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Effect of double-filtration plasmapheresis for antibody-mediated rejection on haemostasis parameters and thrombin generation

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Short title: Plasmapheresis and clotting factors

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Abstract

Introduction: Donor-specific alloantibodies (DSAs) cause kidney-allograft loss in chronic antibody-mediated rejection (CAMR). Treatment relies on blocking antibody-producing cells and removing DSAs by apheresis: e.g., double-filtration plasmapheresis (DFPP).

Materials and Methods: To determine the impact of DFPP (6 or 8 sessions/patient) on clotting factors and natural anticoagulants, and on thrombin generation, we performed a prospective and observational study in five CAMR kidney-transplant patients who received DFPP plus rituximab therapy. Thrombin generation was performed with 5 pM tissue factor without and with 2 nM recombinant human thrombomodulin.

Results: Within the first DFPP session, mean levels of high molecular-weight proteins (fibrinogen, FV, FVIII, FXI, FXIII, von Willebrand factors and α2-MG) decreased significantly to <50% of baseline values, whereas levels of low molecular-weight factors (<100 kDa) were not significantly modified, except for protein S and TFPI. Of note, binding-protein (BP) S, i.e., C4BP, was significantly decreased. Over the course of successive DFPP sessions, both high and lower molecular-weight proteins (<100 kDa) with longer half-lives (>2 days, prothrombin and factor XII) were significantly decreased. DFPP also highly affected thrombin generation in the absence of thrombomodulin but not significantly in the presence of thrombomodulin. After the first DFPP session, mean endogenous thrombin potential (ETP) and peak thrombin (PH) significantly decreased when the thrombin generation assay was performed without thrombomodulin (respectively, 1033 nM.min for ETP and 208 nM for PH after the first DFPP session compared to 1536 nM.min and 274 nM at baseline). In the presence of thrombomodulin, there was no profound decrease in ETP and PH (respectively 737 nM.min, and 160 nM after the first DFPP session compared to 763 nM.min and 170 nM at baseline). After the last session, mean ETP and PH with thrombomodulin decreased respectively to 512 nM.min and 119 nM..

Conclusions: DFPP significantly removed high molecular-weight proteins from the haemostatic system and profoundly decreased levels of protein S and TFPI. Overall thrombin-generation balance was only moderately affected in the presence of thrombomodulin. Nevertheless, high depletion of fibrinogen, FXIII and Von Willebrand Factor may expose patients to an increased risk of bleeding.

Highlights

- Double-filtration plasmapheresis (DFPP) is used to treat chronic antibody-mediated rejection in kidney-transplant patients.
- We assessed the effect of DFPP on the clearance of clotting factors and natural anticoagulants.
- The clearance of hemostatic proteins was highly dependent on their molecular weights, except for protein S and TFPI.
- DFPP induced high depletion of FXIII, fibrinogen and VWF
- DFPP profoundly decreased thrombin generation in the absence of thrombomodulin but thrombin generation in the presence of thrombomodulin was only slightly decreased

Keywords: double-filtration plasmapheresis, chronic antibody-mediated rejection, clotting factors, thrombin generation, thrombomodulin resistance

Abbreviations:

aPTT: activated partial thromboplastin time

α2MG: α2 macroglobulin

CAMR: chronic antibody-mediated rejection

DFPP: double-filtration plasmapheresis

DSA: donor-specific allo-antibody

ETP: endogenous thrombin potential

MFI: mean fluorescence intensity

PH: thrombin peak (peak height)

PT: prothrombin time

SST: serum-separating tubes

TGA: thrombin-generation assay

TM: thrombomodulin

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Introduction

The main cause of kidney-allograft loss, apart from patients dying with a functioning graft, is chronic antibody-mediated rejection (CAMR), which is usually a result of injuries mediated by donor-specific alloantibodies (DSA) within the kidney allograft [1–9]. DSAs may be present at pretransplant: if this is the case, we need to manage HLA-incompatible kidney transplantation. In this setting, the likelihood of developing CAMR is very high [10]. Conversely, DSA(s) may appear at posttransplant, i.e., a *de novo* DSA. This can be caused by inadequate/under-immunosuppression, which may be related to a patient's non-adherence to an immunosuppressive-drug regimen or the physician attempting to minimize immunosuppression [2,8,9,11]. *De novo* DSAs can cause (chronic) antibody-mediated rejection, ultimately resulting in allograft loss.

When CAMR is diagnosed, there are very few possible treatments: these mainly rely on increasing immunosuppression to target B-cells or plasma cells, e.g., rituximab or bortezomib, respectively [12,13]. The clinical efficacy of these interventions may be enhanced by rapid clearance of DSAs from the blood, which can be achieved by apheresis. In this setting, apheresis can consist of plasmapheresis (PP), double-filtration plasmapheresis (DFPP), or semi-specific immunoadsorption. DFPP is a blood purification process that removes pathogenic substances, e.g., auto-antibodies, immune complexes, cholesterol, etc. from the blood using specific filters for specific diseases. Blood is obtained from appropriate brachial, jugular, or femoral veins. The DFPP system includes two filters.

At the beginning of the procedure, blood passes through the first filter, called the ”plasma separator”, which is used to separate the white blood cells, the red blood cells, and platelets from the plasma. The plasma without the cellular components is then passed through the second filter, the “plasma component separator”, where the macromolecules are selectively removed and discarded. This filter includes several special membranes that can be specifically chosen to match different medical disorders.

Semi-specific immunoadsorption enables treatment of large volumes of plasma but the immunoadsorption columns are very expensive. Plasmapheresis can treat low plasma volumes and is associated with clearance of clotting factors. DFPP can treat larger plasma volumes than PP, thus making it potentially more efficient at clearing a DSA(s); however, it is not yet known whether DFPP affects clotting factors (apart from fibrinogen, von Willebrand, and factor XIII [14–17]) The main objective of this prospective study, which focused on kidney-transplant patients experiencing CAMR

and treated with DFPP, was to determine the impact of DFPP sessions on all clotting factors and on natural anticoagulants.

Materials and methods

Patients and methods

This single-center study was performed at the Nephrology and Kidney Transplantation Unit at Grenoble University Hospital (France). The study included six male kidney-transplant recipients that had a mean age of 50 years. The patients had received a transplant kidney on average 17.6 months previously. All presented with CAMR. Mean serum-creatinine level was 191 µmol/L, i.e., estimated glomerular-filtration rate was 39.4 mL/min. Mean proteinuria was 0.72 g/L. Two patients had two DSAs, whereas four patients had only one DSA. The mean fluorescence intensity (MFI) of the DSAs was 6844 (see Table 1).

CAMR was diagnosed in kidney-allograft biopsies performed because of the occurrence of a *de novo* DSA associated with impaired allograft function. When a diagnosis of CAMR was made, patients were offered treatment consisting of six (*n*=3) or eight (*n*=2) sessions of DFPP associated with rituximab therapy (i.e., two injections of 375 mg/m² each) (Supplementary Figure 1). Before and after each DFPP session, routine calcium, fibrinogen, albumin, hemoglobin level, and white blood-cell and platelet counts were assessed. In addition, for the purpose of this study, blood was withdrawn before and after the first and the last DFPP session to assess the clotting factors, as well as thrombin generation. Each patient signed an informed-consent form to have extra blood samples taken for this study.

