



Proteomic Profiling of Age-Related Proteins Following Extracorporeal Apheresis

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ABSTRACT

Lipoprotein apheresis (LA) is often the last option to adequately reduce lipoproteins in patients with familial hypercholesterolemia and lipoprotein (a) hyperlipidemia. Characterized by mild side effects, it is now the most effective method of preventing major cardiovascular events (CVEs). This benefit is due not only to the lowering of lipoprotein levels, but probably also to many other pleiotropic effects that have been extensively described in the literature. These include the reduction of inflammatory signaling substances, fibrinogen, plasminogen or components of the oxidative stress response. Here, we performed a proteomic analysis of 12 patients treated with therapeutic apheresis using two different pore size filters to quantify the effect on age-related plasma proteins. This study showed that important proteins such as α -2-macroglobulin, apolipoprotein C-III, complement C1s subcomponent, C4b-binding protein alpha chain, CD5 antigen-like and pregnancy zone protein, whose role in numerous aging processes has been well described, were significantly reduced by apheresis treatment. We conclude that therapeutic apheresis may be a promising approach to reduce these age-related proteins and that these treatments may become an essential part of managing cardiovascular risk in an aging population.

Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide, and its prevalence increases with age. Aging leads to the accumulation of age-related proteins that play an important role in the progression of CVD [1]. Proteomic studies – the large-scale study of proteins – have shown that thousands of proteins in human plasma and cerebrospinal fluid change with age [2, 3], revealing their impact on cardiovascular health. By profiling the entire set of proteins expressed in tissues or fluids, proteomics

allows researchers to quantify changes in expression, modification, and interaction over time. Many of these age-related proteins are associated with inflammatory processes, lipid metabolism, and endothelial function [4–6]. Aging contributes to several diseases affecting all organ systems and is the major risk factor for heart disease, neurodegeneration, and cancer [7]. Cardiovascular risk reduction in an aging population is primarily achieved through a healthy lifestyle, smoking cessation, and limited alcohol consumption, followed by pharmacotherapy with lipid-modifying drugs such

as statins, ezetimibe, bempedoic acid, or PCSK9 inhibitors. Finally, lipoprotein apheresis (LA) is the last option after all lifestyle and medical measures have been exhausted. LA effectively removes lipoproteins such as low-density lipoprotein cholesterol (LDL-C) and lipoprotein(a) [Lp(a)] from a patient's blood. INUSpherese is a double-filtration process starting with plasma separation that is followed by a filtration of large proteins by size. The high efficacy in preventing the progression of atherosclerosis and further cardiovascular events is not yet fully understood, but additional beneficial effects are increasingly being published. A major advantage of LA appears to be its ability to reduce not only proatherogenic lipoproteins but also other atherosclerosis-associated lipids such as ceramides, inflammatory markers, thrombogenic factors, or components of the oxidative stress response [8–11]. Therefore, it is conceivable that extracorporeal treatments such as therapeutic apheresis may be a promising strategy to remove proteins involved in cell senescence and aging from the blood of patients. This study investigated the acute effect of apheresis therapy on several age-related proteins using a proteomic approach.

Materials and Methods

Study protocol

Patients were recruited from the real-world setting of our routine LA treatment. All patients gave written consent to the study, which was approved by the local Ethics Committee (EK403102014). Patients were on weekly LA therapy for high Lp(a) levels. The LA procedure was performed according to manufacturer's instructions: temperature-optimized double-filtration plasmapheresis using two different sized apheresis filters INUS30 and INUS50 (INUSpherese, Cham). Pre- and post-apheresis plasma samples of 12 patients were collected directly before and after apheresis and analyzed.

In-solution digestion and Data Independent Acquisition (DIA) by nanoflow LC-MS/MS

This was conducted as previously described in Yin et al. [12].

Statistics and bioinformatics

Peptide quantification values were marked as missing when Signal-to-Noise ratio < 5 or q-value > 0.05 [12]. Then peptides with more than 30% missing values were discarded and the remaining missing values were imputed with K nearest neighbors (KNN)-based imputation with K = 3. The precursors from the same peptides with different charges were aggregated by summing their relative quantities. Then for each protein the peptides which were highly correlated (Spearman Rho threshold of 0.4) with the most abundant peptide were kept and the uncorrelated ones were discarded. Protein quantification was performed by summing the relative quantities of the unfiltered peptides for each protein.

