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Precision Medicine Approach for Cardiometabolic Risk Factors in Therapeutic Apheresis

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ABSTRACT

Lipoprotein apheresis (LA) is currently the most powerful intervention possible to reach a maximal reduction of lipids in patients with familial hypercholesterolemia and lipoprotein(a) hyperlipidemia. Although LA is an invasive method, it has few side effects and the best results in preventing further major cardiovascular events. It has been suggested that the highly significant reduction of cardiovascular complications in patients with severe lipid disorders achieved by LA is mediated not only by the potent reduction of lipid levels but also by the removal of other proinflammatory and proatherogenic factors. Here we performed a comprehensive proteomic analysis of patients on LA treatment using intra-individually a set of differently sized apheresis filters with the INUSpheresis system. This study revealed that proteomic analysis correlates well with routine clinical chemistry in these patients. The method is eminently suited to discover new biomarkers and risk factors for cardiovascular disease in these patients. Different filters achieve reduction and removal of proatherogenic proteins in different quantities. This includes not only apolipoproteins, C-reactive protein, fibrinogen, and plasminogen but also proteins like complement factor B (CFAB), protein AMBP, afamin, and the low affinity immunoglobulin gamma Fc region receptor III-A (FcγRIIIa) among others that have been described as atherosclerosis and metabolic vascular diseases promoting factors. We therefore conclude that future trials should be designed to develop an individualized therapy approach for patients on LA based on their metabolic and vascular risk profile. Furthermore, the power of such cascade filter treatment protocols may improve the prevention of cardiometabolic disease and its complications.

Introduction

Therapeutic apheresis is a well-established treatment approach for patients with severe lipid disorders and high cardiovascular risk profiles [1–3]. LA should start as fast as possible when the admission criteria are met to avoid new cardiovascular events [4].

The high efficiency in the prevention of progression of atherosclerosis and further cardiovascular events beyond the success of current lipid-lowering drugs has not been fully acknowledged. One major advantage of LA is the ability to remove not only LDL but also other proatherogenic lipoprotein particles such as lipoprotein(a). Lipidomic profiling revealed that therapeutic apheresis is capable of removing a broad spectrum of inflammatory lipids including ceramides [5, 6].

Furthermore, therapeutic apheresis reduces inflammatory proteins such as C-reactive protein (CRP), which has been implicated in the pathophysiology of cardiovascular disease and the severity of myocardial infarction. However, there are numerous other proatherogenic peptides and proteins involved in the progression of cardiometabolic disease that may be targeted by therapeutic apheresis [7–11]. Therefore, we performed a comprehensive proteomic analysis of more than 400 proteins in plasma samples from patients immediately before and after therapeutic apheresis. By employing different membranes of the INUSpheresis system, the specific effects of INUS-50, INUS-30, and TKM58 columns on plasma proteomic profile were compared.

Materials and Methods

Study protocol

Patients were recruited from the real-world setting of our routine LA [12] 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 manufactures instructions: temperature-optimized double-filtration plasmapheresis (INUSpheresis, Cham). In total, 43 apheresis sessions in 17 patients were analyzed. Patients were treated with routine therapy protocol according to two protocols: 1) sequential LA with intra-individual switches of INUS-30 and TKM58, and 2) three sessions with either INUS-30 or INUS-50. The protocols covered a period of about three months. Pre- and post-apheresis plasma samples were collected directly before and after apheresis session.

In contrast to INUS-30 and INUS-50, TKM58 was not provided for permanent use in LA, but due to its specific properties in removing proinflammatory mediators for a block-wise treatment starting with two initial sessions followed by individualized cycles of treatment in dependency of clinical success.

In-solution digestion

Plasma samples (10 µl) were denatured and reduced in 90 µl denature buffer [8 M urea, 5.56 mM dithiothreitol, 0.1 M triethylammonium bicarbonate (TEAB), pH = 8.5] at 37 °C for 1 hour and alkylated by adding 16 µl 181.25 mM iodoacetamide (final concentration 25 mM) and incubated at room temperature in the dark for 1 hour. Alkylated proteins (6 µl) were diluted with 164 µl 0.1 M TEAB pH = 8.5 and digested by 1.6 µg of trypsin/lysC (Promega). The digestion was carried out in an incubator shaker (Eppendorf) at 37 °C, 180 rpm, overnight and stopped by adding 20 µl 10 % trifluoroacetic acid (TFA) (final concentration 1 %). The digested peptides were cleaned using C18 cartridges (Agilent, P/N 5190-6532) on a Bravo AssayMAP liquid handling system. The eluted peptides were dried using a vacuum centrifuge and resuspended in 20 µl LC-MS solution [2 % acetonitrile (ACN), 0.05 % TFA].

Data independent acquisition (DIA) by nanoflow LC-MS/MS

The samples were separated by a nano flow HPLC (Ultimate 3000 RSLCnano, Thermo Fisher Scientific) on an EASY-Spray C18 column (75 µm × 50 cm, 2 µm) using 120-minute LC gradient (0–1 min, 1 % B; 1–6 min, 1–6 % B; 6–40 min, 6–18 % B; 40–70 min, 18–35 % B; 70–80 min, 35–45 % B; 80–81 min, 45–99 % B; 81–89.8 min, 99 % B; 89.8–90 min, 99–1 % B; 90–120 min, 1 % B; where A = 0.1 % formic acid (FA) in H_2O and B = 0.1 % FA, 80 % ACN in H_2O). The flow rate is 250 nl/min. Column temperature was set at 45 °C. The separated peptides were directly injected into Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific) and analyzed using DIA method with 25 variable isolation windows covering *m*/*z* range 330–1200. Full MS spectra were collected in profile mode on an Orbitrap with resolution 60 000, AGC 1e6 and max injection time 20 ms. All ions in each isolation window were fragmented using higher-energy collisional dissociation (HCD) with collision energy 30 % and MS2 spectra were collected in profile mode on an Orbitrap with resolution 30 000, AGC 1e6 and max injection time 55 ms.

Raw data were analyzed using Spectronaut software (version 15.0, Biognosys), matching against an in-house human plasma proteome spectral library (7851 precursors, 2021.04.15). The peptide signal intensities (fragment ions peak areas) were used for quantitation. All observations with q-value < 0.01 in any samples were considered and quantitative values, Q values, signal to noise ratio from all samples were exported for further quality check and statistical analysis.

Statistical and bioinformatics

Peptide quantification values were marked as missing when signal-to-noise ratio < 5 or q-value > 0.05. 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.

Two-way ANOVA was used to identify significant changes between timepoints (before or after apheresis) and between measurements (different apheresis date), correcting for age, gender, intra-patient variability and number of previous visits. 2-way ANOVA was also used to compare between different intervention methods (INUS-50, INUS-30, and TKM58) considering as timepoints the before or after apheresis. Spearman's correlation and hierarchical clustering was used to identify significant clusters in between of the proteins that were found to be significant for at least one of the intervention methods. Benjamini and Hochberg's FDR corrected q-values were calculated to correct for multiple testing in both 2-way ANOVA and Spearman's correlation analyses. The limma package and the E-bayes method were used to compared between the pre- and post-apheresis samples within each intervention method correcting for age, gender, intra-patient variability and number of previous visits. Statistical analysis and associated figures were generated with R programming environment (version 4.02). Functional and pathway enrichment analysis for the revealed proteins clusters was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) web tool (version 6.8) using a Benjamini and Hochberg's FDR corrected q-value threshold of 0.05 to report significantly enriched terms.

▶ **Table 1** Clinical characteristics.

