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Plasma Exchange Therapy Using Solvent Detergent-Treated Plasma: An Observational Pilot Study on Complement, Neutrophil and Endothelial Cell Activation in a Case Series of Patients Suffering from Atypical Hemolytic Uremic Syndrome

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Keywords
Solvent detergent-treated plasma · Plasma exchange therapy · Atypical hemolytic uremic syndrome · Complement · Nucleosomes

Abstract
Introduction: Plasma exchange therapy (PEX) was standard treatment for thrombotic microangiopathy before eculizumab was available and is still widely applied. However, most PEX patients still ultimately progress to end-stage renal disease (ESRD). It has been suggested that infusion of plasma that contains active complement may induce additional complement activation with subsequent activation of neutrophils and endothelial cells, leading to exacerbation of organ damage and deterioration of renal function. ***Objective:*** This observational pilot study examines the effect of hemodialysis, eculizumab and PEX before and after treatment in plasma of aHUS patients on complement-, neutrophil and endothelial cell activation. ***Methods:*** Eleven patients were included in this pilot study. Six patients were treated with hemodialysis, 2 patients received regular infusions of eculizumab, and 3 patients were on a regular schedule for PEX. Patients were followed during 3 consecutive treatments. Blood samples were taken before and after patients received their treatment. ***Results:*** Complement activation products increased in plasma of patients after PEX, as opposed to patients treated with hemodialysis or eculizumab. Increased levels of complement activation products were detected in omniplasma used for PEX. Additionally, activation of neutrophils and endothelial cells was observed in patients after hemodialysis and PEX, but not in patients receiving eculizumab treatment. ***Conclusion:*** In this pilot study we observed that PEX induced complement and neutrophil activation, and that omniplasma contains significant amounts of complement activation products. Additionally, we demonstrate that hemodialysis induces activation of neutrophils and endothelial cells. Complement activation with subsequent neutrophil activation may contribute to the deterioration of organ function and may result in ESRD. Further randomized controlled studies are warranted to investigate the effect of PEX on complement- and neutrophil activation in patients with thrombotic microangiopathy.

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Introduction

Thrombotic microangiopathy (TMA) is characterized by hemolytic anemia, the presence of schistocytes in the peripheral blood smear, and thrombocytopenia. Furthermore, TMA is often complicated by organ damage due to microvascular thrombosis, resulting among others in acute kidney failure and cerebral ischemia. Activation and injury of endothelial cells is the central pathological event that results in the clinical picture of TMA [1]. However, the etiologies of endothelial cell activation and damage are very broad, ranging from infection, pregnancy, tumors, and drugs to organ and hematopoietic stem cell transplantation [2]. Genetic mutations and/or autoimmune responses may render individuals susceptible to develop TMA as a response to the mentioned triggers [1]. On one end of the spectrum, genetic mutations leading to a complete lack of ADAMTS13 or autoantibodies decreasing ADAMTS13 activity result in an inadequate proteolytic degradation of ultralarge von Willebrand factor (vWF) molecules. This leads to subsequent activation of endothelial cells and platelets, as seen in classical thrombotic thrombocytopenic purpura (TTP) [2]. Atypical hemolytic uremic syndrome (aHUS) at the other end of the spectrum is caused by an acquired and/or congenital defect affecting the function of complement proteins, such as complement regulatory proteins (e.g., factor H, CD46) or complement factors (e.g., gain of function mutation C3) [3]. This results in the activation of and occasionally damage to endothelial cells with subsequent platelet activation and aggregation, a process finally culminating in microvascular thrombosis [1]. As a matter of fact, the etiology of aHUS lies in an overwhelming complement activation caused by inadequate control. However, activation or injury of endothelial cells regardless of the cause as seen in TMA will lead to secondary complement activation, thereby perpetuating endothelial cell damage [4].

Given the high morbidity and mortality, plasma exchange (PEX), also known as therapeutic plasma exchange (TPE), and – in case of acute renal insufficiency – hemodialysis are considered the emergency treatment of patients presenting with acute TMA in order to prevent fatality [4, 5]. Nowadays, complement inhibition using anti-C5 (eculizumab) is considered the long-term standard treatment for aHUS, since it efficiently halts complement-induced renal damage and prevents end-stage renal disease (ESRD) [6–11]. However, PEX has been the standard treatment for aHUS before the availability of complement inhibitors and is still applied as emergency treatment upon presentation and in case complement inhibitors are not available [4, 8]. Although approximately two thirds of the aHUS patients initially show a positive response to PEX with preservation of renal function for multiple years, most patients still finally progress to ESRD [12, 13].

