Novel techniques for volume management and toxin removal in end-stage renal disease

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Novel techniques for volume management and toxin removal in end-stage renal disease

Nieuwe technieken voor volume regulatie en toxine verwijdering in eindstadium nierfalen
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 15 juni 2010 des middags te 2.30 uur

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Walter Jurriaan Brummelhuis

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"Man's mind, once stretched by a new idea, never regains its original dimensions."

Oliver Wendell Holmes Jr.

Contents

Chapter 1

Introduction and outline of this thesis

Chronic renal failure necessitating renal replacement therapy is a growing problem in western societies. In Europe, for instance, the incidence and prevalence of renal replacement therapy between 1997 and 2006 were 125 and 816 per million inhabitants, respectively [1]. Renal replacement modalities involve kidney transplantation, peritoneal dialysis and hemodialysis. The main problems in patients on dialysis are the retention of certain classes of uremic toxins despite dialysis, inducing the uremic syndrome, and intermittent accumulation of salt and water, contributing to hypertension. In the analysis mentioned above, approximately 46% of the patients were treated by hemodialysis. Two specific issues of hemodialysis treatment form the basis of the investigations of this thesis.

1.1 Hemodialysis

In patients with kidney failure, hemodialysis serves two purposes. The first is the removal of toxic substances from the body that cause the uremic syndrome, mainly by diffusion. The second is the removal of excess salt and water from the body by ultrafiltration. Basically, hemodialysis equipment consists of two computer-operated blood pumps and two dialysate pumps, and a dialyzer (Figure 1). Before hemodialysis, a blood tubing system including the dialyzer is placed on the dialysis machine. The patient is connected to the tubing via needles that are inserted into the patient's vascular access (either a surgically created arteriovenous fistula or an implanted synthetic graft) or via a central venous catheter. Blood flows through the semi-permeable fibers in the dialyzer, while dialysate, the cleaning solution, flows in the opposite direction outside these fibers. The dialysate is a mixture of minerals, bicarbonate, and glucose dissolved in water. Differences between dialysate and blood substance concentrations create various concentration gradients over the semi-permeable membrane. As a result, substances diffuse towards the dialysate (e.g. potassium, urea, phosphate) or to the blood (e.g. bicarbonate) and are removed from or added to the body, respectively. In addition, a flow difference can be set between the dialysate pump before and after the dialyzer, e.g. 450 versus 500 ml/min, which creates a negative transmembrane pressure gradient over the fibers. This in turn induces a fluid flow from the blood passing through the dialyzer towards the dialysate compartment (= ultrafiltration) which allows removal of excess fluid retained between dialysis sessions. It is of note that fluid is directly removed from the vascular compartment, and not from the interstitial compartment, where most excess fluid resides (vide infra). Usually, ultrafiltration is applied in a linear, constant

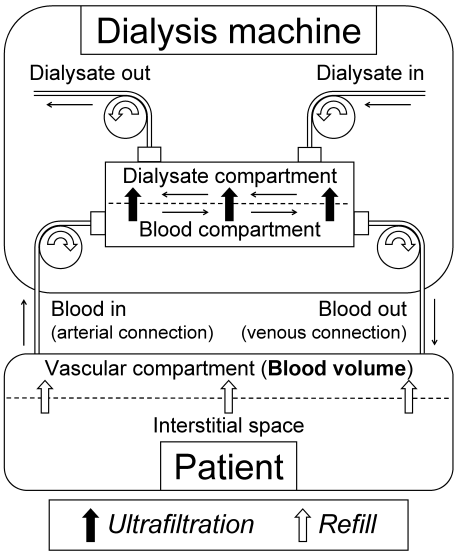

Figure 1. Schematic overview of the relation between ultrafiltration, plasma refilling, and circulating blood volume during hemodialysis.

fashion, implying that the total volume to be removed is distributed equally over the dialysis time. Standard hemodialysis treatment involves 3-4 dialysis sessions per week with a duration of 3-4 hours each. It should be noted that resetting of the volume status therefore has to be performed within an extremely short time interval. This does not come without consequences. Moreover, both removal of fluids and removal of toxins are performed in one step, with one membrane that has been optimized to simultaneously perform these tasks. This comes with limitations too.

1.2 Shortcomings of present hemodialysis treatment

1.2.1 Inadequate removal of toxins

Dictated by the pore size of the dialyzers, mainly small uremic toxins are removed from the body by diffusion using present day hemodialysis techniques. A large group of uremic toxins, known as middle molecules, can not be removed because their size (molecular weight > 500 Da) is larger than the hemodialyzer fibre pore size [2]. Therefore, these compounds accumulate in the body of patients with renal failure despite hemodialysis treatment. They are pro-inflammatory, enhance coagulation, cause endothelial dysfunction and/or smooth muscle cell proliferation, and their retention may contribute to incomplete treatment of the uremic syndrome by dialysis and the severe atherosclerosis present in end-stage renal disease. Likewise, uremic toxins that are bound to proteins can not be removed adequately by hemodialysis.

A new dialysis technique, hemodiafiltration, uses dialyzers with larger pores and a greater water permeability. This technique removes some middle molecules by convection that remain in the circulation during conventional hemodialysis treatment [3]. However, this technique still leaves the majority of middle molecules untouched. Consequently, other extracorporeal techniques are needed to remove these molecules from the body.

A potential way to remove larger substances or substances that are protein-bound from the circulation can be achieved by immunoadsorption [4]. This could be an option to specifically remove substances from the blood in hemodialysis, however, the state of end-stage renal disease is extremely complex. One of the goals of the current investigation was to test whether an immunoadsorptive device could be developed using Variable domains of Llama Heavy-chain antibodies (VHH). We tested these VHH because, unlike conventional antibodies, they can be produced in large quantities. In addition, they have properties that make them very suitable for immunoadsorption. The rationale behind the study is further explained in *Chapter 3*. Models that seemed better suited to test the applicability of immunoadsorption than chronic renal failure were considered, specifically diseases where a relatively stringent relationship between a single causative factor and disease manifestation had been reported. Examples are Goodpasture syndrome and toxic shock caused by bacterial toxin Toxic Shock Syndrome Toxin-1 (TSST-1). Immunoadsorption was tested in a large animal model, using llama antibodies immobilized on a plasma separation filter, and this forms the **first topic of this thesis**. The bacterial toxin TSST-1 was chosen to study the principle that larger toxins can be removed from the body by immunoadsorption. Llama antibodies against this substance were produced in sufficient quantities (*Chapter 2*). As mentioned, an added advantage of studying TSST-1 was that its removal would potentially result in a measurable clinical response. The physical properties of TSST-1, which are of importance for successful removal of the toxin, are reviewed in Chapter 4.

1.2.2 Ultrafiltration-induced hypotension and inadequate vascular refilling

In dialysis patients, the surplus of salt and water that is retained due to insufficient renal excretion is sequestered mainly in the interstitial compartment of the extracellular space (Figure 2). Depending on the intake between treatments, several liters of salt and water have to be removed by ultrafiltration per dialysis treatment. This will result in relatively high ultrafiltration rates, in particular because the time needed to remove small uremic toxins from the body by dialysis has shortened considerably due to increased dialyzer efficiency. As outlined above, fluid is in principle removed from the vascular compartment by ultrafiltration, which will reduce the intravascular volume (blood volume). Refill of fluid from the interstitial to the vascular compartment is needed to remove all the excess fluid and to preserve the blood volume. This topic is extensively reviewed in Chapter 5. Unfortunately, an imbalance between the ultrafiltration rate and the refill rate, where the former exceeds the latter, leads to progressive blood volume depletion. This, in turn, may lead to intradialytic hypotension, an important cause of dialysis morbidity [5]. An adequate refill mechanism is therefore of key importance for patients treated with hemodialysis. However, standardized methods to quantitatively measure the refill mechanism during dialysis treatment are not available. We therefore developed such a method using

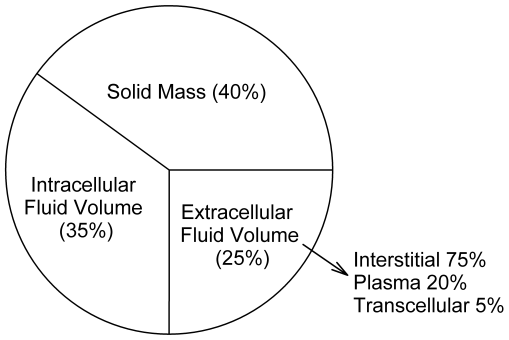

Figure 2. The intracellular water and intracellular water make up the total body water. Most excess fluid accumulates as extracellular water in the interstitial space. Ultrafiltration, however, removes plasma water. Thus, a fluid shift from the interstitial space to the plasma (or blood volume, see Figure 1) ensues during hemodialysis treatment.

feedback-regulated ultrafiltration on the basis of relative blood volume measurements. This method was then used to investigate the refill mechanism under several interventions that are applied in everyday hemodialysis practice. This is the **second topic of this thesis**.

1.3 Outline of this thesis

Part I: Removing substances

In *Chapter 2*, the *in vitro* development and testing of several highly selective Variable domains of Llama Heavy-chain antibodies (VHH) is studied. The main aim of the study was the selection and production of VHH directed against the bacterial toxin Toxic Shock Syndrome Toxin-1 (TSST-1), followed by immunoadsorption of TSST-1 from plasma using this novel method. In *Chapter 3*, we tested one of these VHH in an experimental model of porcine sepsis. The main aim was to test whether timely removal of infused bacterial toxin TSST-1 from the animal's circulation by extracorporeal circulation over a column with VHH directed against TSST-1 would ameliorate the subsequent sepsis syndrome. Issues concerning the removal of TSST-1 and other toxins by immunoadsorption are reviewed in *Chapter 4*.

Part II: Removing excess volume

Chapter 5 reviews the relation of refill with ultrafiltration-induced hypotension, the methods currently available to determine the plasma refill rate and the capillary filtration coefficient during dialysis, and concludes with potential applications of plasma refill rate measurement. *Chapter 6* describes a novel method that enables the quantitative, continuous on-line measurement of the absolute plasma refill rate during dialysis. This method was used to investigate the influence on absolute refill of two commonly used blood-pressure stabilizing interventions (anti-embolic stockings and infusion of a hyperoncotic plasma-expander). In *Chapter 7*, the quantitative refill rate measurement was applied to investigate the effect of cool dialysate and a high sodium dialysate on the plasma refill rate. In *Chapter 8*, we studied the potential use of refill measurements in determining the dry weight in dialysis patients. *Chapter 9* provides a summary of our findings and discusses perspectives for future research.

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Part I

Removing substances

Chapter 2

Specific immunocapturing of the staphylococcal superantigen Toxic Shock Syndrome Toxin-1 in plasma

Abstract Toxic Shock Syndrome is primarily caused by the Toxic Shock Syndrome Toxin-1 (TSST-1), which is secreted by the Gram-positive bacterium *Staphylococcus aureus*. The toxin belongs to a family of superantigens (SAgs) which exhibit several shared biological properties, including the induction of massive cytokine release and V_{β}-specific T-cell proliferation. In this study we explored the possibility to use monoclonal Variable domains of Llama Heavy-chain antibodies (VHH) in the immunocapturing of TSST-1 from plasma. Data is presented that the selected VHH are highly specific for TSST-1 and can be efficiently produced in large amounts in yeast. In view of affinity chromatography, the VHH are easily coupled to beads, and are able to deplete TSST-1 from plasma at very low, for example, pathologically relevant, concentrations. When spiked with 4 ng/ml TSST-1 more than 96% of TSST-1 was depleted from pig plasma. These data pave the way to further explore application of high-affinity columns in the specific immunodepletion of SAgs in experimental sepsis models and in sepsis in humans.

Adams H, Brummelhuis WJ, Maassen BT, van Egmond N, El Khattabi M, Detmers FJ, Hermans PW, Braam B, Stam JC, Verrips CT.

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2.1 Introduction

Staphylococcus aureus is a major human pathogen that produces an array of toxins. Among the secreted toxins, the Staphylococcal superantigens (SAgs) play an important role in the debilitation of the host. Their superantigenic activity has been attributed to the ability to bind simultaneously to MHC class II receptors and the V_β regions of T-cell receptors. The formation of such a trimolecular complex results in the activation of a large population of T-cells, ultimately releasing massive amounts of inflammatory cytokines. This overstimulation of the immune system can eventually lead to a systemic life-threatening response known as Toxic Shock Syndrome (TSS), which is accompanied with a fatality rate of 5% according to the Centers for Disease Control and Prevention. Besides the administration of antibiotics, present therapy is primarily supportive, with fluid resuscitation and vasopressor agents [1]. As current therapies are not effective, novel treatments are eagerly awaited. Among the possible treatments, removal of toxins has hardly been explored.

Since TSST-1 is regarded as the major etiologic agent of TSS, the specific removal of TSST-1 from the blood could be beneficial for patients suffering from TSS. Indeed, there is some evidence to support that plasmapheresis in severe cases of sepsis can ameliorate the course of the syndrome [2, 3]. Apparently, this method, by which toxic components are removed together with the contaminated plasma, can be beneficial. Nevertheless, the specific removal of TSST-1 is preferential, since endogenous defense molecules generated by the host immune system are also removed by plasmapheresis. Various adsorption devices have been developed that were shown to bind to endotoxins, cytokines and enterotoxins including TSST-1. Treatment of animals with experimental sepsis and/or endotoxemia using these devices resulted in reduced mortality compared with the animals which were left untreated [4-7]. A drawback of the adsorption devices used is that they bind a variety of small proteins which are not strictly defined and may also include beneficial molecules. In view of these data we envision that the development of a filtration system which is highly specific for the target of interest is preferable.

Application of antibodies directed against TSST-1 in specific plasmapheresis can be very effective, as antibodies are highly specific and have high affinity for their cognate target. However, large-scale production of antibodies is often tedious and relatively expensive. The potential solution to this problem comes from *Camelidae* (camels, dromedary, and llamas), which possess a unique immune repertoire of fully functional antibodies that lack light chains and the entire CH1 domain [8, 9]. The monoclonal Variable domain of Llama Heavy-chain antibody (VHH) [10] is distinct from the antigen-binding domain of the heavy-chain of conventional antibodies (VH). Interestingly, these camelid heavy chain antibodies have great potential in various biotechnological applications [11] because of their relatively easy production in microorganisms [12], their excellent refolding features [13], and good stability [14]. Furthermore, the interaction with their cognate antigen is with affinities and specificities that resemble those of conventional antibodies [15]. With an eye on the desired application, for example, specific plasma filtration, VHH can be efficiently coupled at high density on different chromatography supports, making them ideal candidates for affinity chromatography. As the purified VHH domain is a very stable protein, the possibility arises to perform regeneration or cleaning-in-place procedures, for

example, flushing the column with NaOH. In this study, we evaluated the applicability of VHH in the scavenging of low-abundant TSST-1 from plasma.

2.2 Materials and methods

2.2.1 Construction of an immune phage display library against TSST-1

For the construction of a VHH phage display library, a llama was immunized three times with 3-week intervals using respectively 100, 50, and 50 μg TSST-1 (Sigma-Aldrich, St. Louis, MO) injected intramuscularly as described [16]. The immune response was followed by titration of serum samples in an enzyme-linked immunosorbent assay (ELISA) with TSST-1 coated at a concentration of 2.5 μg/ml in phosphate-buffered saline (PBS: 10 mM Na_2HPO_4, 150 mM NaCl NaCl, pH 7.4) following the protocol as described [17]. A 150 ml blood sample containing 10^8 peripheral blood lymphocytes (PBL) was taken 55 days after the first boost, and the VHH antibody gene repertoire was cloned as described [17]. Briefly, RNA was extracted from the PBL for the preparation of cDNA using the SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The genes encoding the VHH were amplified by PCR using primers LAM16/LAM17 and LAM07 (annealing to the short hinge region) or LAM08 (annealing to the long hinge region) [17], and subsequently ligated into the phagemid vector pUR8100 using the restriction sites *Pst*I and *Not*I. The ligation mixture was used for the transformation of *Escherichia coli* strain TG1 (*supE thi*-1 Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)5 ($r_k^- m_k^-$) [F′ *traD*36 *proAB lacI*^{q}*Z*ΔM15]).