Filter	TKM58			INUS-30			INUS-50			
n (patients: M/F)	4 (2/2)			8 (5/3)			5 (4/1)			
n (LA sessions)	8			22			13			
Age	65.75 (60.28–71.22)			60.73 (47.5–73.96)			57.00 (48.07–65.93)			
Clinical Chemistry	**Pre**	**Post**	**p-Value**	**Pre**	**Post**	**p-Value**	**Pre**	**Post**	**p-Value**	**ANOVA p-Value**
Erythrocytes (Tpt/l)	4.23 (3.97–4.49)	4.62 (4.25–4.99)	**0.001**	4.32 (4.07–4.58)	4.3 (3.87–4.73)	0.509	4.3 (4.03–4.56)	4.28 (4–4.56)	0.768	0.677
Haemoglobin (mmol/l)	8.4 (7.87–8.93)	9.2 (8.35–10.05)	**0.001**	8.51 (7.91–9.12)	8.45 (7.55–9.36)	0.428	8.15 (7.58–8.73)	8.16 (7.55–8.77)	0.921	0.224
Haematocrit	0.4 (0.37–0.42)	0.43 (0.39–0.47)	**0.002**	0.4 (0.37–0.42)	0.4 (0.35–0.44)	0.467	0.39 (0.36–0.42)	0.38 (0.35–0.41)	0.356	0.611
MCH (pg)	1.99 (1.96–2.01)	1.99 (1.95–2.03)	0.649	1.97 (1.92–2.01)	1.97 (1.92–2.01)	0.918	1.9 (1.86–1.94)	1.9 (1.86–1.95)	0.484	**<0.001**
MCHC (mmol/l)	21.35 (20.77–21.93)	21.49 (20.85–22.13)	0.156	21.42 (20.62–22.21)	21.49 (20.81–22.16)	0.274	21.03 (20.6–21.46)	21.32 (20.82–21.83)	**0.009**	0.255
MCV (fl)	93.13 (91.17–95.08)	92.75 (90.37–95.13)	0.08	91.95 (88.99–94.92)	91.5 (89.07–93.93)	0.086	90.31 (87.3–93.32)	89.38 (86.34–92.43)	**0.008**	0.081
RDW (%)	12.65 (11.67–13.63)	12.6 (11.67–13.53)	0.351	12.49 (11.55–13.43)	12.5 (11.55–13.45)	0.771	13.35 (12.71–13.99)	13.28 (12.68–13.89)	0.088	**0.025**
Leukocytes (Gpt/l)	7.71 (6.71–8.71)	9.59 (7.83–11.35)	**0.001**	7.7 (6.35–9.04)	9.83 (7.72–11.94)	**<0.001**	5.79 (4.49–7.08)	8.08 (6.56–9.6)	**<0.001**	**<0.001**
Platelets (Gpt/l)	241.38 (227.37–255.38)	221 (202.81–239.19)	**<0.001**	247.18 (206.7–287.66)	216.14 (177.69–254.58)	**<0.001**	224.85 (193.21–256.49)	203.08 (172.87–233.29)	**<0.001**	0.192
MPV (fl)	10.06 (9.65–10.48)	10.15 (9.58–10.72)	0.262	10.05 (9.69–10.42)	9.95 (9.48–10.41)	0.084	10.28 (9.7–10.86)	10.16 (9.59–10.74)	0.105	0.346
Quick (%)	105.78 (94.89–116.66)	64.63 (55.14–74.11)	**<0.001**	107.38 (98.33–116.44)	65.53 (47.99–83.08)	**<0.001**	100.77 (93.48–108.06)	67.31 (59.92–74.69)	**<0.001**	0.058
INR	0.98 (0.89–1.07)	1.38 (1.24–1.53)	**<0.001**	0.96 (0.89–1.02)	1.33 (1.17–1.49)	**<0.001**	1 (0.95–1.05)	1.33 (1.22–1.45)	**<0.001**	0.102
Plasma Proteins										
Total protein (g/l)	63.95 (62.34–65.56)	42.49 (38.91–46.07)	**<0.001**	64.27 (60.96–67.59)	50.23 (45.86–54.6)	**<0.001**	66.1 (61.03–71.17)	54.32 (49.69–58.96)	**<0.001**	0.308
Albumin (g/l)	42.61 (40.1–45.12)	29.91 (25.96–33.86)	**<0.001**	42.85 (40.56–45.13)	35.33 (31.91–38.75)	**<0.001**	44.34 (42.42–46.26)	37.75 (35.26–40.24)	**<0.001**	0.119
IgA (g/l)	1.73 (1.04–2.43)	0.86 (0.55–1.17)	**0.001**	1.94 (1.16–2.72)	1.33 (0.75–1.9)	**<0.001**	1.29 (0.91–1.66)	0.96 (0.66–1.26)	**<0.001**	**0.029**
IgM (g/l)	1.04 (0.61–1.48)	0.5 (0.31–0.7)	**0.001**	1.12 (0.57–1.67)	0.37 (0.11–0.64)	**<0.001**	0.83 (0.33–1.33)	0.29 (0.11–0.48)	**<0.001**	0.282
IgG (g/l)	8.03 (6.83–9.24)	4.31 (3.36–5.25)	**<0.001**	8.06 (6.61–9.51)	6.24 (4.96–7.53)	**<0.001**	8.31 (6.53–10.09)	6.72 (5.14–8.31)	**<0.001**	0.877
Fibrinogen (g/l)	2.31 (1.93–2.69)	1.11 (0.85–1.38)	**<0.001**	2.68 (2.39–2.97)	1.34 (1.01–1.68)	**<0.001**	2.75 (2.31–3.19)	1.31 (1.09–1.54)	**<0.001**	0.101
Ferritin (µg/l)	117.86 (40.57–195.15)	80.21 (24.43–136)	**0.002**	106.16 (34.72–177.61)	64.66 (18.75–110.58)	**<0.001**	105.94 (–2.84 to –214.72)	76.98 (–1.04 to –154.99)	**0.01**	0.940
Plasma electrophoresis										
Albumin (%)	64.24 (63.27–65.2)	66.69 (64.02–69.36)	**0.04**	63.41 (61.55–65.28)	65.64 (63.28–67.99)	**<0.001**	64.52 (61.4–67.63)	66.52 (63.33–69.7)	**0.001**	0.334
α1-Globulins (%)	3.96 (3.56–4.36)	4.21 (3.6–4.82)	0.194	4.29 (3.78–4.81)	4.39 (4.01–4.77)	0.317	4.24 (3.89–4.6)	4.23 (4–4.45)	0.865	0.218
α2-Globulins (%)	8.29 (7.26–9.31)	7.64 (6.16–9.11)	0.126	8.74 (7.16–10.32)	7.15 (5.46–8.85)	**<0.001**	8.89 (7.14–10.63)	7.53 (5.93–9.13)	**<0.001**	0.681
β-Globulins (%)	11.39 (10.76–12.01)	11.45 (9.81–13.09)	0.923	11.57 (10.59–12.54)	11.47 (10.42–12.52)	0.618	10.69 (9.96–11.42)	10.63 (9.78–11.47)	0.632	**0.018**

▶ **Table 1** Continued.

Filter	TKM58		INUS-30			INUS-50		
γ-Globulins (%)	12.13 (10.66–13.59)	10.01 (8.27–11.75)	**<0.001**	12 (10.22–13.78)	11.35 (9.56–13.13)	**0.003**	11.66 (10.06–13.27)	11.1 (9.51–12.7)	**<0.001**	0.791
Lipids										
Triglycerides (mmol/l)	1.87 (1.14–2.6)	1.15 (0.71–1.59)	0.033	1.65 (1.01–2.28)	0.71 (0.35–1.07)	**<0.001**	0.93 (0.72–1.14)	0.54 (0.28–0.81)	**<0.001**	**0.001**
Total Cholesterol (mmol/l)	3.97 (3.37–4.58)	1.97 (1.63–2.3)	**<0.001**	3.9 (3.16–4.65)	1.72 (1.36–2.07)	**<0.001**	3.47 (2.85–4.08)	1.8 (1.55–2.05)	**<0.001**	0.147
HDL-Cholesterol (mmol/l)	1.46 (1.2–1.71)	0.78 (0.64–0.91)	**<0.001**	1.45 (1.17–1.73)	1.06 (0.86–1.27)	**<0.001**	1.45 (1.09–1.81)	1.14 (0.93–1.35)	**<0.001**	0.999
LDL-Cholesterol (mmol/l)	2.24 (1.45–3.02)	1.09 (0.69–1.48)	**<0.001**	2.13 (1.42–2.85)	0.61 (0.32–0.89)	**<0.001**	1.77 (1.37–2.17)	0.62 (0.47–0.76)	**<0.001**	0.189
Lipoprotein(a) (nmol/l)	150.75 (100.85–200.65)	71.25 (52.1–90.4)	**<0.001**	164.73 (99.96–229.49)	38.73 (19.59–57.87)	**<0.001**	169.54 (101.25–237.82)	56.62 (28.68–84.55)	**<0.001**	0.800

Continuous data are shown as median (interquartile range). Dichotomous data are shown as n (%). Paired *t*-test statistics has been conducted to compare between before (pre) and after (post) apheresis for each method and significant changes (p-value less than 0.05) have been marked in bold fold. One-way ANOVA analysis have been conducted comparing the pre-apheresis values of each variable for each method. Chi-square test has been conducted for the calculation of the p-values on sex which was the only categorical variable in the dataset. MPV: Mean Platelet Volume; MCH: Mean Corpuscular Haemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; MCV: Mean Corpuscular Volume; RDW: Red blood cell distribution width; INR: International Normalized Ratio.