The main aim of PEX is to substitute defective or deficient plasma proteins, such as ADAMTS13 in case of TTP or factor H in aHUS [1, 2]. However, plasma used for PEX contains a wide range of activatory and inhibitory complement proteins [2]. Although potentially beneficial, the safety of PEX in patients with an activated complement system is debated, as infusion of plasma that contains active complement may induce additional complement activation. This may result in the activation of neutrophils, platelets, and endothelial cells with subsequent exacerbation of organ damage, in particular deterioration of renal function [14, 15]. Indeed, neutrophil activation in the form of neutrophil extracellular traps (NETs) has been reported to play a role in the pathogenesis of TMA [16, 17]. In this observational pilot study, we examined complement, neutrophil, and endothelial cell activation products in plasma of aHUS patients before and after treatment with hemodialysis, anti-C5 infusion (eculizumab) or PEX.

Materials and Methods

Patients

Patients with a very high suspicion of the diagnosis of aHUS (based on clinical and/or histopathological presentation with hemolytic anemia, thrombocytopenia, and acute kidney injury) at an age of 12 years or older treated on a regular schedule with hemodialysis (in case of renal failure), eculizumab, or PEX at the Department of Nephrology at Amsterdam University Medical Centre, location Academic Medical Center (AMC) Amsterdam, were enrolled in the current study. Identification of gene mutations of complement proteins or complement autoantibodies was not required for inclusion. The only exclusion criterion was pregnancy. Informed consent was obtained from all patients or their legal representative. The study was approved by the ethical medical committee of the Amsterdam University Medical Centre location AMC, Amsterdam, the Netherlands.

Study Design

Patients were followed during 3 consecutive treatments. Patients with ESRD were on regular hemodialysis therapy, which meant a hemodialysis session of 4 h 3 times a week. They were dialyzed using a standard filter (F80) and received a prophylactic dose of low-molecular-weight heparin as anticoagulant prior to dialysis. Plasma exchange was performed once or twice a week using centrifugation technique (Spectra Optia, Terumo BCT©). During each treatment, plasma was exchanged for 1.5-fold plasma volume against solvent detergent-treated plasma (omniplasma, OctaplasLG®). Patients on eculizumab (Soliris®) therapy received infusions every other week. All patients were in the maintenance phase including a fixed dose of 1,200 mg every 14 days. Blood samples using EDTA and citrate as anticoagulant were taken 30 min before and after patients received their treatment. From citrate and EDTA tubes, platelet-rich plasma was collected by centrifugation (1,500 *g*, 10 min), and platelets were removed by an additional centrifugation step (1,500 *g*, 10 min). The plasma was aliquoted and stored at −80°C. Serum was collected by allowing the blood to clot at room temperature for 60–90 min with subsequent removal of the clot by centrifuging at 1,500 *g* for 10 min. Serum was aliquoted and stored at −80°C.

Table 1. Patient characteristics

	Treatment		
	hemodialysis	eculizumab	PEX
Number of patients	6	2	3
Age, years; median (IQ range)	50 (45–57)	26 (22–30)	18 (16–18)
Mutation, *n*			
CFH	2	1	2
CFI	1	–	–
CFB	1	–	–
CFHR	–	–	1
No mutation	2	1	–
Kidney transplant, *n*	–	2	1
Hypertension	2	–	1

The age of the patients are presented as median with IQ, range. CFH, complement factor H, CFI, complement factor I, CFB, complement factor B, CFHR, complement factor H related protein.

