Int J Clin Exp Med 2018;11(9):9715-9721
www.ijcem.com /ISSN:1940-5901/IJCEM0075006

Original Article

Proteomic analysis of the rest filtered protein after double filtration plasmapheresis

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Received July 25, 2017; Accepted July 2, 2018; Epub September 15, 2018; Published September 30, 2018

Abstract: To investigate the protein clearance of double filtration plasmapheresis (DFPP) and component of the rest filtered protein, five patients with underlying systemic lupus erythematosus, amyotrophic lateral sclerosis, multiple myeloma or waldenstrom macroglobulinemia were included. All the included patients were treated with DFPP. Proteins of the rest filtrate after DFPP were detected by mass spectrometry. Metascape was performed based on the gene ontology and KEGG database. More than two hundred proteins were detected in the discarded filter plasma of plasmapheresis. Comprehensive proteomic profiling reveals that the filtered proteins are involved in protein activation cascade, regulation of proteolysis, endocytosis and blood coagulation. The clearance of these proteins may have beneficial effects on microcirculation and reduce chronic inflammation, but could also concomitantly induce side effects, such as bleeding risk.

Keywords: Chronic inflammation, DFPP, microcirculation, rheology, proteomic analysis

Introduction

Plasmapheresis is a widely used procedure, in which the extracorporeal separation of blood components would produce filtered plasma [1, 2]. The plasma filtering from the whole blood can be accomplished via the use of semipermeable membranes which mainly depend on the size of different particles [3]. Filtered plasma is discarded and blood cells along with the replacement colloid such as fresh frozen plasma (FFP) or albumin are returned to the patient. In fact, the replacement of the removed plasma needs a voluminous amount of FFP or albumin. Double filtration plasmapheresis (DFPP) was designed to selectively remove the immunoglobulin fraction from the serum to minimize the required volume of substitution fluid. In this procedure, it usually needs 0.4-1.0 L of an 20% albumin solution or 0.2-0.4 L fresh plasma as the replacement fluid, which is equivalent to 2.5-5.0 L of fresh plasma used in regular plasma exchange [3].

DFPP is often employed to rapidly reduce the circulating concentration of auto-antibodies, immune complexes or toxins. DFPP has also been proposed as a useful adjunct to chemotherapy for removing circulating immunoglobulins or immunoglobulin components in multiple myeloma and other dysproteinemias [4]. Moreover, DFPP is also used to treat many diseases. Several studies have shown that DFPP can effectively remove auto-antibodies and may have an important adjuvant role in therapeutic options in the treatment of SLE patients with AITD complications [5, 6]. DFPP could improve myasthenia gravis (MG) through an immunomodulatory mechanism, which could effectively increase Treg cell expression, decrease sICAM-1 and consequently improve MG patients' activity [7]. DFPP infusion itself could also produce beneficial effects. Indeed, there is an evidence to confirm that the replacement of a deficient plasma component may be the principal mechanism for action of plasmapheresis in thrombotic thrombocytopenic purpura (TTP) [8]. Other theoretical beneficial effects on immune function include depletion of complement products, fibrinogen, and some cytokines, alterations in idiotypic/anti-idiotypic antibody balance, and improvement in reticulo-endothelial system function [9]. However, the protein species filtered by DFPP, which could help us to

further understand the mechanism of the effect produced by DFPP, are rarely discussed. Therefore, our study was designed to investigate the protein clearance of DFPP and component of the rest filtered protein with proteomic analysis.

Material and method

Patients and treatment

Five patients were included in this study. Two patients (case 1 and case 3) suffered from systemic lupus erythematosus (SLE). Case 2 was diagnosed as multiple myeloma (MM). Case 4 was diagnosed as amyotrophic lateral sclerosis (ALS). Case 5 suffered from waldenstrom macroglobulinemia (WM). Apheresis data were shown in Supplemental Table 1. The written informed consents were obtained from the patients or their partners.

All the included patients were treated with DFPP under the official guidance of plasmapheresis. The workflow of DFPP was shown in Supplemental Figure 1. For DFPP, the EC-30W column and EC-20W column (Asahi Kaswei Medical, Tokyo, Japan) were applied.