Double-filtration plasmapheresis

We used a DFPP system produced by Asahi Kasei Medical (Tokyo, Japan). The plasma-separation filters were OP-08 W; the secondary filters were EC-30 W for the first session and EC-20 W for the subsequent sessions. All DFPP treatments were performed to remove immunoglobulin (Ig) or substances with a similar molecular weight. Blood-flow rate was ~80 mL/min and plasma separation was ~30% of maximum blood-flow rate. We infused 100 mL of 20% albumin solution at the beginning of each session to replace the volume in the waste bag and to maintain colloidal osmotic pressure of the plasma. The infusion was linked to the DFPP circuit. Anticoagulation was based on ACD-A

anticoagulant citrate-dextrose solution (Macopharma, Mouvaux, France). During DFPP we infused calcium gluconate, i.e., one bolus of 1.5 g, and then 1.0–1.5 g/h according to ionized calcium, which was maintained at ~1.3 mmol/L. Neither fresh frozen plasma nor exogenous clotting factors were administered to patients throughout the study.

Blood sampling and plasma preparation

Venous blood samples were collected immediately before and after the first and the last DFPP sessions into vacutainer tubes (Becton Dickinson, Le Pont de Claix, France) containing 0.109 M trisodium citrate (for all assays except α2-MG assay) and into SST blood tubes (for the α2-MG assay).

Citrated poor platelet plasma (PPP) was prepared by centrifugation at 2,500 *g* for 15 min. Prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen levels were determined immediately. For the other assays, plasma was prepared by double centrifugation at 2,500 *g* for 15 min, collected, quick-frozen, and then stored at –80°C until analysis. The plasma samples were then thawed at 37°C in a water-bath for 5 min. Serum was prepared by centrifugation of SST blood tubes at 2000 *g* for 10 min, collected, and then stored at -80° until analysis.

Assays

Hemostasis assays

Most hemostasis assays were performed using a STA-R Evolution coagulometer (Stago, Asnières, France) using the following reagents: STA®-Neoplastin® CI Plus (Stago) for PT, STA®-PTTA (Stago) for aPTT and STA®-Liquid Fib (Stago) for fibrinogen activity (Clauss method). Prothrombin, FV, FX levels were determined with STA®Neoplastin® CI Plus and, respectively, with STA®-Deficient II (Stago), STA®-Deficient V (Stago), and STA®-Deficient X (Stago). FVII level was determined with Dade® Innovin® (Siemens, Marburg, Germany) and with STA®-Deficient VII (Stago). FVIII, FIX, FXI, and FXII levels were determined with STA®-CK Prest® (Stago) and, respectively, with STA®-Immunodef VIII (Stago), STA®-Immunodef IX (Stago), STA®-Immunodef XI (Stago), and STA®-Imminodef XII (Stago). Antithrombin (activity), protein C (activity) and free protein S (antigen) were determined, respectively, with STA®-Stachrom® Antithrombin (Stago), STA®-Stachrom® Protein C

activity, and STA®- Liatest® Free Protein S (Stago). Von Willebrand antigen level and Von Willebrand activity were, respectively, determined with STA®-Liatest® VWF:Ag (Stago) and with Hemosil® Von Willebrand Activity (Werfen, Le Pré Saint Gervais, France). FXIII antigen levels were determined by immunoturbidimetry on a STA-R coagulometer with K-Assay Fact XIII (Stago). Total TFPI levels were determined with Asserachrom total TFPI (Stago) ELISA kit. α_2-macroglobulin levels were determined on a Optilite® analyzer (The Binding Site, Birmingham, UK) with α_2-macroglobulin Optilite® kit (The Binding Site).

Thrombin generation assay (TGA)

Thrombin generation was assessed using a calibrated automated thrombogram assay (Thrombinoscope BV, Maastricht, Netherlands) on an automated fluorometer (Fluoroscan Ascent, ThermoLab Systems, Franklin, USA). Coagulation was triggered by 5 pM of tissue factor (TF) and 4 µM of pro-coagulant phospholipids (PL) (PPP Reagent® (Thrombinoscope BV)). TGA was performed with and without 2 nM of recombinant human thrombomodulin (Sekisui Diagnostics, Stamford, USA) Thrombomodulin concentration was chosen to halve the mean ETP in healthy subjects. All TGAs were run in triplicate in 96-well plates under standard conditions. Briefly, 80-µL of PPP (poor platelet plasma) was mixed with 20 µL of tissue-factor/phospholipids or with 20 µL of the tissue factor/phospholipids/thrombomodulin mixture. Plates were incubated for 10 min at 37°C in an automated fluorimeter (Fluoroscan Ascent, ThermoLab Systems) before adding the fluorogenic substrate (ZGGR-AMC) and calcium (FluCa Kit®, Stago, France). Raw data were analyzed using Thrombinoscope software, V5.0 (Thrombinoscope BV). Data from Thrombinoscope 5.0 software were exported to GraphPad Prism 5.0 software to superimpose several curves.

C4 binding-protein assay

C4 binding-protein (C4BP) level was quantified using an in-house sandwich ELISA with polyclonal sheep IgG anti-human C4BP (#PC026, The Binding Site) as the capture antibody, and monoclonal mouse anti-human C4BP (#A215, Quidel, San Diego, USA) as the detection antibody.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 5.0 software using paired Wilcoxon rank tests to compare parameters with baseline values. Two-sided significance tests were used throughout.

p_1 denotes the change between baseline and immediately after the first DFPP session. p_2 denotes the change between baseline and immediately before starting the last DFPP session. p_3 denotes the change between baseline and immediately after the last DFPP session.

Results

Impact of DFPP on hemoglobin level, platelets, PT, and aPTT within a session and over a series of sessions

Hemoglobin level was not affected by DFPP, reflecting an absence of hemodilution (Figure 1, Supplementary table 1). PT and aPTT were significantly increased after the first and the last session. Platelet count was not significantly decreased after these sessions.

Impact of DFPP on clotting factors, natural anticoagulants, and von Willebrand factor

After the first DFPP session, plasma levels of high molecular weight proteins (i.e., >150 kDa) were decreased more (fibrinogen, FV, FVIII, FXI, FXIII, α2-MG, and von Willebrand factor) than proteins with a lower molecular weight (<100 kDa) (Figure 2, Supplementary table 1), except for protein S and TFPI.

Repeated DFPP sessions caused a greater decline in the levels of several hemostatic proteins (Figures 3 and 4, Supplementary table 1,). After the last DFPP session, mean levels of prothrombin, FV, FVIII, FX, FXI, and von Willebrand factor were 50% of baseline values. Mean levels of fibrinogen, FXIII, α_2-MG, and protein S and TFPI were even lower (respectively, at 14%, 5%, <15%, 23%, and 22% of baseline) (Figures 3 and 4, Supplementary Table 1).

Some proteins exhibited a slow recovery profile. Compared to baseline, the levels of some factors and inhibitors remained lower before starting the last DFPP session (Figures 3 and 4). For example, before starting the last session, fibrinogen, prothrombin, FX, FXI, FXIII, α2-MG, protein S, and TFPI had mean levels of, respectively, 35%, 69%, 71%, 56%, 19%, 19%, 46%, and 45% of the baseline values (Figures 3 and 4, Supplementary Table 1).

Impact of DFPP on generation of thrombin

After the first DFPP session, mean endogenous thrombin potential (ETP) and peak thrombin (PH) were decreased when TGA was performed without thrombomodulin (respectively, 68% for ETP and 77% for PH compared to baseline values) (Table 3, Figure 5A-B). However, in the presence of thrombomodulin, there was no marked decrease in ETP and PH (respectively, 103% and 99% compared to baseline (Table 2, Figure 5C). ETP ratio (with/without thrombomodulin) increased after the first DFPP session (1.52 times the baseline value) (Table 2, Figure 5D). Of note, one patient (P1) had a high ETP ratio (with/without thrombomodulin) at baseline, possibly explained by a low protein S level at that tome (protein S level at 54%).