The protein quantification was log₂ transformed for statistical analysis. All the statistical analyses were two-tailed. Pre- and post-apheresis comparison was performed using paired differential abundance analysis by applying the EBayes method of the R limma package [13]. p-Values were adjusted for multiple testing using Benjamini-Hochberg (BH) FDR correction. BH adjusted p-value < 0.05 was considered significant. Statistical analysis and asso-

ciated figures were generated with R programming environment (version 4.2.2).

Statistical analyses were performed with IBM SPSS Statistics Windows software (version 29, IBM Corp., USA). Numerical variables were summarized as mean ± standard error of the mean. Significant changes between before versus after LA of clinical data were compared by paired t-test. Differences between the INUS-50 and INUS-30 system were calculated using ANOVA considering only pre-apheresis values. Results with p ≤ 0.05 were considered statistically significant.

Results

Patient clinical measurements

The clinical characteristics of the patients are shown in ► **Table 1**. The study group consisted of 12 patients who were started on LA because of elevated Lp(a) levels (≥ 120 nmol/l) and who had experienced cardiovascular events. All patients did not achieve LDL-C or Lp(a) reduction according to the ESC/EAS lipid guidelines [14] and gave written consent for the study, which was approved by the local ethics committee (EK403102014). Seven patients (4 men and 3 women) with a mean age of 61.7 years (range 33 to 70) were treated with the INUS30 system and five patients (four men and one woman) with a mean age of 55.8 years (range 40 to 68) were treated with the INUS50 apheresis machine. All patients were routinely treated with these apheresis systems once a week. At each treatment, all listed clinical parameters were measured in pre- and post-apheresis plasma samples. Blood samples from all participants were subsequently analyzed at the National Heart and Lung Institute, Imperial College London for proteomic changes in plasma proteins associated with aging. Paired t-test statistics were performed to compare before (pre) and after (post) apheresis for both methods, and significant changes (* p ≤ 0.05) are shown in bold. One-way ANOVA analysis was performed to compare the pre-apheresis values of each variable for the two different INUS columns.

Clinical measurements showed that both methods efficiently removed various lipoproteins and abundant proteins from the patient's blood. Changes in standard blood cell count parameters during the apheresis procedure are listed in ► **Table 1**. Hemoglobin, hematocrit, platelets and erythrocytes were decreased by LA treatment. However, the leukocyte count was significantly increased by both INUS systems (30% to 55% increase after LA). Statistically significant differences comparing pre-apheresis values of INUS30 and INUS50 columns were calculated for MCH (** p ≤ 0.01), the leukocytes count (** p ≤ 0.01) and triglycerides (* p ≤ 0.05).

LA reduced age-related proteins

The data were derived from a re-analysis of a previously published study by Yin et al. [12], focusing specifically on proteins associated with ageing. A paired differential abundance analysis using the EBayes method of the R limma package was applied for the analysis of all patients (n = 12) and for the subgroups of the respective INUS method, that is, INUS50 and INUS30. Significance was based on a Benjamini-Hochberg adjusted p-value < 0.05. Proteins were mainly downregulated after apheresis in each of the three groups. Therefore, a recent age-association study by Oh et al. [5] was lev-

► **Table 1** Clinical characteristics.