2.2.2 Selection and screening of TSST-1 specific VHH

Phage display [18] was utilized to select phages that specifically bind TSST-1. Phages from the short hinge VHH library and from the long hinge VHH library were mixed and preincubated with 50% human plasma (UMCU, Utrecht, the Netherlands) in PBS for 30 min on a rotating wheel. Fifty micrograms TSST-1 per milliliter was captured on a Maxisorp plate (Nunc, Roskilde, Denmark), which was coated with different concentrations (30, 10, or 2 μg/ml) of anti-TSST-1 polyclonal rabbit antibody (Virostat, Portland, ME) at 4 °C overnight, followed by blocking with 50% human plasma in PBS for 1 h. Phage-plasma mixture was incubated with the captured TSST-1 for 2 h at room temperature (RT). Non-binding phages were vigorously washed away by 15 consecutive washes with 0.05% Tween 20 in PBS (PBST), followed by a three times washing with PBS. Bound phages were eluted with 0.1 M Triethylamine for 15 min at RT, followed by neutralization with 1 M Tris-HCl (pH 7.5). Phagemids were rescued by infection of TG1 cells with the eluted phages, and the subsequent selection for ampicillin resistance on Luria Bertani agar plates containing glucose. For the production of VHH-displaying phages, the selected TG1 cells were infected with helper phage VCSM13 (Stratagene, La Jolla, CA) followed by phage production overnight. Phages selected from the highest concentration of captured TSST-1, in the first round, were incubated in wells coated with TSST-1 (20, 4 or 1 μg/ml). Unbound phages were washed away and the bound phages were eluted as indicated above.

Successful selections were designated as those showing an enrichment factor of at least 100-fold phage binding capacity to TSST-1 compared to empty wells. Individual clones from successful selections were isolated in 96-well plates, and phages were produced overnight as described previously [19]. Briefly, supernatants of 96 individual clones were incubated with TSST-1 that was coated into wells, and bound phages were detected using an anti-M13 antibody conjugated to horseradish peroxidase. In addition, the diversity of the selected VHH clones was evaluated by *Hinf*I fingerprint analysis [19]. Briefly, the VHH gene insert was amplified by PCR using primers MPE25WB and M13Reverse. The PCR products were digested with *Hinf*I, and digests were analyzed on 2% agarose gels.

2.2.3 Pull-down assays with anti-TSST-1 VHH

TG1 cells carrying plasmids encoding different anti-TSST-1 VHH were grown until log phase and expression of the VHH genes was induced by the addition of 1mM isopropyl-β-$_D$-thiogalactopyranoside, followed by incubation for 5 h at 37 °C. Subsequently, the periplasmic fraction, containing the VHH, was isolated by a combined Tris-HCl/EDTA treatment and osmotic shock [20]. The VHH were bound by their C-terminal His-tag to Talon metal affinity resin (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's protocol. After extensive washing with PBS, beads containing VHH were incubated with different concentrations of TSST-1 in PBS supplemented with 2% Marvel milk (MPBS) and tumbled for 30 min at RT. The resin was pelleted by centrifugation (2 min at 500 g) and washed three times with 1 ml PBS. Twenty microliter beads were mixed with an equal volume of Laemmli sample buffer, boiled for 10 min, and analyzed by SDS-PAGE on 15% polyacrylamide gels. Proteins were visualized by Coomassie brilliant blue staining and Western blot analysis.

2.2.4 Western blot analysis

Proteins were transferred to Immobilon Polyvinylidene Fluoride (PVDF) membranes (Millipore, Billerica, MA) using a Mini Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA). Immuno-incubations were performed as described [19], using polyclonal antibodies directed against TSST-1 as a primary antibody and horseradish peroxidase-conjugated affinity pure goat anti-rabbit IgG (GARPO; Jackson Immuno-Research Laboratories, West Grove, PA) as secondary antibody.

2.2.5 Surface plasmon resonance (SPR) measurements

In a typical SPR experiment, one of the binding partners (in this case TSST-1) is immobilized on a sensor surface and the other interactant (the VHH) is injected over the surface. The interactions are recorded in resonance units (RU), which are proportional to the mass accumulation on the surface. For the analysis of the VHH-TSST-1 interactions, TSST-1 (between 700 and 2.300 RU) was immobilized on the channels of a CM5 sensor chip (Biacore, Chalfont St. Giles, UK) using NHS-coupling chemistry according to the manufacturer's recommendations. SPR measurements were performed at RT using a Biacore 3000 apparatus (Biacore AB), with HBS-EP buffer (0.01 M Hepes, pH 7.4: 0.15 M NaCl; 3 mM EDTA; 0.005% Surfactant P20; GE Healthcare, Chalfont St. Giles, UK) in

the mobile phase. Different purified VHH were tested for TSST-1 binding. The injection time of the VHH samples was 3 min followed by injection of running buffer for 15 min. An irrelevant VHH was used as a negative control. Regeneration of TSST-1 coated surfaces was achieved after each measurement by 3 min pulses of 10 mM HCl (pH 3), followed by equilibration with HBS-EP buffer. Data were evaluated using BIA evaluation software (Biacore, GE Healthcare).

2.2.6 Large-scale production and purification of VHH

In order to obtain highly purified VHH preparations devoid of endotoxins, His-tagged VHH were produced in the yeast *Saccharomyces cerevisiae* and purified from the extracellular medium using a combination of immobilized metal affinity and ion-exchange chromatography as described before [21]. For this purpose the VHH genes were cloned into the shuttle vector pUR8569 using *Pst*I and *Bst*EII restriction sites. Integration of the VHH genes into the genome of *S. cerevisiae* was as described [22, 23].

2.2.7 Coupling of VHH to sepharose matrices

To prepare a chromatography support with covalently coupled VHH, 2 ml of 1 mg/ml VHH in buffer (0.1 M Hepes, 0.5 M NaCl pH 8.0) was immobilized on 2 ml of NHS-activated Sepharose 4B Fast Flow (GE Healthcare) according to the manufacturer's protocol. After treatment with blocking buffer (0.1 M Tris, 0.5 M NaCl, pH 8.0), the affinity support was washed in an alternating fashion with PBS (pH 7.4), 0.1 M glycine (pH 2.0), and PBS (pH 7.4). Coupling efficiency was checked by SDS-PAGE analysis (data not shown).

2.2.8 Detection of TSST-1 in plasma samples

TSST-1 was immunoprecipitated from depleted fractions and from standards, used for the calibration of the TSST-1 amounts, with 10 ml 50% (v/v) anti-TSST-1 T32-C6 coupled to NHS-Sepharose (1 mg/ml) in a final volume of 500 μl. The samples were tumbled for 2 h head-over-head at RT. The beads were collected by a short spin, followed by washing with PBST and PBS, and finally resuspended into 5 μl Laemmli sample buffer. After incubation at 95 °C for 5 min, the samples were separated on 12% SDS-PAGE gels, followed by Western blot analysis as described above. Human plasma (UMCU, Utrecht, the Netherlands) and pig plasma spiked with 50, 25, 12.5, 6.25, and 0 ng/ml TSST-1 were used as standards.

For the detection of TSST-1 in plasma by ELISA, a Maxisorb-96 wells plate was coated overnight at 4 °C with 1 μg/well sheep polyclonal antibody to TSST-1 (Abcam, Cambridge, UK). The next day, the plate was washed three times with PBS, followed by blocking of the wells with 4% MPBS supplemented with 0.05% Tween 20 for 1 h. From the depleted plasma samples or standards, 100 μl was added to each well, and incubated for 2 h at RT. Subsequently, the plate was washed with PBST and PBS. Captured TSST-1 was detected using anti-TSST-1 polyclonal rabbit antibodies and GARPO.

2.2.9 Pilot depletion experiments

Fast performance liquid chromatography (FPLC) was used to monitor the depletion of TSST-1 from plasma. Human or pig plasma was spiked with TSST-1 and applied onto 1 ml anti-TSST-1 VHH functionalized Sepharose (1 mg VHH/ml matrix), which was poured into a Tricorn 5/50 (Amersham Biosciences, Chalfont St. Giles, UK) high performance column. Loading of the column was monitored on an ÄKTA Prime (Amersham Biosciences) using Unicorn software (Amersham Biosciences). After removal of TSST-1 from plasma, the column was washed with PBS, and bound TSST-1 was released with 0.1 M glycine pH 2.

2.3 Results

2.3.1 Generation of monoclonal llama single-chain anti-TSST-1 VHH antibodies by phage display

For the generation of high-affinity and highly specific VHH directed against TSST-1, a llama was immunized with TSST-1, and the immune response was followed in time. As is shown in Figure 1, a strong increase in the immune response was observed at day 34 and day 55 after the first injection of the llama immunized with TSST-1. Peripheral blood cells were isolated and a VHH-phage library was constructed which is derived from the heavy-chain IgG immune repertoire. The library had a diversity of more than $1.5x10^8$ clones and an insert percentage of 100%. Since the final application involves immunodepletion of plasma, we performed two rounds of enrichment in the presence of 50% plasma. After these two rounds, a high enrichment factor of 25.000-fold, compared to non-coated wells, was calculated. Ninety-six individual clones were tested and all were shown to bind TSST-1 as determined by phage ELISA (data not shown). From 24 selected clones, which had different *Hinf*I fingerprint patterns, twenty clones with unique amino acid sequences were obtained.

2.3.2 Selection of the highest affinity antibodies suitable for immunodepletion

Since we aim at affinity chromatography as the main application of the anti-TSST-1 VHH, we reasoned that immunoprecipitation experiments should shed more light on the potential applicability of the antibody fragments in specific apharesis to deplete TSST-1 from plasma of infected individuals. After production of the VHH in *E. coli*, the antibody fragments were bound to Talon beads through the C-terminal His-tag. The VHH bound to Talon beads were used to immunoprecipitate TSST-1 from spiked 2% MPBS. All 20 selected clones were able to precipitate TSST-1 (data not shown). To test the difference in affinity of VHH to TSST-1, we gradually decreased the concentrations of VHH and TSST-1 in immunoprecipitation experiments (data not shown). The high affinity of the anti-TSST-1 VHH monoclonals is shown in Figure 2, demonstrating that the anti-TSST-1 VHH clones were capable of immunoprecipitating TSST-1 even when only 8 ng of VHH was used. In total seventeen of the 20 selected VHH show high affinity to TSST-1.

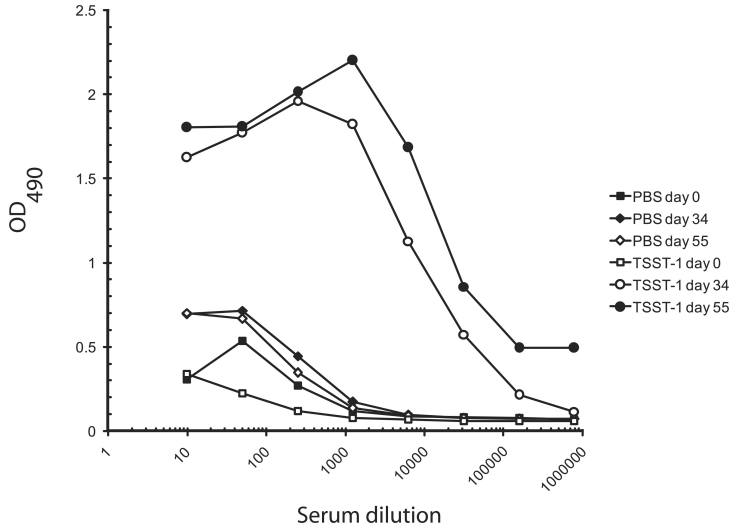

Figure 1. ELISA measurement of the immune response against TSST-1. Maxisorb plate wells were coated with 0.25 μg TSST-1 at 4 °C overnight. After blocking with 4% MPBS, dilutions of the llama serum taken after immunization with TSST-1 at the indicated days were added in 2% MPBS to each well. Heavy chain antibodies were detected with a polyclonal anti-llama heavy chain rabbit antiserum and GARPO.

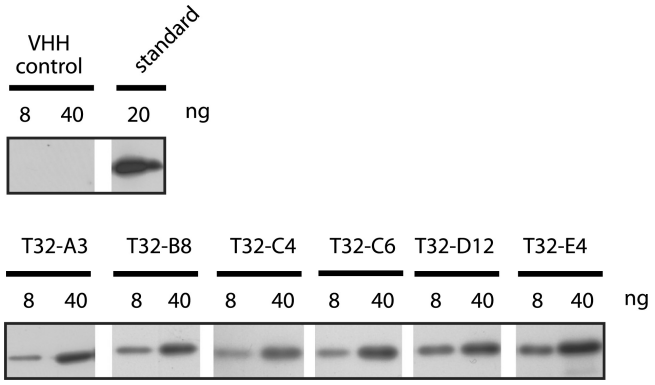

Figure 2. Immunoprecipitation of TSST-1. Two percent MPBS was spiked with 50 ng TSST-1 per ml, and 8 or 40 ng of VHH was used to precipitate TSST-1. The beads were taken up in sample buffer and loaded on a 12% SDS-PAGE gel and analyzed by Western blotting and immunodetection using anti-TSST-1 polyclonal antibodies and GARPO.

2.3.3 *In-depth analysis of VHH clones: binding-efficiency and leakage from the Ab-Ag complex*

The best VHH candidate that will scavenge TSST-1 from plasma of patients should bind the target toxin very tightly even at low concentrations. An important feature of a column intended for scavenging TSST-1 from plasma is, next to efficient binding of the antigen, a very low leakage of the antigen from the antibody-antigen complex. To evaluate the clones for this feature, SPR experiments were conducted with a series of VHH clones that immunoprecipitated TSST-1 with high efficiency.

For a semi-quantitative determination of the VHH-TSST-1 binding, TSST-1 was immobilized by NHS-coupling to the sensor surface. Purified VHH were diluted with running buffer and injected in the Biacore apparatus. The sensorgrams corresponding to the injections of the different VHH are shown in Figure 3. All VHH tested showed a very low dissociation rate, and any binding of the irrelevant control VHH with the TSST-1-coated surface was not observed, demonstrating the high affinity of the VHH for TSST-1. A potential irreversible binding of the VHH to TSST-1 was not observed as regeneration of the chip, using brief washes with low pH buffer, showed removal of all VHH bound (data not shown). After the recorded signal returned to its baseline, we concluded that the VHH antibody fragment was quantitatively removed from the TSST-1-coated Biacore chip.

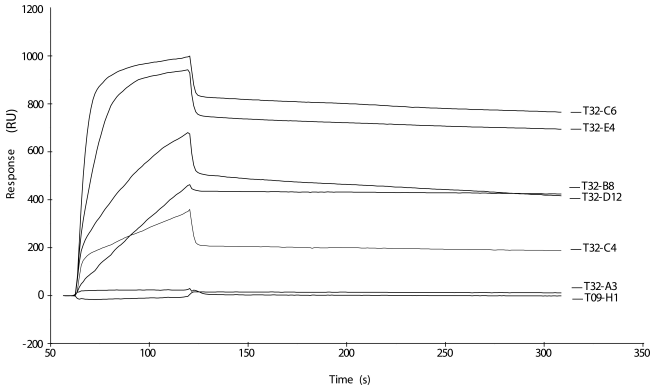

Figure 3. SPR experiments with anti-TSST-1 VHH. Shown are the sensorgrams corresponding to the injections of different VHH on a surface with immobilized TSST-1. T09-H1 is an irrelevant VHH control. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

2.3.4 Production of high-affinity clones in yeast

For a more detailed analysis of the anti-TSST-1 VHH clones, we chose five VHH on the basis of phylogenetic relationship (data not shown) and with unique amino acid sequences, and we cloned those five VHH genes in a yeast expression system. In a previous study [21], we observed unwanted endotoxin contaminations in VHH preparations isolated from *E. coli*. It has been demonstrated that production of VHH in yeast and their subsequent purification using ion exchange chromatography yields a protein preparation lacking detectable levels of endotoxin [21]. The anti-TSST-1 VHH clones were purified under endotoxin-free conditions and chemically coupled to NHS-Sepharose beads.