Before starting the last DFPP session, ETP and PH had decreased to, respectively, 51% and 71% of baseline values (Figure 5B, Supplementary Table 1). In the presence of thrombomodulin, there was no decrease in ETP and PH (Figure 5C). ETP ratio (with/without thrombomodulin) was increased to 1.63 times baseline values (Table 3, Figure 5D).

After the last DFPP session, ETP and PH were even lower without thrombomodulin: respectively, 41% and 53% of baseline values (Table 2, Figure 5B). In the presence of thrombomodulin, the decrease was less pronounced with ETP and PH at, respectively, 72% and 74% of baseline values (Table 2 Figure 5C). In the presence of thrombomodulin, ETP and PH remained within the normal range defined for healthy subjects (Table 2).

In addition, LT was shortened after DFPP sessions with no thrombomodulin (2.89 min at baseline, 2.16 min after the first DFPP session, 1.90 min after the last DFPP session) or when thrombomodulin was included (2.91min at baseline, 2.13 min after the first DFPP session, 1.82 min after the last DFPP session) (Table 2).

Discussion

DFPP resulted in a significant decrease in high molecular-weight hemostatic proteins, protein S, TFPI, and α2-MG

DFPP induced a large depletion of high molecular-weight proteins from the hemostatic system, in particular fibrinogen, FVIII, FXIII, α2-MG, and von Willebrand factor. Smaller proteins were somewhat less depleted, whereas free protein S and TFPI were increased more (i.e., protein S and TFPI were reduced, respectively, to 45% and 52% after session 1 and, respectively, to 23% and 22% after the last session compared to baseline data) despite their low molecular weights (70 kDa for protein S and ~40 kDa for TFPI). Because 60% of protein S is bound to the larger C4BP protein (i.e., 570 kDa),[18] we assumed that the high molecular-weight complex formed by C4BP and protein S was adsorbed onto the DFPP membrane, resulting in the decreased level of plasma protein S. We have shown that C4BP is strongly removed by DFPP procedures (Supplementary Table 1) with a mean decrease in C4BP level to 28% of baseline values after the first DFPP session and to 12% of baseline values after the last DFPP session (Supplementary Table 1). It has also been previously shown that a decrease in C4BP level induced a marked decrease in protein S half-life [19] The high depletion of TFPI, despite its low molecular weight, may be because most circulating TFPI (~80%) is bound to lipoproteins (low- and high-density lipoproteins, which have high molecular weights) [20].

DFPP results in a profound decrease of plasma fibrinogen and FXIII

Depletion of fibrinogen by DFPP has been reported previously [14]. Hanafusa and colleagues have also shown that FXIII is efficiently removed by DFPP [15–17]. Our data confirm the slow recovery of FXIII and fibrinogen, particularly FXIII, which resulted in profound depletion of these factors when DFPP was performed several times (Figure 3, Supplementary Table 1).

FXIII, a transglutaminase, plays an essential role by establishing covalent bonds between fibrin molecules, which convert soluble fibrin into insoluble fibrin. FXIII is also important in wound healing [21]. DFPP-induced FXIII deficiency may increase bleeding complications during/after surgery or invasive procedures (i.e., a kidney biopsy), particularly when DFPP is repeated to desensitize ABO-incompatible kidney transplants and when the last DFPP session is scheduled for the day before transplantation.

In the absence of an invasive procedure, the recommended FXIII trough levels are low (i.e., 2–5%) [22] when prophylaxis is given for spontaneous bleeding in patients with congenital FXIII deficiency. However, when there is a high risk of bleeding during/after surgery: a higher FXIII trough level (>30%) is usually recommended for these patients [23,24]. Because FXIII deficiency cannot be detected by routine coagulation tests (PT or aPTT), a FXIII assay could be useful, but is currently not widely available.

For fibrinogen, the minimal trough level for a surgical procedure is 1–1.5 g/L [25–27]. Fibrinogen level can be rapidly determined at any time in a hospital where DFPP procedures are available. If needed, patients can be rapidly supplemented before an invasive procedure.

Repeated DFPP sessions increase the depletion of fibrinogen, prothrombin, FX, FXI, FXII, FXIII, α2-MG, protein S, and TFPI

Our data confirm the previously reported slow recovery of FXIII and fibrinogen levels after repeated DFPP sessions [15–17]. We also found that recovery rates of prothrombin, FX, FXI, FXII, α2-MG, protein S, and TFPI levels were slow. Mean fibrinogen, prothrombin, FX, FXI, FXII, α2-MG, protein S, and TFPI levels were reduced, respectively, to 35%, 69%, 71%, 56%, 79%, 19%, 46%, and 45% of baseline values before starting the last DFPP session. The levels of FV and FVIII, although significantly decreased after each session, became rapidly re-normalized just before the last session of DFPP (respectively, 84%and 84% of baseline values) (Figures 2 and 3, Supplementary Table 1). Recovery was (logically) highly correlated with the reported half-lives of clotting factors (~10 days for FXIII, ~5 days for fibrinogen and α2-MG, ~3-5 days for lipoproteins bound to TFPI, ~3 days for prothrombin, ~2.5 days for FXI and FXII, ~2 days for FX and protein S). Indeed, from a theoretical

point of view, after complete depletion of a protein from plasma, the time for the level to reach a steady state (corresponding to the time for a full recovery) was approximately five halve-lives, if we consider that the synthesis of this protein was not affected. Factors that were most strongly removed by the DFPP sessions were those with high molecular weights and longer half-lives.

DFPP decreased thrombin generation in the absence of thrombomodulin, but did not profoundly decrease thrombin generation in the presence of thrombomodulin

To assess the overall coagulation balance, we performed TGA, which is more relevant and comprehensive than the usual laboratory tests. Indeed, the generation of thrombin reflects the combined effect of pro- and natural anticoagulants [28,29]. To sensitize TGA to the protein C pathway, we also performed TGA with thrombomodulin [30]. The thrombin-generation assay in the presence of thrombomodulin, may better reflect *in vivo* thrombin generation because this assay takes into account all of the pro-coagulant and anticoagulant players.

After the first DFPP and the last sessions, ETP and PH of thrombin were profoundly decreased when TGA was performed without thrombomodulin. This decrease may have been caused by depletion of numerous clotting factors, especially FVIII and prothrombin.

However, in the presence of thrombomodulin, ETP and PH were not significantly modified after the first DFPP session (mean ratio (ETP after S1/ ETP at baseline **=** 1.03; ratio of S1/PH at baseline = 0.99). After repeated sessions, ETP and PH were nevertheless moderately decreased (mean ratio: ETP after S1/ ETP at baseline = 0.72; ratio PH after S1/PH at baseline = 0.61). Nevertheless, absolute values of ETP and PH remained within the normal range (ETP 596 nM/.min after the last DFPP session, normal range: 459--1218 nM/min). The lower relative decrease in ETP and PH in the presence of thrombomodulin may be explained by the decreased protein S and, to a lesser extent, the decrease in TFPI and protein C. This counterbalanced the decrease in TG induced by the decreased pro-coagulant factors as TGA, in the presence of thrombomodulin, sensitized the assay to the protein C/protein S system. Indeed, thrombomodulin is a cofactor for thrombin-mediated protein C activation, and protein S is a cofactor of activated protein C, which inactivates FVa and FVIIIa via proteolytic cleavage [29]. Protein S has also been shown to be a cofactor of protein TFPI (31).

