Heparin-induced lipoprotein precipitation apheresis in dyslipidemic patients: A multiparametric assessment

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Abstract
Low-density lipoprotein (LDL) apheresis (LA) selectively eliminates lipoproteins containing apolipoprotein B 100 (ApoB100) on patients affected by severe dyslipidemia. In addition to lowering lipids, LA is thought to exert pleiotropic effects altering a number of other compounds associated with atherosclerosis, such as pro- and anti-inflammatory cytokines or pro-thrombotic factors. More knowledge needs to be gathered on the effects of LA, and particularly on its ability to modify blood components other than lipids. We performed a multiparametric assessment of the inflammatory, metabolic and proteomic profile changes after Heparin-induced lipoprotein precipitation (H.E.L.P.) apheresis on serum samples from nine dyslipidemic patients evaluating cholesterol and lipoproteins, plasma viscosity and density, metabolites, cytokines, PCSK9 levels and other proteins selectively removed after the treatment. Our results show that H.E.L.P. apheresis is effective in lowering lipoprotein and PCSK9 levels. Although not significantly, complement and inflammation-related proteins are also affected, indicating a possible transient epiphenomenon induced by the extracorporeal procedure.

KEYWORDS
cytokines, metabolomics, PCSK-9, proteomics, proton nuclear magnetic resonance

1 | INTRODUCTION

Disorders of lipoprotein metabolism (dyslipidemias) are a major risk factor for atherosclerotic cardiovascular disease (ACVD), and may involve a combination of: increased total cholesterol, low-density lipoprotein cholesterol (LDL-C), lipoprotein (a) (Lp(a)) and triglyceride levels; or decreased high-density lipoprotein cholesterol (HDLC). Some dyslipidemic patients are affected by familial hypercholesterolemia (FH), an autosomal dominant inherited disease leading to high levels of LDL-C and increased risk of premature ACVD. Pharmacological and lifestyle interventions are the first choices to improve lipid profiles in these subjects. In most cases, LDL-C can be reduced by means of HMG-CoA reductase inhibitors (statins). However, for some patients, the medication is not effective or the patients do not tolerate the side effects. Recent data on modern lipid-lowering agents, such as proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, seem to be effective in reducing LDL in patients non responder to statins.
Nevertheless, for some dyslipidemic patients LDL apheresis (LA) is still mandatory.\(^8\)

LA systems are extracorporeal treatments that mainly exploit the use of a column that selectively removes apolipoprotein B 100 (ApoB100)-containing lipoproteins from blood or from plasma.\(^9\) Among these, heparin-induced lipoprotein precipitation apheresis (H.E.L.P.) has been established as a valuable treatment option not only for the treatment of FH, but also for mixed dyslipidemias and for patients with high Lp(a).\(^9\) H.E.L.P. apheresis works on the principle of specific precipitation of LDL, Lp(a), and fibrinogen at an acidic pH in the presence of heparin.\(^7,10\) In addition to lowering lipids, H.E.L.P. apheresis lowers plasma viscosity due to the elimination of serum cholesterol and proteins.\(^8\)

Many studies have investigated so-called “pleiotropic effects” of H.E.L.P. and other LA systems: these non-selective effects are mainly associated to changes in pro- and anti-inflammatory factors and potentially contribute to the beneficial effects of this treatment in advanced atherosclerosis.\(^10\) It is hitherto unclear whether the effect of LA on circulating inflammatory markers is related to the direct removal of inflammatory substances or to altered cytokine expression.\(^11-14\)

LA treatment seems to affect also plasma concentration of PCSK9, which might be of additional benefit for patients.\(^15\)

Aim of our study was to evaluate the ability of H.E.L.P. apheresis to modify lipids and other blood components (i.e., proteins and inflammatory factors, or metabolites) on the dyslipidemic patients currently undergoing LA treatment at the Azienda USL-IRCCS of Reggio Emilia: results will contribute to a better understanding of the pleiotropic effect of LA for each single patient, which is relevant in the frame of the strong effort to potenitate a personalized medicine approach.

2  | MATERIALS AND METHODS

The study was approved by the Ethics Committee of the province of Reggio Emilia (Italy) in June, 13th 2016 (protocol number 2016/0014691). All patients included in this study were eligible for the treatment with H.E.L.P. apheresis (B. Braun, Melsungen AG) according to Italian regulations, and gave their written informed consent according to the Declaration of Helsinki.

