Effect of Low-Density Lipoprotein Apheresis on Circulating Endothelial Progenitor Cells in Familial Hypercholesterolemia

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Key Words
Endothelial progenitor cells • Apheresis, LDL • Low-density lipoprotein • Familial hypercholesterolemia

Abstract
Background: Long-term treatment with low-density lipoprotein (LDL) apheresis (LA) has been shown to reduce the incidence of cardiovascular events in patients affected by familial hypercholesterolemia (FH). Data from experimental studies suggest that circulating endothelial progenitor cells (EPCs) can repair the vascular lesions caused by atherosclerosis. Since a reduction of these cells has been demonstrated to predict atherosclerosis progression, the aim of this study was to verify whether LA can increase the percentage of EPCs.

Methods: In 15 patients affected by FH periodically treated with LA, the percentage of EPCs was determined before and after performing LA, and compared with the values of 15 control subjects and 15 hypercholesterolemic patients treated with statins.

Results: Significant differences were found in FH patients between the pre-apheresis percentages of CD34+/KDR+, defined as EPCs by a wide consensus of opinion, and the values found 24 h after the procedures (0.00868 ± 0.003 vs. 0.01009 ± 0.002%, p < 0.005). Instead, the percentages of CD34+/KDR+/CD133+, considered as an immature subset of EPCs, remained substantially unchanged. However, a significant reduction in the percentage of EPCs was observed in both patient groups as compared to the controls, at all the assessment times.

Conclusion: In the short-term LA seems to stimulate mobilization of CD34+/KDR+ cells. Hypercholesterolemic patients show a lower percentage of EPCs than controls. There were no differences in the EPCs percentages between the 2 patients groups, despite the fact that LDL cholesterol levels were higher in the group undergoing LA.

Introduction
The endothelium plays a fundamental role in vascular homeostasis. Its functions can undergo alterations as a result of assaults of the endothelial cells by traditional and newly identified cardiovascular risk factors that are
the prime movers of atherosclerotic lesions and the basic cause of vascular occlusion and organ damage [1].

Because resident endothelial cells are not always able to proliferate and repair the damage [2], recent research in the cardiovascular field has been focused on endothelial progenitor cells (EPCs) derived from bone marrow that have been shown to be able to differentiate into endothelial cells and are therefore ideal candidates for vascular regeneration tasks [3, 4].

Many experimental studies have confirmed the beneficial effect of EPCs in the repair of vascular lesions and in slowing the progression of atherosclerosis [5–7]: they circulate in the peripheral blood, homing in on damaged sites and exerting a neoangiogenetic action [8].

In humans, the role of EPCs is less clear. It has been seen that the accumulation of cardiovascular risk factors, or an overall condition of increased risk, are associated with a reduced number of EPCs [9–11]. Moreover, in clinical conditions known to be associated with an increased cardiovascular risk such as diabetes mellitus, the number and/or function of the EPCs is diminished [12].

Cholesterol has long been recognized as an important cardiovascular risk factor [13]. Many studies have confirmed this assumption, revealing an increased incidence of cardiovascular events in patients affected by familial hypercholesterolemia (FH) [14, 15]. In these subjects, when medical therapy is unable to maintain low-density lipoprotein (LDL) cholesterol within the limits indicated in the guidelines, aggressive therapy with LDL apheresis (LA) procedures is recommended to remove the harmful cholesterol from the bloodstream. LA has been shown not only to achieve a rapid, marked reduction in the cholesterol values but also to reduce the long-term incidence of cardiovascular events [16–18]. It has still to be seen how much this depends on the reduction of harmful cholesterol alone, and how much on the influence of pleiotropic effects: we have already attempted to verify this question in a disturbance of the ocular microcirculation [19].