Filter	INUS-30			INUS-50			ANOVA p-value INUS30 vs. INUS50
	Pre	Post	p-Value	Pre	Post	p-Value	
Erythrocytes (Tpt/l)	4.33 ± 0.11	4.28 ± 0.13	0.761	4.42 ± 0.1	4.42 ± 0.13	0.887	0.591
Haemoglobin (mmol/l)	8.57 ± 0.25	8.41 ± 0.28	0.683	8.4 ± 0.26	8.46 ± 0.31	0.921	0.653
Haematocrit	0.40 ± 0.01	0.39 ± 0.02	0.722	0.40 ± 0.02	0.39 ± 0.02	0.804	0.921
MCH (pg)	1.98 ± 0.02	1.96 ± 0.02	0.582	1.90 ± 0.02	1.92 ± 0.02	0.569	0.01
MCHC (mmol/l)	21.5 ± 0.3	21.5 ± 0.3	0.973	21.1 ± 0.2	21.6 ± 0.2	0.04	0.314
MCV (fl)	92 ± 1	92 ± 1	0.643	90 ± 1	89 ± 1	0.48	0.25
RDW (%)	12.5 ± 0.4	12.6 ± 0.4	0.939	13.3 ± 0.2	13.2 ± 0.2	0.898	0.163
Leukocytes (Gpt/l)	7.48 ± 0.53	9.74 ± 0.64	0.02	5.22 ± 0.36	8.12 ± 0.95	0.022	0.009
Platelets (Gpt/l)	255 ± 17	221 ± 17	0.191	209 ± 12	195 ± 13	0.431	0.075
MPV (fl)	10.1 ± 0.1	10.0 ± 0.2	0.757	10.3 ± 0.2	10.1 ± 0.2	0.464	0.397
Quick (%)	106 ± 4	66 ± 3	<0.001	100 ± 4	69 ± 5	0.001	0.302
INR	0.96 ± 0.02	1.36 ± 0.06	<0.001	1.00 ± 0.03	1.31 ± 0.08	0.005	0.318
Total protein (g/l)	65.4 ± 1.4	52.1 ± 1.9	<0.001	66.3 ± 2.6	54.5 ± 2.8	0.02	0.76
Albumin (g/l)	43.0 ± 1.1	36.6 ± 1.7	0.01	44.1 ± 0.7	37.2 ± 1.3	0.002	0.494
IgA (g/l)	2.06 ± 0.28	1.45 ± 0.19	0.095	1.37 ± 0.18	1.03 ± 0.17	0.208	0.089
IgM (g/l)	1.15 ± 0.19	0.39 ± 0.10	0.004	1.03 ± 0.29	0.37 ± 0.11	0.073	0.708
IgG (g/l)	8.94 ± 0.37	7.09 ± 0.38	0.004	8.49 ± 0.93	6.89 ± 0.88	0.246	0.62
Fibrinogen (g/l)	2.67 ± 0.13	1.36 ± 0.13	<0.001	2.95 ± 0.24	1.41 ± 0.12	<0.001	0.285
Ferritin (µg/l)	116.8 ± 28.8	77.2 ± 21.1	0.288	135.1 ± 64.2	95.7 ± 47.8	0.636	0.779
Albumin (%)	63.0 ± 0.5	64.9 ± 0.5	0.03	64.1 ± 2.1	66.5 ± 1.9	0.437	0.545
α1-Globulins (%)	4.1 ± 0.2	4.2 ± 0.1	0.588	4.1 ± 0.2	4.3 ± 0.1	0.481	1
α2-Globulins (%)	8.6 ± 0.6	7.0 ± 0.6	0.08	9.7 ± 1.1	7.9 ± 0.9	0.252	0.376
β-Globulins (%)	11.4 ± 0.3	11.3 ± 0.2	0.945	10.4 ± 0.4	10.4 ± 0.5	1	0.094
γ Globulins (%)	12.9 ± 0.5	12.5 ± 0.5	0.61	11.7 ± 1.0	10.9 ± 1.0	0.619	0.259
Triglycerides (mmol/l)	1.82 ± 0.32	0.66 ± 0.12	0.006	0.92 ± 0.08	0.59 ± 0.15	0.093	0.05
Total Cholesterol (mmol/l)	3.86 ± 0.29	1.71 ± 0.10	<0.001	3.64 ± 0.21	1.88 ± 0.08	<0.001	0.599
HDL-Cholesterol (mmol/l)	1.39 ± 0.09	1.04 ± 0.06	0.008	1.47 ± 0.18	1.16 ± 0.10	0.167	0.673
LDL-Cholesterol (mmol/l)	2.10 ± 0.29	0.61 ± 0.11	<0.001	1.87 ± 0.16	0.65 ± 0.08	<0.001	0.544
Lipoprotein(a) (nmol/l)	166 ± 31	38 ± 6	0.001	178 ± 38	62 ± 17	0.024	0.822