Results

Patient clinical measurements

The clinical characteristics of the patients are shown in ▶ **Table 1**. In total, there were 17 patients (11 men and 6 women) included in this study. Each patient was chronically treated with LA over a 2-year period during 2020–2021. In each treatment, all the listed clinical parameters were measured in pre- and post-apheresis plasma samples. Then the same plasma samples were sent to King's College London for proteomics analysis. Continuous data are shown as median (interquartile range). Dichotomous data are shown as n (%). Paired *t*-test statistics have been conducted to compare between before (pre) and after (post) apheresis for each method and significant changes (p-value less than 0.05) have been marked in bold font. One-way ANOVA analysis have been conducted comparing the pre-apheresis values of each variable for each method.

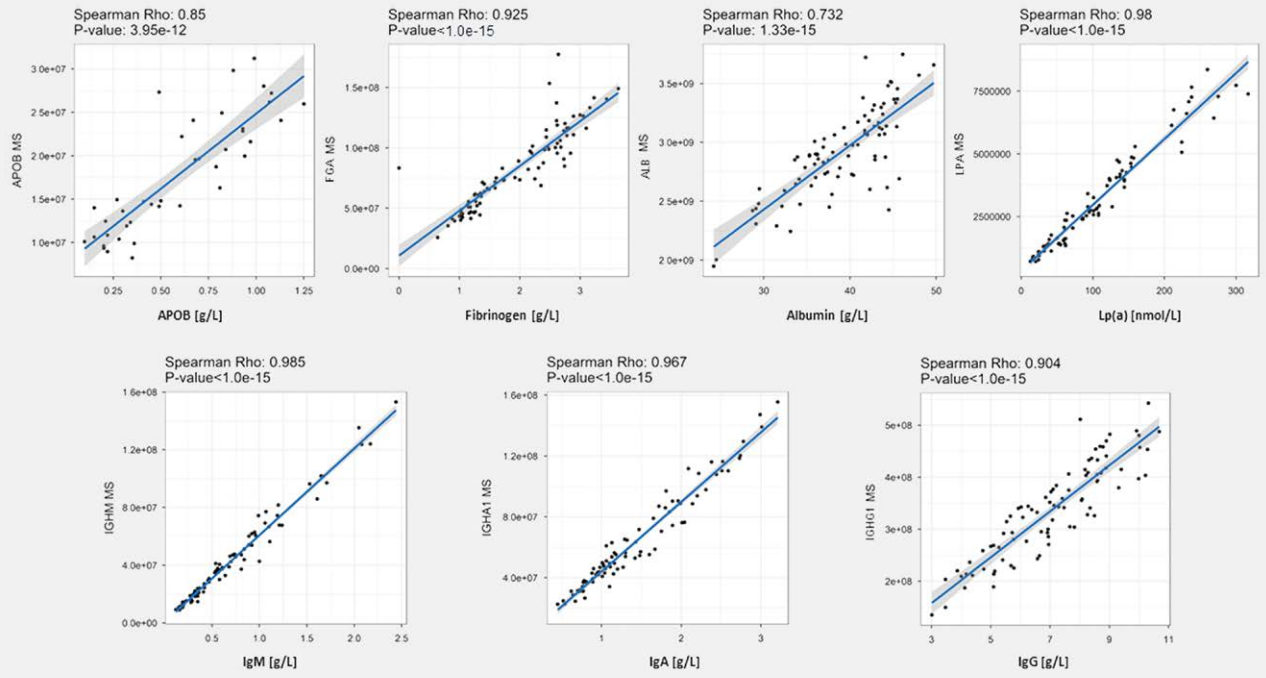
The clinical measurements revealed that the three methods all efficiently removed various lipids and abundant proteins. They also had a similar effect on leukocytes count (24–40 % increase after LA) and platelet count (10 % decrease after LA). However, the degree of changes was different when using different LA setups. TKM58 induced a more pronounced reduction of albumin and total protein. Both parameters, however, recovered within 48 hours to pre-apheresis values.

Summary of the proteomics results

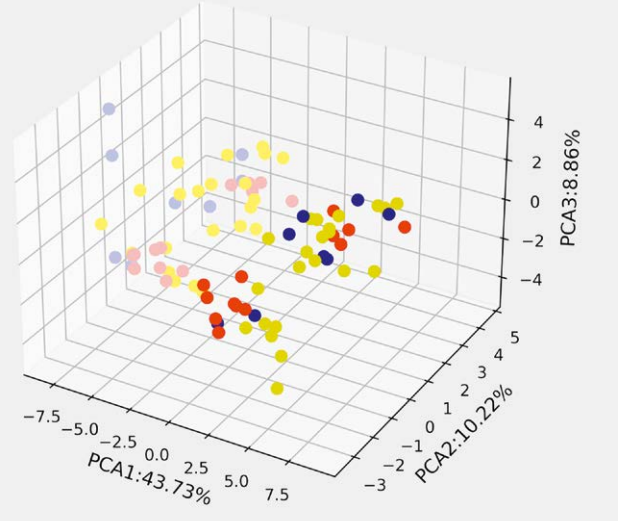
Next, data-independent LC-MS/MS was performed to quantify the plasma proteins of the pre- and post-LA samples. In total, 468 proteins were identified in the plasma samples (n = 86). A total of 255 proteins were reliably quantified (**Supplemental Table 1**) but 193 proteins remained after filtering protein quantifications which were based on variable regions of immunoglobulins (**Supplemental Table 2**). Protein measurements were compared with corresponding clinical measurements in these samples. The correlation scores were high, confirming that the mass spectrometry-based proteomics method provides comparable data to the conventional clinical methods (▶ **Fig. 1**). The principal component analysis (PCA) (▶ **Fig. 2**) showed clear separation between the pre samples (darker colored dots) and post samples (lighter colored dots), but there is no clear separation among the 3 different LA methods.

LA reduced lipid-related apolipoproteins

Regarding the relevant lipid species and apolipoproteins, all three LA methods achieved an adequate lowering reduction of up to 80 % (▶ **Fig. 3a**) as expected. TKM58 (blue) showed comparable performance as INUS-30 (yellow), and both were better than INUS-50 (red). Several proteins (APOA1/2/4, APOC1, APOD, APOF, APOH, APOM, CETP, LCAT, PLTP) were reduced more by TKM58 and another group of proteins (APOB, APOC2/3, APOE, CLU, LPA) were reduced more by INUS-30. Surprisingly the LPA reduction was smaller when using the TKM58 method in comparison to the other 2 methods. The hierarchical clustering of the apolipoproteins and related proteins showed a high correlation amongst most apolipoproteins except for APOH, which is the least reduced one after LA and is reduced significantly more with the TKM58 method (▶ **Fig. 3b**).



▶ **Fig. 1** Proteomics data highly correlating with corresponding clinical measurements: Spearman correlation was conducted between the proteomics relative quantities of the proteins against their corresponding clinical measurements for proteins for which such a clinical measurement was available: APOB: Apolipoprotein B; LPA: Lipoprotein(A); ALB: Albumin; FGA: Fibrinogen Alpha Chain; IGHM: Immunoglobulin Heavy Constant Mu; IGHA1: Immunoglobulin Heavy Constant Alpha 1; IGHG1: Immunoglobulin gamma-1 heavy chain. The correlation analysis for APOB was conducted using only 40 samples because clinical APOB measurement was only available for these samples. Y-axes show the relative quantities of each protein in each sample using Mass Spectrometry and X-axes show the corresponding clinical measurements.



▶ **Fig. 2** Principal component analysis (PCA) analysis of plasma samples before and after INUS-50 (red), INUS-30 (yellow), TKM58 (blue) apheresis: Post-apheresis samples are shown in lighter colors.

