mann-Whitney U test).

Although patients receiving heparin as a bolus and by continuous infusion (n = 21) received a higher total dose of heparin (8360 ± 2200 I.U., mean ± SD) compared to those anticoagulated only by a bolus (n = 11, 2350 ± 1258 I.U.), they did not differ in the relative decrease of triglycerides and the relative change of all LDL subtypes during apheresis (P > 0.28, Mann-Whitney U test). Furthermore, the total heparin dose was not correlated with the degree of small, dense LDL reduction during apheresis (P > 0.1).

Patients with Lp[a] levels above 60 mg/dL (n = 7) did not significantly affect the results found in the entire group.

**Influence of plasma lipids, medication, and anticoagulation on the distribution of LDL subfractions and its change with apheresis**

Patients with CHLP compared to patients with FH showed higher amounts of small, dense LDL (Table 2, P < 0.01) and higher triglycerides (Table 1, P < 0.01, Mann-Whitney U test), whereas a higher proportion of larger LDLs was found in patients with FH (Mann-Whitney U test, P < 0.01).

In patients with heterozygous FH (n = 22, Fig. 1) LDL subfraction 1 increased after apheresis (P < 0.01) and LDL subfractions 5 and 6 decreased (LDL-5, P = 0.16; LDL-6, P = 0.18). After exclusion of 6 patients with elevated Lp[a] levels (> 60 mg/dL), the reduction of LDL subfractions 5 and 6 was significant (LDL-5, P = 0.02; LDL-6, P = 0.03). In patients with CHLP (n = 6), LDL subfractions 1, 2, and 3 increased (P < 0.05), whereas LDL-6 decreased after apheresis (P < 0.05). The number of patients with isolated Lp[a]-HLP (n = 4) was too small for statistical analysis. The mean values are listed in Table 2. In this group there was also a trend toward an increase in LDL subfractions 1 and 2 and a decrease in LDL subfractions 5 and 6.

**Distribution of LDL subtypes in patients with different hyperlipoproteinemias (Table 2, Fig. 1)**

Patients with CHLP compared to patients with FH showed hypercholesterolemia, independent of the apheresis system used (P > 0.5, Mann-Whitney U test). Table 3 shows the distribution of LDL subfractions before and after apheresis in patients treated by different apheresis systems. Independent of the apheresis system used, there was a relative increase in large, buoyant LDL and a relative decrease in small, dense LDL with apheresis. However, only in patients with immunoadsorption or dextran sulfate adsorption were these changes statistically significant (Table 3). When all aphereses (n = 37) were analyzed simultaneously, a highly significant increase of large, buoyant LDL (LDL-1, 2), LDL-3, and a highly significant decrease of small, dense LDL could be observed (Table 3).
TABLE 3. Relative amount of LDL subfractions (%) before and after apheresis in patients treated by immunoadsorption (Immuno), HELP-apheresis (HELP), dextran sulfate adsorption (Dextran), and cascade-filtration (Cascade).

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<tr>
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<th>LDL-1</th>
<th>LDL-2</th>
<th>LDL-3</th>
<th>LDL-4</th>
<th>LDL-5</th>
<th>LDL-6</th>
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<td>n</td>
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<tr>
<td>Immuno</td>
<td>14</td>
<td>4.0 ± 2.3</td>
<td>5.1 ± 3.3</td>
<td>8.1 ± 1.6</td>
<td>9.2 ± 2.6</td>
<td>24.5 ± 6.5</td>
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<tr>
<td>Dextran</td>
<td>10</td>
<td>5.1 ± 2.5</td>
<td>5.6 ± 2.6</td>
<td>8.8 ± 1.8</td>
<td>9.4 ± 1.5</td>
<td>21.5 ± 6.6</td>
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<tr>
<td>HELP</td>
<td>10</td>
<td>4.6 ± 1.4</td>
<td>5.1 ± 1.4</td>
<td>9.3 ± 3.7</td>
<td>9.5 ± 1.9</td>
<td>22.5 ± 8.7</td>
</tr>
<tr>
<td>Cascade</td>
<td>3</td>
<td>3.5 ± 2.2</td>
<td>4.4 ± 2.0</td>
<td>7.5 ± 1.5</td>
<td>9.1 ± 0.8</td>
<td>22.7 ± 9.7</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>4.4 ± 2.1</td>
<td>5.1 ± 2.6</td>
<td>8.6 ± 2.4</td>
<td>9.3 ± 2.0</td>
<td>22.9 ± 7.7</td>
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</table>

Values given as mean ± SD; density: LDL-1, 1.020–1.024 g/mL; LDL-2, 1.025–1.029 g/mL; LDL-3, 1.030–1.034 g/mL; LDL-4, 1.035–1.040 g/mL; LDL-5, 1.041–1.047 g/mL; LDL-6, 1.048–1.057 g/mL.

a P < 0.01.

b P < 0.05.
during apheresis and the degree of small, dense LDL reduction. Thus, heparin-induced activation of lipoprotein lipase is unlikely to account for our observation.

In a subgroup analysis we found that the shift in the LDL subtype distribution during apheresis was much more pronounced in patients with CHLP compared to patients with FH. This observation may be explained by the different characteristics of hyperlipidemia, as FH and CHLP both represent genetically determined metabolic disorders that differ in the amount of small, dense LDL subtypes. CHLP is a disorder known to be associated with hypertriglyceridemia and a high proportion of small, dense LDL (25), whereas FH is characterized by normal triglyceride concentrations and a normal LDL subtype distribution (25, 26). As expected, we found elevated triglyceride levels (Table 1) and higher amounts of small, dense LDL (Table 2) in patients with CHLP compared to patients with FH. Furthermore, baseline TG levels correlated positively with the proportion of small, dense LDL in the entire group.

Changes in triglyceride concentration induced by drugs or life style modification are related to changes in the distribution of LDL subtypes (27). We therefore tested whether the different degree of small, dense LDL reduction between patients with FH and CHLP may be related to differences in triglyceride reduction achieved with apheresis. However, no difference in the relative triglyceride reduction between both groups was observed. Moreover, in the entire group there was no correlation between the extent of triglyceride and small, dense LDL reduction.

As the Lp[a] concentration was slightly different between both groups of patients (median of 30 mg/dL in the FH group versus median of 21 mg/dL in the CHLP group) an unequal contamination of small, dense LDL subfractions might have contributed to the observed phenomenon. However, the findings were not substantially changed after exclusion of patients with high Lp[a] concentrations. In the group of patients with an Lp[a] concentration ≤60 mg/dL, LDL subclass-6 showed only minimal amounts of Lp[a] which are very unlikely to influence the amount of cholesterol determined in this subclass. Therefore, this factor cannot explain the observed difference in the extent of small, dense reduction between patients with FH and CHLP.

One could also hypothesize that small, dense LDL in FH and CHLP are derived from different metabolic sources. Generally speaking, small, dense LDL could be derived directly from IDL or from larger, more buoyant LDL. It is possible that in different forms of hyperlipoproteinemia the contribution of each of these pathways is different. As the rebound of small, dense LDL will depend on its metabolic source, rebound kinetics may differ in different forms of hyperlipoproteinemia. However, this hypothesis can only be tested with kinetic studies.

In summary, LDL apheresis induces a relative decrease in small, dense LDL and a relative increase in large, buoyant LDL and thus, not only decreases LDL mass, but also improves LDL density profile. However, this effect was found to be much more prominent in patients with CHLP compared to those with FH, indicating that small, dense LDL may be metabolically different in these diseases.

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