Sample collection

The discarded filter plasma was collected from the plasma separator column after extracorporeal circulation (Case 1, 3, 4, 5 for EC-30W and Case 2 for EC-20W). Then the five patients' samples were mixed and prepared for the next experiment.

Protein extraction and digestion

The collected plasma of plasmapheresis was homogenised in RIPA lysis buffer (Fitgene, China), and then centrifuged at 12,000 rpm at 4°C for 20 min. The supernatant was precipitated in acetone overnight, then centrifuged at 12,000 rpm at 4°C, and redissolved in RIPA buffer. The protein concentration was measured with a BCA assay kit (Sigma, USA). 200 μg of each protein sample was reduced and alkylated. Then, Proteins were digested with trypsin (Promega, USA) at 37°C at a ratio of 1:50 (enzyme-to-substrate) overnight.

High-pH reversed-phase chromatography

Peptides were resolved in eluent A (20 mM $HCOONH_4$) (pH 10) and separated using an Ultimate 3000 RSLCnano system equipped with a Phenomenex columns (Gemini-NX 3u C18 110A; 150 * 2.00 mm). Peptide fractionation was performed using a gradient from 5 to 40% B (20 mM $HCOONH_4$, 80% ACN) (pH 10) at 0.2 ml/min over 40 min. Finally, the column was washed at 95% B for 5 min and returned to 5% B for 10 min. The UV detector was set at 214/280 nm, and fractions were collected every 1 min. In total, 5 fractions were pooled, desalted by Strata-X SPE column and dried by vacuum centrifuge for subsequent nano-reversed phase liquid chromatography (nano-LC) fractionation.

RPLC-MS analysis

Each fraction was resuspended in loading buffer (0.1% FA, 2% ACN) and loaded onto a C18 reverse phase column (100-μm inner diameter, 10-cm long, 3-μm resin from MichromBioresources, Auburn, CA). The peptides were separated using the following parameters: 1. mobile phase A: 0.1% FA; 2. mobile phase B: 0.1% FA, 80% ACN; 3. flow rate: 300 nl/min; 4. gradient: B-phase increased from 4% to 50%, 40 min.

The eluted peptides were detected by Q Exactive and MS data were acquired using a data-dependent top20 method, dynamically choosing the most abundant precursor ions from the survey scan (350-1800 m/z) for HCD (high-energy collisional dissociation) fragmentation. Determination of the target value was based on Automatic Gain Control (AGC). Survey scans were acquired at a resolution of 70,000 at m/z 200, and resolution for HCD spectra was set to 17,500 at m/z 200. Normalized collision energy was 30 eV and the under-fill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum-fill time, was defined as 0.1%. The instrument was run with the peptide recognition mode enabled.

The peptide data were analyzed using Protein Pilot Software 5.0 (AB SCIEX, CA), and the main parameters were set as follows: Cys alkylation: MMTS; ID focus: biological modifications; Digestion: typsin; Database: HUMAN_uniprot_2015.8.3.fasta; Search effort: thorough.

Table 1. LC-MS/MS analysis result of the discarded plasma proteins (top 19)

Protein Name	Gene Name	Accession (Swiss-port, TrE MBL)	Score	Peptides (95%)	Mass
Apolipoprotein B-100	APOB	P04114	407.29	344	515605
Serum albumin	ALBU	P02768	201.03	655	69367
Complement C3	CO3	P01024	192.67	196	187148
Alpha-2-macroglobulin	A2MG	P01023	181.6	276	163291
Fibronectin	FINC	P02751	145.02	138	262625
Complement C4-A	A0A0G2JPR0	A0A0G2JPR0	120.33	126	192876
Ig gamma-1 chain C region	A0A087WV47	A0A087WV47	100.98	341	51154
Serotransferrin	TRFE	P02787	99.7	150	77064
Fibrinogen alpha chain	FIBA	P02671	88.68	162	94973
Complement factor H	CFAH	P08603	87.59	76	139096
Ceruloplasmin	CERU	P00450	80.05	65	122205
Fibrinogen beta chain	FIBB	P02675	72.76	219	55928
Ig mu chain C region	A0A087WYJ9	A0A087WYJ9	71.21	143	65701
Complement C5	CO5	P01031	67.67	45	188305
Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	Q14624	65.65	65	103357
Apolipoprotein A-I	APOA1	P02647	65.13	105	30778
Alpha-1-antitrypsin	A1AT	P01009	64.81	93	46737
Plasminogen	PLMN	P00747	62.93	49	90569