2.1  | Patient population

We recruited nine dyslipidemic patients that regularly undergo H.E.L.P. apheresis as part of their routine care. Six of them presented a Dutch Lipid Clinic Network Score > 6, indicating probable/definite Familial Hypercholesterolemia. Two patients undergo apheresis due to high Lp(a), and one due to statin intolerance.

We collected the familial and medical history, the cardiovascular adverse events, the current therapies and, when available, the molecular diagnosis of the disease from the dyslipidemic patients participating to the study. Patients’ characteristics are shown in Table 1.

2.2  | Blood samples collection

Blood samples (15-20 mL) were obtained before and after (15 minutes) three consecutive H.E.L.P. apheresis sessions. 5 mL were collected in tubes containing anticoagulant for routine complete blood count and lipid profile. A 2 mL blood aliquot was centrifuged at 2500 g for 10 minutes at room temperature (RT) to obtain plasma for density and viscosity assays. The remaining 7-8 mL of blood were collected in separgel-containing tubes, allowed to coagulate at RT for 1 hour and then centrifuged at 2000 g for 10 minutes at 4°C. Serum samples were recovered and stored at −80°C for cytokine and PCSK9 quantification, proteomic, and metabolomic analyses.

2.3  | Lipid profile

Routine analyses, performed at the clinical laboratory of Azienda USL-IRCCS of Reggio Emilia, comprised complete blood count, glycemia, fibrinogen, total cholesterol (CHOL), HDL, LDL, triglycerides, ApoA, ApoB, and Lp(a).

2.4  | Plasma density and viscosity

Plasma density, dynamics and kinematic viscosity were assessed on 2 mL aliquots collected immediately before and after the apheresis procedure, using a Lovis/DMA viscosimeter (Anton Paar, GmbH).

| TABLE 1 | Characteristics of the nine patients under study |
|----------|-----------------------------------------------|
| Age (y ± SD) | 62.1 ± 9.8 |
| Gender (males/females) | 6/3 |
| BMI ± SD | 25.5 ± 2.9 |
| Weight ± SD | 77.7 ± 17.7 |
| Height ± SD | 171.8 ± 9.9 |
| Familiarity (yes/no) | 5/4 |
| Coronary heart disease | 7/9 |
| Smoker (yes/no) | 3/6 |
| Lipid lowering drugs (yes/no) | 6/3 |
| Days between LA treatment ± SD | 14.2 ± 5.5 |

Abbreviation: y, years.
2.5 | Cytokines by magnetic-immunosorbent assay—Luminex assay

Serum cytokines were assessed using a customized human magnetic Luminex multiplex assay (R&D Systems) containing the following six analytes: IL-1α, IL-1 receptor antagonist (IL-1RA), IL-6, IL-10, TNF-α, and vascular endothelial growth factor (VEGF). Analyses were carried out in triplicate.

2.6 | Quantitative assessment of PCSK9

PCSK9 expression was evaluated by means of ELISA assay (R&D Systems) according to manufacturer instructions. Calibration curve and sample absorbance was measured with a GloMAX plate reader (Promega).

2.7 | Two-dimensional gel electrophoresis (2-DE)

The analysis was performed on serum aliquots previously treated with Protease Inhibitor Cocktail (Sigma-Aldrich) to avoid protein alterations, and stored at −80°C. First, the two most abundant serum proteins were removed by the ProteoPrep Immunoaffinity Albumin and IgG depletion kit (Sigma-Aldrich). Total protein content was then measured spectrophotometrically at λ 595 nm, using bovine serum albumin (BSA, Sigma Aldrich) as calibration standard. Afterwards, the 2-DE analysis was performed as previously published.16 Briefly, the first-dimension separation was carried out with IPG strip (Bio-Rad), pH range 3-10, length 17 cm, loading 80 μg of proteins premixed with the rehydration buffer. In second-dimension separation, 8%-16% polyacrylamide gradient gels were used to separate protein spots, that were visualized by a silver-nitrate staining protocol, as previously described.17

All gel images were acquired by a calibrated densitometer (model GS-800, Bio-Rad) and analyzed by the PD Quest 2-D analysis software (version 7.3.1, Bio-Rad), to detect protein spots modifications. The spots of interest were cut from the gel and the included proteins were subjected to a process of digestion by trypsin16 prior to identification by mass spectrometry (MS).