It has already been reported in the literature that some therapeutic procedures can increase the number of EPCs. Therapy with statins [20], erythropoietin [21], estrogens [22], antagonists of the angiotensin II receptor [23], as well as constant, moderate physical activity [24] have been demonstrated to increase circulating EPCs. It can thus be presumed that the beneficial effect of these treatments in the prevention of cardiovascular attacks is exerted partly by means of stimulating the mobilization of the EPCs.

Starting from these considerations, in the present study we aimed to verify whether treatment with LA, that has also been shown to be able to reduce the incidence of cardiovascular events, could have an influence on the number of circulating EPCs.

**Materials and Methods**

**Study Population**

The study included 15 patients (49 ± 8 years, 9 females and 6 males) affected by heterozygous FH who underwent chronic LA treatment twice weekly for 6 ± 2 years (group 1). All subjects were taking statins (atorvastatin 9/15, simvastatin 4/15, rosuvastatin 2/15).

Two other age- and gender-matched groups of 15 subjects were enrolled: patients with hypercholesterolemia who had been taking statins for more than 6 months (simvastatin 7/15, atorvastatin 8/15; group 2), and healthy subjects with no traditional risk factors for cardiovascular disease (smoking, arterial hypertension, diabetes mellitus, hyperlipidemia, obesity), nor taking any therapy (group 3). Informed consent to take part was obtained from all patients and controls, and the study protocol was approved by the Ethics Committee of the University of Bari.

Blood samples were taken to determine the EPC values at the following time intervals: time 0 = immediately before the apheresis session for group 1 or at baseline for groups 2 and 3; time 1 = 10 min after apheresis for group 1, and time 2 = 24 h after apheresis for group 1 or 24 h after baseline for the other 2 groups. In addition, hematocrit determinations were made to measure the hemodilution induced by the procedure, as well as the plasma concentrations of the molecules targeted by the procedure (total cholesterol and fractions, triglycerides, fibrinogen).

**Apheresis Procedure**

The Heparin-induced Extracorporeal Low-density lipoprotein Precipitation (HELP) system (Braun, Melsungen, Germany) was employed. The technique operates by increasing the positive charges on LDL and Lp(a) particles at low pH (5.12), allowing them to form a network with heparin and fibrinogen in the absence of divalent cations [25]. In the first step, plasma is obtained by filtration of whole blood through a plasma separator. This is then mixed continuously with an acetate buffer at pH 4.85 containing 100 IU heparin/ml. Sudden precipitation occurs at pH 5.12 and the suspension is circulated through a polycarbonate filter to remove the precipitated LDL, Lp(a) and fibrinogen. Excess heparin is completely adsorbed by passage through an anion-exchange column which binds only heparin at the given pH. Finally, the plasma buffer mixture is subjected to bicarbonate dialysis and ultrafiltration to remove excess fluid and to restore the physiological pH, before mixing the plasma with the blood cells and returning the final volume to the patient.

The procedure was performed at the Nephrology Division of Bari University Hospital using a Plasmal Futura device. We treated a volume of 3,000 ml plasma/session, corresponding to a mean of 1.2 ± 0.4 plasma volume/patient, with a blood flow of 80–100 ml/min, obtained using (17-gauge) needles inserted in the peripheral veins of the right and left forearms, and a plasma flow of...
25–30 ml/min. The mean duration of the procedure was 2 h ± 27 min. Arterial pressure and heart rate were measured every 30 min. The blood samples collected before the procedure were taken through the arterial line access needle before connecting up the patient. The post-apheresis values were obtained 10 min after the end of the session, through the same needle left in situ, after aspirating 20 ml of blood which was re-injected into the patient after setting aside the blood sample.