Before starting the last DFPP session, thrombin generation in the absence of thrombomodulin was particularly reduced (mean ETP was 51% of the baseline value) despite normalized levels of numerous factors (except for fibrinogen, FXIII, prothrombin, FX, FXI, and FXII). The persistent decrease in prothrombin level (69% of the baseline value) partially explains the decrease in ETP, as a proportional relationship is well-established between these two parameters [32]. Another explanation for the decrease in thrombin generation could be the significant decrease in fibrinogen level. Indeed, *in vitro* plasma defibrination can cause a 30% decrease in thrombin generation [33]. It has been shown that fibrinogen slows down the decay of thrombin: i.e., adsorbed thrombin on fibrin(ogen) may be less susceptible to interacting with its inhibitors compared to free thrombin [(33)]. In addition, fibrinogen has been shown to increase the conversion of prothrombin into thrombin [36], even though this mechanism has still not been elucidated. Decreased thrombin generation has also been reported in patients with congenital FXIII deficiency [37]. However, more recently, the opposite has also been reported [35].

Finally, we observed that DFPP induced a shortening in lag times without or with thrombomodulin (Table 2), which may be explained by the decrease in TFPI levels as TFPI is an inhibitor of coagulation initiation.

In conclusion, DFPP partially removes numerous coagulation factors and inhibitors, as well as von Willebrand factor, from plasma. Repeated DFPP sessions strongly depleted factors that had high molecular weights and long half-lives, Natural anticoagulants of high-molecular weight (α2-MG) or bound to a high molecular-weight ligands (protein S and TFPI) were also strongly depleted. Although, ETP repeated after each DFPP sessions was profoundly diminished using TGA without thrombomodulin, ETP was only slightly reduced despite repeated DFPP sessions in the presence of thrombomodulin. If though TGA performed with thrombomodulin may better reflect *in vivo* thrombin generation, the overall coagulation balance was only slightly altered. However, as DFPP removes large amounts of fibrinogen, patients with FXIII and VWF may have an increased bleeding risk, particularly during/after an invasive or surgical procedure, if it is not supplemented.

Addendum:

R. Marlu co-designed the study, analyzed the data, and contributed to writing the manuscript.

P. Malvezzi co-designed the study, recruited the patients, and contributed to writing the manuscript.

L. Seyve performed some of the tests (thrombin generation and clotting factors).

T. Jouve performed the statistical analyses.

J. Maurizi performed the DFPP sessions.

F. Defendi performed the C4BP tests.

P.L. Carron performed the DFPP sessions.

M. Christophe performed the clotting-factor assays.

A. Le Gouellec performed the α2 macroglobulin tests.

B. Polack finalized the manuscript.

L. Rostaing co-designed the study and finalized the manuscript.

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Conflict of interest disclosure: None.

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Figures:

Figure 1: Course of prothrombin time, aPTT, platelet count, and hemoglobin level

PT and aPTT, platelet counts were determined immediately before the first and last DFPP session. *p*-values were calculated according to the model described in the Materials and Methods. p_1 denotes the change after the first DFPP session. p_2 denotes the change between baseline and immediately before starting the last DFPP session. p_3 denotes the change between baseline and immediately after the last DFPP session.

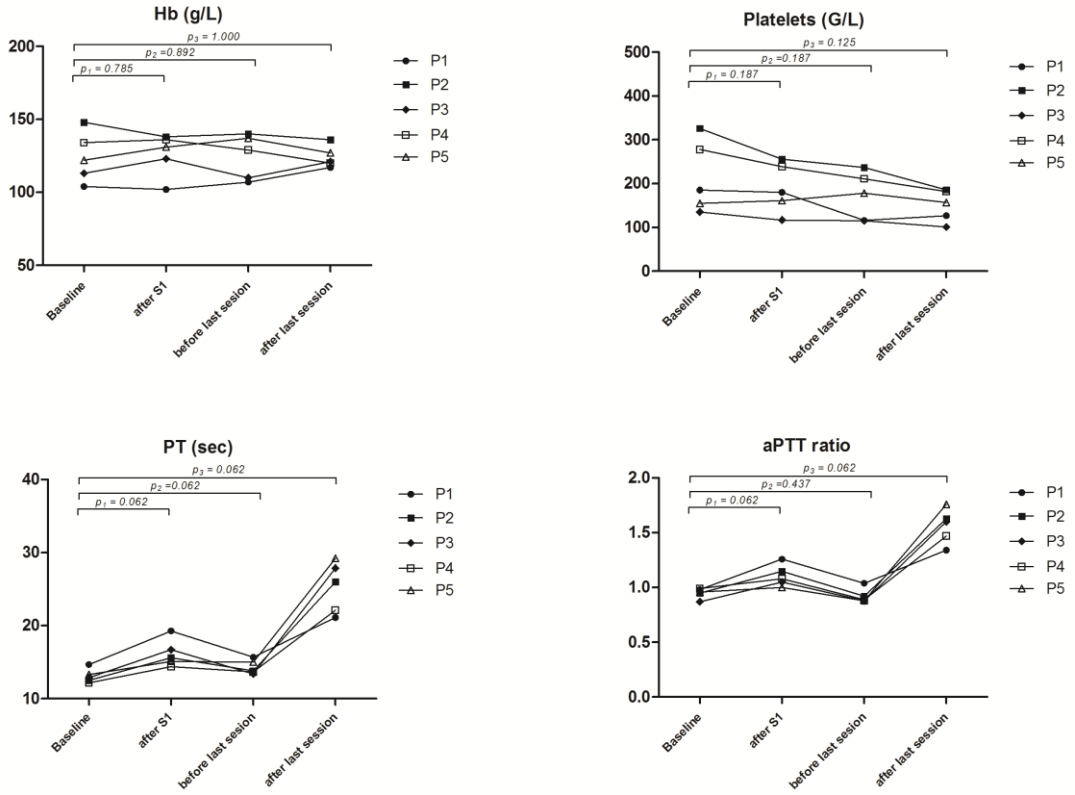


Figure 2: Correlation between molecular weights and decreases in factors and inhibitor levels after the first DFPP session.

Molecular weights are listed below: Fibrinogen 340 kDa, prothrombin (FII) 72 kDa, FV 183 kDa, FVII 46 kDa, FVIII 280 kDa, FIX 55 kDa, FX 59 kDa, FXI 160 kDa, FXII 79 kDa, FXIII 320 kDa, VVF 500 kDa for dimers, anti-thrombin 58 kDa, protein C 62 kDa, protein S 70 kDa, TFPI 41 kD, α2-macroglobulin 720 kDa, and C4BP 570 kDa.