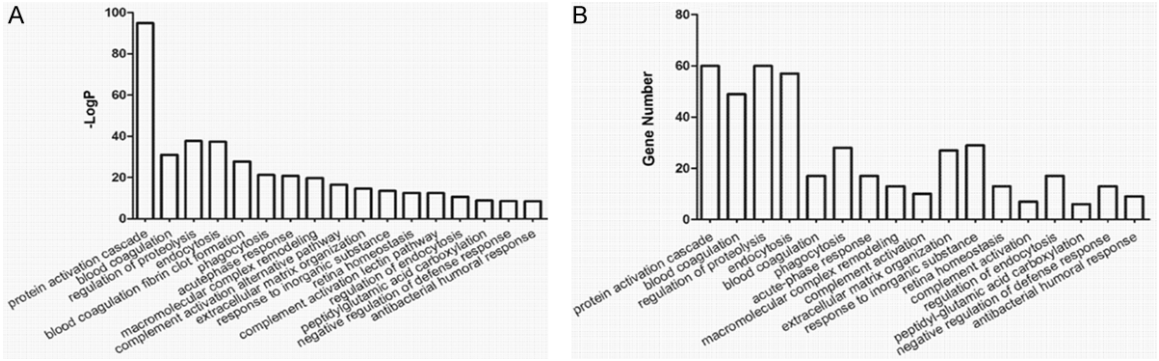

Figure 1. (Write and black) GO analysis of the detected proteins. Functional annotations of the discarded filter plasma of plasmapheresis. (A) is divided by the -Log (*P*) value. -Log (*P*) values for negative logarithm of *P* value, greater value represents more enrichment of significance. (B) is divided by the gene number which trends as (A).

Binformatics analysis

For functional analyses of proteomics of DFPP, GO term analysis was applied to organize genes into categories on the basis of biological processes. Biological pathways defined by Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were identified by Metascape (http://metascape.org/gp/index.html). Metascape online provides a set of functional annotations of a large number of genes. *P*-value was adjusted by the method of Benjamini-Hochberg to control the false discovery rate (FDR). In current study, GO terms and signaling pathways were selected with the threshold of significance being defined as $P < 0.01$, FDR < 0.05, minimum count > 3 and enrichment factor (the ratio between observed count and the count expected by chance) > 1.5.

Result

Proteomics of the discarded plasma after DFPP

LC-MS/MS was firstly applied for protein identification and 292 creditable proteins were detected. ProteinPilot™ Software 5.0 was used

Table 2. KEGG analysis of the detected proteins

Term	Description	LogP	Log (q-value)	InTerm/InList	Signification
hsa04610	Complement and coagulation cascades	-81.06214661	-77.181	50/79	*****
hsa05150	Staphylococcus aureus infection	-23.97770097	-21.511	19/55	*****
hsa05020	Prion diseases	-15.26131559	-13.008	12/35	*****
hsa05322	Systemic lupus erythematosus	-13.52090518	-11.338	17/134	*****
hsa05133	Pertussis	-12.25135215	-10.114	13/75	*****
hsa04977	Vitamin digestion and absorption	-5.443119445	-3.689	5/24	***
hsa05146	Amoebiasis	-3.299434116	-1.746	6/99	**
hsa03320	PPAR signaling pathway	-3.180464649	-1.636	5/69	**
hsa04611	Platelet activation	-2.825151085	-1.327	6/122	*
hsa04975	Fat digestion and absorption	-2.090796201	-0.710	3/41	*

to screen the protein. All the protein name, gene symbol name, number of experimental-measured peptide masses matching the theoretical ones obtained from Swiss-Prot/TrEMBL entries, percentage of the protein sequence covered by the matching peptides (95%) and probabilistic score were listed in Supplemental Table 2. The confidence level of detected protein was determined by the score value > 1 and matched peptides number > 1.