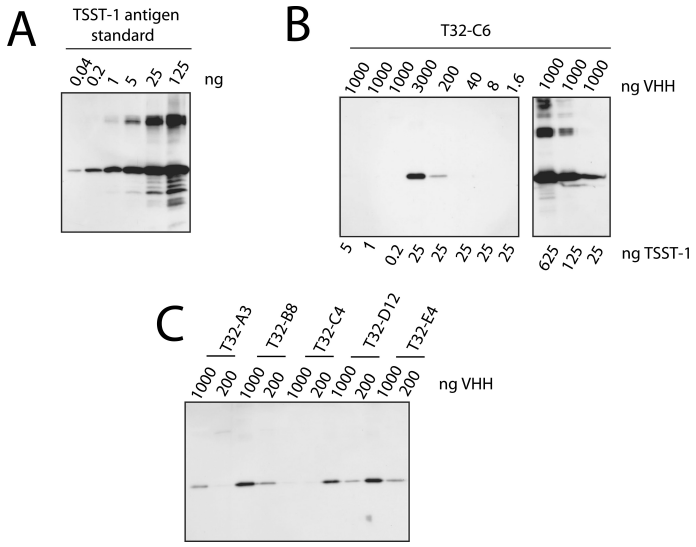

Figure 4. Immunoprecipitation analysis of TSST-1 from human plasma with anti-TSST-1 VHH chemically coupled to NHS-sepharose. Shown are Western blots using polyclonal rabbit anti-TSST antibodies as detection reagent. **A**: Calibration series with the indicated amount of TSST-1 blotted on PVDF membrane. **B**: The immunoprecipitation conditions were optimized with T32-C6 to be able to discriminate the best binding clones. **Left panel**: 1 μg of covalently coupled T32-C6 was used to immunoprecipitate the indicated amount of TSST which was spiked in 1 ml plasma. **Right panel**: with 1000 ng of immobilized T32-C6, immunoprecipitation was performed on the indicated concentrations of TSST-1. **C**: Immunoprecipitation of TSST-1 with 1000 and 200 ng of covalently coupled VHH clones using human plasma spiked with 25 ng TSST-1.

Since the immunoprecipitation experiments described above were conducted with VHH immobilized to Talon beads (i.e. oriented coupling), we tested the NHS-coupled VHH (i.e. high degree of random coupling) again using immunoprecipitation experiments. Two amounts (1 and 0.2 μg) of VHH coupled to NHS-Sepharose were used to immunoprecipitate TSST-1 from human plasma, which was spiked with 50 ng TSST-1/ml. The reaction conditions were optimized using T32-C6 (Figure 4B), followed by analysis of the other VHH clones. As can be seen in Figure 4, three out of six clones were able to precipitate TSST-1 efficiently when 0.2 μg of VHH was used. It must be noted that Sepharose beads alone were not able to precipitate the TSST-1 molecule from the spiked plasma (data not shown), which clearly shows the specificity of the NHS-coupled VHH matrices. To substantiate the data, we also performed a detailed SPR analysis (Table I). As can be observed from these data, clone T32-E4 was the best ligand candidate for the affinity column with respect to its affinity constants.

Table 1. SPR analysis

TSST clone	K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_A (M^{-1})	K_D (nM)
T32-B8	3.6 x 10^5	1.08 x 10^{-4}	3.33 x 10^9	0.30
T32-D12	2.39 x 10^5	1.10 x 10^{-3}	2.17 x 10^8	4.60
T32-E4	1.26 x 10^6	3.58 x 10^{-4}	3.52 x 10^9	0.28

Equilibrium constants as evaluated for the interaction of anti-TSST-1 VHH with immobilized TSST-1.

2.3.5 Depletion of TSST-1 from human plasma by FPLC with an anti-TSST-1 VHH column

The anti-TSST-1 clone T32-E4, which showed the highest binding in the SPR analysis and good results in immunoprecipitation, was chosen for a more detailed study of TSST-1 depletion from plasma using FPLC. To test if covalently immobilized anti-TSST-VHH can efficiently remove TSST-1 from undiluted plasma, 4 ml of undiluted plasma was spiked with 40 μg of TSST-1 and passed over a 1 ml matrix coated with T32-E4. As shown in Figure 5, already 1 ml of column material could efficiently remove this amount of TSST-1 within 2 min, and no TSST-1 could be detected in the flow through fractions. Thus, these data indicate rapid depletion and show that the capacity of the column is more than sufficient to deplete plasma of patients from the concentrations of TSST-1 that are observed in sepsis (i.e. 4 ng/ml TSST-1).

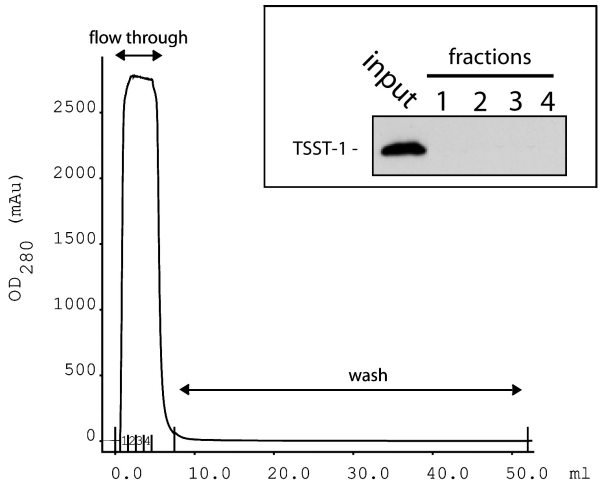

Figure 5. Efficiency of TSST-1 depletion from spiked human plasma. A column with 1 ml NHS-sepharose, which was functionalized with T32-E4 (1 mg VHH/ml), was used to deplete 40 μg TSST-1 from 4 ml of human plasma. Ten microliter of the 100-fold diluted input and flow through samples was analyzed by SDS-PAGE and Western blotting. Input corresponds to 1 ng TSST-1.

Since binding of such low concentration of antigen is much more demanding to the system, we tested if the avidity of the immobilized T32-E4 is sufficient to capture low concentrations TSST-1 from plasma. Direct detection of low amounts of TSST-1 by Western blotting was not possible since the samples had to be diluted to prevent overloading of the SDS-PAGE gels with abundant plasma proteins. Therefore the fractions were first subjected to immunoprecipitation before Western blot analysis. Four milliliters of human plasma NP3 was spiked with 400 ng TSST-1 (100 ng/ml), and Sepharose columns, which were functionalized with T32-E4, were used to deplete TSST-1 from this sample. The amount of TSST-1 in the flow through was immunoprecipitated using T32-C6 and was quantified by comparing it to the amounts, which were immunoprecipitated from calibration samples (Figure 6). Approximately 85% of the TSST-1 was removed. We tried to remove the remaining 15% by sequential reloading of the depleted fractions on a regenerated column but this did not lead to any detectable further removal of TSST-1 (Figure 6, lanes 3 and 4). This is most likely not due to limitations of the immobilized T32-E4 (see below) but might be due to the levels of anti-TSST-1 antibodies present in almost all human plasmas (see Discussion), which compete with the VHH for the free TSST-1.

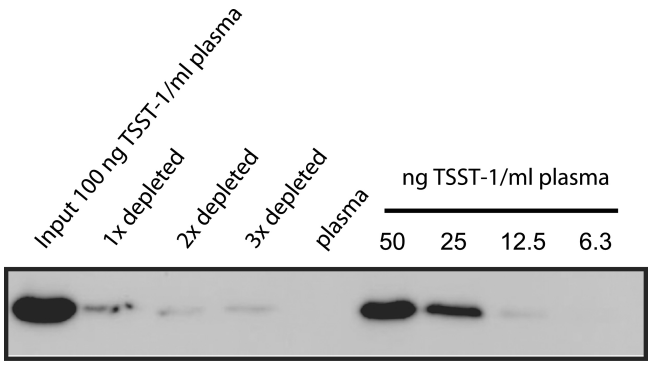

Figure 6. Detection and depletion of low concentration TSST-1 in human plasma. Input: 100 ng/ml TSST-1 in human plasma. The T32-E4 column was used for the depletion of human plasma containing 100 ng/ml TSST-1. The depleted fractions were reloaded twice on the ÄKTA Prime. Input and fractions obtained after depletion were subjected to immunoprecipitation with immobilized T32-C6 VHH, followed by SDS-PAGE, Western blot analysis and immunodetection. The TSST-1 calibration samples were subjected to the same procedure. The detection limit of this assay was 12.5 ng/ml.

2.3.6 Depletion of TSST-1 from pig plasma using FPLC with an anti-TSST-1 VHH column

It has been demonstrated that devices capable of capturing toxins from plasma resulted in reduced mortality among septic animals compared to the untreated animals [4- 7]. In these experiments a toxin-concentration-controlled animal model was used in which pigs were infused with TSST-1, and monitoring was performed for both the infusion period and a subsequent 1 h post-infusion period. From these experiments it was concluded that TSST-1 plays an important role in the pathogenesis of Gram-positive bacterial sepsis by inducing multiple organ dysfunction [24]. Since our aim is to perform *in vivo* trials with an anti-TSST-1 apheresis unit in a pig sepsis model, we tested the applicability of the column in pig plasma at pathologically relevant concentrations of TSST-1 of 4 ng/ml. Four milliliters of pig plasma was spiked with 16 ng TSST-1, and depleted with a T32-E4 functionalized column (5 mg VHH/ml matrix) on an ÄKTA Prime. The amount of TSST-1 in the flow through was quantified by immunoprecipitation using T32-C6 VHH and compared to the amounts, which were immunoprecipitated from the calibration samples, using SDS-PAGE and Western blotting (Figure 7A). Strikingly, in contrast to the 85% removal from human plasma, more than 96% of the TSST-1 was depleted from the pig plasma (Figure 7A and C).

To corroborate these results, an ELISA assay was newly developed for rapid and accurate measurement of TSST-1 levels. A 96-wells Maxisorb plate was coated with polyclonal antibodies directed to TSST-1, in which samples from depleted fractions and calibration samples were tested. The lowest detection limit of TSST-1 in human plasma was relatively high (higher than 4 ng/ml). Remarkably, in pig plasma it was 160 pg/ml (Figure 7B). The ELISA signal of the depleted fractions was in the background, implying that these

fractions contain less than 160 pg/ml of TSST-1. More important, by a combination of immunoprecipitation and Western blot analysis as well as by ELISAs (Figure 7C), our data show that columns of immobilized anti-TSST-VHH are well capable to rigidly deplete pathologically relevant amounts of TSST-1 from pig plasma.

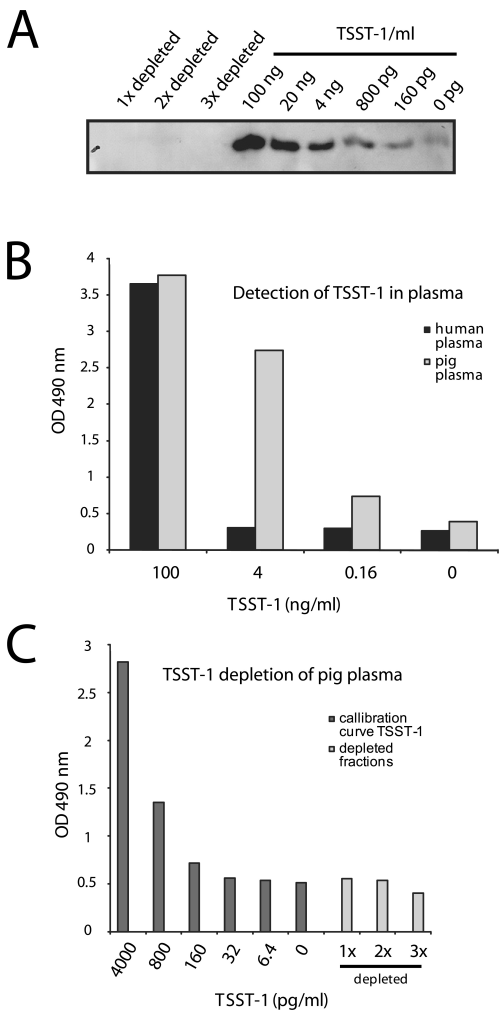

Figure 7. Detection and depletion of TSST-1 in pig plasma. **A**: Depletion of pig plasma containing 4 ng/ml TSST-1 using FPLC, followed by TSST-1 detection using immunoprecipitation and Western blot as described in the legend of Figure 6. Detection limit was 160 pg TSST-1/ml. **B**: Calibration of TSST-1 detection in swine and human plasma. **C**: Depletion of TSST-1 from pig plasma. Input: 4000 pg TSST-1/ml swine plasma. Detection limit of this assay is 160 pg TSST-1/ml.

2.4 Discussion

Toxic Shock Syndrome is a condition characterized by a sepsis syndrome often companied by multiple organ failure and potentially lethal shock. Since the clinical syndrome is mainly caused by the bacterial SAg TSST-1, we reasoned that removal of TSST-1 from spiked plasma will be advantageous in the treatment of the condition. Consistent with this rationale, previous experiments with SAg absorbing devices showed a beneficial effect in sepsis models [4- 7]. These devices are based on polystyrene and are therefore regarded to be aspecific. In our study, we focused on the construction and characterization of an affinity chromatography column for the specific removal of TSST-1 from plasma.

As ligand for the matrix, we chose for the use of the antigen-binding domain of heavy-chain antibodies, and in order to obtain high-affinity antibodies against TSST-1, we immunized a llama with purified TSST-1. Using phage display techniques, we were able to select high-affinity TSST-1 binders. SPR analysis demonstrated that the VHH could easily be applied in scavenging chromatography and we observed very efficient coupling of the VHH to the Sepharose matrix.

During the development of the ELISA tests we observed that the detection limit in pig plasma was lower than in human plasma (160 pg/ml in pig plasma versus 15 ng/ml in human plasma). A plausible explanation is that the natural immune repertoire of most humans contains antibodies directed against TSST-1. If these antibodies compete with the immobilized VHH on the matrix for the same binding site on TSST-1, the TSST-1 molecules which are in complex with these antibodies will not be depleted from the plasma by the column. As pigs do not have such antibodies in their circulation [6], we assume that this is the reason for the measured difference. Taken together, we developed an immunocapturing/Western blotting detection method, as well as an ELISA method, that can both be used for a rapid and reliable test for the determination of residual TSST-1 in plasma after plasmafiltration.

In view of a therapy for the treatment of TSS, in future experiments we will study the use of an immunocapturing hemofiltration filter. This method is based on the extracorporeal blood purification in diseases in which pathological proteins or cells have to be eliminated. We expect that such specific plasma treatment is more efficient in toxin removal than unselective plasma exchange. In addition, it does not require donor plasma or a substitution fluid containing albumin. Specific plasmapheresis has been used in the treatment of rheumatoid arthritis (using Prosorba or Protein A on silica, Fresenius) and dilative cardiomyopathy (Ig-Therasorb, Baxter, Immunosorba, Fresenius). Although the latter methods were used for the removal of abundant proteins, these examples show the viability of specific apheresis (or hemofiltration with functionalized filters). In our study, we obtained proof-of-principle that high-affinity and highly specific VHH can be applied for the removal or scavenging of minor proteins from plasma. We even show that toxic agents at very low concentrations can quantitatively be removed. In addition to the immunodepletion of TSST-1 from human plasma, we believe that these columns can be equipped with other ligands in order to deplete other contaminants from plasma. Consistently, a VHH has been recently discovered which can be used to deplete the bulk plasma protein IgG [25]. This VHH display improved depletion over the classical method based on protein A depletion, and the authors concluded that the VHH can be used for

antibody depletion in the auto-immune diseases Goodpasture's syndrome and systemic lupus erythematosus.

In the present study, we have discovered a selective TSST-1 Camelid VHH. The antibody fragment has a very high affinity, which is essential for its intended purpose. Using Sepharose immobilized VHH, we have successfully depleted the harmful toxin TSST-1 from spiked pig and human plasma. In summary, the affinity columns are able to remove pathologically relevant amounts of TSST-1 (e.g. 4 ng/ml) from pig plasma, yielding a concentration that is less than 160 pg/ml. In addition, we have shown that 1 ml of anti-TSST-1 resin is capable of capturing 40 μg of TSST-1 from plasma in one run through the column. As the total amount of TSST-1 in infected patients is less than 20 μg, it is clear that the binding capacity of the column is more than sufficient for its intended purpose. Moreover, we can produce the antibody fragment in such high quantities and in a cost-effective way, that a potential application to absorb TSST-1 from human plasma *in vivo* would be possible. These features give VHH fragments more advantages over the use of conventional antibodies. To broaden the applicability of the procedure, hemofiltration filters, in which blood cells are directly separated from plasma, will be used to explore the feasibility of extracorporeal TSST-1 depletion in septic animals and to evaluate its consequences on hemodynamics and survival.