2.8 | Mass spectrometry protein identification

Peptide mixtures obtained after trypsin digestion were dissolved in a solution composed of water/acetonitrile/formic acid (95:3:2, vol/vol/vol) and analyzed by an Electrospray Ionization-Quadrupole-Time of Flight Liquid Chromatography/MS (ESI-Q-ToF LC/MS, Model 6520 Agilent Technologies Inc., California) coupled with a Nano HPLC-Chip (Model 1200 microfluidic device, Agilent Technologies Inc., California). Samples with a volume of 4 μL were injected into the system and analyzed as previously fully described.16 MS data were generated and processed by the MassHunter Qualitative Analysis software (version B.05.00), while Mascot MS/MS ion search program (www.matrixscience.com) was employed for peptide sequence searching within the UniProt database, with the following restrictions: Human species (Homo sapiens taxonomy), peptide tolerance ±20 ppm, MS/MS error tolerance ±0.1 Da, one allowed missed tryptic cleavage, cysteine alkylation and methionine oxidation (as fixed and variable modifications, respectively). Proteins were identified with a minimum of two significant peptide sequences, considering the highest score hits among Mascot search results.

2.9 | Metabolomic profile using proton nuclear magnetic resonance

Serum samples were filtered using a cutoff 3 kDa membrane (Merck Millipore) to eliminate proteins and lipids, which may interfere with the quality of the 1H-NMR spectrum. 570 μL of each protein-depleted sample was mixed with 30 μL of 1% trimethylsilpropanoic acid (TSP, internal reference standard) in D2O. pH was adjusted to 7.4 by addition of 10 μL of phosphate buffer 1 M.18 Proton nuclear magnetic resonance (1H-NMR) spectra were acquired on a JEOL 600 MHz ECZ600R spectrometer using the following parameters: 15 000 Hz spectral width, 128 scans, 64 k points, 5 seconds relaxation delay, at 25°C using the first increment of Noesy pulse sequence. Metabolites identification and quantification were carried out using Chenomx NMR suite 8.3 software (Chenomx Inc., Edmonton, Alberta, Canada).

2.10 | Statistics

Numerical data are presented with mean ± SD or SE of the mean (SEM) values. Markers levels before and after LA treatment were compared by unpaired t tests. All tests were two-tailed, and results with a P < 0.05 were considered statistically significant. Analyses were carried out using GraphPad Prism6 (GraphPad Software, San Diego, California).

3 | RESULTS

In this study, we evaluated the effect of H.E.L.P. apheresis on blood samples from the nine dyslipidemic patients currently undergoing LA treatment within our hospital. The
results of laboratory analyses, performed on patients’ blood before and after H.E.L.P. apheresis sessions, are shown in Table 2 as mean of the three consecutive procedures.

Plasma density, dynamics and kinematic viscosity were assessed immediately before and after each procedure as described in Section 2, and results are reported in Figure 1. We found a reduction of all the analyzed parameters, although not statistically significant ($P < .05$). Delta percentage ($\Delta\%$) was 10% for blood viscosity and 2% for density.

### TABLE 2  Lipoprotein reduction after heparin-induced lipoprotein precipitation apheresis

|                  | Pre apheresis Mean ± SD | Post apheresis Mean ± SD | % of reduction |
|------------------|-------------------------|--------------------------|----------------|
| CHOL             | 225.07 ± 1.70           | 124.40 ± 2.80            | 45             |
| HDL              | 59.37 ± 0.60            | 46.00 ± 1.14             | 23             |
| LDL              | 151.59 ± 2.46           | 63.18 ± 0.76             | 58             |
| Triglycerides    | 128.37 ± 8.75           | 76.33 ± 24.93            | 41             |
| ApoA             | 146.29 ± 6.44           | 115.29 ± 2.18            | 21             |
| ApoB             | 108.15 ± 1.62           | 48.70 ± 1.93             | 55             |
| Lp(a)            | 65.18 ± 2.33            | 28.55 ± 6.29             | 56             |

In order to characterize the protein species removed by LA, we first performed a proteomic analysis. We prepared two pools (PRE and POST) for all the enrolled patients, using the serum samples obtained before and after three consecutive apheresis sessions. The evaluation of total protein content for each pool, previously depleted of albumin and IgG, showed a statistically significant decrease (*$P$ value < .0003) in POST-LA ($2.85 \pm 0.106 \mu g/\mu L$) compared to PRE-LA ($3.47 \pm 0.086 \mu g/\mu L$), corresponding to a percentage reduction of $18.04\% \pm 3.72\%$ (Figure 2A).

The 2-DE/MS analysis revealed 27 differentially expressed protein spots between PRE-LA and POST-LA, detected by the evaluation of spot intensity modifications (details are reported in Table ISI). These proteins resulted to be involved in different biological functions, particularly inflammation and immunity (Figure 2B). We assessed the extent of protein removal as difference ($\Delta\%$) between the spot intensity values measured in PRE-LA and POST-LA. Of note, we found no significant difference concerning the total protein depletion among the different functional protein clusters (Table ISI).