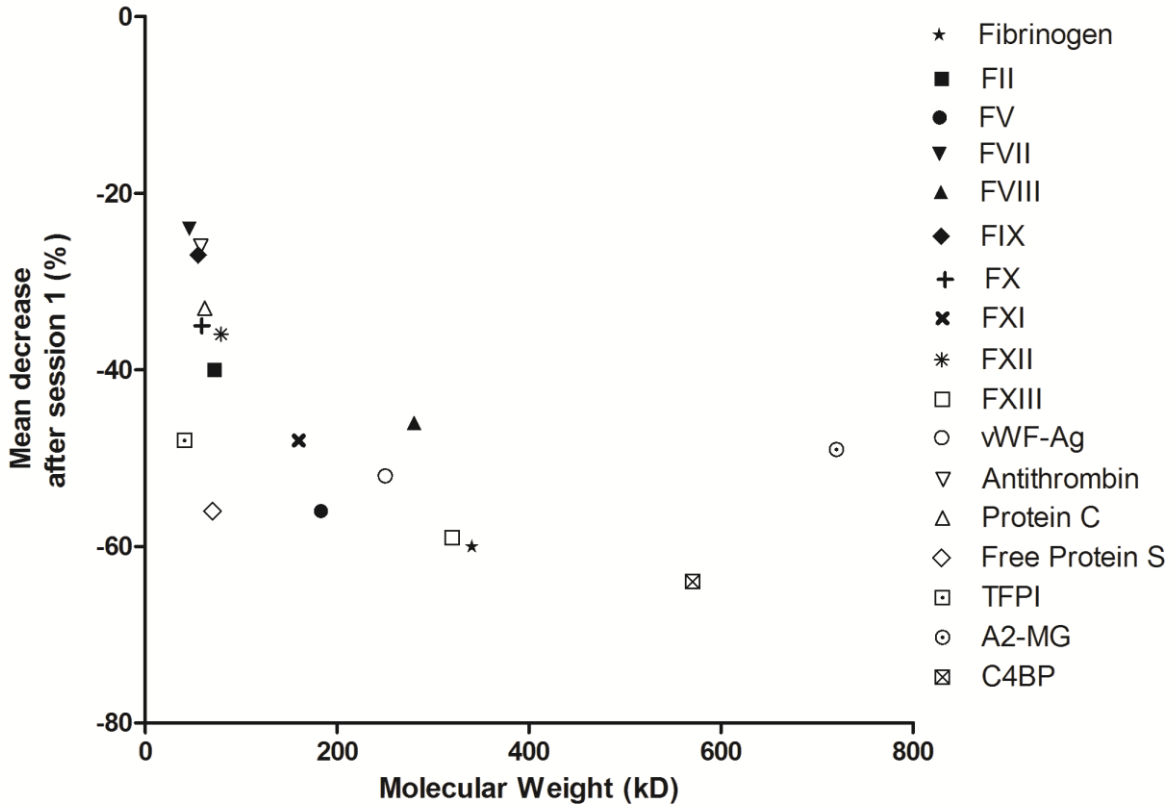


Figure 3: Course of clotting-factor levels

Levels of clotting factors were determined immediately before the first and last DFPP sessions. *p*-values were calculated according to the model described in the Materials and Methods. p_1 denotes the change between baseline and after the first DFPP session. p_2 denotes the change between baseline and immediately before starting the last DFPP session. p_3 denotes the change between baseline and immediately after the last DFPP session.

In the “Fibrinogen” panel, the horizontal dashed line corresponds to the limit of measurement (<0.4 g/L)

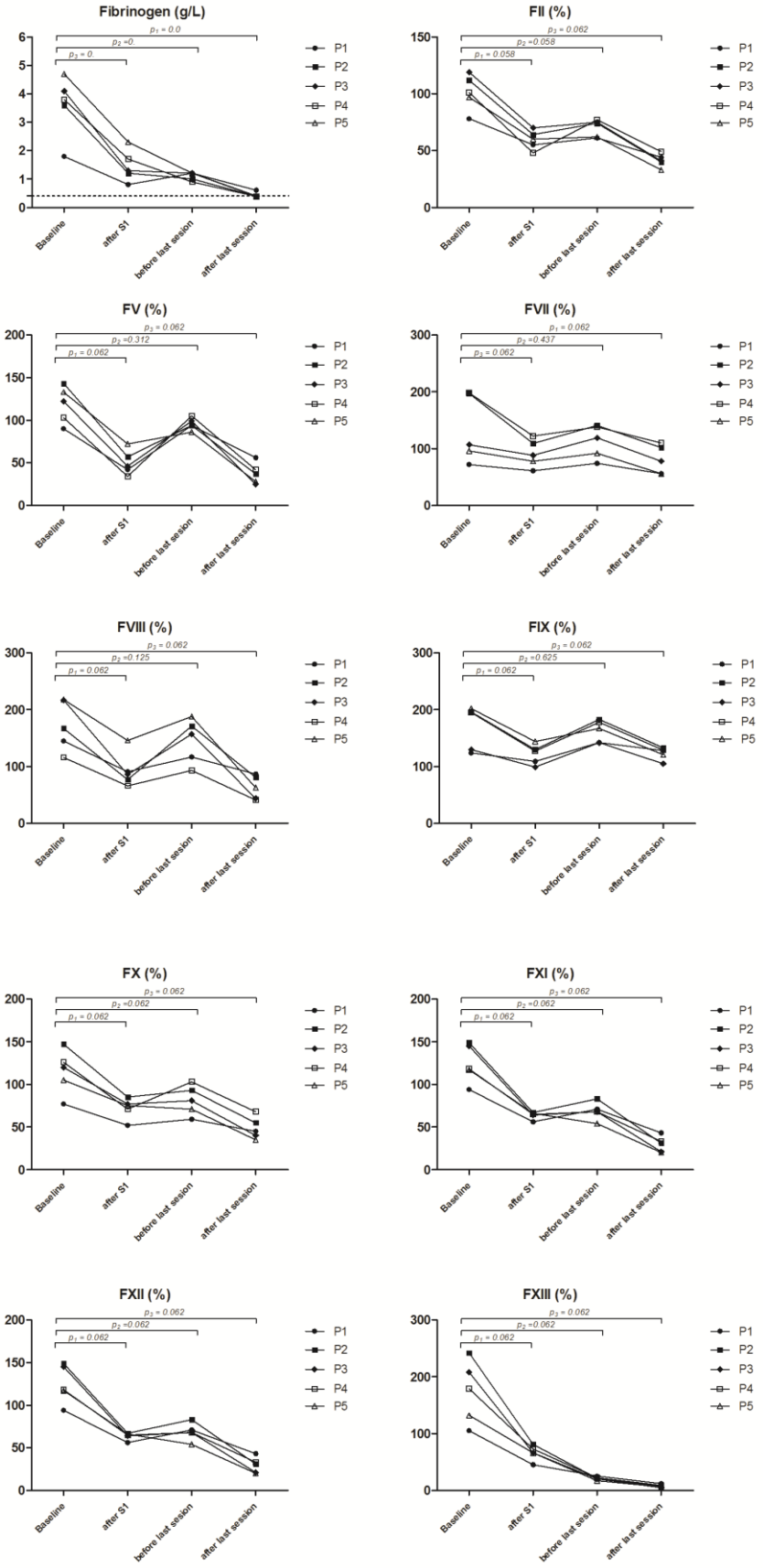
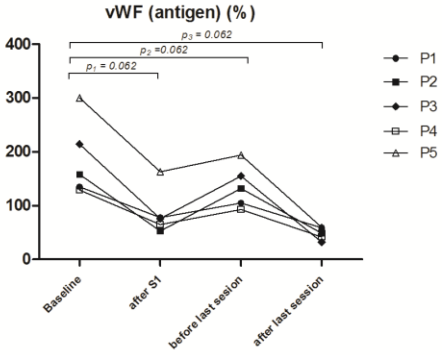
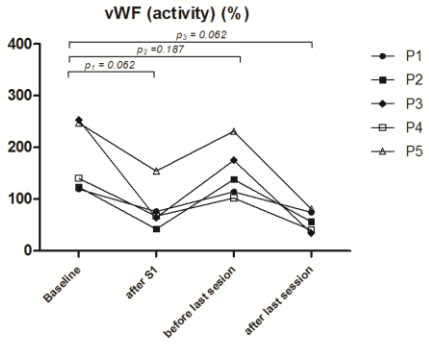
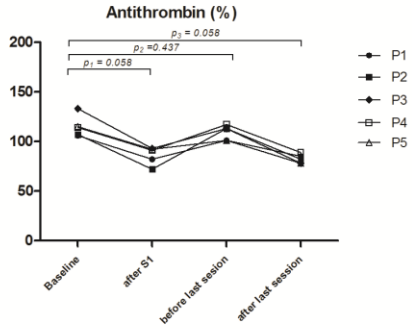


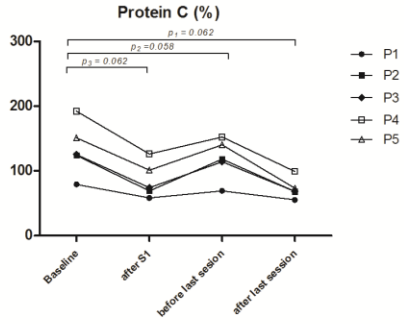
Figure 4: Levels of von Willebrand factor, coagulation inhibitors, α2-MG and C4BP. Levels were determined immediately before and after each DFPP session. p_1 denotes the change between baseline and after the first DFPP session. p_2 denotes the change between baseline and immediately before starting the last DFPP session. p_3 denotes the change between baseline and immediately after the last DFPP session.