Top 19 proteins were listed in **Table 1**, while the whole creditable proteins list could be found in Supplemental Table 3. Judging from the result, APOB (apolipoprotein-B), ALB (*alb*umin), C3 was adequate in the clearance of proteins after DFPP which was probably due to the size of separation column.

GO (Gene Ontology) analysis

The function of the detected protein was analyzed using GO annotation with the Metascape online. When setting the cutoff standard at *P* value < 0.01, minimum count > 3, enrichment factor > 1.5 and FDR > 0.05, 346 GO terms were found in the biological processes (Supplemental Table 4). In addition, cluster analysis was also conducted to search for the enrichment of target represented by the negative logarithm of the *P* value (-Log (P)) in the ontology of biological processes. The top 17 functional classes were shown in **Figure 1**. According to cluster analyses for biological functions, the majority of the proteins were involved in protein activation cascade, blood coagulation, regulation of proteolysis etc. These functions are known to be strongly associated with microcirculation and chronic inflammation [10-12].

KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis

To gain a better understanding of the function and regulatory mechanism of the discarded plasma protein, we searched for target enrichment in KEGG by Metascape database. There were 292 proteins in 10 pathways with a cutoff standard of $P < 0.01$ and Q value < 0.05 by KEGG pathway analysis (**Table 2**). These results revealed that the discarded plasma protein were associated with infection and immune activation. Some important signaling pathways were involved in digestion and absorption, including vitamin, fat etc. Moreover, the PPAR pathways were enriched according to -Log (*P*) values. The detail discarded proteins participated in the most significance pathway were shown in Supplemental Figure 2.

Protein-protein interaction network in the discarded plasma after DFPP

It is known that the majority of protein do not act as independent entities [13]. They form either transient or stable complexes with other protein that act as scaffolds or regulate the protein activity. To further elucidate the correlation among the discarded plasma proteins, protein-protein interaction networks were visualized by Cytoscape (**Figure 2**). Relationships among proteins were analyzed by integrating the top GO analysis results with the KEGG pathway data. We identified all statistically enriched terms, accumulative hypergeometric *p*-values < 0.01 and enrichment factors > 1.5 were calculated and used for filtering. Remaining significant terms were then hierarchically clustered into a tree based on Kappa-statistical similarities among their gene memberships. 0.3 kap-

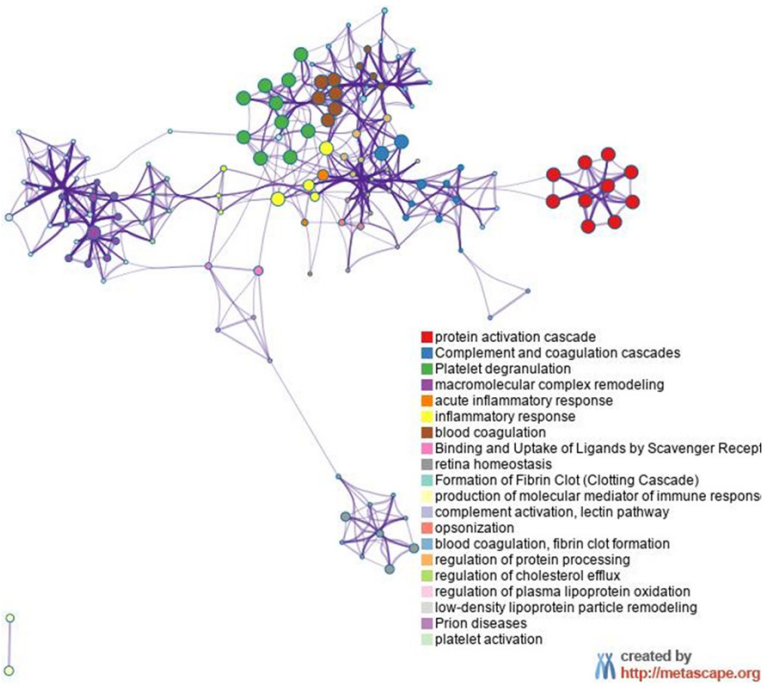

Figure 2. (color) PPI analysis of the detected proteins. The different color circles represent different clusters which combine the top GO terms with KEGG pathway result. As the map indicated, APOB is the largest node in the network.