On the whole, results showed that: (a) H.E.L.P. apheresis modifies the serum proteome of dyslipidemic patients; (b) the proteome modification is not uniform in all patients; (c) proteins most frequently removed were Complement C3
(75%) and Apolipoprotein C-III (83%), both considered cardiovascular risk factors; (d) the other removed proteins are mostly involved in inflammatory and immune response. The “non-uniformity” of the serum proteome alteration could be justified by the fact that the analyzed patients showed different clinical features.

To investigate in depth the LA effect on inflammation markers, we measured IL-6, TNF-α, IL-1α, IL-1RA, and VEGF concentrations on each patient’s serum before and after three consecutive sessions of H.E.L.P. apheresis, by means of magnetic Luminex assay (Figure 3, see Section 2 for details). Although not statistically significant, we found an increase of TNF-α, IL-1α, and IL-6; on the contrary, IL-1RA and VEGF levels decreased after the procedure (Figure 3).

We also performed a metabolomic analysis using 1H-NMR, where we identified and quantified about 50 metabolites (MW < 1500 Da) of different classes (aminoacids, nucleotides, purines, organic acids) (Figure 1SI). H.E.L.P. apheresis had no impact on removal of intermediate and final products of metabolic pathways, but we observed an increase of ketone bodies

**FIGURE 3** Cytokines expression. Serum cytokines concentration (pg/mL) before and after LDL-apheresis (mean values of three consecutive sessions)
in the samples after apheresis, which can be explained with the fasting during procedure.

Finally, serum levels of PCSK9 significantly decreased (≈50%, \( P < .05 \)) after H.E.L.P. apheresis for eight of the recruited patients (Figure 4A). One of them (homozygous FH, patient 9) was undergoing contemporary PCSK9 inhibitor (Evolocumab, Repatha) treatment, Figure 4B. The mean amount of PCSK9, calculated on the remaining eight patients, was 282.5 ± 15.3 pg/mL before and 137.5 ± 5.6 pg/mL after H.E.L.P. apheresis. Apheresis, however, was effective also for the homozygous FH patient, which showed much higher pre-apheresis levels of PCSK9 compared to the others (PRE-LA 1734.3 ± 40.7 pg/mL; POST-LA 991.15 ± 33.1 pg/mL); therefore, the efficacy of the procedure on removing PCSK9 demonstrated to be independent from the initial concentration (Figure 4B).

4 | DISCUSSION

With the aim of investigating the effect of H.E.L.P. apheresis in removing lipids and other biomolecules, we assessed the changes of a large variety of compounds in the nine dyslipidemic patients currently undergoing LA treatment at our hospital.

Our results confirm that H.E.L.P. apheresis halves the plasma levels of LDL, ApoB and Lp(a), and reduces total cholesterol and triglycerides of around 40%.\(^21\) Literature studies reported a range for mean LDL reduction after apheresis: 57%-75% for patients with homozygous FH, and 58%-63% for patients with heterozygous FH.\(^22\) On the contrary, HDL levels show only a minor reduction after the procedure.

These results are accompanied by a reduction of blood viscosity and density, supporting the beneficial effect of apheresis treatment. Blood viscosity, indeed, has been reported to be strongly predictive of cardiovascular events, and is a major risk factor for atherosclerosis.\(^23,24\)

It is also positively associated with high levels of LDL-C and hyperfibrinogenemia.\(^23\)

In our patients, H.E.L.P. apheresis induced a 56% mean reduction of Lp(a), confirming that LA systems are the most effective available interventions able to significantly reduce plasmatic Lp(a).\(^25\)

Proteomic assessment of serum samples confirmed the routine analyses results that are a decrease of LDL-related apolipoproteins. Importantly, we also observed that inflammation-related proteins (such as complement factors) are retained during the procedure. Inflammation plays a key role in the progression of atherosclerosis, and atherosclerotic plaques are characterized by inflammatory infiltrates.\(^1,26,27\) Our results support the hypothesis that, besides removal of cholesterol, LA has pleiotropic beneficial effects on the inflammatory processes that drive atherosclerosis.