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Chapter 3

Llama heavy-chain antibody fragments efficiently remove Toxic Shock Syndrome Toxin-1 (TSST-1) from plasma *in vitro* but not in experimental porcine septic shock

Abstract *Staphylococcus aureus* produces the superantigen Toxic Shock Syndrome Toxin-1 (TSST-1). When the bacterium invades the human circulation, this toxin can induce life-threatening Gram-positive sepsis. Current sepsis treatment does not remove bacterial toxins. Variable domains of llama heavy-chain antibodies (VHH) against Toxic Shock Syndrome Toxin-1 (α-TSST-1 VHH) were previously found to be effective *in vitro*. We hypothesized that removing TSST-1 with α-TSST-1 VHH hemofiltration filters would ameliorate experimental sepsis in pigs. After assessing *in vitro* whether timely removing TSST-1 interrupted TSST-1-induced mononuclear cell TNF-α production, VHH-coated filters were applied in a porcine sepsis model. Clinical course, survival, plasma IFN-γ, and TSST-1 levels were similar with and without VHH-coated filters as were TSST-1 concentrations before and after the VHH filter. Plasma TSST-1 levels were much lower than anticipated from the distribution of the amount of infused TSST-1, suggesting compartmentalization to space or adhesion to surface not accessible to hemofiltration or pheresis techniques. Conclusions: Removing TSST-1 from plasma was feasible *in vitro*. However, the α-TSST-1 VHH adsorption filter-based technique was ineffective *in vivo*, indicating that improvement of VHH-based hemofiltration is required. Sequestration likely prevented the adequate removal of TSST-1. The latter warrants further investigation of TSST-1 distribution and clearance *in vivo*.

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Accepted in *Shock*

3.1 Introduction

Sepsis is a systemic inflammatory response syndrome in the presence of circulating infective agents and their toxins [1]. Mortality rates remain high at approximately 20%. The incidence of sepsis still rises (currently circa 0.3% of the American population [2]). Gram-positive bacteria cause most cases. While gram-negative cell wall components are called endotoxins, e.g. lipopolysaccharide (LPS), gram-positive bacteria produce and secrete very pathogenic exotoxins such as Staphylococcal enterotoxin B (SEB) and F (SEF). The latter is also known as Toxic Shock Syndrome Toxin-1 (TSST-1). These toxins are called superantigens (SAgs), because they bind with very high affinity to both the T-cell receptor and the MHC-class II complex on antigen-presenting cells (APC). This leads to sustained formation and release of pro-inflammatory cytokines such as interferon-γ with the sepsis syndrome as a result [3, 4].

In current sepsis treatment, respiratory support, intravenous fluid administration, and/or vasoactive drugs maintain adequate tissue oxygenation and perfusion while antibiotics eradicate the infectious agents. However, the SAgs that initiate the systemic response most likely sustain the deranged immune reaction. Immediate and specific removal of SAgs from the blood could interrupt this cascade. One way of achieving this would be by applying antibodies (Abs) aimed directly at the SAgs. However, isolating, selecting, and producing conventional Abs is often difficult and expensive. A sub group of Camelidae (camels, dromedary and llamas) Abs lack the light chain and CH1 domain of conventional Abs. Recently, a technique has been developed that enables easy, low-cost, large-scale selection and production of the Variable domain of the Heavy chain of Heavy chain Camelidae Abs (VHH) [5]. These VHH are small (12-15 kDa) and preserve highly specific binding capacity. In addition, they have excellent refolding features and biological stability; all features making them suitable for biotechnological applications. This new technology now requires *in vivo* exploration before clinical application.

The hypothesis of this study was that timely removing TSST-1 with highly selective VHH against Toxic Shock Syndrome Toxin-1 (α-TSST-1 VHH) could interrupt the TSST-1 induced inflammatory cellular response. We first developed an α-TSST-1 VHH that demonstrated highly specific TSST-1 binding *in vitro* [6]. We then certified that timely removing the toxin interrupted a TSST-1 induced *in vitro* inflammatory response. Finally, an α-TSST-1 VHH plasmapheresis filter was tested in a porcine TSST-1 sepsis model.

If these tests, and follow-up survival studies in animals, are successful, then a following step will be to test the device in human sepsis. Ideally our novel approach will facilitate direct, therapeutic removal of sepsis-inducing toxins instead of the aforementioned traditional supporting treatments. Early intervention in the sepsis cascade with our device would potentially prevent severe tissue damage and improve patient survival.

3.2 Materials and methods

3.2.1 Binding TSST-1 in vitro with highly specific α-TSST-1 VHH

Previous experiments [6] showed the α-TSST-1 VHH protein-ligand had a very high affinity (0.28 nM). Coupled to sepharose beads, the VHH could successfully remove TSST-1 from

human and pig plasma. *In vivo*, a separate plasma separation step would be necessary before depletion by such sepharose beads. We decided to remove this step and design a filter that separates plasma and depletes TSST-1 at the same time. We coupled α-TSST-1 VHH via carbodiimid to acrylacid-grafted microporous polyethersulphone (PESU) fibres (Gambro Dialysatoren GmbH, Device Research, Hechingen, Germany). After gamma sterilization, the fibers' TSST-1-depleting capacity was investigated. A mini filter containing 77 fibers was tested on an ÄKTA prime chromatography device (Amersham Biosciences, Freiburg, Germany) to deplete TSST-1 from pig plasma (4 ng/ml TSST-1). After washing and equilibrating the filter with phosphate buffer solution (PBS) pH 7.4, TSST-1 was eluted with glycine pH 2. The output fraction was reloaded once and TSST-1 was measured by a custom ELISA (see Supplement).

3.2.2 *Interrupting the TSST-1-induced inflammatory response* in vitro *by removing the toxin*

Rationale
If TSST-1 binds with such a high affinity to its target cells that removing TSST-1 from the cell solution does not alter the equilibrium of free versus cell bound TSST-1, then *in vivo* application of highly selective α-TSST-1 VHH in experimental sepsis will be very difficult. To investigate the TSST-1 equilibrium, we adapted previously described experiments of TSST-1-induced human peripheral blood mononuclear cells (PBMC) TNF-α production [7, 8]. In this setup, we tested whether timely removing TSST-1 dampened the immune response.

PBMC isolation and cell culture
Informed consent was obtained and venous blood was collected. PBMC were isolated and cultured at a concentration of $1x10^6$ cells/well in RPMI 1640 with 10% autologous plasma (see Supplement).

PBMC challenge and cytokine analysis
At different TSST-1 concentrations and stimulus duration, the optimum inflammatory response in terms of TNF-α production was determined. PBMC were challenged at TSST-1 (Sigma-Aldrich, Inc., Saint Louis, MO) concentrations of 0, 10 and 30 ng/ml for 2, 4, 8 and 24 h. TNF-α, measured by enzyme-linked immunosorbent assay (ELISA Max™, Biolegend, San Diego, CA), was chosen as primary read-out. At the optimum response time (24 h), the dose-response curve was determined using TSST-1 concentrations of 0, 3, 10, 30, 100, 300 and 1000 ng/ml. All incubations and cytokine assays were performed in triplicate.

Experimental design
After 24 h of stimulation, PBMC (obtained from 5 donors) showed an increasing TNF-α production with increasing TSST-1 concentration, reaching a plateau of circa 2000 pg/ml TNF-α at TSST-1 concentrations of 30 and 100 ng/ml TSST-1. These concentrations were chosen to investigate if tenfold reduction of the TSST-1 load would reduce TNF-α production to sub-maximum levels, hence demonstrating that timely removal of TSST-1

is beneficial for stopping the immune response. PBMC were incubated at TSST-1 concentrations of 0, 30 and 100 ng/ml. On separate occasions, at t = 1 h or 4 h, 0.9 ml of spiked culture medium was aspirated in a 1 ml Micro-Fine^{TM} insulin needle (Becton Dickinson France S.A., le Pont de Claix, France) and clean culture medium was gently added. Particular care was taken not to aspirate cells. The time points of 1 h and 4 h were chosen because in the *in vivo* experiments (see below), which lasted 10 h, the α-TSST-1 VHH filter would be applied before t = 4 h. At the same points in time (1 h and 4 h), in an identical PBMC system containing an equal amount of PBS instead of TSST-1, 0.9 ml culture medium was withdrawn, discarded, and an equal volume of TSST-1 laden culture medium was added. This was done to investigate whether culture medium thus handled was still biologically active. TNF-α concentrations were determined as described above in all culture media 24 h after incubation start (23 h and 20 h after replacement of the culture media).

3.2.3 Application of highly selective α-TSST-1 VHH in a TSST-1 porcine sepsis model

Animal study
The Utrecht University Board for experimental animal studies approved the study protocol. All animal use was in compliance with the principles of Laboratory Animal Care (NIH, No. 85-23, 1985) and the Dutch law on Experimental Animal Care. Twenty-four germ-free, female Dalland pigs (Waiboerhoeve, Lelystad, the Netherlands) were housed until start of the experiments. One day prior to experimentation, the pigs were fasted. The pigs weighed between 25 and 35 kilograms on the experimental day.

Extracorporeal circuit and α-TSST-1 VHH filter
An AK100 dialysis machine (Gambro AB, Lund, Sweden) was used for continuous hemofiltration using a customized extracorporeal circuit. This included a modified hemofiltration filter, containing fibers coated with either α-TSST-1 VHH or uncoated filter (α-TSST-1 VHH concentration 33 ± 5 mg (mean ± SD) per filter; fibre material polyethersulphone (PESU); 1300-1400 fibers per filter; fibre diameter 320 μm; effective fiber length 15 cm; surface area circa 0.2 m^2). Assuming a 1:1 molar ligand/TSST-1 ratio, this filter VHH concentration should be more than sufficient to remove the ca. 0.9 mg infused TSST-1 per animal (see below). When blood flowed through the fibers, the small pore size trapped all solid blood components with a diameter > 0.2 μm, while allowing passage of plasma to the outside of the fiber lumen. The created plasma flow thus came into close contact with the α-TSST-1 VHH on the inner and outer surface of the fibers before returning to the animal. Arterial blood flow was 100 ml/min, and plasma flow was 20 ml/min. The extracorporeal circuit including the filter had a volume of 217 ml and was primed with sterile saline (37 °C) before connecting it to the animal. Air bubble detectors prevented passage of air into the animal.

Toxin
Highly purified (> 95% pure by SDS-PAGE, Coomassie blue stain) TSST-1 was obtained from Toxin Technology, Inc. (Sarasota, FL, USA). The TSST-1 contained < 30 IU/mg

endotoxin, determined by colorimetrix LAL assay. Before each experiment, solid TSST-1 was dissolved in 1 ml sterile saline at 1 mg/ml per vial.

Experimental protocol
Pigs were anesthetized throughout the experiment. Anticoagulation was maintained with heparin (see Supplement). Care was taken to minimize bleeding, especially at sites of cannulation. The study groups and experimental procedure are depicted in Table 1 and Figure 1. The effect of the α-TSST-1 VHH filter on plasma TSST-1 levels and clinical outcome (see below) was tested in the two toxin treated groups (Toxin/Sham and Toxin/Ligand). The two control groups (Sham and Ligand) were included to obtain baseline control values and to rule out any unwanted effects of the VHH, respectively. Based on previous pig experiments by Miwa *et al*. [9], a continuous infusion of TSST-1 at a concentration of 2 μg/kg/h was used. In their model, Miwa *et al*. aggravated the immunological reaction to TSST-1 by adding LPS infusion [9]. However, in our study we aimed for a clinically severe sepsis model, solely based on TSST-1 infusion in germ-free, LPS-naive, animals. To increase the severity of the model, we added a bolus infusion of 10 μg/kg TSST-1 at t = 0 h. The TSST-1 solutions for bolus and continuous infusion were prepared directly beforehand in 50 ml sterile saline in two separate syringes (Becton Dickinson BV, Etten-Leur, the Netherlands). After the extracorporeal circuit was connected to the pig and had run without hemofiltration for 30 min (only passage of blood through the filter), at t = 0 h hemofiltration and TSST-1 infusion were started simultaneously. The bolus solution was infused in 30 min while the continuous infusion lasted for 10 h until the end of the experiment. The syringe for continuous infusion was cooled externally with ice wrapped in aluminum foil to minimize toxin degradation.

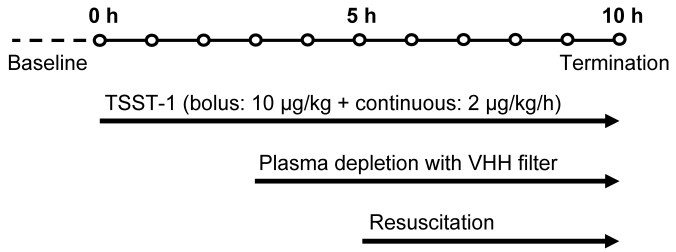

Figure 1. Experimental procedure. Abbreviations: TSST-1, Toxic Shock Syndrome Toxin-1; VHH, Variable domains of llama heavy-chain antibodies.

At t = 3 h, hemofiltration was briefly interrupted by stopping the pumps. Arterial, venous and plasma tubing connected to the filter was clamped after which the Sham filter was substituted by the α-TSST-1 VHH coated filter (= Ligand filter). Hemofiltration was started again, allowing blood to come into contact with the α-TSST-1 VHH inside the filter. During the experiment, all filters were flushed every 30 min with 50 ml sterile saline in order to prevent clotting. Resuscitation was started at t = 5 h. The main resuscitation goal was to keep the mean arterial pressure $\geq$ 35 mmHg by infusing sterile saline, 10%

Table 1. Study group characteristics

Group	No. of Animals	Mean Weight (kg)	TSST-1 Administered		Hemofiltration Filter	
			Bolus (μg/kg)	Continuous (μg/kg)	0-3 h	3-10 h
Sham (S)	2	33 ± 10	None	None	Sham	Sham
Ligand (L)	2	28 ± 1	None	None	Sham	α-TSST-1 VHH
Toxin/Sham (TS)	4	29 ± 2	10	2	Sham	Sham
Toxin/Ligand (TL)	5	32 ± 6	10	2	Sham	α-TSST-1 VHH

All data mean $\pm$ SD. Abbreviations: α-TSST-1 VHH = variable domains of llama heavy-chain antibodies (VHH) against TSST-1; TSST-1, Toxic Shock Syndrome Toxin-1.

hydroxy-ethylated starch (HAES, Fresenius Kabi BV, 's- Hertogenbosch, the Netherlands; MW 200 kDa) and norepinephrine (Centrafarm BV, Etten-Leur, the Netherlands) (Figure 2). All animals were terminated at t = 10 h by a thiopental overdose.

In Vivo *Measurements*
Plasma TSST-1 levels, animal survival, systemic vascular resistance (SVR), mean arterial pressure (MAP) and cardiac output (CO) were chosen as primary outcome variables. Resuscitation need, heart rate, percent neutrophil count and plasma INF-γ levels were secondary outcome variables.

Histology
Post-experimental lung, liver, kidney and spleen tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissues were sectioned at 3 μm, stained with Hematoxylin and Eosin (H&E) and Periodic Acid-Schiff (PAS), and microscopically examined (H&E: 200x magnification; PAS: 100x). Total neutrophil count (PAS, 10 fields) and thrombi count (H&E, 20 fields) were averaged per group.

3.2.4 Statistical analysis

All results are expressed as mean $\pm$ SD. All comparisons were performed using SigmaStat version 3.10 (Systat Software Inc., San Jose, CA, 2004). Comparisons were made within each group and between groups using two-way RM-ANOVA, and, where significant interaction was found, a SNK post hoc test was applied. A $p < 0.05$ was considered significant.