Flow Cytometry
The EPC number was analyzed by flow cytometry analysis of peripheral blood CD34+/VEGFR2+/CD133+ cells. The blood (10 ml) was collected in EDTA-coated Vacutainer tubes. The first 3 ml of blood were discarded to avoid contamination with circulating endothelial cells due to vascular trauma. Samples were kept on ice and processed within 2 h after blood collection. The monoclonal antibodies: anti-CD133 PE-conjugated (Miltenyi Biotec, Teterow, Germany), anti-VEGFR2/KDR APC-conjugated (R&D Systems, Minneapolis, Minn., USA) and anti-CD34 FITC-conjugated (Becton Dickinson, Mountain View, Calif., USA), were added to aliquoted blood. Negative controls were FITC, PE and APC-conjugated murine monoclonal antibodies unrelated to human leukocyte antigens. Viability was assessed by propidium iodide exclusion. Cytometric analysis was made using a Becton-Dickinson FACSComp TM II flow cytometer, equipped with BD FACSDiva Software, and adopting a multiparametric method. Before analysis the flow cytometer was cleaned to re-establish the lymphocyte gate.

Laboratory Parameters
Serum concentrations of total, LDL, HDL cholesterol and triglycerides were assayed by standard routine methods on the Automatic Analyzer. Fibrinogen was determined with the Biopool Fibrinogen Assay Kit (Trinity Biotech plc, Bray, Ireland), according to Clauss.

Statistical Analysis
Results are presented as mean ± SEM for the EPCs and as mean ± SD for the other parameters. Continuous variables were tested for normal distribution by the Kolmogorov-Smirnov test. Univariate comparisons of continuous variables within groups were performed by paired t test or nonparametric Wilcoxon rank sum test in cases of non-normally distributed variables.

ANOVA was used for multiple comparisons between groups. For post hoc analysis the Bonferroni correction was applied. Statistical significance was assumed when a null hypothesis could be rejected at p < 0.05.

Procedure-induced hemodilution was calculated according to the following equation: Fdil = (1 – Htc before)/(1 – Htc after). Post-LA values were corrected for hemodilution: Xcorrected = Xmeasured / Fdil. Statistical analyses were performed with the use of SPSS software, version 11.5, for Windows.

Table 1. Characteristics of the patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>49 ± 8</td>
<td>49 ± 11</td>
<td>47 ± 10</td>
</tr>
<tr>
<td>Sex, F/M</td>
<td>9/6</td>
<td>8/7</td>
<td>8/7</td>
</tr>
<tr>
<td>BMI</td>
<td>23 ± 4.4</td>
<td>27 ± 0.9</td>
<td>23 ± 4.5</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>254 ± 66</td>
<td>206 ± 23</td>
<td>174 ± 17</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>190 ± 56</td>
<td>133 ± 21</td>
<td>104 ± 14</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>45 ± 4</td>
<td>51 ± 13</td>
<td>53 ± 6</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>115 ± 43</td>
<td>112 ± 30</td>
<td>89 ± 51</td>
</tr>
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</table>

Group 1 = Hypercholesterolemia and LDL apheresis; group 2 = hypercholesterolemia with statins; group 3 = controls.

Results
The patient and control group characteristics are shown in table 1. The patients in group 2 had a significantly higher BMI than the patients undergoing LA and the controls (p < 0.01). The patient groups 1 and 2 had significantly higher levels of total and LDL cholesterol than the control group; in addition, LDL cholesterol was significantly higher in patient group 1 also undergoing LA than in group 2 taking only statins (table 1).

Figures 1 and 2 show the mean percentages of CD34+/KDR+ cells and CD34+/KDR+/CD133+ cells. In group 1, a statistically significant increase (p < 0.005) was found in the percentage of CD34+/KDR+ cells assessed 24 h after the apheresis procedure as compared to the pre-apheresis values (fig. 1). Instead, no significant differences were found in the percentages of CD34+/KDR+/CD133+ cells between the pre- and post-apheresis values, nor among these and the values found 24 h after the procedures (fig. 2).

CD34+/KDR+ cells were found to be statistically significantly lower in groups 1 and 2, both at time 0 and time 2, than in group 3 (p < 0.001; fig. 1). Similarly, CD34+/KDR+/CD133+ cells were significantly reduced in both groups as compared to group 3, both at time 0 and time 2 (fig. 2).