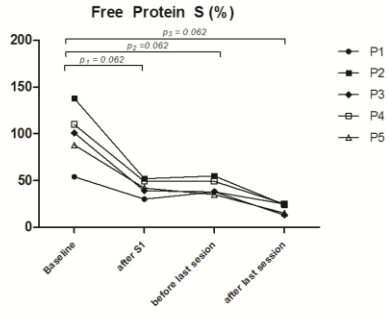
In the "α2-MG" and "C4BP" panel, the horizontal dashed line corresponds to the limit of measurement (<0.19 g/l for α2-MG and <10% for C4BP).

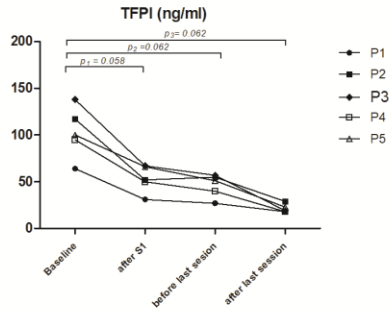


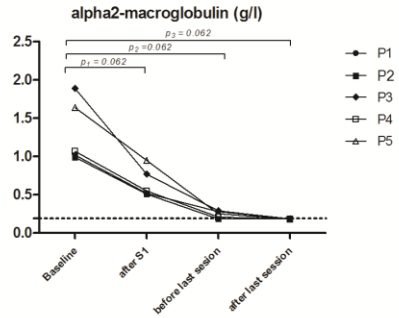












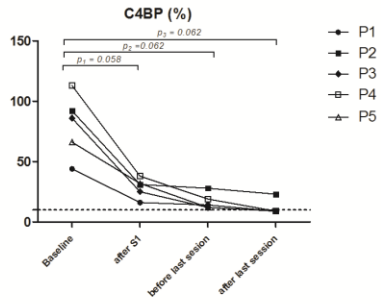
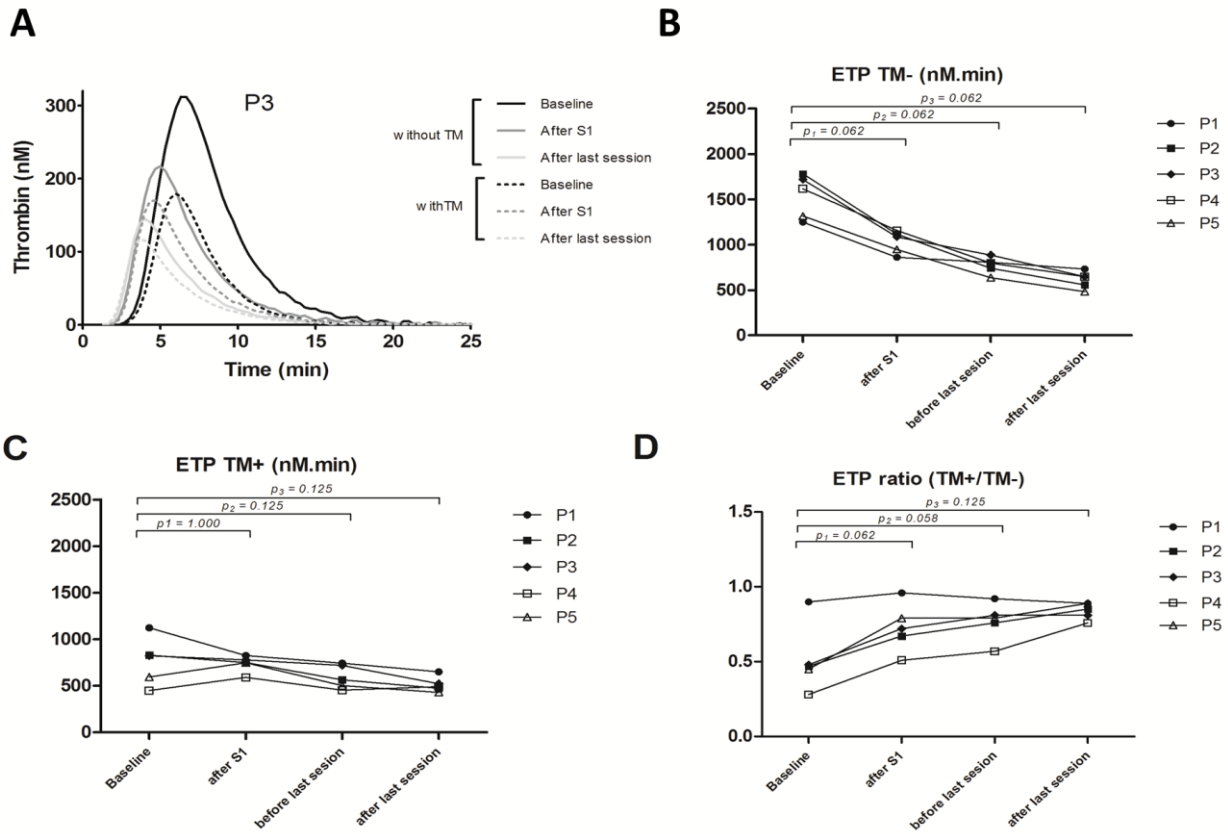
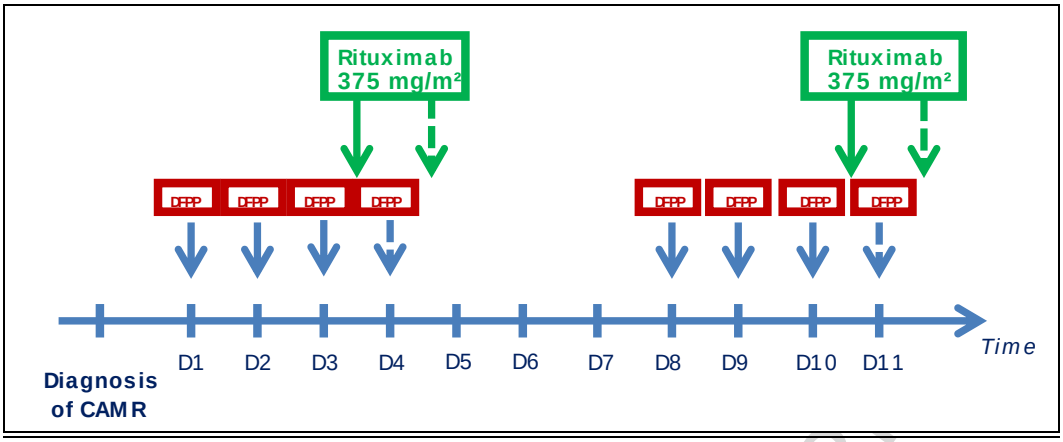


Figure 5: Effect of DFPP on thrombin generation. TGA was performed with/without thrombomodulin, immediately before and after the first DFPP session, and immediately before and after the last DFPP session (session 6 for patients 1, 2, 3, and session 8 for patients 4 and 5). (**A**) TGT curve from a representative patient (P3). (**B**) Course of ETP (endogenous thrombin potential) without thrombomodulin. (**C**) Course of ETP with thrombomodulin. (**D**) Course of ETP ratio (with/without thrombomodulin). TM = thrombomodulin.

p_1 denotes the change between baseline and after the first DFPP session. p_2 denotes the change between baseline and immediately before starting the last DFPP session. p_3 denotes the change between baseline and immediately after the last DFPP session.