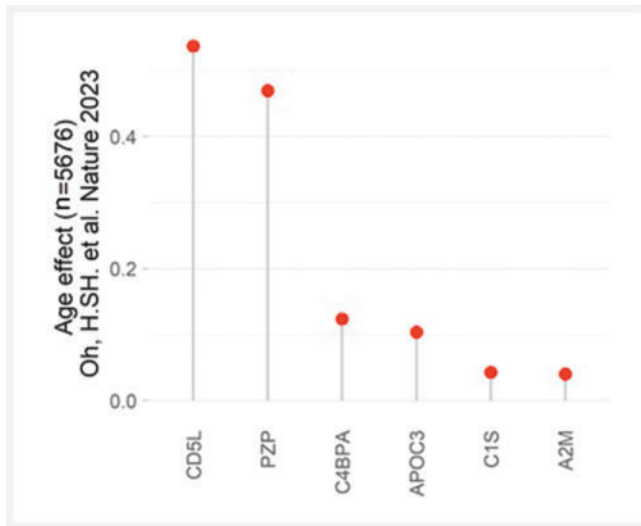
Data are shown as mean ± S.E.M. Paired t-test statistics has been conducted to compare between before (pre) and after (post) apheresis for each method. Significant changes (*p ≤ 0.05) have been highlighted in bold. One-way ANOVA analysis have been conducted comparing the pre-apheresis values of each variable for each method. MPV: Mean Platelet Volume; MCH: Mean Corpuscular Haemoglobin; MCHC: Mean Corpuscular Haemoglobin Concentration; MCV: Mean Corpuscular Volume; RDW: Red blood cell distribution width; INR: International Normalized Ratio.

eraged and example proteins were used to show the reversal of directionality for these age-associated proteins post apheresis. The effect of INUSpheresis on these proteins in all 12 patients is shown in ► **Fig. 1**. Five differentially expressed proteins were identified, namely α-2-macroglobulin (A2M), apolipoprotein C-III (APO-C3), complement C1s subcomponent (C1S), C4b-binding protein alpha chain (C4BPA), CD5 antigen-like (CD5L), and pregnancy zone protein (PZP) which were reduced during apheresis (► **Fig. 1**). Oh and co-workers calculated an increased expression of these proteins with age (► **Fig. 2**) [5]. The greatest reduction was observed for C4BPA and A2M. A comparison of the two INUS systems showed

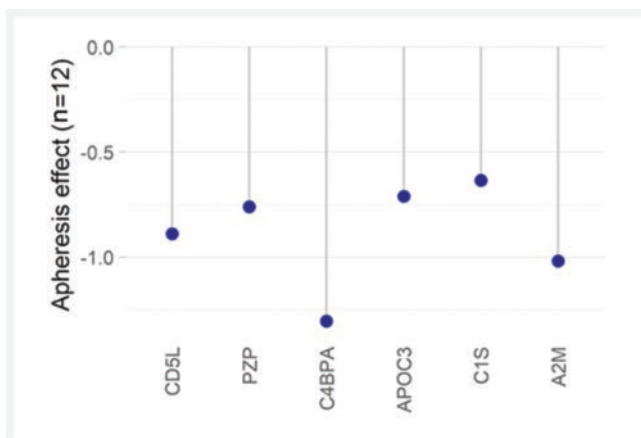
that INUS30 reduced ApoC3 significantly more than INUS50 (−1.04 vs. −0.25). The opposite effect was observed for PZP, which was more effectively reduced by the INUS50 system (−1.05) compared to INUS30 (−0.55) ► **Table 2**.