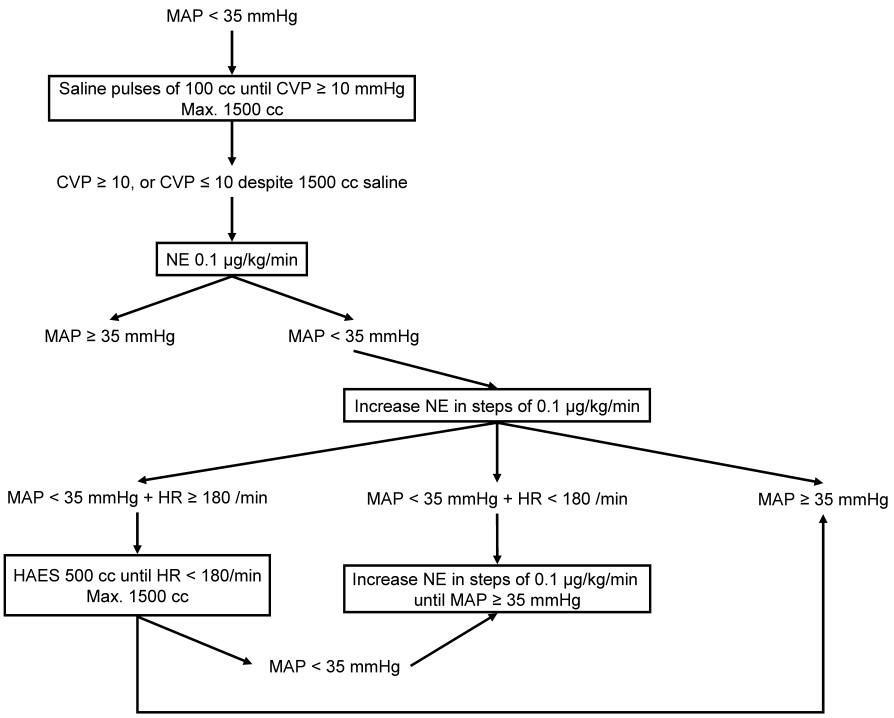

Figure 2. Resuscitation scheme of the *in vivo* experiment. After t = 5 h, resuscitation was allowed. The main resuscitation goal was to keep the MAP $\geq$ 35 mmHg. Abbreviations: CVP, central venous pressure; HAES, 10% hydroxy-ethylated starch; HR, heart rat; MAP, mean arterial pressure; NE, norepinephrine.

3.3 Results

3.3.1 Binding of TSST in vitro *using highly specific α-TSST-1 VHH*

After one pass over the α-TSST-1 VHH filter, the TSST-1 signal in the depleted fraction fell within the background signal ($\leq$ 160 pg TSST-1 per ml; ELISA, data not shown). Given the input of 4000 pg/ml, at least 96% of the TSST-1 was depleted in one pass. Reloading the sample on the ÄKTA prime gave the same result.

3.3.2 Interruption of the TSST-1-induced inflammatory response in vitro *by removal of TSST-1*

In the time-course experiment, TSST-1 induced highest PBMC TNF-α production after 24 h (data not shown). The dose-response curve at 24 h showed a maximum inflammatory response at TSST-1 concentrations of 30 and 100 ng/ml (Figure 3A). Culture medium substitution experiments were performed under these conditions (24 h incubation with 30

and 100 ng/ml TSST-1). Reducing the TSST-1 load tenfold at t = 1 h (30 ng/ml to 3 ng/ml) lowered the 24 h TNF-α response considerably, and, in accordance with the 24 h dose-response level of 30 ng/ml TSST-1, to the level of 3 ng/ml TSST-1 (Figure 3B, 1). This was also the case when 100 ng/ml TSST-1 was reduced tenfold at t = 1 h (Figure 3B, 2). Thus, substitution of toxic medium by clean medium reduced the toxic stimulus, indicating that TSST-1 is not irreversibly bound to PBMC. In addition, aspired culture medium was able to induce TNF-α production when added to non-stimulated cells (Figure 3B, 3 and 4).

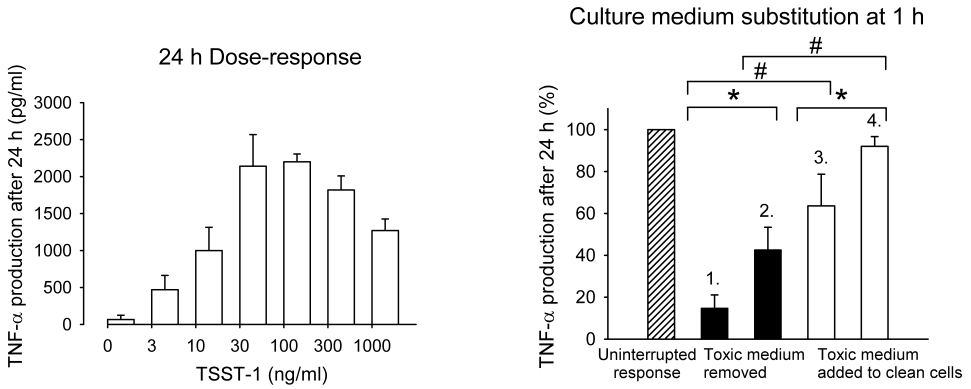

Figure 3. **(A)** TSST-1 stimulated PBMC TNF-α production in a dose-dependent fashion, reaching maximum inflammatory response after 24 h stimulation with 30 and 100 ng/ml TSST-1. **(B)** The *in vitro* inflammatory response could be interrupted by tenfold reduction of the toxin load at t = 1 h. Maximum response (100%) at 30 and 100 ng/ml TSST-1, 24 h incubation (uninterrupted response, striped bar). **1.** Strong reduction of the 30 ng/ml TSST-1 inflammatory response when replacing 90% of TSST-1 laden culture medium with clean medium after 1 h of toxic incubation. **2.** Identical reaction when reducing 100 ng/ml to 10 ng/ml. **3.** The aspired medium of 1. induced a marked response when added to cells unexposed to TSST-1. **4.** Idem when adding the medium of 2. to unexposed cells.
All TNF-α readout after 24 h incubation. # $p < 0.01$, * $p < 0.05$.

This indicated that TSST-1 was still in the supernatant, and was active. Repeating these experiments with 4 h of initial toxic incubation gave similar results (Figure S1, see Supplement). The negative control showed no TNF-α production (data not shown). These findings imply that TSST-1 reversibly binds to PBMC. Thus, timely TSST-1 removal potentially can reduce a TSST-1 induced inflammatory response *in vivo*.

3.3.3 Application of highly selective α-TSST-1 VHH in the porcine TSST-1 model

Effect on hemodynamics, resuscitation need and survival

There were no significant differences in clinical measurements between the two toxin-treated groups (Toxin/Sham and Toxin/Ligand) (Table 2; see also Table S1, Supplement). Both toxin groups showed a similar fall in MAP, while the two control groups remained stable. This was also the case for SVR. Cardiac output in Toxin/Sham was slightly

higher than in Toxin/Ligand (NS). Heart rate increased similarly in both toxin groups, as did neutrophil count and plasma IFN-γ levels. Resuscitation need was equal in Toxin/Ligand and Toxin/Sham (Table 3). The 10 h survival of the pigs in Toxin/Ligand and Toxin/Sham was not significantly different (60% vs. 25% at t = 10 h, NS) (Figure 4). Due to the small group sizes at t = 10 h, this point was omitted in the analysis.

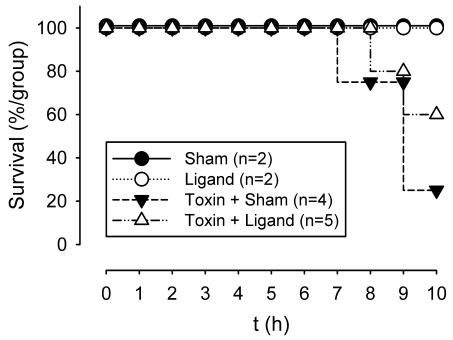

Figure 4. Survival of the animals in the *in vivo* experiment. Survival was not significantly different between Toxin/Sham and Toxin/Ligand.

Effect of the α-TSST-1 VHH filter on plasma TSST-1 levels
Application of the filter with attached α-TSST-1 VHH failed to affect the course of the sepsis syndrome. We then investigated plasma TSST-1 levels and TSST-1 removal by the filter. Plasma TSST-1 levels were not different between animals treated with the α-TSST-1 VHH filter and the sham filter (Figure 5). There was no detectable TSST-1 in the Sham and Ligand group (data not shown). TSST-1 levels were the same before and after the filter in both Toxin-treated groups (Figure S2, see Supplement). Note that the plasma levels were much lower than expected from the infusion scheme (see calculated distribution volume below).

Microscopic findings
Compared to the Sham and Ligand filter groups, neutrophil infiltration increased under toxin infusion (Table 4 and Figure S3). Analysis of variance indicated a significant effect of toxin infusion ($p < 0.01$). Their was no significant effect of the ligand or interaction between toxin and ligand. Toxin also tended to increase the thrombi count, but this was not significant. Histological examination of liver, kidney, and spleen showed no evident pathological findings.

Calculations regarding distribution volume of TSST-1
Since the plasma levels were much lower than anticipated, we estimated the distribution volume and the anticipated plasma TSST-1 levels. A pig weighing 30 kg received a TSST-1 bolus of 10 μg/kg and 2 μg/kg/h continuously. The blood volume of a 30-kg pig is circa

Table 2. Clinical and laboratory measurements in the animal experiments

Measurement	Group	0 h	3 h [a]	5 h [b]	9 h [c]	Interaction between Groups and Time
SVR (dyn·s·cm^{-5})	S	1822 ± 992	1439 ± 443	1951 ± 888	1829 ± 933	$p < 0.001$
	L	1513 ± 442	1348 ± 209	1471 ± 530	1000 ± 581	
	TS	1547 ± 579	1393 ± 267	1161 ± 372	659 ± 140 ** #	
	TL	1587 ± 593	1085 ± 308*	757 ± 403** #	743 ± 138** #	
MAP (mmHg)	S	67 ± 22	63 ± 20	60 ± 23	68 ± 28	NS
	L	57 ± 3.5	55 ± 5.7	58 ± 21	54 ± 22	
	TS	64 ± 6.8	52 ± 14	47 ± 16	43 ± 11	
	TL	56 ± 11	51 ± 8.1	40 ± 10	41 ± 19	
CO (l/min)	S	2.8 ± 0.3	3.3 ± 0.1	2.4 ± 0.3	2.7 ± 0	$p = 0.045$
	L	2.5 ± 0.1	3.3 ± 1	2.9 ± 1.7	2.7 ± 0.8	
	TS	3.3 ± 1	2.8 ± 0.4	3 ± 0.3	4.6 ± 2.2	
	TL	2.9 ± 1	3.3 ± 0.7	4 ± 1.9	3.5 ± 1.1	
HR (min^{-1})	S	74 ± 3.5	67 ± 13	62 ± 15	68 ± 10	$p = 0.043$
	L	59 ± 1.4	67 ± 13	71 ± 4.9	141 ± 13	
	TS	74 ± 12	74 ± 33	140 ± 35* #	140 ± 59* #	
	TL	73 ± 15	96 ± 48	129 ± 51*	173 ± 10** #	
Neutrophil count (% of total WBC)	S	33 ± 1.8	36 ± 5.7	30 ± 6.0	31 ± 0.1	$p = 0.003$
	L	28 ± 4.9	27 ± 4.5	43 ± 5.1	45 ± 4.9	
	TS	23 ± 6.8	44 ± 19*	47 ± 18*	59 ± 21**	
	TL	19 ± 7.6	41 ± 14*	35 ± 13*	59 ± 12**	
IFN-γ (pg/ml)	S	21 ± 29	0 ± 0	0 ± 0	0 ± 0	$p = < 0.001$
	L	8.5 ± 13	11 ± 11	21 ± 7.5	14 ± 7.5	
	TS	11 ± 11	327 ± 227*	630 ± 406** #	520 ± 349**	
	TL	23 ± 31	484± 285**	685 ± 251** #	653 ± 224** #	

All data mean ± SD. Abbreviations: CO = cardiac output; HR = heart rate; IFN-γ = interferon-gamma; MAP = mean arterial pressure; SVR = systemic vascular resistance; TSST-1 = toxic shock syndrome toxin-1; WBC = White blood cells.
For group explanation, see Table 1.

[a] Sham filter replaced by either another Sham filter (S, TS), or by Ligand filter (TL)
[b] Resuscitation start
[c] Last point in time where group sizes were large enough for comparison (1 h before termination)

* $p < 0.05$ versus t = 0 h; ** $p < 0.001$ versus t = 0 h; # $p < 0.05$ versus Sham

8% of its body weight (2400 ml), the hematocrit is circa 0.35. This leads to a calculated plasma TSST-1 concentration at t = 10 h of:

$$\frac{(30[\text{kg}]\cdot 10[\mu\text{g/kg}]) + (30[\text{kg}]\cdot 2[\mu\text{g/kg/h}]\cdot 10[\text{h}])}{30[\text{kg}]\cdot 0.08\cdot 0.65} = 577\ \mu\text{g/l} = 0.577\ \mu\text{g/ml} = 577\ \text{ng/ml}$$

Table 3. Resuscitation need

Intervention	Group	6 h	7 h	8 h	9 h	Interaction between toxin and filter
Fluid infusion (ml)	S	0	0	0	0	NS
	L	0	0	0	0	
	TS	1125 ± 854	625 ± 629	667 ± 764	583 ± 144	
	LS	400 ± 418	110 ± 219	380 ± 415	380 ± 415	
Norepinephrine (μg)	S	0	0	0	0	NS
	L	0	0	0	0	
	TS	188 ± 164	424 ± 351	382 ± 95	630 ± 494	
	TL	5 ± 12	81 ± 111	523 ± 654	665 ± 836	

All data mean ± SD. For group explanation, see Table 1.

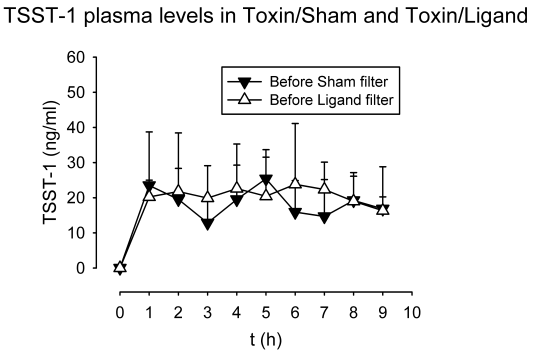

Figure 5. Plasma TSST-1 levels did not differ between Toxin/Sham and Toxin/Ligand group. Abbreviations: TSST-1, Toxic Shock Syndrome Toxin-1.

Table 4. Lung histology

Group	Total Neutrophil Count (10 fields)	Interaction between toxin and filter	Total no. of Thrombi (20 fields)	Interaction between Toxin and Filter
S	141 ± 76	NS	16 ± 3	NS
L	102 ± 67		12 ± 6	
TS	293 ± 156		21 ± 10	
TL	777 ± 250		21 ± 11	

All data mean ± SD. For group explanation, see Table 1.

However, in Toxin/Sham however, the plasma TSST-1 concentration is constant at 20 ng/ml, or $\approx$ 3.5% of the infused amount of toxin at t = 10 h. This low plasma level makes the removal of toxin nearly impossible: assuming a 1:1 molar α-TSST-1 VHH/TSST-1 filter

ratio, with a plasma flow of 20 ml/min, the amount of TSST-1 that theoretically could be removed per minute is (20 ng/ml x 20 ml/min =) 400 ng/min, or 24 μg/h. The total amount infused TSST-1 was 900 μg. So, even under assumption of a 1:1 molar ratio, removal would take (900 μg / 24 μg/h =) 37.5 h. Assuming TSST-1 is not distributed solely in the circulation, but also in the extracellular fluid volume (ECFV, circa 20% of bodyweight), the plasma TSST-1 concentration should be 150 ng/ml. Assuming that there is no substantial (and fast) clearance, the distribution volume of TSST-1 cannot be the ECFV because the largest part of infused TSST-1 remains unaccounted for. Recalculated from the plasma TSST-1 levels, the TSST-1 distribution volume should be 45 liters (in a $\approx$ 30 kg pig).

3.4 Discussion

In the present study, we tried to remove the bacterial toxin TSST-1 using highly selective, specifically developed α-TSST-1 cameloid VHH. First, we showed *in vitro* that the VHH was capable of removing TSST-1 from a plasma solution. Second, when TSST-1 was added to PBMC, it strongly induced TNF-α production after 24 h, reaching a plateau at a TSST-1 concentration of 30 and 100 ng/ml. Further increase of the TSST-1 dose reduced TNF-α levels, most likely via enhanced PBMC apoptosis, although this was not investigated in detail. Since we wanted to know whether TSST-1 was adsorbed by or adhered to PBMC, we then tested *in vitro* if the TSST-1 induced PBMC TNF-α production could be interrupted by removing the TSST-1. This was indeed the case, and aspired supernatant was able to induce a response in unexposed PBMC. These data predicted that the α-TSST-1 VHH could bind and remove TSST-1 *in vivo*, and that TSST-1 would not be taken up by or adhere to PBMC. Consequently, we proceeded to test whether a filter functionalized by chemically coupled α-TSST-1 VHH was able to alter the course of events in a reported TSST-1 porcine model. Severe sepsis was allowed to develop within three hours before toxin removing interventions were started. This mimicked accelerated human sepsis course and treatment. During the last hours of the experiment, TSST-1 infusion was lethal in several animals, while sham-treated animals all survived. The resuscitation after 5 hours was introduced in order to prevent the animal's death before the end of the experiment, and to preserve group size. Resuscitation need was one of the primary outcome variables. Although it is tempting to speculate that the volume and epi findings are supportive for a beneficial effect of the filter, none of the other parameters supports such a conclusion. The filter was unable to reverse the TSST-1 induced sepsis syndrome. When further investigating the TSST-1 concentrations in the plasma, levels were substantially lower than anticipated, which may suggest sequestration into a compartment not directly in equilibrium with the plasma. Furthermore, at the observed low plasma concentrations, the filter was unable to remove measurable amounts of TSST-1. We decided not to expand group sizes since the intervention obviously did not seem to be effective and the ligand filter did not appreciably effect the other parameters.