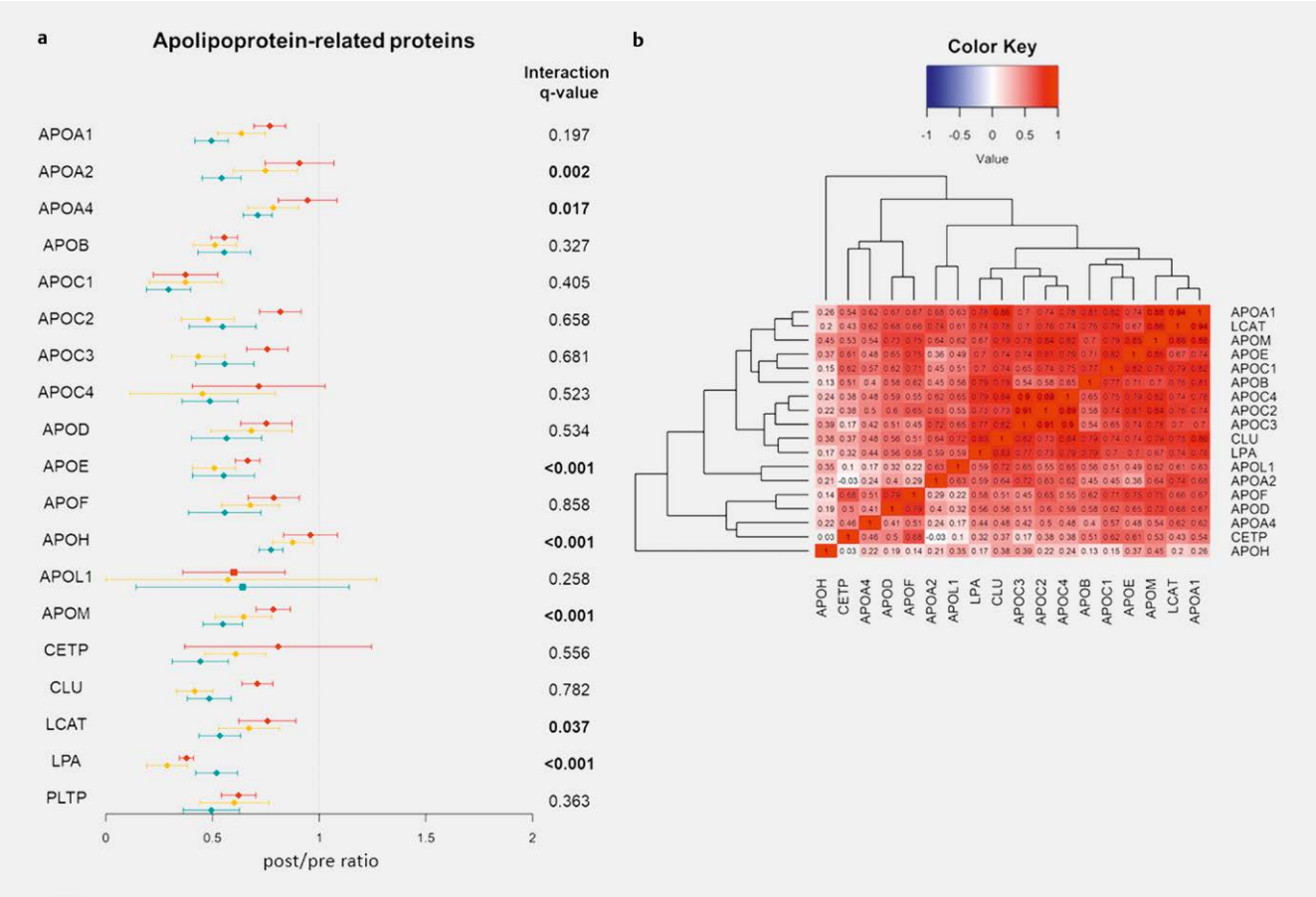
Response of coagulation system to LA

As the platelet count and prothrombin time (Quick/INR) both showed impaired coagulation function after the LA, the proteins involved in coagulation were carefully checked (▶ **Fig. 4**). Same as clinical measurements, the fibrinogens (FGA, FGB and FGG) were all reduced to 50 % after LA (▶ **Fig. 4a**). Some of the coagulation factors were not reduced much (F9, F12) but some others were consistently reduced to 50 % by all 3 methods (F5, F13A1, F13B). In general, serpins were not reduced much after LA and the 3 methods performed equally except for SERPIND1 and SERPING1. The hierarchical clustering showed 2 clear groups (▶ **Fig. 4b**). One includes the proteins that behave similarly to fibrinogens, which did not show a reduction difference between the 3 methods. Another group includes the proteins which were differentially reduced by different LA methods.

Immune system changes after LA

As the major abundant proteins in the plasma, immunoglobulins were all reduced from 10 % when using INUS-50, to 25 % using INUS-30, and to 50 % using TKM58, except IGHM and JCHAIN, which were reduced to ~50 % by all 3 methods (▶ **Fig. 5a**). This may be due to the large size of the IgM complex (heavy chains are linked by joining chain to form the pentamer), which cannot pass any size filters easily during the LA. In the hierarchical clustering, the proteins which were interacting with others were clustered together (▶ **Fig. 5b**), for example, IGHA1 and IGHA2, IGHM and JCHAIN, hence they were removed in the same way.

For the factors of the complement system, there is a clear separation of the degree of reduction when using INUS-30 or INUS-50 (▶ **Fig. 6a**), for example, C1QA, C1QB, and C1QC were reduced by > 60 % while C2, C3, C5, C6, and C7 were only reduced by 10–20 %



▶ **Fig. 3** Effect of apheresis on apolipoprotein-related proteins: **a**: Protein abundance ratio between before (pre) and after (post) apheresis using INUS-50 (red), INUS-30 (yellow), and TKM58 (blue). Interaction q-value < 0.05 indicates the 3 methods have significant differences. **b**: Spearman's correlation and hierarchical clustering analyses of the selected proteins. The values are Spearman's Rho.

when using INUS-30 or INUS-50. This difference was not seen when using the TKM58 setup. When looking at the clustering pattern, there is a cluster in the middle, including the proteins which were not reduced more when using TKM58 than using INUS-30 or INUS-50, such as C1QA, C1QB, C1QC. The cluster on the right side includes the proteins which were reduced much more when using TKM58 than using the other 2 methods, such as C2, C3, C5 (▶ **Fig. 6b**).

LA may weaken the immune system of the patients by removing the immunoglobulins and most of the complement factors, especially when using TKM58.

Other inflammation-related proteins

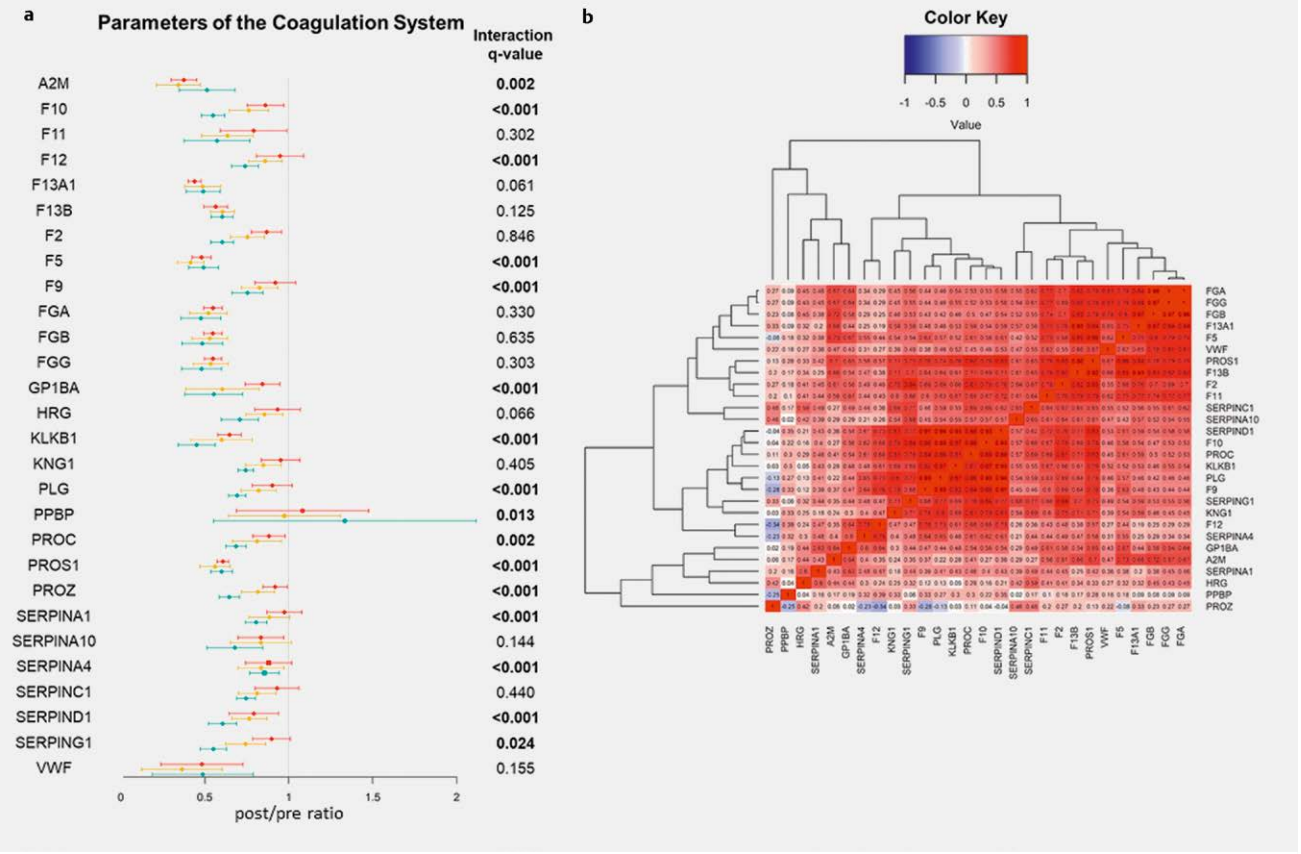
No clear pattern was observed for the remaining inflammation-related proteins, which were not included in the complement and coagulation systems protein groups. Some proteins showed similar gradual reduction when using INUS-50, INUS-30, and TKM58, for example, CD44, CP, FCGR3A, LYZ, PON1, SAA4 (▶ **Fig. 7a**). The hierarchical clustering showed 3 separated clusters (▶ **Fig. 7b**). The rightmost cluster includes proteins that showed different reductions when using the 3 methods, with TKM58 reduced the most except CD5L.