Supplementary Figure 1: Treatment of chronic antibody-mediated rejection using DFPP + rituximab.



Abbreviations: D, day; DFPP, double-filtration plasmapheresis; CAMR, chronic antibody-mediated rejection. Blue dashed arrows denote when eight DFPP sessions were performed. Green dashed arrows denote the timing of rituximab infusions when eight DFPP sessions were performed. Three patients had six DFPP sessions, whereas two patients had eight DFPP sessions.

Tables

Table 1: Demographic data of the study population

	Gender (M/F)	Age (yrs)	Duration of KT (mo)	Serum creatinine (µmol/L)	eGFR (mL/min)	Proteinuria (g/L)	Type of DSA	MFI
Patient 1	M	39	1	115	69	0.12	DBP1* 04:01	8,050
Patient 2	M	51	25	155	44	2.65	DQ5	6,236
Patient 3	M	47	23	243	26	0.23	DR53	12,371
Patient 4	M	41	14	194	36	0.05	DQA2 DQ9	5,538 3,800
Patient 5	M	72	25	247	22	0.57	DPB1*04:01 DPB1*13:01	8341 9800
Mean ± SD	**NA**	**50**	**17.6**	**191**	**39.4**	**0.72**	**NA**	**6844**

Abbreviations: M, male; F, female; yrs, years; mo, months; eGFR, estimated glomerular-filtration rate according to CKD-EPI formula; DSA, donor-specific alloantibody; MFI, mean fluorescence intensity; KT, kidney transplantation.NA: not applicable.

Table 2: Impact of DFPP sessions on thrombin-generation parameters. TGA was performed without and with thrombomodulin and before and after the first DFPP session, and also before and after the last DFPP session (session 6 for patients 1, 2, 3 and session 8 for patients 4, 5).

Normal range was determined in 20 healthy volunteers (10 males and 10 females) as mean +/- 2 SD.

. p_1 denotes the change between baseline and after the first DFPP session. p_2 denotes the change between baseline and immediately before starting the last DFPP session. p_3 denotes the change between baseline and immediately after the last DFPP session.

Abbreviations: TGA: thrombin-generation assay; TM: thrombomodulin; ETP: endogenous thrombin potential

		Baseline	After session 1	*Ratio (after session1 / baseline)*	p_1	Before last session	*Ratio (before last session / baseline)*	p_2	After last session	*Ratio (after last session /baseline)*	p_3
	Parameter (normal range)	**Mean/ Median (min-max)**	**Mean/ Median (min-max)**	***Mean/ Median (min-max)***		**Mean/ Median (min-max)**	***Mean / Median*** *(min-max)*		**Mean/ Median (min-max)**	***Mean/ Median (min-max)***	
Without TM	**Lag time (min)** (2.6-3.1)	**2.99 / 3.33** (1.67-3.67)	**2.16 / 2.33** (1.50-2.6)	***0.74 / 0.70*** *(0.66-0.90)*	*0.048*	**1.97 / 2.00** (1.67-2.33)	***0.69 / 0.63*** *(0.60-1.00)*	*0.058*	**1.90 / 2.00** (1.63-2.11)	***0.67 / 0.60*** *(0.55-1.00)*	*0.062*
	ETP (nM.min) (1096-2341)	**1536 / 1616** (1249-1780)	**1033 / 1084** (861-1158)	***0.68 / 0.69*** *(0.63-0.72)*	*0.062*	**773 / 797** (637-887)	***0.51 / 0.49*** *(0.42-0.64)*	*0.062*	**613 / 646** (483-732)	***0.41 / 0.38*** *(0.31-0.59)*	*0.062*
	Peak (nM) (229-389)	**274 / 264** (241-312)	**208 / 210** (194-220)	***0.77 / 0.81*** *(0.64-0.84)*	*0.062*	**193 / 186** (183-212)	*0.71 / 0.70* *(0.66-0.73)*	*0.062*	**144 / 143** (127-161)	***0.53 / 0.53*** *(0.45-0.64)*	*0.058*
With TM	**Lag time (min)** (1.9-3.4)	**2.91 / 3.11** (1.83-3.33)	**2.13 / 2.33** (1.67-2.33)	***0.74 / 0.70*** *(0.66-0.91)*	*0.054*	**1.95 / 2.00** (1.67-2.33)	***0.69 / 0.60*** *(0.59-0.91)*	*0.058*	**1.82 / 1.78** (1.63-2.00)	***0.65 / 0.60*** *(0.53-0.91)*	*0.062*
	ETP (nM.min) (459-1218)	**763 / 822** (450-1121)	**737 / 748** (590-824)	***1.03 / 0.95*** *(0.74-1.31)*	*1.00*	**596 / 564** (453-742)	***0.81 / 0.85*** *(0.66-1.01)*	*0.125*	**512 / 490** (474-648)	***0.72 / 0.64*** *(0.57-1.09)*	*0.125*

	Peak (nM) (92-273)	**170 / 179** (96-239)	**160 / 172** (120-185)	***0.99 / 0.96*** *(0.77-1.25)*	*0.625*	**153 / 158** (109-179)	***0.94 / 0.99*** *(0.72-1.14)*	**0.375**	**119 / 117** (105-145)	***0.74 / 0.65*** *(0.61-1.09)*	*0.125*
ETP ratio **(with/without TM)** (0.24-0.76)		**0.51 / 0.4** (0.28-0.90)	**0.73 / 0.72** (0.51-0.96)	***1.52 / 1.50*** *(1.07-1.83)*	*0.062*	**0.77 / 0.79** (0.57-0.92)	***1.63 / 1.69*** *(1.03-2.04)*	*0.058*	**0.84 / 0.85** (0.76-0.89)	***1.84 / 1.83*** *(0.99-2.72)*	*0.125*

Supplementary Table 1: Impact of DFPP sessions on hemostasis parameters.

(α) Fibrinogen level was below the limit of measurement (<0.40 g/L) in three patients after the last DFPP. (β) α2 macroglobulin level was below the limit of measurement (<0.19 g/L) in all patients after the last DFPP. (ɣ) C4BP level was below the limit of measurement (<10%) in four patients after the last DFPP.