There are several animal models (e.g. pig [9, 10], mice [11], and rabbit [12, 13]) for studying TSST-1 induced sepsis. We opted for a model in a larger animal because we wanted to investigate variables measured via the Swan-Ganz catheter. Also, the equipment to perform the hemofiltration was not compatible with smaller animals. For these reasons,

we chose a porcine sepsis model as published previously by Miwa *et al*. [9]. To increase its severity, we added a 10 μg/kg TSST-1 i.v. bolus at the beginning of the protocol in addition to the continuous 2 μg/kg/h i.v. infusion. This TSST-1 regimen indeed resulted in a severe systemic inflammatory response with hemodynamic instability. Systemic vascular resistance decreased and the animals invariably became hypotensive, necessitating volume resuscitation combined with vasoconstrictor infusion. This coincided with an increase in percent neutrophil count. As such, the model was very similar to the model reported previously by Miwa *et al*. [9] in which a positive effect of their filter was claimed. It should be mentioned that our *in vivo* plasma TSST-1 levels (20.000 pg/ml) were much higher than in Miwa *et al*.'s combined TSST-1/LPS model (3000 pg/ml [10]), and human toxic shock syndrome (440 pg/ml [14]). It is known that LPS increases the host's susceptibility to TSST-1 dramatically [15, 16]. Since our model did not include LPS exposure before (germ-free animals) or during the experiments, a much higher plasma TSST-1 concentration was needed in order to develop toxic shock syndrome.

A filter functionalized by chemically coupled α-TSST-1 VHH failed to influence the development of the sepsis model. As mentioned, we did not increase the number of animals in each group because we were unable to demonstrate any beneficial effect of the α-TSST-1 VHH filter on hemodynamic parameters, cytokine levels, and resuscitation need. This unanticipated finding led us to explore TSST-1 levels. First, plasma levels of TSST-1 were not affected by the α-TSST-1 VHH filter. This suggested that no substantial amount of TSST-1 was removed from the animal. Second, when plasma samples, taken from the arterial inflow and reconstituted blood after the filter were compared, TSST-1 concentrations were unchanged, regardless of the presence or absence of ligand on the filter membrane.

There are several possible explanations for this *in vivo* observation. One is a difference between plasma and blood. In the *in vitro* experiments, plasma was spiked with TSST-1, while in the *in vivo* experiments the TSST-1 resided in blood. Our cell experiments revealed that TSST-1 is not substantially adsorbed by PBMC, however, TSST-1 may have bound to other cells present in the blood or in the vascular wall (i.e. endothelial cells). Another explanation is that the plasma or blood contained sepsis factors (e.g. cytokines and complement) that interfered with the technique. These could have affected the binding of the TSST-1 to the VHH in the filter or could have bound to the TSST-1, thereby shielding the toxin from the VHH. Alternatively, assuming that the VHH's high affinity for TSST-1 applies to plasma rather than to blood, the difference between the experimental settings (namely blood used *in vivo* and plasma used *in vitro*) results in less effective binding *in vivo*. Finally, the *in vitro* experiments and the complex *in vivo* situation are different with respect to the circulation time. *In vitro*, the TSST-1 spiked plasma recirculated once over the filter (lasting approximately 30 min), while the *in vivo* experiment lasted for 10 h with continuous recirculation. This could have saturated the VHH or even fouled the filter with other blood components such as plasma proteins or specific factors of the sepsis cascade. All in all, the α-TSST-1 VHH-coated filter did not result in substantial TSST-1 removal from blood of septic animals *in vivo*.

Another issue is the unexpectedly low plasma concentration of TSST-1. Miwa *et al*. [9, 10] demonstrated that a TSST-1 recovery of $\approx 1\%$ after 5 hours of continuous infusion (2 μg/kg/h; no bolus) could be calculated. Although our recovery was slightly higher ($\approx 3.5\%$; see 'Calculations regarding distribution volume of TSST-1' above), it was obvious that in

both series of experiments most of the infused TSST-1 had disappeared from the circulation. Hence, TSST-1 was either rapidly degraded or rapidly cleared. There is surprisingly little literature available about the fate of TSST-1 that enters the blood. It is known that the toxin, although containing a high percentage of hydrophobic amino acids, is highly water-soluble. In addition, it is very resistant to heat, proteolysis and trypsin [15]. This makes spontaneous degradation unlikely. Hepatic and renal clearance are unclear. Another possibility is that TSST-1 sequesters into space that does not equilibrate with blood plasma. Although it has been confirmed *in vitro* that TSST-1 crosses tissue (vaginal mucosa [17]) and binds to endothelial [18, 19], epithelial [20], and splenic cells [21], the precise distribution space *in vivo* is unknown. Because plasma TSST-1 levels were far lower than anticipated, the estimated distribution space would be much larger than the extracellular fluid volume. Even if the α-TSST-1 VHH filter would have been effectively removing TSST-1, it is not likely that the clinical course would have been ameliorated. Admittedly, the study revealed uncertainties about the clearance, action, and distribution space of TSST-1 that were not foreseen. It follows from our (and Miwa's) data, that the fate of TSST-1 (and probably other septic superantigens as well) is strongly underrated and needs investigation. We suggest that this insight is of great importance for future attempts to clear TSST-1 from organisms.

Removing substances directly from the blood using highly selective VHH is a technique that could be useful in several diseases where the causal factor is known. Removal of this factor will, in theory, directly 'cure' the patient or ameliorate the course of disease. Our findings strongly suggest that despite their attractiveness, the utility of such techniques needs to be carefully explored for each causal factor.

In the literature it has been argued that once the cascade of the events leading to the sepsis syndrome has started, the removal of the causing agent may not be relevant anymore. Solid proof for such a hypothesis should come from experiments like ours, where we have removed the sepsis causing agent in cell experiments, and have been able to attenuate the cellular response. *In vivo*, we run into an interesting situation: the agent 'disappears' into a compartment not easily accessible to techniques aiming to remove the substance. Unfortunately, this leaves the basic question unresolved. Nevertheless, our study enlightens a pivotal aspect of the problem of toxin-induced sepsis: the distribution of the TSST-1 to a compartment not easily accessible to technologies that aim to remove the toxin.

3.5 Conclusions

In summary, TSST-1 infusion induced a serious sepsis syndrome in pigs. Application of hemofiltration filters coated with specific α-TSST-1 VHH did not ameliorate the clinical course of the syndrome. Moreover, TSST-1 levels were substantially lower than anticipated and TSST-1 levels before and after the filter were unchanged. The low plasma levels suggest substantial compartmentalization to space not accessible for hemofiltration or pheresis techniques. We conclude that removal of TSST-1 using α-TSST-1 VHH is feasible *in vitro*. The VHH adsorption filter-based technique did not seem effective *in vivo*, however, the sequestration of TSST-1, observed in this study and previously by others [9, 10], prevents adequate removal. Our study reveals complete lack of information about distribution of TSST-1, which may well be important for further development of therapeutic strategies.

3.6 Acknowledgements

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3.8 Supplement

3.8.1 Supplemental materials and methods

TSST-1 ELISA
The ELISA for detecting TSST-1 consisted of sheep polyclonal α-TSST-1 Ab (cat. no. 15936, Abcam, Cambridge, UK), rabbit α-TSST-1 Ab (cat. no. 6831, Virostat, Portland, ME, USA), and peroxidase-conjugated Affinity Pure Goat Anti-Rabbit IgG (GARPO, cat. no. IR 111-035-045, Jackson Immunoresearch laboratories, West Grove, PA, USA) Detection with ortho-phenylenediamine (OPD, MP Biomedicals, Illkirch, France). The lowest detection limit was 160 pg/ml.

PBMC isolation and cell culture
Venous blood was collected from healthy volunteers in 10 ml Vacutainer™tubes (Becton Dickinson, Plymouth, UK; sodium-heparin concentration 17 IU/ml). Plasma was obtained by centrifugation at 500 g for 10 min. The remaining cell/plasma mixture was reconstituted with an equal amount of sterile PBS. PBMC were isolated by Ficoll-Pague™ plus gradient (GE Healthcare, Uppsala, Sweden; endotoxin concentration < 0.12 IU/ml) and counted in a Cell-Dyn© 1800 cell counter (Abbott Diagnostics Division, Abbott Park, IL). The cells were incubated in 24-well, flatbottomed plates (Costar©, Corning Incorporated, Corning, NY) at 37 °C in 5% CO_2 in air. Each well contained $1x10^6$ cells and 1 ml culture medium (RPMI 1640 supplemented with 2 mM L-Glutamate, 25 mM HEPES (Gibco©, Paisley, UK), 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Merelbeke, Belgium), and 10% autologous plasma).

Preparation of animals
Each pig was sedated with an intramuscular bolus of 5 mg/kg Dormicum© (midazolam 5 mg/ml; Roche BV, Woerden, the Netherlands), 4 mg/kg Stresnil© (azaperone 40 mg/ml; Janssen-Cilag BV Tilburg, the Netherlands) and 0.5 mg atropine (atropine sulphate 0.5 mg/ml; Janssen-Cilag BV, Tilburg, the Netherlands). An intravenous catheter was placed in the ear vein, through which a bolus of 25 mg/kg Pentothal© (thiopental 25 mg/ml; Hospira Enterprises BV, Hoofddorp, the Netherlands) was given to anesthetize the pig. The ear vein catheter functioned as a general intravenous access port throughout the experiment. The pig was intubated with an endotracheal tube, which was then connected to a gas vaporization and delivery station (Datex-Ohmeda BV, Hoevelaken, the Netherlands) that provided an air mixture containing 33% O_2 and 1.5% to 2.0% isoflurane (Forane©, Abbott Laboratories, North Chicago, IL). The latter was chosen as maintenance inhalation anesthetic because most infused anesthetics are protein-bound and thus can be cleared by hemofiltration. The carotid artery, jugular vein, femoral artery, and femoral vein were surgically exposed. A 14 G catheter was inserted into the femoral artery to infuse Sufenta© (sufentanil 5 μg/ml; Janssen-Cilag BV, Bergen, the Netherlands) at a continuous rate of 3.5 ml/h, which served as a pain depressant. A Swan-Ganz catheter (Criticath™ SP 5105H TD 5 French catheter, Becton Dickinson BV, Breda, the Netherlands) was inserted through the jugular vein and threaded into the right atrium. Catheters (14 G) were placed in the carotid artery and femoral vein. These catheters were connected to the arterial and venous sides of the

extracorporeal circuit.

An i.v. bolus injection of 75.000 IU Heparin Leo© (heparin 5.000 IU/ml; LEO pharma BV, Breda, the Netherlands) was given as an anticoagulant. Five min after this injection the activated clotting time (ACT) was measured using a HemoTec ACT 38 system (Medtronic, Inc, Englewood, CO) and, when needed, additional heparin boluses of 25.000 IU were given until an ACT value of 999 was achieved. Continuous heparin infusion was started at a rate of 25.000 IU/h on the venous access port in the ear vein to maintain complete anticoagulation, after which ACT was measured each hour. In most experiments this heparin scheme was sufficient to maintain complete anticoagulation, although in some cases one to three extra boluses had to be administered during the experiment.

Measurements

Cardiac output was measured with a thermodilution cardiac output monitor (Starcom, Spectramed Inc., Oxnard, CA). Heart rate (HR), systolic and diastolic blood pressure, pulmonary artery pressure and central venous pressure were measured and stored by a physiological monitor (Spacelabs Medical, Inc., Redmond, WA, USA). Every hour plasma (3 ml) was drawn from the plasma flow after the filter and 5 ml arterial blood was sampled from the femoral artery. This blood was then centrifuged at 4000 rpm for 10 min to obtain plasma that was stored at -80 °C for later analysis for IFN-γ by ELISA (Endogen, Cambridge, MA, USA). Neutrophil count was determined on an Advia 120 hematology analyzer (Bayer, Mijdrecht, the Netherlands). Plasma TSST-1 concentrations were measured by ELISA (see above).

3.8.2 Supplemental figures

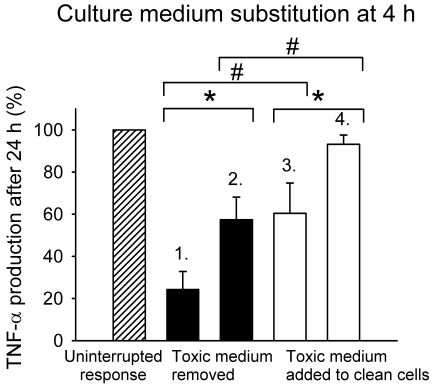

Figure S1. Longer initial incubation (4 h instead of 1 h) gave results similar to Figure 3B. All data mean ± SD. # $p < 0.01$, * $p < 0.05$. There were no significant differences between substitution times. Abbreviations: PBMC, peripheral blood mononuclear cell; TNF-α, tumor necrosis factor-alpha; TSST-1, Toxic Shock Syndrome Toxin-1.

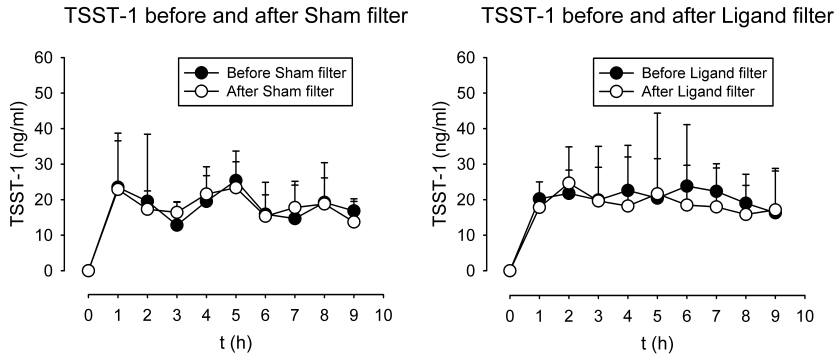

Figure S2. TSST-1 levels were the same before and after the filter in both Toxin/Sham and Toxin/Ligand group. Abbreviations: TSST-1, Toxic Shock Syndrome Toxin-1.

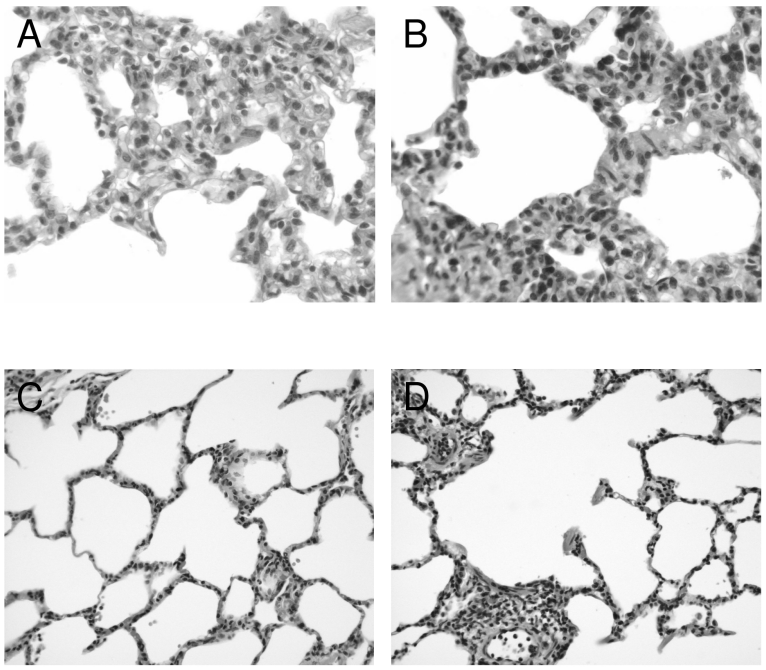

Figure S3. Lung histology. (**A-B**: Periodic Acid-Schiff; 200x magnification) Neutrophil infiltrate increased in Toxin/Ligand **(B)** (or Toxin/Sham, not shown) compared with Sham **(A)** (or Ligand, not shown). (**C-D**: Hematoxylin and Eosin; 100x magnification) Thrombi count was not significantly different between Toxin/Ligand **(D)** (or Toxin/Sham, not shown) and Sham **(C)** (or Ligand, not shown).