Two-way ANOVA analysis considering time point and INUS setup

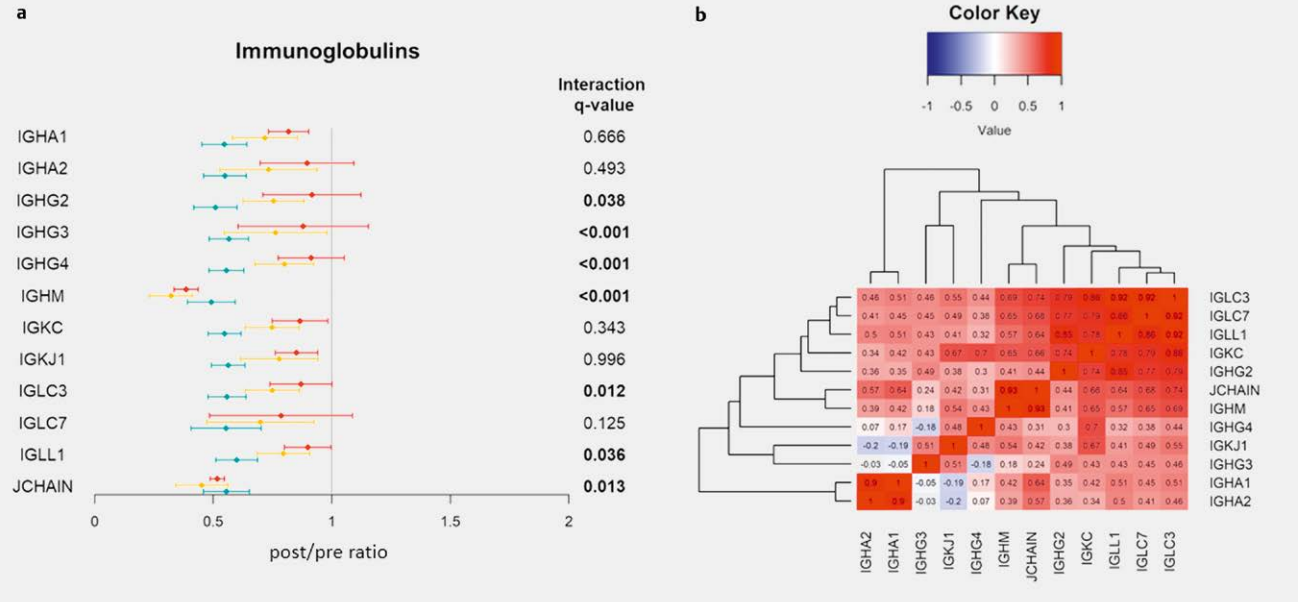
Two-way ANOVA analysis was carried out to discover the different protein change pattern among the 3 LA methods. Hierarchical clustering revealed a group of 14 proteins (▶ **Fig. 8**, dashed rectangle), which were decreased substantially more after LA in TKM58 compared to INUS-30 and INUS-50. The detailed abundance changes for each of the 14 proteins were displayed in ▶ **Fig. 9** and listed in **Supplemental Table 2**. Functional and pathway enrichment analysis showed that this list of proteins is significantly enriched with complement and coagulation cascade KEGG pathway (q-value: 0.0053), while the proteins are secreted (q-value: 3.4E-10) and present in the extracellular space (q-value: 1.2E-9) and blood microparticles (q-value: 1.0E-7). Most of these proteins are involved in atherosclerosis and metabolic disease. The remaining proteins mostly decreased more in TKM58 and INUS-30 compared to INUS-50.

Discussion

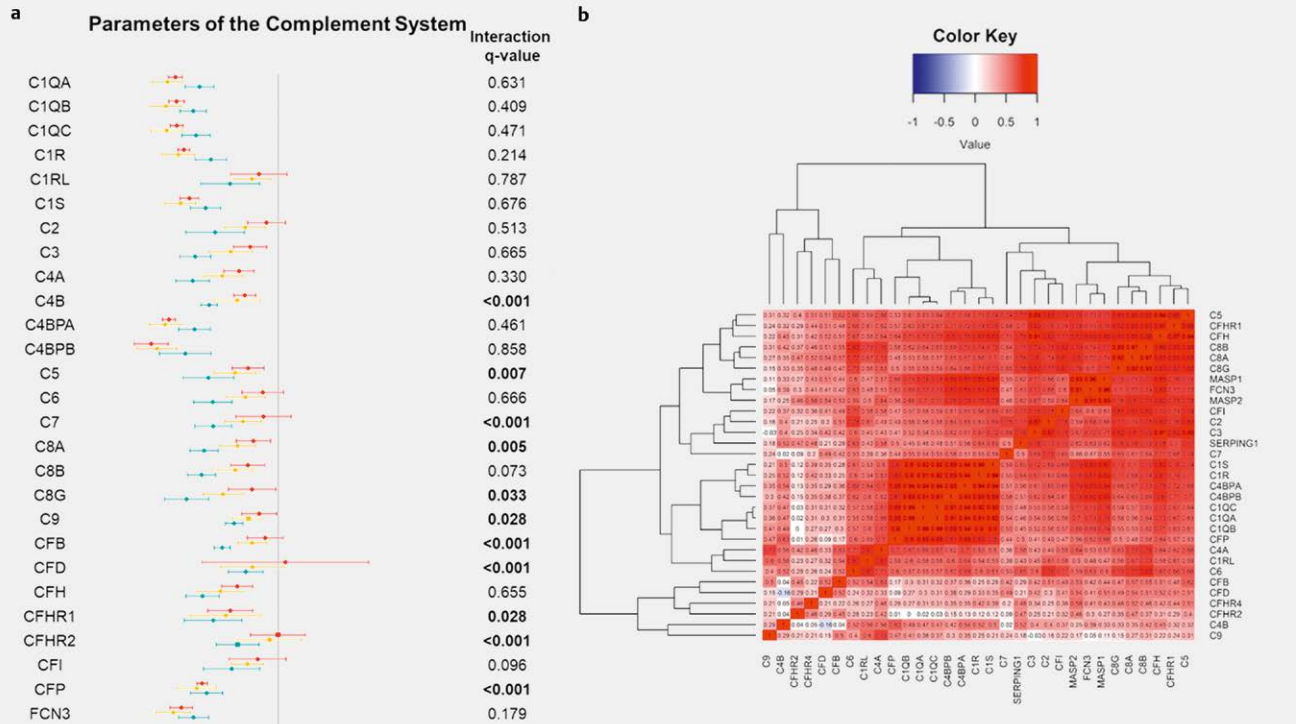
Therapeutic apheresis is an established treatment option for patients with severe lipid disorders, which could not be adequately



▶ **Fig. 4** Effect of apheresis on coagulation system: **a**: Protein abundance ratio between before (pre) and after (post) apheresis using INUS-50 (red), INUS-30 (yellow), and TKM58 (blue). Interaction q-value < 0.05 indicates the 3 methods have significant differences. **b**: Spearman's correlation and hierarchical clustering analyses of the selected proteins. The values are Spearman's Rho.