Our data are in accordance with literature reporting a significant depletion of more than 70 functional proteins including peptides involved in the coagulation system and with adhesive (fibronectin), rheological (fibrinogen) and immunological/inflammatory properties (complement components).\(^28,29\)

Based on proteomic results, we investigated in depth the role of H.E.L.P. in affecting inflammation by measuring selected cytokines. We found slightly increased serum levels of the pro-inflammatory factors TNF-\(\alpha\) and IL-1\(\alpha\) after H.E.L.P. apheresis, whereas the anti-inflammatory cytokine IL-1 receptor antagonist IL1-RA was reduced. IL-6, which has both pro- and anti-inflammatory properties, slightly increased after the procedure. The modulation of inflammation factors after H.E.L.P. apheresis could be, in part, a consequence of the blood contact with membranes involved in plasma separation during extracorporeal circulation.\(^30\) Pro-inflammatory cytokines, and particularly those belonging to the IL-1 pathway (which is considered a central mediator of inflammatory reactions), have an important role in atherogenesis by a multitude of mechanisms,\(^25,27,31\) and their modulation during apheresis might bring beneficial effects on the inflammatory status of the patient. Our data, however, do not seem to confirm this hypothesis.

On the contrary, in our patients we demonstrated that H.E.L.P. apheresis reduces VEGF serum levels of 19%
of metabolic products. Our 1H-NMR metabolomic analysis did not observe an impact of H.E.L.P. apheresis on the removal of metabolic products. Our 1H-NMR metabolomic analysis was performed in conditions that allow the detection of water-soluble molecules only (that are unlikely retained by the hydrophobic filters of H.E.L.P. machine). This data suggests that H.E.L.P. apheresis does not induce changes to the metabolic profile of patients, preserving the vital metabolites required to maintain the intermediate metabolism.

In our patients, we also observed a significant impact of H.E.L.P. apheresis on PCSK9 levels, which approximately decrease of 60% after the procedure. The reduction is not influenced by the initial concentration of the protein, since pre-apheresis values are highly variable among the nine patients under study. In particular, the homozygous FH patient had very high pre-apheresis levels of the protein, although he was the only one undergoing contemporary treatment with Evolocumab. A paradoxical effect of Evolocumab on PCSK9 levels in hoFH patients, however, was already reported, probably due to the entrapment in the hydrophobic filters of H.E.L.P. machine. This data suggests that H.E.L.P. apheresis does not induce changes to the metabolic profile of patients, preserving the vital metabolites required to maintain the intermediate metabolism.

We are aware that our study has some limitations: the patient population, which is small due to the rareness of the pathology, lacks complete genetic characterization. The study is also monocentric, with an observational approach on data routinely obtained. Anyway, it proposes a wide investigation of what really happens to patient’s blood during the H.E.L.P. apheresis procedure. We believe that a deeper understanding of the pleiotropic effects of LA could be of great help in the choice of the most suitable lipid-lowering treatment option. Indeed, literature on the effects of LA on dyslipidemic patients is mainly based on clinical experience or, at most, on few studies on single compounds or classes of compounds.

To date, this is the first study exploiting a multidisciplinary approach to shed light on the effects of apheresis in dyslipidemic patients. We evidenced that H.E.L.P. apheresis is not merely a selective procedure that lowers ApoB100-containing lipoproteins, as it affects several aspects of to the complex area of atherosclerosis pathophysiology, that is, vascular endothelium, immunity, inflammation, and blood viscosity. Other LA systems, with different working principles, might affect these aspects in a different manner; an in-depth study of patients’ blood on its whole should be encouraged for all of them.

Although LA is considered a safe procedure that results in a reasonably good quality of life (QoL), its main drawback is the rebound effect on LDL levels that forces the patients to undergo cycles of apheresis every 1-2 weeks. Our results suggest that the combination of LA with a pharmacological treatment with novel lipid-lowering agents, such as Evolocumab, may be promising to reduce CVD risk.

The clinical challenge we are facing with H.E.L.P. apheresis is to manage the procedure with pharmacotherapy and drugs. An in-depth knowledge of the H.E.L.P.-induced plasma modifications could be of great advantage in future studies on the effects of different combined therapeutic options, and might contribute to improve the personalized approach to dyslipidemia, improving patients’ QoL and reducing adverse effects.

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AUTHOR CONTRIBUTIONS
T.A.P. and R. B. designed the study; E.D.B. supervised the procedures. I.S. controlled treatment, blood sampling, and handling. D. F. wrote the project for Ethics Committee approval. A. L., L.M., C. M. performed the experimental analyses. E. Q. performed 1H-NMR analyses. S. B., E. B., E. M., A. T. performed 2-DE and MS analyses. T. F. performed routine laboratory analyses. All authors evaluated the result. L. M. and C. M. drafted the manuscript and analyzed the results. T. A. P. and R. B. coordinated the study and critically revised the article. All authors approved the final version.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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