3.8.3 Supplemental tables

Table S1. Additional clinical and laboratory measurements in the animal experiments

Measurement	Group	0 h	3 h [a]	5 h [b]	9 h [c]	Interaction between Groups and Time
T_{animal} (°C)	S	36.9 ± 0.5	35.9 ± 0.1	36.4 ± 0.5	31.7 ± 0.4	NS
	L	36.8 ± 0.1	36.2 ± 0.2	36.7 ± 0.3	37.1 ± 0.8	
	TS	36.5 ± 0.3	35.8 ± 1.1	36.5 ± 1.4	36.9 ± 1.2	
	TL	37.5 ± 0.4	35.9 ± 0.5	36.2 ± 0.9	36.4 ± 1.2	
CVP (mmHg)	S	4.5 ± 6.4	5.0 ± 1.4	3.0 ± 0.0	4.0 ± 1.4	$p = 0.028$
	L	10.0 ± 8.5	8.5 ± 7.8	8.0 ± 7.1	7.5 ± 7.8	
	TS	5.8 ± 3.9	7.0 ± 7.5	4.0 ± 6.9	6.7 ± 2.1	
	TL	5.4 ± 2.8	7.0 ± 6.4	9.8 ± 7.4	15.3 ± 6.7**	
MPAP (mmHg)	S	13.5 ± 6.4	12.5 ± 6.4	12.0 ± 8.5	15.5 ± 6.4	$p = 0.026$
	L	21.5 ± 4.9	16.5 ± 0.7	16.5 ± 0.7	16.5 ± 0.7	
	TS	9.0 ± 3.6	16.8 ± 3.8**	16.3 ± 3.9**	17.3 ± 2.9**	
	TL	9.6 ± 2.3	16.2 ± 3.7*	22.4 ± 4.8**	20.0 ± 9.1**	
Hemoglobin (mmol/L)	S	6.6 ± 1.2	5.9 ± 0.5	5.7 ± 0.9	5.3 ± 0.6	NS
	L	6.3 ± 0.6	6.0 ± 0.1	5.9 ± 0.2	5.3 ± 0.2	
	TS	6.4 ± 1.5	6.3 ± 0.7	6.2 ± 0.8	2.9 ± 2.1	
	TL	6.5 ± 0.7	5.4 ± 1.9	5.9 ± 1.2	4.8 ± 2.1	
Hematocrit (%)	S	31 ± 5	28 ± 1	27 ± 3	25 ± 2	NS
	L	30 ± 3	28 ± 0	28 ± 1	24 ± 1	
	TS	30 ± 7	29 ± 3	29 ± 3	14 ± 10	
	TL	30 ± 4	25 ± 8	28 ± 6	22 ± 10	
Platelet count ($\cdot 10^9$/l)	S 1	62 ± 30	200 ± 32	215 ± 10	217 ± 11	$p < 0.001$
	L	131 ± 19	161 ± 24	215 ± 2	261 ± 55	
	TS	151 ± 60	153 ± 68	167 ± 84	98 ± 43*#	
	TL	139 ± 47	173 ± 51	122 ± 30	122 ± 39 #	
Na^+ (mmol/l)	S	139 ± 4	137 ± 1	136 ± 0	139 ± 2	NS
	L	141 ± 2	141 ± 2	140 ± 0	142 ± 4	
	TS	141 ± 3	141 ± 2	142 ± 1	142 ± 1	
	TL	142 ± 2	141 ± 1	142 ± 1	142 ± 1	
K^+ (mmol/l)	S	3 .3 ± 0.3	3.8 ± 0.2	4.3 ± 0	3.9 ± 0.4	NS
	L	3.2 ± 0.1	3.6 ± 0.7	3.9 ± 0.6	4.3 ± 1.2	
	TS	3.5 ± 0.5	3.7 ± 0.5	4.0 ± 0.7	3.9 ± 0.7	
	TL	3.0 ± 0.2	3.4 ± 0.3	3.8 ± 0.6	4.5 ± 0.7	

All data mean ± SD. Abbreviations: CO = cardiac output; HR = heart rate; IFN-γ = interferon-gamma; MAP = mean arterial pressure; SVR = systemic vascular resistance; TSST-1 = toxic shock syndrome toxin-1; WBC = White blood cells.
For group explanation, see Table 1.

[a] Sham filter replaced by either another Sham filter (S, TS), or by Ligand filter (TL)
[b] Resuscitation start
[c] Last point in time where group sizes were large enough for comparison (1 h before termination)

* $p < 0.05$ versus t = 0 h; ** $p < 0.001$ versus t = 0 h; # $p < 0.05$ versus Sham

Chapter 4

Removing TSST-1 in sepsis: why is this an ongoing challenge?

Abstract Severe sepsis is characterized by rapid development of multiple organ failure associated with high mortality. Bacterial toxin release triggers a sequence of events that activates intracellular pathways to produce inflammatory mediators and nitric oxide. There have been numerous attempts to interrupt this devastating cascade by removing toxins, removing or inhibiting mediators, and by blocking receptors of mediators. This review considers toxin properties (with a strong focus on Toxic Shock Syndrome Toxin-1 (TSST-1), a potent staphylococcal toxic shock toxin), and the potential of various removal technologies related to these properties. The various toxins' distribution volumes form a key issue: not only are these poorly defined, but, at least for TSST-1, available estimates suggest a high degree of compartmentalization to a space not accessible by pheresis or immunoabsorption technologies. At least for TSST-1, available information strongly suggests that further attempts to remove toxins in order to treat sepsis are futile if we cannot access this space. When extrapolated to other toxins, a set of general requirements has to be made in order to facilitate succesful toxin removal by a pheresis technique.

Brummelhuis WJ, Joles JA, Verrips CT, Braam B.

Submitted

4.1 Introduction

Bacterial infections that progress to a sepsis syndrome are relatively common and associated with high mortality and short- and long-term disabilities in those patients who survive [1, 2]. Very strong activation of the systemic inflammatory response, primarily aimed to defend the organism against the invader, has secondary consequences that may be detrimental or fatal for the organism [3]. On the one hand the response is aimed at activating the immune system, importantly via TNF-α, by destroying the bacteria by cellular actions, and by releasing large amounts of nitric oxide [4, 5]. On the other hand, it is aimed at protecting cells against the attack and the consequences of this response. Mechanisms that participate in protection are blood pressure-increasing hormones such as arginine vasopressin [6, 7] and endothelin-1 [8, 9], scavengers of damaging factors such as gelsolin [10] and anti-oxidative enzymes such as superoxidase dismutase [11], and antioxidants such as glutathione [12]. Altogether, the response is so strong, that frequently the host suffers fatal damage.

Initiators of the inflammatory response are constituents of the bacteria (usually the bacterial cell membrane), or toxins secreted by the bacteria. There are a large variety of such toxins, all with specific chemical and physical characteristics [13-15]. They differ in protein binding affinity, molecular weight, lipophylic properties, and clearance. This importantly determines how these toxins are distributed throughout and cleared from the body compartments. One of the most pathogenic toxins is the staphylococcal exotoxin Toxic Shock Syndrome Toxin-1 (TSST-1). It is a superantigen (SAg) and causes very strong immune response activation. We will use this archetypical toxin to guide our exploration of removal strategies.

In the last 30 years, there have been numerous attempts to use hemofiltration and more specific strategies (such as polymyxin B-coated columns and immunocapturing) to remove endotoxin, superantigens (e.g. TSST-1 and streptococcal pyrogenic exotoxin), and inflammatory mediators from the circulation [16]. All in all, such attempts have had surprisingly little success. This supposedly is due to inefficient toxin removal and concomitant removal of protective factors, in particular by non-selective techniques.

This review focuses on the methods that are available to remove TSST-1. We will summarize what we know about the physical, chemical and biological properties of TSST-1, analyze current (specific) technologies to remove such toxins (and their inflammatory mediators), and review the literature on (clinical) experiments with these techniques.

4.2 Physical, chemical, and biological properties of TSST-1

The superantigens are a group of proteins that activate a large population of specific T-cells, in such a way that the ensuing inflammatory cascade is harmful to the host. They are produces by various bacteria (e.g. *Staphylococcus aureus*, *Mycobacterium tuberculosis*), viruses (e.g. *Herpes virus*, *Rabies virus*), and fungi (*Candida albicans*). Within each group, they show a striking resemblance in structural morphology and function [17]. In this review, we focus on the SAgs of *Staphylococcus aureus*, and more in particular on TSST-1. Because of the extensive homology between the various SAgs [18] much of the matter discussed below most likely also applies to other SAgs. The streptococcal and staphylococcal SAgs, for instance, show a striking resemblance in structural morphology and function. The SAgs

of *S. aureus* are more commonly referred to as staphylococcal enterotoxins because they have strong emitic activity, causing food poisoning. In addition, when they are present in the circulation, sustained inflammatory activation ensues resulting in the sepsis syndrome. TSST-1, which is also a member of the *S. aureus* SAg family, does not have emetic activity but is a prominent sepsis-inducing SAg. It causes sepsis by invasion of the circulation via primarily the skin and (vaginal) mucosa. SAgs bind with varying affinity (10^{-8} - 10^{-6} M) to both the V_β chain of the T-cell receptor and the MHC-class II on antigen-presenting cell (APC; i.e. macrophages and monocytes). This leads to a high and sustained release of inflammatory components from both cell types, resulting in the development of sepsis. Although this reaction is aimed to protect the host against the invading micro-organism, it is so overwhelming that it endangers the host. TSST-1 is a water-soluble, intermediate sized (22 kDa) molecule of 194 amino acids [18]. It is produced inside the *Staphylococcus aureus* bacterium, and secreted into the circulation.

An essential question for the therapeutic options discussed in this paper is where does TSST-1 go in the infected organism? In patients suffering from toxic shock syndrome, maximum reported TSST-1 concentrations were 1.8 nM in breast milk [19], 5450 pg/ml (mean 440 pg/ml) in serum [20], and 41 pg/ml in plasma [21]. We have recently reported on experiments in pigs infused with TSST-1. Strikingly, the plasma levels of TSST-1 were about much lower than expected solely on the basis of extracellular fluid volume distribution (calculated TSST level: 150 ng/ml, actual level 20 ng/ml) [Brummelhuis *et al*. 2010. Accepted in Shock Journal; see *Chapter 3*]. From these observations, we argued that there must have been substantial sequestration to a space other than the extracellular fluid volume. When inspecting the other studies that performed infusion of TSST-1, a similar pattern was observed: only a fraction of the infused TSST-1 could be accounted for when related to levels in the plasma [22, 23].

The few *in vitro* studies on TSST-1-cell binding (other than to T-cells and APC) report that TSST-1 binds to endothelial [24, 25], epithelial [26, 27], and spleen cells [28], and crosses the vaginal mucosa [29] and the placenta [19]. Binding to tonsillar B-cells was also reported [30]. The toxin can transverse a layer of human colon (Caco-2) cells [31] by receptor-mediated transcytosis, and can enter elastic and collagen tissue after crossing endothelial cells [24]. It does not, however, cross the choroidal epithelial barrier *in vitro* (= the blood-cerebrospinal fluid barrier) [32]. Although such findings in cultured cells are important, studies *in vivo* on the physiological distribution (i.e. adherence to cells and distribution between the intracellular and extracellular compartments) of TSST-1 are still lacking. The potential distribution over the various body compartments is represented in Figure 1.

Furthermore, whether excretion or degradation occurs by the kidney or liver is unknown. Clearly, detailed investigation into the TSST-1-cell interactions (other than T-cells and APC) is necessary. Without this, it will be difficult to determine the effect of specific removal strategies in experimental and clinical trials.

TSST-1 binds very strongly to T-cells expressing MHC-II. This has two consequences. This mechanism might 'sequester' the TSST-1 so that it can not be removed by plasmapheresis or immunocapturing. Moreover, T-cell binding can commence a cascade of events that might not be interruptible by removal of the toxin. Irreversible binding to the T-cell is unlikely. In experiments in our lab, we first exposed peripheral blood

mononuclear cells (PBMC, monocytes and lymphocytes), freshly isolated from healthy humans, to TSST-1. We then tested the TNF-α production after 24 h in cells that were continuously exposed to TSST-1 for 24 h, cells of which the supernatant with the TSST-1 was replaced with clean cell medium after 1 h of exposure to TSST-1, and non-stimulated cells that were exposed to the TSST-1-laden supernatant that had been removed from the second group. The latter displayed a significantly lower TNF-α production after 24 h, while the unstimulated cells that were exposed to the aspired toxic supernatant showed a marked TNF-alpha production. This indicates that binding to the T-cells was not irreversible, and the cascade of cytokine production and amplification of this response could be interrupted. Moreover, it indicates that the monocyte and lymphocyte pool is not the compartment that internalizes the TSST-1 [Brummelhuis *et al*. 2010. Accepted in Shock Journal; see Chapter 3].

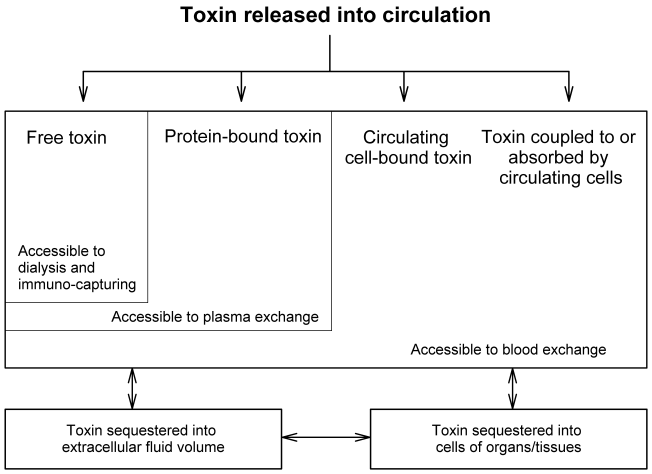

Figure 1. Potential distribution of staphylococcal TSST-1 over the various body compartments.

4.3 Limited success of removing TSST-1 and its mediators in human sepsis

There are several reasons why experimental mediator removing techniques in sepsis have yet failed to convince as a potential treatment option. First, although limited success has been reported in small trials and case reports, there are not enough randomized controlled trials to confirm the success of novel therapies. Second, not all trials contain precise blood cultures and if they do, the specific subset of bacterial species (e.g. *S. aureus* producing staphylococcal enterotoxin A (SEA), SEB, TSST-1 etc.) is often omitted. Third, within one study, the treatment specifity sometimes varies per patient. Cruz *et al*., for instance, employed a polymyxin B fiber column to reduce blood endotoxin levels in septic patients [33]. However, blood cultures indicated that a substantial number of patients had a primary

Gram-positive sepsis (against which polymyxin B is not active).

The absence of randomized, clearly-defined studies make the interpretation of novel therapies difficult. We will now briefly discuss techniques that aim to improve sepsis treatment. Note that these studies were usually not aimed at a specific sepsis mediator, but rather at removing all 'harmful' substances (including high cytokine levels) from the circulation.

Initial successes were reported on the application of hemofiltration in experimental sepsis in pigs [34, 35]. A decrease in plasma levels of TNF-α and several other pro-inflammatory cytokines was reported. In concordance, infusion of hemofiltrate of septic pigs into healthy pigs was associated with a decrease in cardiac performance, supporting that hemofiltration removed a cardiodepressant. However, treatment with hemofiltration may be more complex than solely substance removal [36].