▶ **Fig. 5** Effect of apheresis on immunoglobulins: **a**: Protein abundance ratio between before (pre) and after (post) apheresis using INUS-50 (red), INUS-30 (yellow), and TKM58 (blue). Interaction q-value < 0.05 indicates the 3 methods have significant differences. **b**: Spearman's correlation and hierarchical clustering analyses of the selected proteins. The values are Spearman Rho.



▶ **Fig. 6** Effect of apheresis on complement system: **a**: Protein abundance ratio between before (pre) and after (post) apheresis using INUS-50 (red), INUS-30 (yellow), and TKM58 (blue). Interaction q-value < 0.05 indicates the 3 methods have significant differences. **b**: Spearman's correlation and hierarchical clustering analyses of the selected proteins. The values are Spearman's Rho.

treated by routine lipid-lowering measures. LA has been a powerful tool for retarding, preventing or even reducing the atherosclerotic process in patients with lipid disorders refractory to medical treatment [1–3]. Based on the new European Society of Cardiology (ESC) and European Atherosclerosis Society (EAS) guidelines most patients with familial hypercholesterolemia currently do not reach the LDL-C target values even on a maximal dosage of statin/ezetimibe. Many patients are candidates for PCSK9 inhibitor therapy, and a considerable number of patients would require apheresis to reach target lipid levels [13].

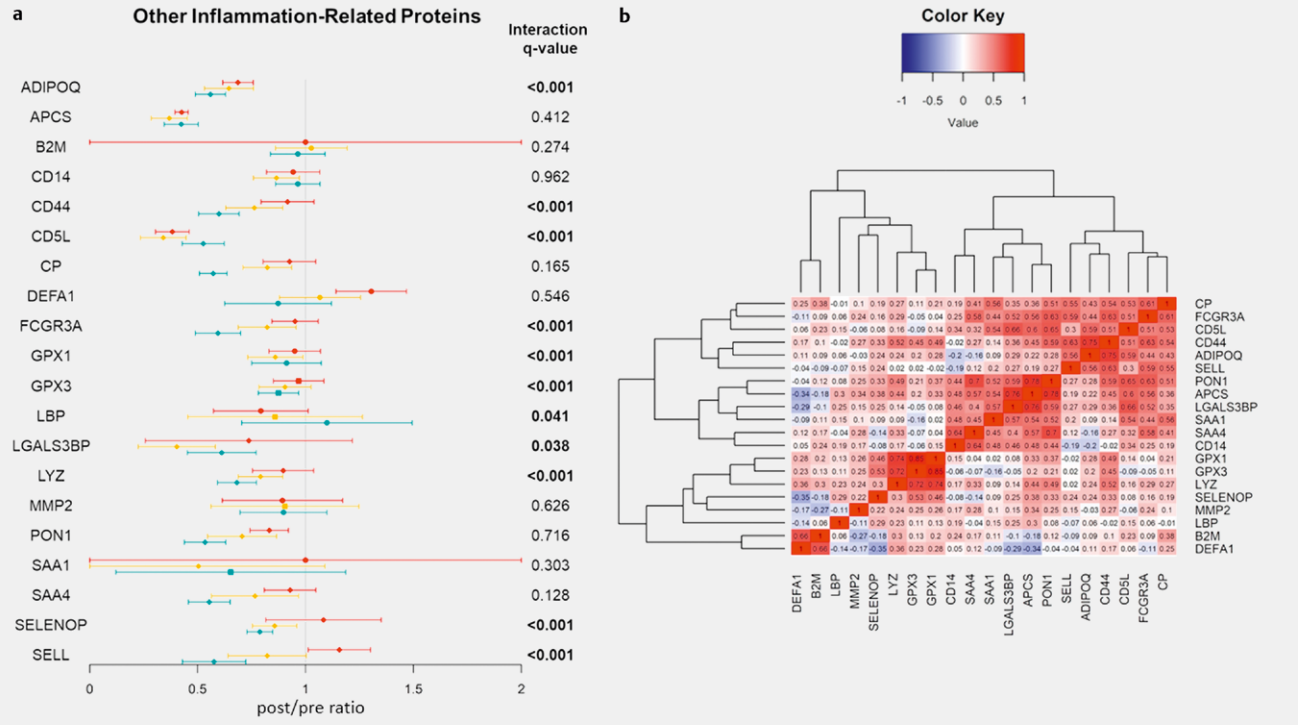
Clearly, beyond its lipid-lowering capacity, LA has been shown to affect several pro- and anti-inflammatory factors, which are known to promote or trigger cardiometabolic disease. This includes the complement cascade, the cytokine network and various other inflammatory molecules [7–11]. So far, it is however not known if the profile of those risk factors can be removed in a differential manner by using different filters in therapeutic apheresis.

In our analysis, we could clearly demonstrate a filter-dependent effect on plasma protein patterns in patients following therapeutic apheresis. Proteomics analysis correlated well with clinical parameters measured in our routine laboratory. Each filter clearly provided a different pattern of protein reduction suggesting the possibility for a precision medicine approach in therapeutic apheresis.

Whereas all filters provided an efficient reduction in LDL, INUS-30 provided an optimal profile on apolipoproteins, fibrinogen and inflammatory proteins. On the other hand, TKM58 exhibited a more significant reduction of several proteins known to play a role in metabolic and cardiovascular disease.

Several key factors involved in the alternative complement pathway were primarily reduced following apheresis with TKM58.

Interestingly complement factor B (CFB) has been demonstrated to be a determinant of both metabolic and cardiovascular features of the metabolic syndrome. CFB correlates significantly with fasting glucose and circulating lipids, risk of endothelial dysfunction and coronary heart disease [14]. Furthermore, CFB mediates podocyte injury and thus may promote diabetic kidney disease [15, 16] and ocular angiogenesis [17]. CFB exerts its pro-angiogenic effect by influencing the vascular endothelial growth factor (VEGF) signaling pathway [17]. Moreover, CFB promotes adipocyte maturation and insulin resistance [18]. CFB plays a key role in the late phase of adipocyte differentiation and subsequent lipid droplet formation [18]. Analysis of the human type 2 diabetes-specific visceral adipose tissue proteome and transcriptome in obesity identified enrichment of CFB [19]. Therefore, CFB may mediate dysfunctional proatherogenic visceral adipose tissue in obese patients with