Parameter	Baseline (before session 1)	After session 1	*Ratio (after session1 / baseline)*	Before last session	*Ratio Before last session/ baseline)*	After last session	*Ratio (after last session/ baseline)*
	Mean / Median (min–max)	*Mean / Median (min–max)*	*Mean / Median (min–max)*	*Mean / Median (min–max)*	*Mean / Median (min–max)*	*Mean / Median (min-max)*	*Mean / Median (min-max)*
Hemoglobin (g/L)	**124 / 122** (104-148)	**126 / 131** (102-138)	***1.02 / 1.01*** *(0.93-1.09)*	**125 / 129** (107-140)	***1.01 / 0.97*** *(0.95-1.12)*	**124 / 121** (117-136)	***1.01 / 1.04*** *(0.90-1.13)*
Platelets (G/L)	**216 / 185** (135-326)	**191 / 180** (117-256)	***0.90 / 0.87*** *(0.79-1.04)*	**171 / 178** (115-237)	***0.82 / 0.76*** *(0.63-1.15)*	**151 / 157** (101-186)	***0.73 / 0.69*** *(0.57-1.01)*
PT (s)	**13.1 / 12.9** (12.2-14.7)	**16.2 / 15.6** (14.4-19.3)	***1.23 / 1.18*** *(1.14-1.31)*	**14.3 /13.8** (13.4-15.7)	***1.09 / 1.10*** *(1.04-1.13)*	**25.3 / 26.0** (21.1-29.2)	***1.94 / 2.08*** *(1.44-2.20)*
aPTT (s)	**0.95 / 0.96** (0.87-0.99)	**1.11 / 1.08** (1.00-1.26)	***1.17 / 1.21*** *(1.04-1.29)*	**0.92 / 0.89** (0.88-1.04)	***0.97 / 0.97*** *(0.90-1.06)*	**1.56 / 1.60** (1.34-1.76)	***1.65 / 1.72*** *(1.37-1.84)*
Fibrinogen (g/L)	**3.60 / 3.80** (1.8-4.7)	**1.46 / 1.30** (0.9-1.5)	***0.41 / 0.44*** *(0.32-0.49)*	**1.10 / 1.20** (0.9-1.2)	***0.35 / 0.28*** *(0.24-0.67)*	**0.43 / <0.4** [α] (<0.4-0.6)	***0.14 / 0.10*** *(0.09-0.33)*
II (%)	**101 / 101** (78-119)	**59 / 60** (48-70)	***0.59 / 0.59*** *(0.48-0.71)*	**70 / 74** (61-77)	***0.69 / 0.66*** *(0.63-0.78)*	**41 / 41** (35-49)	***0.42 / 0.36*** *(0.34-0.56)*

V (%)	**118 / 122** (90-143)	**50 / 46** (34-72)	***0.42 / 0.40*** *(0.33-0.54)*	**96 / 94** (86-105)	***0.84 / 0.81*** *(0.65-1.04)*	**38 / 37** (25-56)	***0.39 / 0.35*** *(0.20-0.62)*
VII (%)	**134 / 107** (72-198)	**92 / 88** (61-122)	***0.73 / 0.81*** *(0.55-0.85)*	**113 / 119** (74-141)	***0.90 / 0.96*** *(0.70-1.11)*	**80 /78** (56-110)	***0.63 / 0.58*** *(0.52-0.78)*
VIII (%)	**173 /167** (116-218)	**93 / 86** (66-146)	***0.54 / 0.57*** *(0.40-0.67)*	**145 / 138** (93-188)	***0.84 / 0.81*** *(0.72-1.02)*	**63 /63** (41-87)	***0.39 / 0.35*** *(0.20-0.60)*
IX (%)	**169 / 195** (124-202)	**122 / 127** (99-144)	***0.73 / 0.71*** *(0.65-0.88)*	**162 / 132** (142-183)	***0.98 / 0.93*** *(0.83-1.15)*	**123 /128** (105-133)	***0.76 / 0.68*** *(0.60-1.03)*
X (%)	**115 /120** (77-147)	**72 / 75** (52-85)	***0.63 / 0.64*** *(0.56-.0.71)*	**81 / 81** (59-103)	***0.71 / 0.68*** *(0.63-0.82)*	**49 / 45** (35-68)	***0.43 / 0.37*** *(0.33-0.58)*
XI (%)	**125 / 118** (94-149)	**64 / 65** (56-67)	***0.52 / 0.55*** *(0.44-0.60)*	**69 / 68** (54-83)	***0.56 / 0.56*** *(0.46-0.76)*	**30 / 31** (20-43)	***0.25 / 0.21*** *(0.14-0.46)*
XII (%)	**99 / 108** (49-121)	**62 / 68** (32-74)	***0.64 / 0.65*** *(0.56-0.72)*	**79 / 88** (34-101)	***0.79 / 0.81*** *(0.69-0.86)*	**49 / 53** (27-60)	***0.50 / 0.49*** *(0.44-0.56)*
XIII (antigen)(%)	**173 /179** (105-242)	**66 / 66** (45-81)	***0.40 / 0.41*** *(0.33-0.50)*	**21 / 21** (17-25)	***0.19 / 0.13*** *(0.09-0.41)*	**8 / 7** (5-12)	***0.05 / 0.05*** *(0.02-0.11)*
Antithrombin (%)	**115 /114** (106-133)	**86 / 91** (72-93)	***0.75 / 0.77*** *(0.67-0.80)*	**112 / 113** (101-117)	***0.98 / 1.01*** *(0.85-1.06)*	**82 / 82** (78-89)	***0.72 / 0.77*** *(0.59-0.80)*
Protein C (%)	**134 /125** (79-192)	**86 / 74** (58-126)	***0.64 / 0.66*** *(0.56-0.73)*	**119 / 118** (69-152)	***0.89 / 0.91*** *(0.79-0.95)*	**73 / 68** (55-99)	***0.56 / 0.54*** *(0.48-0.70)*
Free protein S (%)	**98 /101** (54-138)	**42 / 42** (30-52)	***0.45 / 0.45*** *(0.38-0.56)*	**43 / 38** (35-55)	***0.46 / 0.40*** *(0.38-0.70)*	**20 / 24** (13-25)	***0.23 / 0.17*** *(0.13-0.46)*
Total TFPI (ng/ml)	**103 /100** (64-138)	**53 / 52** (31-67)	***0.52 / 0.49*** *(0.44-0.66)*	**46 / 51** (27-57)	***0.45 / 0.42*** *(0.41-0.51)*	**21 /19** (18-29)	***0.22 / 0.23*** *(0.14-0.28)*

α2-Macroglobulin (g/l)	**1.32 /1.07** (0.99-1.89)	**0.66 / 0.55** (0.51-0.95)	**0.51 / 0.52** (0.41-0.58)	**0.24 / 0.22** (0.18-0.29)	**0.19 / 0.19** (0.15-0.27)	**<0.19 / < 0.19** [β] (<0.19-<0.19)	**<0.15 / 0.17** (0.11- 0.18)
VWF-activity (%)	**150 /160** (98-218)	**81 / 67** (42-154)	***0.48 / 0.48*** *(0.32-0.64)*	**152 / 138** (102-231)	***0.92*** *(0.73-1.12)*	**57 / 56** (34-81)	***0.37 / 0.33*** *(0.17-0.62)*
VWF-antigen (%)	**166 /140** (119-247)	**87 / 76** (53-163)	***0.46 / 0.50*** *(0.34-0.58)*	**136 / 132** (93-194)	***0.74*** *(0.65-0.84)*	**48 / 49** (32-60)	***0.28 / 0.31*** *(0.15-0.44)*
C4BP (%)	**80 / 86** (44-113)	**28 / 31** (16-38)	***0.36 / 0.34*** (0.29-0.48)	**17 / 14** (12-28)	***0.22*** *(0.14-0.32)*	**12 - <10** (<10-23)	***0.15 / 0.14*** *(0.08-0.25)*

Abbreviations: PT: prothrombin time; aPTT: aPTT: activated partial thromboplastin time; TFPI: tissue-factor pathway inhibitor; VWF: von Willebrand factor; C4BP: C4 binding protein