Discussion

Age is the major risk factor for chronic diseases such as atherosclerosis. Studies of heterochronic parabiosis and plasma exchange in animals have shown that circulating proteins can causally influence in aging phenotypes throughout the body, including skeletal mus-



► **Fig. 1** Effect of lipoprotein apheresis in N = 12 individuals. Only examples of differentially expressed proteins are shown. Paired differential expression (pre- and post-apheresis) was performed by applying the Ebayes method of the R limma package. Significance was based on Benjamini–Hochberg adjusted p-value < 0.05. Proteins were measured using mass spectrometry. Plots were generated using the ggplot package from R ver 4.2.2. A2M: Alpha-2-Macroglobulin; APO-C3: Apolipoprotein C-III; C1S: Complement C1s subcomponent; C4BPA: C4b-binding protein alpha chain; CD5L: CD5 antigen-like and PZP: Pregnancy zone protein.



► **Fig. 2** Age effect in n = 5676 individuals as computed by Oh et al. [5]. Age effect was computed using the least absolute shrinkage and selection operator (LASSO). Proteins were measured using SomaScan assay. Plots were generated using the ggplot package from R ver 4.2.2.

cle, heart, and brain [3]. A number of studies have demonstrated that the manipulation of blood composition can lead to a reduced biological age and improved health status. For example, therapeutic plasma exchange (TPE) has been shown to significantly improve the outcome of various medical therapies [4]. LA contributes to slow the progression of atherosclerosis by significantly reducing established risk factors such as lipoproteins and other proatherogenic proteins in the patient's blood. This effect is supported by the reduction of complement cascade factors, the cytokines and

other inflammatory molecules [12]. We have now investigated the effect of LA on age-related proteins.

Apolipoprotein C3 (APO-C3) has emerged as a key regulator of triglycerides (TG) and LDL-C. Mutations in APO-C3 are associated with lower TG levels, and APO-C3 inhibitors have shown promising lipid-lowering effects in clinical trials [15]. Beyond lipid regulation, APO-C3 influences organ damage and inflammation, impairing endothelial repair and renal function through NLRP3 and caspase-8 pathways [16]. Elevated APO-C3 levels are associated with cardiovascular events in aging populations and with prothrombotic conditions, particularly diabetes and coronary calcification [17, 18]. Therapeutically, small interfering RNAs (siRNAs) such as volanesorsen and olezarsen are used to reduce APO-C3 levels [19, 20]. APO-C3 also inhibits lipolysis by interfering with the interaction of lipoprotein lipase (LPL) with triglyceride-rich lipoproteins, resulting in impaired lipid clearance [21]. This suggests that the reduction of APO-C3 by LA contributes to the protective effects against cardiovascular disease [2].

Complement component C4b-binding protein (C4BP) is another molecule that has gained attention in the context of aging and inflammation-related cardiovascular risk. C4BP is a heparin-binding protein involved in both the complement system and the coagulation system, which regulates blood clotting. C4BP levels increase with age and are associated with higher erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), both markers of inflammation. Elevated C4BP levels have been associated with increased thrombotic risk, particularly in inflammatory conditions such as rheumatoid arthritis and cardiovascular disease [22]. Although C4BP may protect against serious infections, its elevated levels in older adults may lead to vascular damage [23]. C4BP has been detected in diseased aortic valves, synovial membranes of rheumatoid arthritis patients, and myocardial infarction sites during the acute inflammatory phase [24–27]. This suggests that C4BP is a key player in both promoting inflammation and contributing to vascular damage, particularly during the aging process.

The combined roles of APO-C3 and C4BP in lipid metabolism, inflammation and aging highlight the potential benefits of targeting these proteins for therapeutic intervention. By reducing APO-C3 levels, apheresis therapies could have the dual benefit of lowering TG and LDL-C levels while reducing inflammation and preventing organ damage. Similarly, targeting C4BP may help to reduce age-related thrombotic risk and vascular damage.

Alpha-2-macroglobulin (A2M) has gained interest in aging research due to its potential role in age-related diseases and systemic changes. A2M is an aging biomarker of human fibroblasts [28] and is associated with higher future risk of coronary artery disease. Plasma levels of A2M in plasma typically increase with age. Rheopheresis, a double filtration plasmapheresis that removes a defined spectrum of high molecular weight proteins such as fibrinogen, A2M, LDL-C and IgM, has been shown to improve blood flow and microcirculation [29]. It has been noted that rheopheresis is a specific therapeutic approach with acute rheological as well as chronic functional and structural effects and is therefore considered an effective treatment in patients with age-related macular degeneration [30]. The same may be true for INUSpheresis, given the observed reduction in A2M.