The mean pre- and post-apheresis values of lipids, fibrinogen and hematocrit are shown in table 2, with the relative percentages of reduction. The post-apheresis values were normalized for the degree of LA-induced hemodilution. A statistically significant decrease in all the parameters was observed.
Aim of this work was to investigate whether LA is able to increase the percentage of circulating EPCs. As reported by Asahara et al. [6], the so-called endothelial progenitor cells are characterized by the surface markers CD34 and vascular endothelial growth factor receptor 2 or the kinase domain receptor (KDR).

In our study, this cell type showed a percentage increase 24 h after the end of the apheresis session (fig. 1).

**Fig. 1.** CD34+/KDR+ percentages. Group 1 = Hypercholesterolemia and LDL apheresis; group 2 = hypercholesterolemia with statins; group 3 = controls. Time 0 = Baseline or before apheresis; time 1 = 10 min after apheresis; time 2 = 24 h after baseline or apheresis. \( ^{a} p < 0.001 \) vs. group 3, time 0; \( ^{b} p < 0.001 \) vs. group 3, time 2; \( ^{c} p < 0.001 \) vs. group 3, time 0; \( ^{d} p < 0.001 \) vs. group 3, time 2; \( ^{*} p < 0.005 \) in group 1, time 2 vs. time 0.

**Fig. 2.** CD34+/KDR+/CD133+ percentages. Group 1 = Hypercholesterolemia and LDL apheresis; group 2 = hypercholesterolemia with statins; group 3 = controls. Time 0 = Baseline or before apheresis; time 1 = 10 min after apheresis; time 2 = 24 h after baseline or apheresis. \( ^{a} p < 0.05 \) vs. group 3, time 0; \( ^{b} p < 0.01 \) vs. group 3, time 2; \( ^{c} p < 0.01 \) vs. group 3, time 0; \( ^{d} p < 0.01 \) vs. group 3, time 2.
Hematocrit, % 35.8
Fibrinogen, mg/dl 282
Triglycerides, mg/dl 115
LDL cholesterol, mg/dl 190
HDL cholesterol, mg/dl 45
Total cholesterol, mg/dl 254

Table 2. Lipids, fibrinogen and hematocrit before and after LDL apheresis (group 1)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before apheresis</th>
<th>After apheresis</th>
<th>Reduction, % p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>254 ± 66</td>
<td>133 ± 19</td>
<td>46, 0.007</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>45 ± 4</td>
<td>36 ± 4</td>
<td>24, 0.002</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>190 ± 56</td>
<td>78 ± 21</td>
<td>58, 0.003</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>115 ± 43</td>
<td>70 ± 39</td>
<td>40, 0.03</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>282 ± 13</td>
<td>137 ± 50</td>
<td>50, 0.003</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>35.8 ± 3</td>
<td>35.4 ± 3</td>
<td>1, NS</td>
</tr>
</tbody>
</table>

However, despite this increase the patients in group 1 did not reach comparable percentages of CD34+/KDR+ cells to those in the control group (group 3, time 2), and these values remained statistically lower than in the controls (fig. 1). Moreover, in both groups 1 and 2, the values of CD34+/KDR+ cells were significantly lower than in the controls (fig. 1).

In addition to CD34+KDR+ endothelial progenitor cells, we measured CD133+ cells, which are considered as a subset of immature EPCs [26]. For this cell type, that is characterized by triple staining (CD34+/KDR+/CD133+) and is nowadays considered as an early EPC or a cell that has just migrated from the bone marrow into the systemic circulation [27], we found no post-apheresis increase as compared to the pre-apheresis percentages (fig. 2). Nor were any percentage differences in these cells found between the 2 groups of hypercholesterolemic patients at the various times of observation. Instead, the percentages of this type of EPC were again significantly reduced in the 2 patient groups as compared to the controls, at the different observation times (fig. 2).