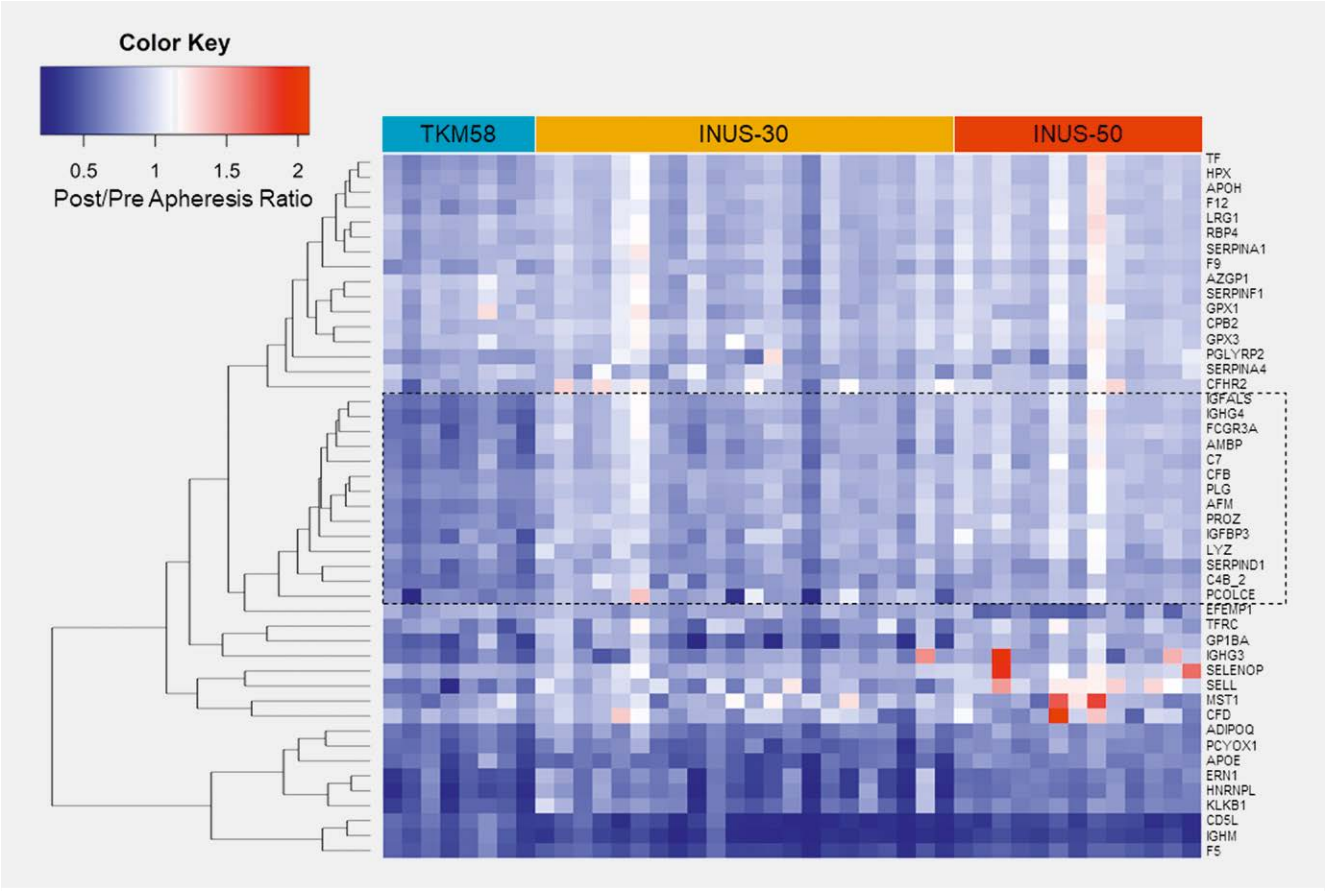


▶ **Fig. 7** Effect of apheresis on other inflammation-related proteins: **a**: Protein abundance ratio between before (pre) and after (post) apheresis using INUS-50 (red), INUS-30 (yellow), and TKM58 (blue). Interaction q-value < 0.05 indicates the 3 methods have significant differences. **b**: Spearman's correlation and hierarchical clustering analyses of the selected proteins. The values are Spearman's Rho.

diabetes [19]. CFB depletion improves glucose homeostasis, adipose tissue function, reduces blood pressure and cardiac hypertrophy and stress [20]. Finally, CFB was associated with incident cardiovascular disease even after adjustment for diabetes. Therefore, targeting CFB will be a valuable approach for the treatment of cardio-renal and metabolic disease. Based on the individual risk profile of patients with metabolic disease and severe lipid disorders the use of an apheresis filter such as TKM58 capable of significantly reducing the circulating levels of CFB may provide additional therapeutic benefits.

Another protein that was reduced most efficiently by the TKM58 filter is afamin. In previous studies using regular LDL apheresis, afamin levels were only mildly reduced by about 10 % [21]. Afamin is a liver produced glycoprotein and early marker of metabolic syndrome [22]. Patients with prediabetes and diabetes exhibit high serum afamin levels. Afamin is closely related to hepatic lipid accumulation, liver damage and insulin resistance. Furthermore, afamin constitutes a predictive and diagnostic biomarker for gestational diabetes and may contribute to the progression of the disease [23]. In a pooled analysis in more than 20.000 individuals, plasma concentrations of afamin were found to be associated with prevalent and incident type 2 diabetes [24]. This association was independent of other major metabolic risk factors and therefore afamin may be a promising novel marker and target for the identification and management of individuals at high risk for type 2 diabetes [24]. Consistent with these findings it has been shown that early restoration of insulin sensitivity after biliopancreatic diversion was associated with reduction of both apolipoprotein A-IV and afamin [25]. Moreover, urinary afamin levels were found to be associated with progression of diabetic nephropathy[26]. The urinary afamin to creatinine ratio was significantly higher in patients who progressed to a more severe stage of diabetic nephropathy [26]. Finally, afamin levels correlated negatively with high-density lipoprotein cholesterol (HDL) and positively with high blood pressure [27]. Therefore, a more effective removal of afamin may provide additional benefits for patients on LA treatment with progression of comorbidities of the metabolic syndrome. An apheresis regimen allowing reduction of afamin may help in prevention of diabetes, steatosis hepatis, hypertension and development of diabetic kidney disease clearly defining therapeutic benefits beyond the mere reduction of lipids.

Another factor that was selectively reduced by the TKM58 filter was FcγRIIIa (CD16). Previous studies have reported that Fc receptor IIIA of immunoglobulin G is involved in the development of coronary heart disease [28]. Thus, the enhancement of FcγRIIIa on monocytes correlated with the induction of inflammatory signals leading to atherosclerosis [28]. FcγRIIIa polymorphism has been identified as a risk factor for coronary artery disease [29]. Finally, increased soluble FcγRIIIa has been found in plasma of patients with CAD [30]. The FcγRIIIa-level was related to the number of affected coronary arteries and positively with LDL cholesterol [30]. Therefore, reduction and inhibition of FcγRIIIa or its signaling has been suggested as another



▶ **Fig. 8** Protein cluster with the most differential trajectory in TKM58 compared to INUS-30 and INUS-50: 2-Way ANOVA analysis corrected for age, sex, patient and number of previous apheresis interventions has been performed and the ratios of post vs pre apheresis significantly changing proteins (Q-value of interaction < 0.0001) were visualized in a heatmap performing hierarchical clustering to reveal significant groups of proteins. Hierarchical clustering revealed a group of 14 proteins (dashed rectangle) which were decreased substantially more after apheresis in TKM58 compared to INUS-30 and INUS-50. Functional and Pathway enrichment analysis showed that this list of proteins is significantly enriched with Complement and Coagulation cascade KEGG pathway (q-value: 0.0053), while the proteins are secreted (q-value: 3.4E-10) and present in the extracellular space (q-value: 1.2E-9) and blood microparticles (q-value: 1.0E-7).

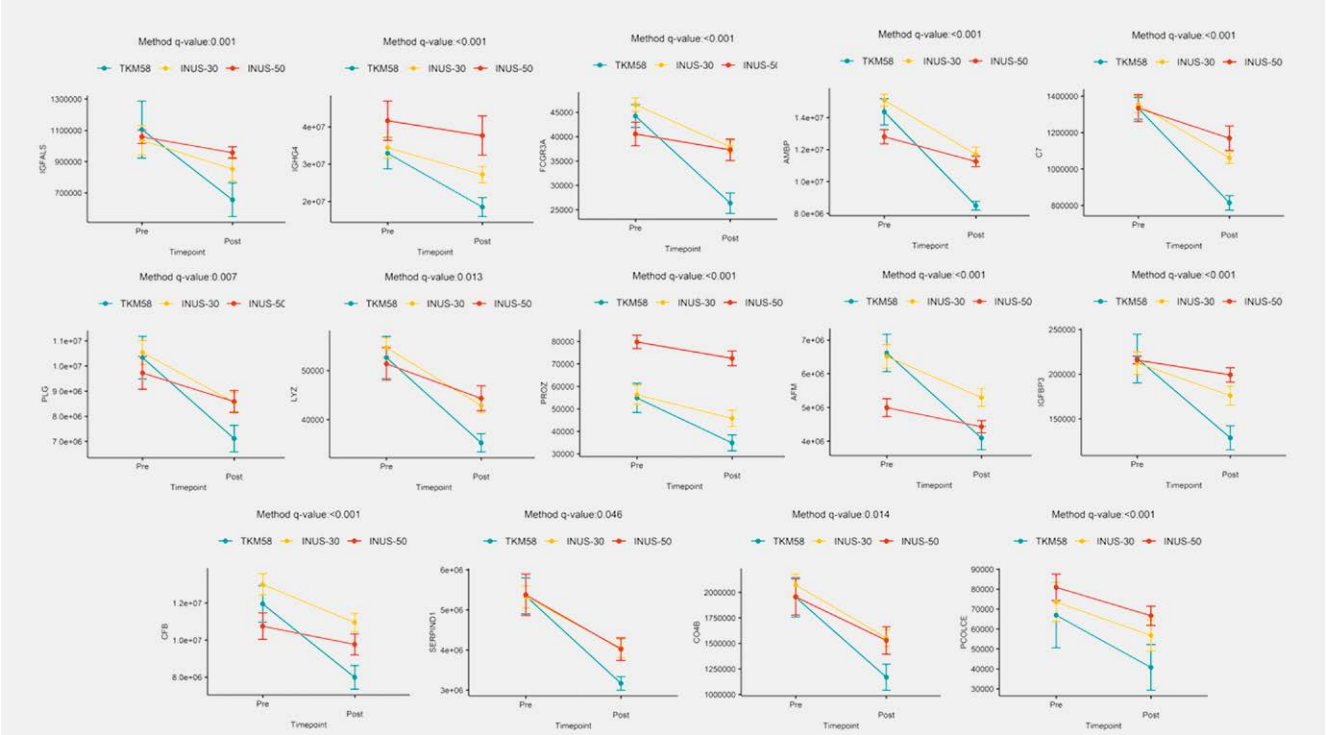
promising approach for the prevention and treatment of coronary heart disease [27]. The use of apheresis filters capable of reducing these factors in high-risk patients with cardiovascular disease may provide a novel potent therapeutic modality.