Complement Assays

C3 antigen levels have been determined by nephelometric measurement in the routine diagnostic laboratory. The levels of C3 and C4 activation products (C3b/c and C4b/c) were determined by ELISA [18]. For determination of C3d levels, plasma was treated with 22% polyethylene glycol (to remove macromolecules such as C3) for 2 h at 4°C. Afterwards, the samples were centrifuged at 3,800 rpm for 20 min at 4°C. The supernatant, which now only contained low-molecular-weight proteins or protein degradation products, was used in the ELISA as previously described [18]. The serum pool was used as standard and arbitrarily set as 100%. Levels of C3a were determined by ELISA. In brief, microtiter plates were coated with 1 μg/mL monoclonal anti-C3a (#HM1072, Hycult, USA) diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.6). After 5 washes with phosphate-buffered saline 0.02% Tween-20, samples diluted in phosphate-buffered saline Tween 0.1% gelatin 0.2% (PTG)-ethyl-diamino-tetra-acetic acid (EDTA) (10 mM) were added. After 5 washes, biotinylated rabbit polyclonal anti-C3a (IgG fraction: Behring, Marburg) diluted in PTG (0.5 μg/mL) was added to the wells. After 5 washes, streptavidin-polymerized horseradish peroxidase diluted 10,000 times in high-performance ELISA buffer was added to the wells. After another 5 washes, plates were developed by adding 100 μg/mL tetramethylbenzidine and 0.003% (v/v) hydrogen peroxide in 0.11 M sodium acetate buffer (pH 5.5). The reaction was stopped by adding 2 M H_2SO_4. Absorbance was measured at 405 nm. The serum pool was used as standard and arbitrarily set as 100%.

Endothelial Cell and Neutrophil Activation Markers

Human neutrophil elastase-α_1-antitrypsin complex (EA) and human lactoferrin levels were determined by ELISA [19]. Cell-free DNA levels in the form of nucleosomes were measured by ELISA [20]. vWF antigen and vWF propeptide levels were determined as previously described [21].

Quantification of Mitochondrial DNA

Mitochondrial (mt)DNA was purified from patient plasma samples or omniplasma using the QIAamp DSP Virus kit (#60704, Qiagen). Patient DNA samples were subsequently diluted (1:5) in DNase-free water. A digital droplet PCR was performed according to the manufacturer's instructions: in this study, the digital droplet (dd)PCR system included an automated droplet generator and reader from Biorad (QX200 Droplet Digital PCR, Biorad, Hercules, CA, USA) and a T100 thermal cycler (Biorad). For mtDNA quantification, a primer and probe targeting the mitochondrial encoded NADH dehydrogenase 1 (MT-ND1) (NADH dehydrogenase 1 (ND1), human (FAM), Biorad, unique assay ID dHsaCNS669425578) was used. Results were analyzed using the Quantasoft software, and absolute values of mtDNA (ND1) (copies/μL) were calculated for each DNA sample.

Statistical Analysis

Measurements of patient's samples of 3 consecutive treatments and of plasma products are individually shown. All results are indicated as median and interquartile range. Statistical analyses were performed using a paired Wilcoxon test in GraphPad Prism 8 software. $p < 0.05$ was considered significantly different from the null hypothesis.

Results

Patient Characteristics

In total, 11 patients were included in this pilot study. Six patients were treated with hemodialysis, 2 patients received regular infusions of eculizumab, and 3 patients were on a regular schedule for PEX. Table 1 presents the basic characteristics of the 11 patients who completed the study according to the protocol. Based on the clinical and/or histopathological presentation with microangiopathic hemolytic anemia, thrombocytopenia, and acute kidney injury all patients were diagnosed with aHUS. In 8 patients genetic analysis for mutations in complement factors associated with aHUS revealed a mutation. The majority ($n = 5$) had factor H mutations, 1 patient had a mutation in factor I, 1 patient had a mutation in factor B, and 1 patient had a deletion in factor H-related proteins 1 and 3. Patients within the hemodialysis group were older (median 50, range 45–57 years) compared to patients treated with eculizumab (median 26, range 22–30 years) or PEX (median 18, range 16–18years). Three patients received a kidney transplant, and 3 patients suffered from arterial hypertension.

Complement Activation Products Increased after PEX

First, we measured complement activation in all patients before and after treatment. Complement C3 activation products C3b/c, C3a, and C3d were significantly increased in the circulation of the patients after PEX, whereas levels of these markers did not change when comparing before and after eculizumab treatment (shown in Fig. 1a–c). After hemodialysis treatment we observed a significant increase in C3d, but not of C3b/c or C3a (Fig. 1a–c). In addition, classical complement activation as evidenced by C4 activation products (C4b/c) could be detected after PEX (Fig. 1d), whereas no difference could be detected before and after treatment with either eculizumab or hemodialysis. There was no difference in complement regu-