pa score was applied as the threshold to cast the tree into term clusters (Supplemental Table 5). According to the clusters presentation, APOC3, LPA, LYZ, SERPINA1, HRG, KNG1, APOB, CD44 and SELL are the pivots that may be the key mechanism, which could be used in the modification of substitute components to get better therapeutic effect during DFPP.

Discussion

DFPP is known to capture proteins depending on their size [14]. Plasma proteins with a tertiary structure > 15 nm are eliminated by the lipid filter [15]. Although the correlation between protein MW and diameter is not significant, it is tempting to speculate an additional clearance in DFPP independent of the protein size alone. Based on the GO terms and KEGG terms, it was conspicuous that the discarded plasma proteins consist of lipoproteins, adhesive, rheological and inflammation relevant proteins.

Plasma exchange has been widely used for the treatment of autoimmune, metabolic, and hematological disorders [16], although its clinical efficacy has been documented by randomized controlled studies only for a limited number of diseases [17]. The efficacy of plasma exchange was mostly determined clinically without concomitant monitoring of the pathogenic substances cleared during plasma exchange treatment. The choice of treatment protocol is often empiric due to lack of data from objective and controlled comparisons among different protocols [18]. A major disadvantage of plasma exchange is the unselected removal of all plasma proteins, which often requires replacement of exogenous proteins. The use of plasma as a replacement fluid contributes to the occurrence of major complications in reported studies [19, 20]. DFPP selectively removes high-molecular weight substances including immunoglobulins (Ig) and immune complexes, therefore, it minimizes the loss of albumin and the subsequent need for substitution fluids [21, 22]. Optimal protocols for DFPP treatment, nevertheless, remain undetermined.

Plasma exchange removes all components in the serum such as albumin, globulins, fibrinogen, Igs, and lipids by a constant percentage. The cumulative effects of serial plasma exchange depend on both the extent of removal of plasma constituents during each session and the rate of recovery between exchanges [23]. Keller et al. reported that plasma fibrinogen concentrations fell to a mean of 25.0% of initial levels during an individual plasma exchange session and were sequentially reduced to 10.7% after 5 consecutive daily exchanges [24]. However, DFPP is semi-selective for the removal of larger proteins. The removal rates of serum substances by a single DFPP session were closely related to their molecular weights: highest for IgM and lowest for albumin [25]. It is likely that various serum substances may have different cumulative clearance after DFPP. Lin et al. [26] investigat-

ed the cumulative effects of fibrinogen and von Willebrand factor (vWF) clearance after an intensive course of DFPP; the clearance rate of fibrinogen was approximately 66% in each session and 86% after 5 consecutive sessions of treatment. In contrast, the vWF remained at 50% of the baseline level after several sessions and usually returned to the level before DFPP treatment in the next session. The longer half-life of fibrinogen as compared to vWF accounted for its higher clearance rate during DFPP treatment.

In our study, we have investigated the discarded plasma proteins by DFPP method with the help of proteomics. Our results suggested that APOC3, LPA, LYZ, SERPINA1, HRG, KNG1, APOB, CD44 and SELL proteins maybe the mainly the key point to influence the therapeutic effect by DFPP, which could help us to optimize the use of DFPP and extend its use according to the international guidelines, and, in our opinion, a greater attention to the growing therapeutic indications will increase its application and will improve the therapeutic efficacy. Our results indicate that proteomic technique is a useful approach for the investigation of proteins surface-adsorbed onto hemodialysis membranes, and may provide a molecular base for the interpretation of the efficacy and safety of anticoagulation treatment during renal replacement therapy.