Other proteins that have been implicated in metabolic-vascular disease that are differentially regulated by the 3 different filters include α1-microglobulin/bikunin precursor (AMBP) and insulin-like growth factor binding protein 3 (IGFBP-3). An association between single nucleotide polymorphism of AMBP and risk of atherothrombotic stroke has been shown [31]. High levels of AMBP may reflect oxidative stress. Proteomics analysis of adipose tissue in chronic kidney disease revealed significantly elevated levels of this protein [32]. IGFBP-3 in turn has been identified as a key factor of the secretome of senescent mesenchymal stromal cells and is able to regulate cell proliferation and apoptosis [33]. IGFBP-3 measured by mass spectroscopy was correlated with incident chronic kidney disease in patients with diabetes [34].

However, continuous use of filters, such as TKM58, capable of removing factors shown to play a critical role in metabolic-vascular disease may not be advocated since regenerative intervals are needed. First of all, such a continuous use in patients on LA treatment on a weekly basis will lead to a significant chronic loss of total protein, immunoglobulins and albumin which may be harmful. Secondly, other protective proteins that have been shown to exert an anti-atherosclerotic effect such as heparin cofactor II [16, 35, 36] are removed at an increased level by the use of TKM58.

In conclusion, our data show that proteomics analysis is a reliable method clearly correlating with routine clinical chemistry. Moreover, the method is suited to detect new biomarkers and risk factors.

Therapeutic apheresis is a powerful method to reduce significantly not only established risk factors such as lipoproteins and other proatherogenic peptides and proteins from the plasma of patients. This may explain the significant additional benefit of therapeutic apheresis seen in our patients with familial lipid disorders in preventing further major cardiovascular events. The key message of this study relates to the fact that the use of different filters may allow an even better removal of major cardiovascular risk factors.



▶ **Fig. 9** Most pronounced reduction differences after LA using 3 different methods: Scatterplots of proteins belonging to the cluster with the most differentially proteins among TKM58, INUS-30 and INUS-50 columns. Statistical analysis was performed using 2-way ANOVA and correcting for Age, Sex, Patient and number of previous repeated apheresis interventions, as well as for multiple testing using Benjamini-Hochberg method. Average relative quantities and their standard error pre and post are shown in figures. All interaction q-values were less than 0.0001. IGFALS, Insulin-like growth factor-binding protein complex acid labile subunit; IGHG4, Immunoglobulin heavy constant gamma 4; FCGR3A, Low affinity immunoglobulin gamma Fc region receptor III-A; AMBP, Protein AMBP; C7, Complement component C7; CFB, Complement factor B; PLG, Plasminogen; AFM, afamin; PROZ, protein Z; IGFBP3, Insulin-like growth factor-binding protein 3; LYZ, Lysozyme C; SERPIND1, Heparin cofactor 2; C4B, Complement C4-B; PCOLCE, Procollagen C-endopeptidase enhancer 1.

Therefore, we propose for the future to design a study comparing the use of a cascade filtration model using different filters with the current model of continuous apheresis with the same filter. Here we should embark on a strategy of precision medicine in therapeutic apheresis. This will allow for a more individualized approach for our patients on LA based on their individual risk factors, thus opening the door for new indications for this powerful therapeutic paradigm for other high-risk metabolic-vascular patients.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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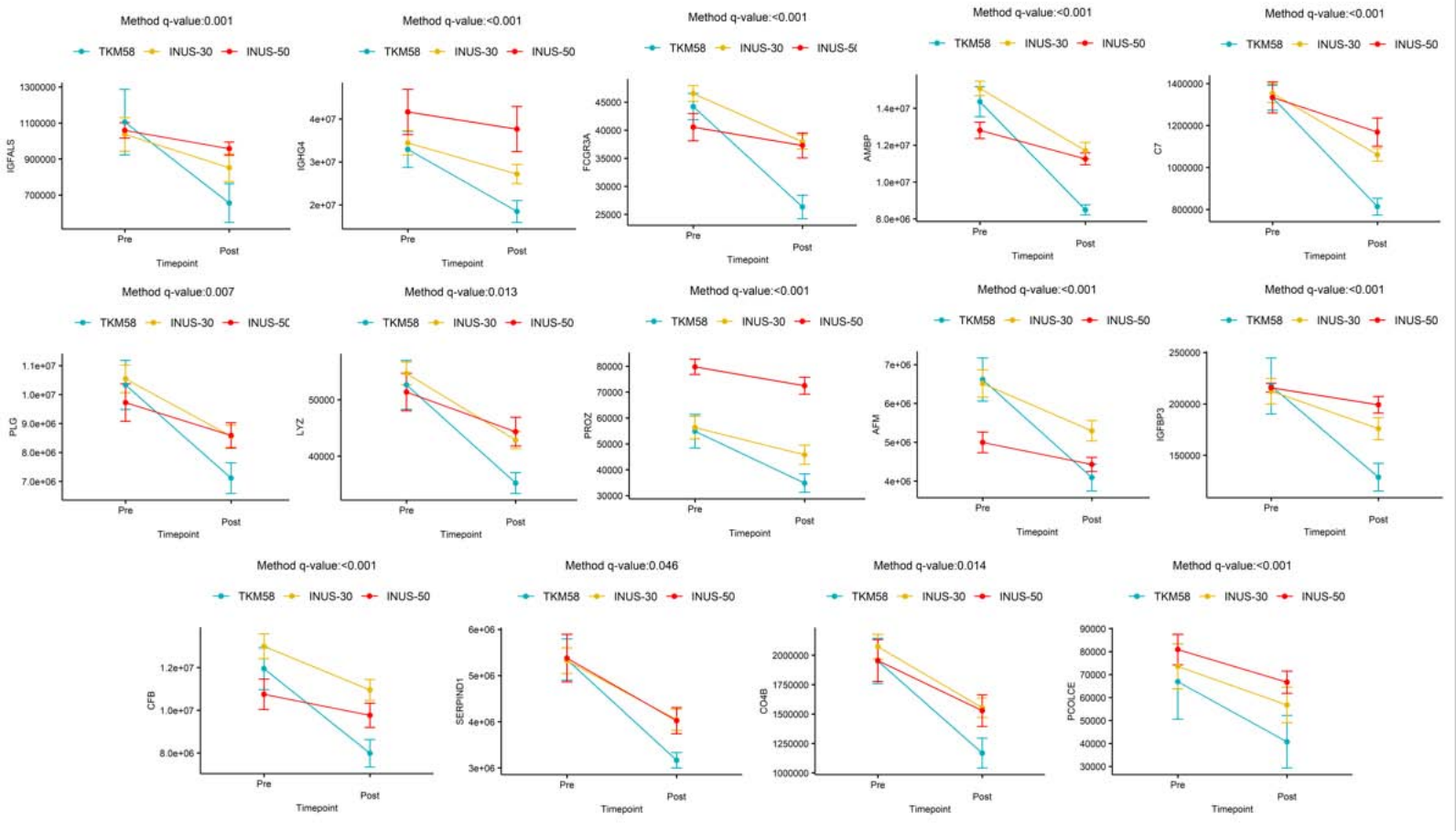
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