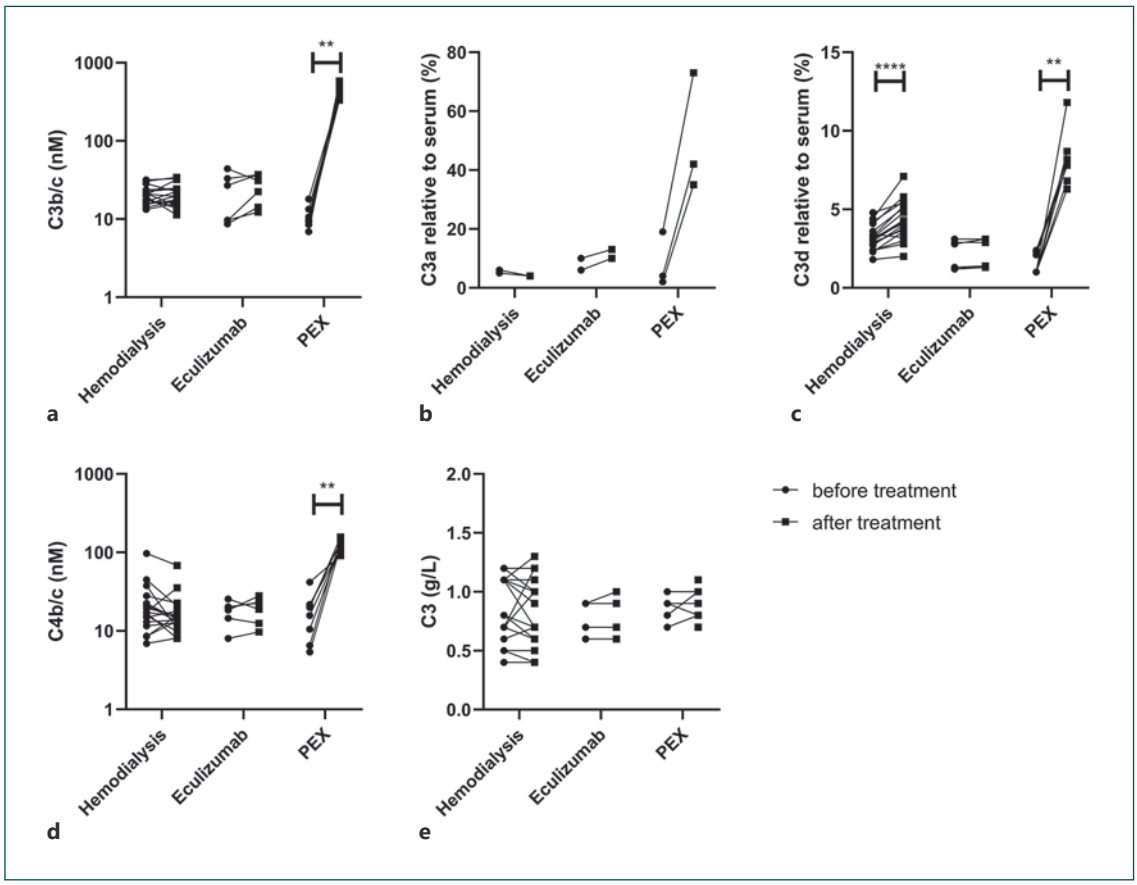

Fig. 1. Complement activation is elevated in patients after PEX. **a** C3b/c. **b** C3a. **c** C3d. **d** C4b/c. **e** C3. Concentrations were measured in patients before and after they received treatment. $n = 18$ hemodialysis (3 consecutive treatments of 6 patients), $n = 6$ eculizumab (3 consecutive treatments of 2 patients), $n = 8$ PEX (3 consecutive treatments of 2 patients and 2 consecutive treatments of 1 patient). $^{**}p < 0.01$, $^{****}p < 0.0001$.

lator factor H and B levels before and after treatment in all groups (data not shown). C3 antigen levels remained comparable before and after treatment in all 3 groups indicating that measurements of total complement protein levels are not reflecting activation (Fig. 1e).

In a next step, we investigated whether the increase in complement activation products after PEX could be caused by complement activation products present in the plasma products used for PEX. Therefore, we assessed complement activation products (C3b/c, C3a, C3d, and C4b/c) in 10 omniplasma units that were administered to 2 patients. Interestingly, C3b/c, C3a, C3d, and C4b/c levels were considerably increased in 10 omniplasma units compared to patients before PEX treatment (Fig. 2a–d). These results identified the complement activation products in omniplasma as a potential source of the complement activation products observed in patients after PEX.