It seems, therefore, that although LA can cause an increase in CD34+/KDR+ cells, this increase does not persist up to the following session, as demonstrated by the lower pre-apheresis values. Instead, no difference in the percentages of CD34+/KDR+/CD133+ cells seems to be brought about by LA.

The increase in CD34+/KDR+ cells observed 24 h after apheresis is difficult to interpret. It is possible that it could be the result of an inflammatory reaction occurring during the course of LA, due to contact with the blood line materials and/or filters, even if the plasma levels of C-reactive protein were not increased after the procedure (data not shown). This same phenomenon was observed after hemodialytic sessions, when the number of EPCs [28] was shown to be increased. Another hypothesis could be a stimulatory effect induced by the LA treatment on the mobilization of these cells from the bone marrow. In fact, physiological stress secondary to tissue injury results in the release of angiogenic factors, including vascular endothelial growth factor, which promote the mobilization of stem cells into the circulation [29, 30]. If we accept this hypothesis, allied with the capacity of LA to recruit EPCs, this could justify the improved cardiovascular prognosis observed in patients affected by FH in long-term LA treatment. To confirm this hypothesis further studies need to be performed, concentrating on long-term rather than short-term changes in EPC values, and trying to link EPC changes to the progression/regression of the atherosclerosis process.

Moreover, hypercholesterolemia, a known cardiovascular risk factor, has been shown to reduce the number and function of EPCs [11, 31]. It has been reported that the level of circulating CD34+/KDR+ cells is predictive of the occurrence of cardiovascular events and death from cardiovascular causes, being lower in patients with a higher incidence of cardiovascular events [32, 33]. Our study confirms that patients suffering from hypercholesterolemia have a reduced percentage of EPCs. Even if some studies have emphasized the ability of statins to increase the number of circulating CD34+/KDR+ cells [19, 34], a recent report [35] described a reduction in the number of circulating EPCs associated with prolonged treatment with statins (>8 weeks). This finding was statistically significant at high drug dosages. Our patients with hypercholesterolemia, both group 1 and 2, had been in treatment with high-dose statins (simvastatin 40 mg/day, atorvastatin 40 mg/day, rosuvastatin 20 mg/day) for long periods (>1 year). Thus, in chronic treatment with statins, hypercholesterolemic patients likely have a lower percentage of EPCs than normal subjects. Despite the selectiveness of LA in removing ApoB, we know that a small percentage of HDL cholesterol is acutely reduced [36]. In fact, in our study too, the mean values of HDL cholesterol in group 1 were 45 ± 4 mg/dl before apheresis and 36 ± 4 mg/dl immediately after apheresis: this difference, although minimal, was statistically significant (p = 0.0002; table 2).

We know that multiple prospective studies support an inverse correlation between plasma levels of HDL cholesterol and coronary artery disease [37, 38]. In any case, we have to bear in mind that inflammation induces major changes in the levels and composition of HDL cholesterol. Navab et al. [39] suggested that the pro-inflammatory/anti-inflammatory properties of HDL cholesterol...
may be indirectly measured through its inability/ability to inhibit LDL-induced monocyte chemotactic activity. A recent study analyzing the plasma inflammatory HDL cholesterol levels in 13 FH patients before and after chronic LA therapy showed that 37% of the total reduction of HDL cholesterol (16%) was of the pro-inflammatory type [40]. Thus, the mild reduction in HDL cholesterol induced by LA, that in any case largely affects the pro-inflammatory type, does not seem to have harmful effects.

In conclusion, our study demonstrates that in patients affected by FH, LA may induce an increase in circulating CD34+/KDR+ cells 24 h after the procedure, but seems to have no effect on CD34+/KDR+/CD133+ cells. The results also show a lower percentage of circulating EPCs in hypercholesterolemic patients as compared to controls.

These results pave the way for studies aimed at evaluating whether the benefits of long-term therapy with LA in terms of cardiovascular prevention could be linked to long-term changes in the EPC values.

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References


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