There are also some limitations in our study. Firstly, the number of patients undergoing the particular DFPP method was small. Secondly, patients could not be randomized to treatment options due to historic individual side effects following the treatment with one or more hemodialysis method. Thirdly, the impact by individual drug therapy in some patients on the component change in discarded plasma could not be excluded.

In conclusion, this results prove not only the reliability of DFPP therapy, but also could be the selection evidence of substitute component for plasma exchange to gain the better therapeutic effect.

Acknowledgements

This work was supported by grants from South Wisdom Valley Innovation Research Team Program (2014CXTD04); The EU FP7 Program, UroSense 286386 (2011); ISN GO R&P Grant (06-019) (2014); Science and Technique Program of Tianhe District, Guangzhou (NO. 114KW041); Science and Technology Planning Project of Guangdong Province, China (NO. 2014A0202-12196) and Medical scientific research fund project in Guangdong Province (NO. B2013260).

Disclosure of conflict of interest

None.

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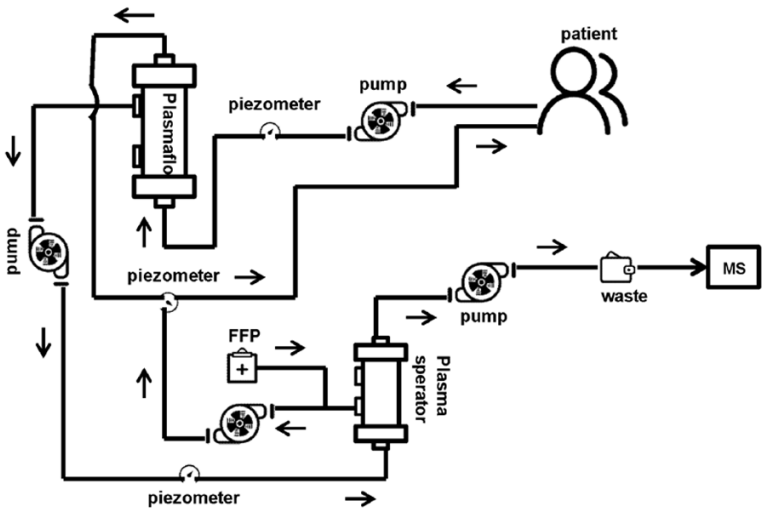
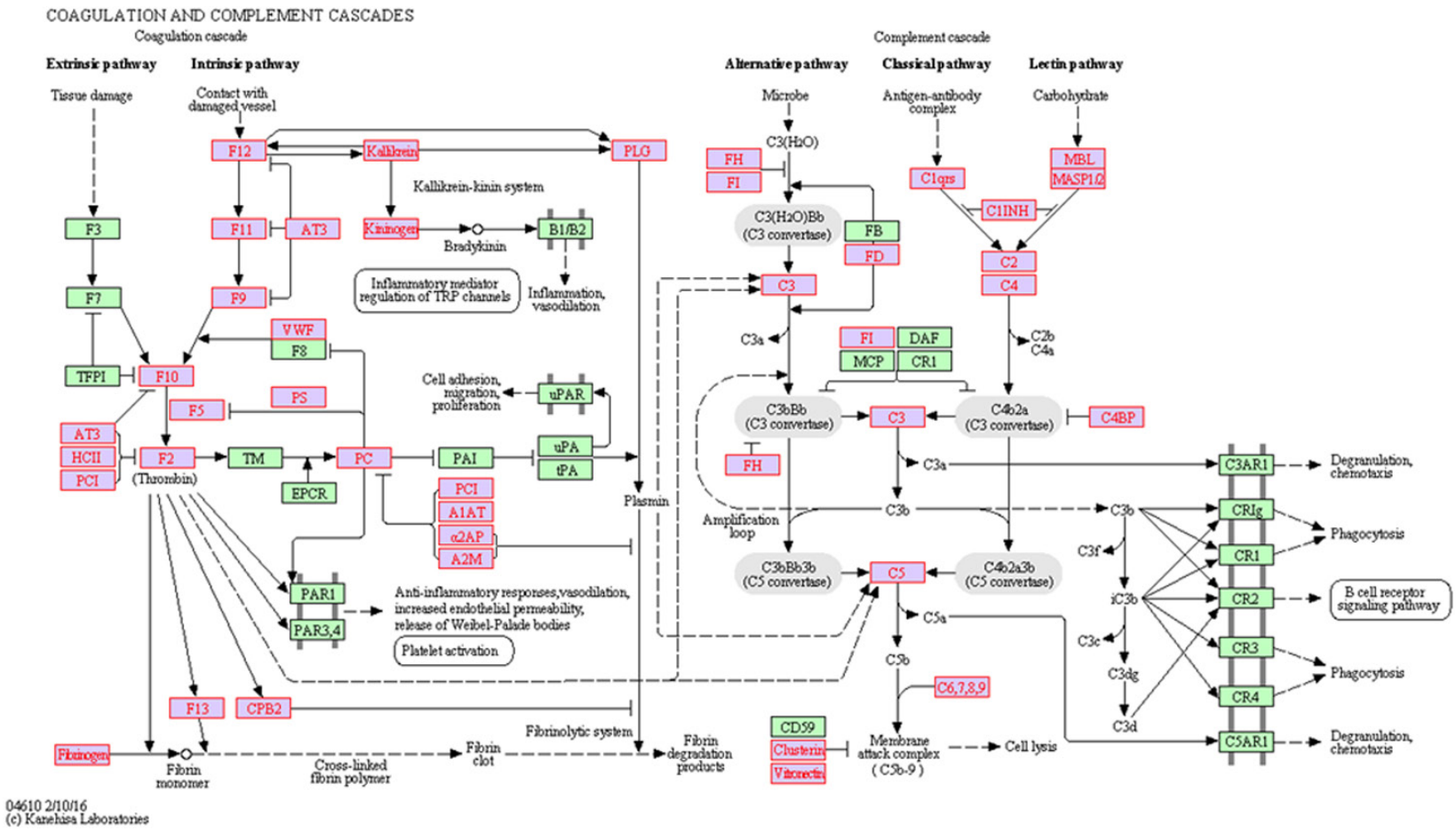
Supplemental Table 1. The detail informations of participation patients were shown, including the demographic data, apheresis data (mainly the parameters of modified DFPP therapy)