Activation of Endothelial Cells and Neutrophils after Hemodialysis and PEX

Subsequently, we investigated the effect of the 3 treatment modalities on endothelium by measurement of the direct markers for endothelial cell activation vWF antigen (vWF:Ag) and vWF-propeptide (vWFpp). Hemodialysis resulted in a significant increase in vWF:Ag and vWFpp (Fig. 3a, b), whereas the levels remained comparable before and after treatment with PEX and eculizumab, respectively.

Neutrophil activation was determined using plasma levels of EA, lactoferrin, and cell-free DNA in the form of nucleosomes (Fig. 3c–e). Interestingly, all 3 markers of neutrophil activation were significantly increased in the circulation of patients after PEX and hemodialysis, whereas there was no change observed for EA and lactoferrin levels after eculizumab treatment. In contrast, nucleosome levels significantly decreased upon treatment with eculizumab (Fig. 3e). Additionally, we could detect neutrophil activation products, such as EA and lactoferrin, in the omniplasma products (EA: median 619.0 ng/mL, range 591.3–644.0; lactoferrin: median 1,160.0 ng/mL, range 1,103.0–1,220.0), whereas nucleosome levels in omniplasma products were not increased as compared to control plasma (median 2.3 U/mL, range 2.3–2.7; control plasma <10 U/mL) (data not shown).

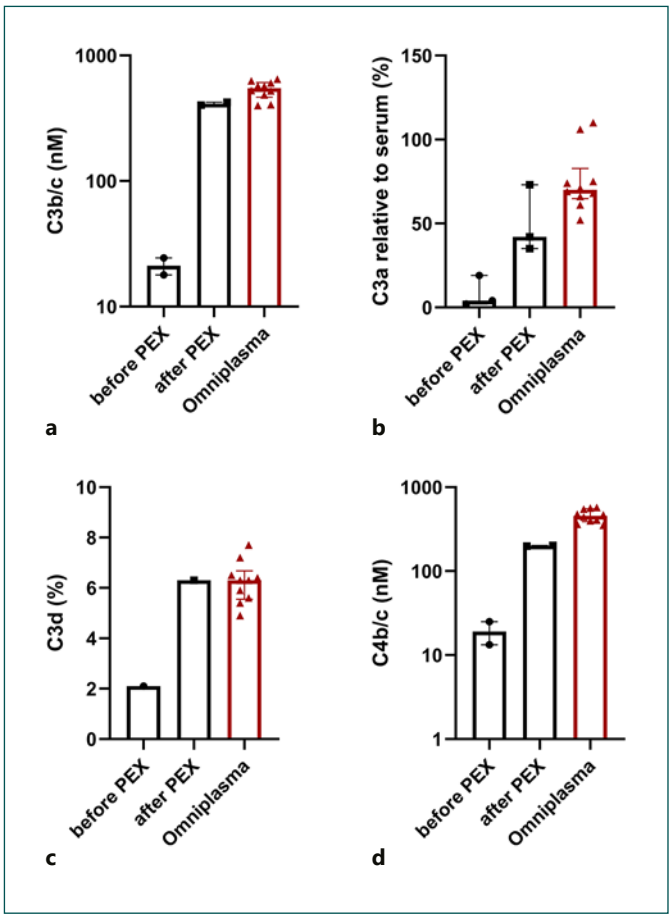

Fig. 2. Omniplasma contains high levels of activated complement products. **a** C3b/c. **b** C3a. **c** C3d. **d** C4b/c. Concentrations were measured in plasma before and after treatment of 2 patients and in 10 omniplasma units administered to these 2 patients subjected to PEX.

We also measured mtDNA, which is released from platelets upon activation. We observed no differences in levels of mtDNA before or after treatment in the plasma of hemodialysis, PEX or eculizumab patients (Fig. 4a), and the level of mtDNA in omniplasma was low (median 132 copies/μL, range 114–182, Fig. 4b). Finally, we assessed plasma levels of markers for hemolysis before and after treatment. There were no significant changes in plasma levels of haptoglobin, hemopexin and lactate dehydrogenase in hemodialysis, PEX or eculizumab patients, ruling out significant hemolysis (data not shown).