► **Table 2** Quantitative changes of different age-related proteins.

Gene	IN-US30-log-FC	IN-US30-CI.L	IN-US30-CI.R	INUS30 - p value	IN-US30-adj. p value	IN-US50-log-FC	IN-US50-CI.L	IN-US50-CI-R	INUS50 -p value	IN-US50-adj. p value
CD5L	-0.95	-1.58	-0.33	0.007	0.0471	-0.8	-1.43	-0.17	0.0219	0.1216
PZP	-0.55	-1.05	-0.05	0.033	0.1365	-1.05	-1.27	-0.83	5.99E-05	0.0022
C4BPA	-1.28	-1.59	-0.97	2.67E-06	0.0004	-1.33	-1.5	-1.16	4.87E-06	0.0005
APO-C3	-1.04	-1.4	-0.69	6.00E-05	0.0017	-0.25	-0.44	-0.05	0.0216	0.1216
C1S	-0.64	-0.82	-0.45	1.49E-05	0.0015	-0.63	-0.84	-0.42	0.0006	0.0086
A2M	-1.03	-1.37	-0.69	4.36E-05	0.0015	-1	-1.1	-0.9	1.43E-06	0.0002

PZP decreases more with INSU50 (n = 5 patients); APO-C3 decrease is higher with INSU30 (n = 7 patients).

Pregnancy zone protein (PZP) is a novel biomarker of DNA damage-induced senescence [31]. PZP is capable of suppressing T cell function during pregnancy to prevent fetal rejection, but has recently been implicated in airway infection and neutrophil extracellular trap formation [32]. Given its involvement in aging and immune dysfunction, reducing PZP levels by therapeutic apheresis may have potential clinical benefits, although further research is needed to determine the long-term impact of such interventions.

CD5 antigen-like (CD5L) protein is a predominantly macrophage-secreted protein with functions that include regulation of lipid and cholesterol homeostasis, metabolism, and resolution of inflammation [33]. Castelblanco et al. [34] hypothesized that high CD5L levels could serve as a biomarker for increased risk of CV events and mortality in individuals with chronic kidney disease.

Taken together, APO-C3 and C4BP play critical roles in lipid metabolism, inflammation and the aging process, all of which contribute to cardiovascular and cerebrovascular risk. Reducing these age-related proteins or other senescent proteins such as alpha-2-macroglobulin, PZP or CD5L by therapeutic apheresis could further reduce the risk of vascular damage and improve individual outcomes for the aging population. The INUS systems differ in their effect on specific proteins. INUS30 shows a stronger reduction in APO-C3, which is strongly associated with atherosclerosis and coronary disease. INUS50, on the other hand, reduces PZP more significantly, which is more closely associated with immune disorders. Extracorporeal treatments, particularly LA, have emerged as promising strategies for reducing lipoproteins that contribute to cardiovascular disease. The lipid-lowering effect has been suggested to be the main mechanism responsible for these clinical benefits of LA. Well-documented pleiotropic effects of LA include reduction of blood viscosity, reduction of oxidative stress and microalbuminuria, and improvement of endothelial function through effects on circulating endothelial and progenitor cells. These effects are achieved not only by lowering apolipoprotein B-rich lipoproteins, but also by removing many circulating proteins such as fibrinogen, von Willebrand factor, immunoglobulins, antithrombin, various coagulation factors or inflammatory molecules [9, 11, 12, 35, 36]. Even the reduction of extracellular vesicles (EVs) by LA has been

described [37]. Thus, there is no exclusive reduction of only these age-associated proteins, but this reduction occurs as part of the acute reduction of many proteins during apheresis treatment. However, it is possible that the reduction of these proteins contributes to the overall positive pleiotropic effects of LA. Further long-term studies are needed to clarify whether the lowering of these proteins contributes to a reduction in age-associated diseases.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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