	Patients				
	Case 1	Case 2	Case 3	Case 4	Case 5
Demographic data					
Age (years)	27	58	23	54	79
Sex	F	F	M	M	M
Underlying Disease	SLE	MM	SLE	ALS	WM
Apheresis data					
Treated plasma volumn (ml)	3400	2600	3200	3600	2200
Session duration (min)	140	110	105	120	90
Blood pressure (mmHg)	172/96	118/74	144/98	121/78	160/82
Sphygmus (per/min)	80	96	75	72	64
Heparin [prime dose/total dose] (mg)	20/40	22/35	34/54	36/54	24/36
Plasma Separation Flow Speed (L/h)	1.5	1.5	1.8	1.7	1.5
Returned Plasma Flow Speed (L/h)	1.5	1.5	1.8	1.7	1.5
Disuesd Plasma Flow Speed (L/h)	0.4	0.4	0.2	0.25	0.3
Blood Flow (ml/min)	100	100	100	100	100
Blood Aceess	Right Femoral Vein	Right Internal Jugular Vein	Right Internal Jugular Vein	Left Internal Jugular Vein	Right Internal Jugular Vein
PlasmaFlo	OP-08	PE-08	OP-08W	PE-08	PE-08
Plasma Seperator	EC-30	EC-20W	EC-30W	EC-30W	EC-30W



Supplemental Figure 1. (Write and black) The modified DFPP workflow. The detected proteins come from the discarded plasma components when the plasma go through the plasma seperator.



Supplemental Figure 2. (color) The Complement and Coagulation Cascades Pathway. The red box stand for the partial proteins which detected in discarded plasma. The first and second row are the pathway category; the third and fourth row are the enrichment of pathways represented by Log (*P*) values and Log (q-value). The last row stands for the degree of signification.