Discussion

Since recently, complement inhibition using eculizumab is now an established and standard treatment of atypical HUS in most high-income countries. However, in many countries, PEX is still widely applied and in case of renal insufficiency, patients are on hemodialysis. In fact, aHUS treatment using PEX may prevent acute complication, ESRD finally occurs in many of these patients. In the present observational pilot study, we noticed that complement is activated in patients after PEX and that omniplasma used for PEX contains high amounts of complement activation products. No complement activation was observed in patients treated with eculizumab. In addition, we observed activation of neutrophils and endothelial cells in patients after hemodialysis and PEX, which was not observed in patients receiving eculizumab treatment.

The observation of complement activation products after PEX is in line with previous studies reporting complement activation after plasma exchange and apheresis [22–26]. Most probably, the etiology of complement activation observed in our study after PEX is multifactorial. It has been suggested that the plasma separator and filtration steps may induce in complement activation [22, 23, 27]. PEX using centrifugation technique also results in complement activation, but to a lower degree compared to combined techniques including centrifugation and filtration, respectively [28]. Centrifugation is the physical technique used to perform PEX in our study, which may account for at least a part of complement activation detected after PEX. In addition, we also detected complement activation products in omniplasma products. Indeed, the presence of complement activation products in quarantine plasma as well as in Solvent Detergent (S/D) plasma has been described [27, 29, 30]. Plasma apheresis using centrifugation technique to collect plasma from donors may in part be responsible for the complement activation detected in the plasma products [28]. In addition, next to the apheresis procedure, the S/D process step may also induce significant complement activation in the product [29]. Unfortunately, it remains difficult to establish to what degree PEX itself contributes to the measured complement activation in our patients, as the exchanged apherisate for complement measurement has not been collected. Therefore, omniplasma administered to the patient itself is not only a source of complement proteins but also of complement activation products. S/D plasma, especially omniplasma, has an excellent safety profile, which is reflected by the very low incidence of allergic reactions and TRALI [31]. The most common adverse effect in patients of plasma exchange therapy, hypotension, was suggested to be caused by the complement activation products C3a and C5a [25]. It has been suggested that high plasma concentration of complement activation products in the treated patients may influence the clinical outcome of the treatment [23]. Patients with aHUS are characterized by ongoing complement activation, that results in damage to endothelial cells with subsequent microvascular thrombosis resulting in organ failure [4]. Especially the kidneys, where endothelial and epithelial integrity is strictly dependent on the availability of

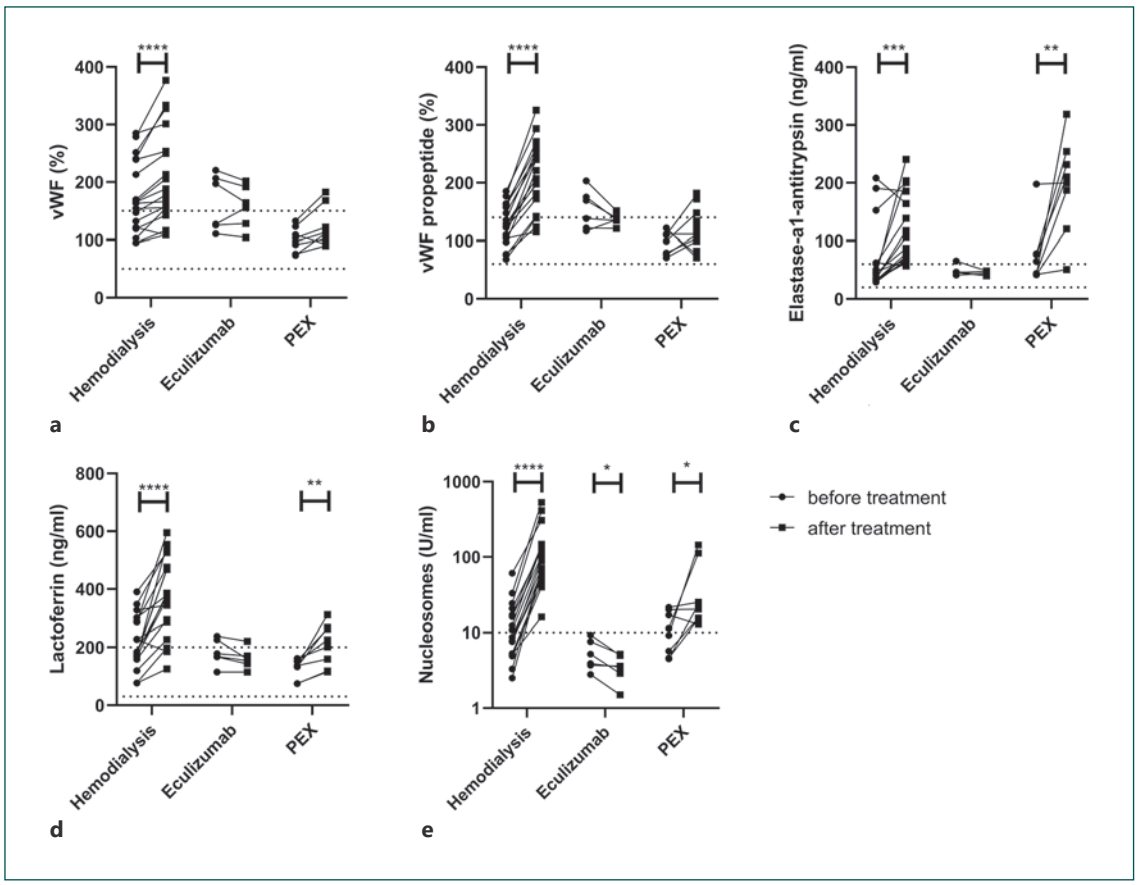

Fig. 3. Endothelial and neutrophil activation were observed after hemodialysis and PEX. **a** Von Willebrand factor antigen. **b** Von Willebrand factor propeptide. **c** Elastase-α_1-antitrypsin complexes. **d** Lactoferrin. **e** Nucleosomes. All were measured in patients before and after they received treatment. $n = 18$ hemodialysis (3 consecutive treatments of 6 patients), $n = 6$ eculizumab (3 consecutive treatments of 2 patients), $n = 8$ PEX (3 consecutive treatments of 2 patients and 2 consecutive treatments of 1 patient). The dotted line represents the normal value or range in plasma. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

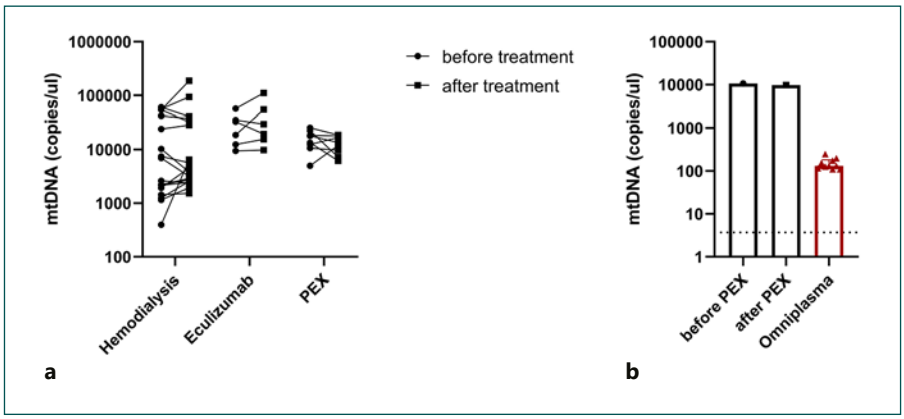

Fig. 4. mtDNA is not affected by treatment in aHUS patients. **a** mtDNA concentrations were measured in patients before and after they received treatment. $n = 18$ hemodialysis (3 consecutive treatments of 6 patients), $n = 6$ eculizumab (3 consecutive treatments of 2 patients), $n = 8$ PEX (3 consecutive treatments of 2 patients and 2 consecutive treatments of 1 patient). **b** mtDNA concentrations were measured in plasma before and after treatment of 2 patients and in 10 omniplasma units administered to these 2 patients subjected to PEX.

functional fluid-phase complement regulators such as, FH, are prone to complement-mediated attack [4]. By introducing complement activation products in the patient's circulation, PEX may theoretically not cease but rather maintain the ongoing complement activation. Administration of plasma that already contains complement activation products may therefore cause more harm than benefit. This is supported by the fact that-although PEX delays deterioration of renal function-a large proportion of aHUS patients finally still progress to ESRD [12, 13].

In the current study, we report that C3d levels were significantly upregulated in patients after hemodialysis. However, although significant, the levels did not increase to biological relevant concentrations. Moreover, there were no other signs of relevant ongoing complement activation as reflected by low C3bc values in patients after hemodialysis. In the past, using cellulose membranes for hemodialysis, inflammatory activation products of complement components C3 and C5 were demonstrable after dialysis [22]. However, this issue was resolved with the use of advanced filter technology (e.g., application of hydrophilic polysulfone hemodialysis membrane), which is in accordance to our results not demonstrating relevant ongoing complement activation [32].

Additionally, we observed neutrophil -and endothelial cells activation after hemodialysis and PEX, while no neutrophil and endothelial cell activation was observed in patients treated with eculizumab. In a recent study in Paroxysmal Nocturnal Hemoglobinuria (PNH) patients we demonstrated neutrophil activation to be dependent on complement activation and that treatment with eculizumab completely abrogated neutrophil activation in these patients [33]. Our results are in line with these earlier findings, since no complement- and neutrophil activation were observed in aHUS patients treated with eculizumab. Therefore, although neutrophil activation observed after PEX is most probably a multifactorial process, complement activation in this activation process might play an important role [34, 35]. For the last decades hemodialysis membranes have been extensively studied and were found to activate the alternative pathway of the complement system; activate platelets; induce cytokine release; induce neutrophil degranulation; and activate monocytes [36–38]. Complement as the main inducer of neutrophil activation in our hemodialysis patients can be ruled out since there was no relevant complement activation observed after hemodialysis, apart from a limited increase in C3 activation product C3d, suggesting no ongoing activation. Interestingly, vWF and vWFpro concentrations were increased after hemodialysis and to a lesser extent after PEX. Increased levels of markers of endothelial damage have been demonstrated in patients with ESRD on hemodialysis [39, 40]. Therefore, hemodialysis and probably PEX may affect endothelial cell homeostasis resulting in activation of endothelial cells.

The clinical consequences of neutrophilic mediators released during PEX and hemodialysis are not entirely clear. There is evidence that Neutrophil Extracellular Trap (NET) formation plays an important role in the pathogenesis of thrombotic microangiopathy [16, 41]. Neutrophil activation in the form of NETs may result in the release of pro inflammatory and cytotoxic molecules, such as histones, and induce a procoagulant state [35, 42]. Neutrophils have been reported to release NETs upon storage of red blood cell concentrates [43]. Therefore, it cannot be ruled out that neutrophils may release NETs during the production process of blood products. The S/D pool plasma production process includes a filtration step using a membrane with pores of 0.2 μm in diameter which at least will remove DNA-fragments, such as (poly) nucleosomes [44]. However, neutrophilic products such as elastase and lactoferrin will not be removed by this filtration step. The present observational pilot study, however, is too small to answer the question on whether these identified markers correlate with adverse events that occur during hemodialysis and PEX. Interestingly, we observed no difference in mtDNA levels before or after treatment, whereas we did observe a difference in mtDNA levels between the different treatments. Given the large range of these values, an interpretation of these findings remains difficult, especially when considering the limited number of measurements.

Here we have to point out, that in the patient population in this observational pilot study is very heterogeneous regarding the underlying forms as well as the treatment of aHUS, which impedes to draw firm conclusions on the definite therapeutically consequences of our findings.

In summary, we detected activation products of complement and neutrophils in patients after PEX and in S/D plasma products in this small cohorts of aHUS patients. In addition, we observed neutrophil activation in hemodialysis patients. It remains surprising that in this observational pilot study PEX, a therapy used to treat complement-mediated diseases, in fact seems to increase the amount of complement activation products in patients. Moreover, it seems remarkable that the products used for this procedure contain complement activation products. Since this pilot study contained only a very small and heterogeneous number of patients future studies will have to determine the relevance of the activation of complement.

Acknowledgement

We would like to acknowledge all the patients that participated in this study.

Statement of Ethics

This study protocol was reviewed and approved by METC AMC, Amsterdam UMC, approval No. 2012_255, NL No. 41,994.018.12. Informed consent was obtained from all patients or their legal representative.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

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Author Contributions

Y.W., A.R., G.M., and F.B. substantially contributed to the conception of the work, data acquisition, analysis, and interpretation, and drafted the manuscript. D.W., A.B., and S.S. contributed to the analysis and interpretation of the data and revised the work critically for important intellectual content. All authors approved the final version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author. The data that support the findings of this study not publicly available due to information that could compromise the privacy of research participants but are available from the corresponding author upon reasonable request.

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