Toft *et al*. could not demonstrate any effect of hemofiltration on cytokine levels in sepsis patients. Although cytokines were excreted at low concentrations in the ultrafiltrate, the plasma concentrations did not change [37]. Other groups had similar findings [38-40]. Any decrease in cytokine levels by hemofiltration might have been counteracted by activation of leukocytes by the hemofiltration membrane. There is clear data from cardiopulmonary bypass surgery that complement can be activated [37]. More recently, the study by the VA/NIH Acute Renal Failure network has reported that intensive renal replacement therapy in critically ill patients has no additional benefit over less intensive therapy [41]. In that study, a substantial number of patients suffered from sepsis (about 63% of both the intense strategy and the less-intense strategy groups). A small trial that assessed the effects of high volume venovenous hemofiltration was stopped early because of a more prolonged course on hemodynamic and ventilatory support and because of increased mortality [42]. This study was also unable to show changes in plasma levels of IL-6, MCP-1 and IL-1ra. These observations do not support the idea that bacterial toxins and inflammatory mediators are all in an accessible compartment, are unbound, and have other properties that facilitate the removal by filtration through conventional hemodialysis membranes. Furthermore, there are no clinical studies that specifically measure changes in toxin levels in all enrolled patients. In summary, hemofiltration is unable to bring clear benefit in the treatment of sepsis.

Regarding plasmapheresis and plasma exchange for TSST-1 mediated sepsis, only a single case report is available. Kohro *et al*. describe a case report of a 14-year-old boy who presented with toxic shock syndrome [43]. He was treated with combined plasma exchanges and filtering of the blood with a polymyxin B column. After the first plasma exchange, the plasma TSST-1 level had decreased significantly (106 to 2 pg/ml). Levels of SEA remained around 10 pg/ml, endotoxin was not present. After 4 days his clinical course has improved enough to be discharged from hospital.

In some instances, blood exchange has been tried in severe sepsis, although no reports specifically for TSST-1 have been reported. Since these reports only involved a few patients, and studies were not controlled, the results are mentioned very briefly. Christensen *et al*. used blood exchange in neonates with sepsis, and found a slight clinical benefit [44]. Van Deuren *et al*. applied blood exchange (n = 6) or plasma exchange (n = 9) in children and several adults with meningococcal sepsis, and reported a strong decrease in endotoxin levels over the course of 36 h after initiation of the therapy [45]. One report in TSST-1 infused pigs indicated a better preservation of cardiac output in animals treated with leukapheresis

versus controls, however, blood pressure and pulmonary function were not improved by this treatment [46]. Thus, these studies do not convincingly show or even suggest that leukocyte removal is beneficial in the treatment of sepsis.

Miwa *et al*. reported immunocapturing using a polystyrene-based chemically modified composite fiber reinforced with polypropylene. This device was able to bind TSST-1, SEA, SEB, SEC, IL-6, streptococcal pyrogenic exotoxin B, β2-microglobulin, vancomycin and (to a certain degree) endotoxin from plasma [47]. Septic rabbits (combined TSST-1/LPS model) showed increased survival after two days. Subsequent experiments with septic pigs (also using TSST-1/LPS infusion, followed by direct hemoperfusion of the immunocapturing device) showed that mortality decreased in the treatment group, despite a marginal lowering of plasma TSST-1 levels [22]. Surprisingly, in the control group (as well as the treated group) there was a striking discrepancy between the amount of infused TSST-1 and the recovered plasma levels: this should have been about 192 ng/ml after 5h, but was only 2.6 ng/ml (or about 1.4% of the TSST-1 amount originally infused). We also found a similar low plasma TSST-1 recovery (20 ng/ml recovered from plasma where 577 ng/ml was expected) in our porcine experiments, in which we aimed to remove infused TSST-1 from the circulation of septic pigs with llama antibody fragments (TSST-1-infusion model; see Chapter 3). Our survival did not improve by the anti-TSST-1 column.

4.4 Prerequisites for succesful application of plasmapheresis and immunocapturing techniques in sepsis

There are several categories of prerequisites for efficient removal of substances from the circulation. The first relates to the substance itself. In fact, classical pharmacologic rules apply here. We have already mentioned distribution space. Bacterial toxin compartmentalization could be extremely complex, and has not been studied for TSST-1. Given our and others' data, two or more compartments are likely. Beside the number of compartments, application of removal technologies is quite dependent upon the speed by which TSST-1 is distributing over these spaces. Since one of the ways that TSST-1 might leak into a separate space is via the endothelium, it should be realized that sepsis comes with progressive transcapillary leak. From our report [Brummelhuis *et al*. 2010. Accepted in Shock Journal], and the report by Miwa *et al*. [22], it seems that this redistribution for TSST-1 is quick. Obviously, studies using radioisotopes (e.g. iodine-125 labeled TSST-1 [48]) would be very helpful. A general issue with any removal technique is whether the compound binds to proteins, albumin in particular. Remarkably, protein binding of TSST-1 has not yet been studied.

The second category concerns the removal strategy per se. The membrane can be 'passive' or 'active'. A passive membrane will only be able to remove a substance if it has the electrical, chemical and physical driving forces to move it from the plasma site to the exit site. When considering TSST-1, such characteristics have to our knowledge not been studied in detail. We studied the *in vivo* characteristics of an 'active' membrane and encountered an interesting problem, in that the membrane underperformed *in vivo* while *in vitro* it worked perfectly [Brummelhuis *et al*. 2010. Accepted in Shock Journal]. This suggests that the constitution of the blood (and this could also include components of the sepsis cascade such

as cytokines) somehow affects the potential of the membrane to bind TSST-1. This could be related to fouling of the membrane in a general sense, or to more specific inhibition of binding between TSST-1 and the anti-TSST-1 llama antibody fragments (VHH) on the membrane. The latter is certainly possible, and could involve purely physiochemical issues such as local pH, or binding of non-specific proteins to the (*in vitro* highly selective) VHH. Alternatively, the VHH, after coupling to the fibers as in our *in vivo* model, cross-linked to the fiber material itself, or were coupled in such a way that the active groups were not completely free to bind TSST-1.

When developing applications for the removal of disease-causing agents from the circulation, the following seems important:

Regarding the substance

- a direct causative relation between the agent and the disease has been proposed
- physical and chemical properties, and binding of the causative factor to proteins and cells of a variety of tissues should be studied before application in humans
- *in vitro* experiments are followed by an animal model
- questions when studying the fate of the toxin in an animal model:
  - what is the binding to various cell types (e.g. endothelial cells, proteins), and can a mass balance of the toxin be determined?
  - does the toxin stay in the circulation or is there e.g. compartmentalization of the agent to compartments not accessible by the removal strategy?
  - what is the equilibrium between the bound and free agent *in vivo*, and is it to be expected that the equilibrium will be influenced by removing the unbound form?
- finally, removing the agent should be able to influence the animal model before proceeding to clinical studies

Regarding the removal strategy

- the toxin-carrying and antibody-carrying matrix are equal in the *in vitro* and *in vivo* experiments
- *in vitro*, the setup contains elements that are formed during the *in vivo* tests (including cytokines, nitric oxide, changing pH, etc), preferably in a dose- and time-dependant fashion
- knowledge about the antibody stability and the carrier matrix, and about potential leakage of antibody into the host is essential

4.5 Elucidating the properties of TSST-1, and other toxins, is key for the development of succesful treatment

The structure of TSST-1 (and other superantigens) has been studied in much detail by X-ray crystallography [49]. This has revealed that epitopes of the toxin binds to the MHC-class-II complex, which will facilitate the development of new sepsis treatment options, such as specific antibodies and vaccines against TSST-1. When developing new strategies, however, two issues are potentially relevant. First, different *S. aureus* strains produce different toxins. Currently, 19 *S. aureus* strains have been identified, each with its own specific toxin, but this number will undoubtedly rise [17]. Second, it is not uncommon that several bacteria are active simultaneously in sepsis, e.g. TSST-1 is often accompanied by the SEB toxin. Successfully removing TSST-1, while SEB remains present, will therefore not necessarily help the patient. Therefore, it seems important to uncover the properties of all the sepsis-inducing *S. aureus* strains for specific antibody development (although some common epitopes do exist). Moreover, before treatment is started, obtaining blood cultures of sepsis patients could serve to assess the *S. aureus* subtype(s) and to identify the specific (mix of) toxin(s). There is increasing evidence that TSST-1 not only causes harm by inducing a cytokine storm, but that also direct and indirect effects of TSST-1 on for instance endothelium contribute to the severity of sepsis. Information about the consequences of the binding between TSST-1 and various cell types is limited.

Before TSST-1 (and other sepsis-inducing superantigens) can be removed successfully in a clinical setting, more information is needed on the superantigen's distribution space. This could be studied by e.g. infusing and monitoring labeled TSST-1 in an animal model. It is possible to do this with radio- [48] or dye-labeled TSST-1, the latter with infrared readout. In addition, such studies about distribution could be combined with studies on hepatic and renal TSST-1 clearance.

4.6 Conclusions

Although many attempts have been done to remove toxic mediators in sepsis, there is a lack of clear clinical benefit for this approach. The complex inflammatory mechanisms involved and the severity of the disease can potentially explain this failure. However, when it comes to the toxin's properties that could be of importance for developing novel removal strategies, we are still in the dark. In this review we identify important knowledge gaps with respect to the characteristics of TSST-1. These include the toxin's bio-activity, distribution, and *in vivo* clearance. We argue that such information is pivotal for developing a rational removal strategy, and we have formulated some prerequisites before any removal strategy is tested in clinical settings. Although focused on TSST-1, a number of these considerations also apply to other toxins. As such, investing in further unraveling of the sepsis pathophysiology will form the basis for more effective therapeutic strategies.

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Part II

Removing excess volume

Chapter 5

Plasma refill rate during hemodialysis and ultrafiltration: physiology, measurement and clinical implications

Abstract The discrepancy between ultrafiltration and plasma refilling from the interstitial space to the circulation causes a blood volume decline during hemodialysis, often accompanied by hypotension. Refill occurs at the tissue level, where Starling's law incorporates hydrostatic and oncotic forces into net capillary fluid transport. Little is known about the absolute value and the dynamics of the plasma refill rate during hemodialysis. We discuss several papers that investigated refill either directly by measuring absolute plasma volumes during hemodialysis or isolated ultrafiltration, or indirectly by comparing ultrafiltration rates to the change in relative blood volume. A few papers also analyzed the indirectly measured capillary ultrafiltration coefficient. Finally, the importance of absolute plasma refill rate measurements and the potential applications are discussed.

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Submitted

5.1 Introduction

Since the late 40's of the previous century, hemodialysis is available to replace renal function in patients with kidney failure. Over the years, improved dialyzer design has reduced the time required for removing waste substances from the circulation. The resulting shortened treatment time allows less time for removing the excess fluid that accumulates in the interdialytic interval. This has led to an increased frequency of hypotensive episodes during hemodialysis [1]. At the heart of this problem lies the fact that the rate at which fluid is removed from the circulation by ultrafiltration (UF) during short dialysis sessions generally exceeds the rate at which fluid is returned to the circulation from the interstitial compartment (plasma refill rate). The resulting decrease in blood volume (BV) is the main cause of dialysis-induced hypotension.

As the plasma refill rate is a very important factor in conserving BV and blood pressure during combined UF and hemodialysis, it is relevant to have methods that allow easy and reproducible measurement of the plasma refill rate. Using such technology would increase the knowledge on the dynamics of refill during dialysis sessions. This could help to adapt the UF rate to the actual refill capacity of patients, thus reducing the risk of intradialytic hypotension. In addition, having methods to measure refill quantitatively would allow the direct evaluation of strategies aimed at improving refill. Finally, it has been proposed that cessation of refill during hemodialysis and UF reflects the situation in which the interstitial compartment has reached a state of normal volume [2]. If this would be correct, the direct assessment of refill could be a new tool to better determine the dry weight in dialysis patients. This review focuses on the techniques that have been used to obtain quantitative information on plasma refilling during hemodialysis.

5.2 Ultrafiltration during hemodialysis

The hemodialysis machine consists of two computer-operated blood and two dialysate pumps, and a dialyzer (Figure 1). Before hemodialysis, a blood tubing system including the dialyzer is placed on the dialysis machine. The patient is connected to the tubing via needles that are inserted into the patient's vascular access (either a surgically created arteriovenous fistula or an implanted synthetic graft) or via a central venous catheter. Blood flows through the semi-permeable fibers in the dialyzer, while dialysate, the cleaning solution, flows in the opposite direction outside these fibers. The dialysate is a mixture of minerals, bicarbonate, and glucose dissolved in water. Differences between dialysate and blood substance concentrations create various concentration gradients over the semi-permeable membrane. As a result, substances diffuse towards the dialysate (e.g. potassium, urea, phosphate) or to the blood (e.g. bicarbonate) and are removed from or added to the body, respectively. Importantly, a flow difference can be set between the dialysate pump before and after the dialyzer, e.g. 450 versus 500 ml/min, which creates a negative transmembrane pressure gradient over the fibers. This in turn induces a fluid flow from the blood passing through the dialyzer towards the dialysate compartment (= UF) which allows removal of excess fluid retained between dialysis sessions. Usually, UF is applied in a linear, constant fashion, implying that the total volume to be removed is distributed equally over the dialysis time. In attempts to reduce the incidence of intradialytic hypotension, some have advocated

the use of UF profiles, e.g. pulsed UF or the application of very high UF rates in the initial stages of dialysis followed by a gradual or stepwise reduction in UF rate. The clinical usefulness of such profiles, however, has not been demonstrated [3].

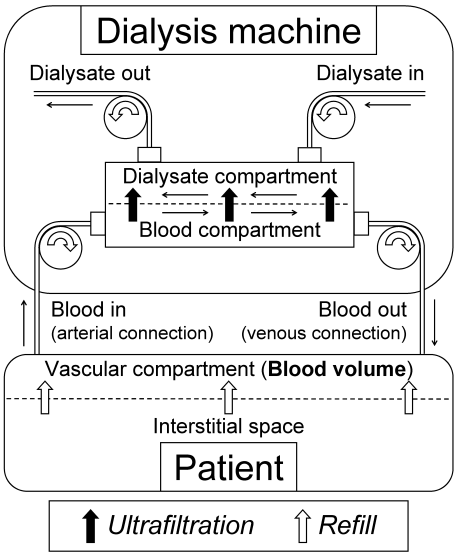

Figure 1. Schematic overview of the relation between ultrafiltration, plasma refilling, and circulating blood volume during hemodialysis.

5.3 Intradialytic hypotension and its relation to ultrafiltration and plasma refilling

Most of the excess fluid that is retained in the interdialytic interval in dialysis patients migrates to the interstitial space, whereas a smaller proportion resides in the circulation [4]. This excess volume is an important contributor to interdialytic hypertension in dialysis patients. It may amount to several litres and has to be removed by UF during dialysis in a relatively short time (3-4 hours), which results in UF rates (15-20 ml/min) that are high relative to the refill capacity (see below).

As outlined above, UF removes fluid directly from the intravascular compartment. As a consequence, the BV starts to fall as soon as UF is initiated. Initially, enhanced refill from the interstitial compartment, where most of the excess fluid resides, attenuates the UF-induced fall in BV. In addition, the impact of the BV reduction on the arterial blood pressure is mitigated by the activation of the sympathetic nervous system and the renin-angiotension system. However, these compensatory mechanisms can be overwhelmed by a continuing decline in BV, and eventually hypovolemic hypotension may develop. Consequently, approximately 15-25% of dialysis patients regularly suffer from intradialytic

hypotension [5, 6]. Although there are contributing factors, such as diastolic heart failure and autonomic neuropathy, the imbalance between plasma refilling and UF is the primary cause of intradialytic hypotension. The consequence of intradialytic hypotension is that UF is interrupted, intravenous volume replacement is sometimes necessary, and the UF goal is not reached. The patient does not achieve dry weight and remains overhydrated at the end of dialysis and in the interdialytic interval.

5.4 Transcapillary fluid transport: physiology

Refill takes place at the capillary level in the tissues and by return of excess tissue fluid to the vascular compartment through the lymphatics. Fluid transport over the capillary membrane is the resultant of hydrostatic and oncotic forces in the capillary lumen and the interstitial space (Figure 2). Ernest H. Starling described these forces in his important 1896 paper [7]. Together with the intrinsic capillary permeability characteristics, they play a crucial role in the dynamics of refill during dialysis and the pathogenesis of dialysis-induced hypotension. The following formula describes the effect of these forces on the transcapillary fluid flow (J_v):

$$J_v = K_f[(P_c - P_i) - \sigma(\pi_c - \pi_i)]$$

In this formula, $[(P_c - P_i) - \sigma(\pi_c - \pi_i)]$ is the net driving force, consisting of capillary and interstitial hydrostatic pressures (P_c and P_i), and capillary and interstitial oncotic pressures (π_c and π_i), σ is the reflection coefficient, and K_f is the filtration constant for the capillary membrane.

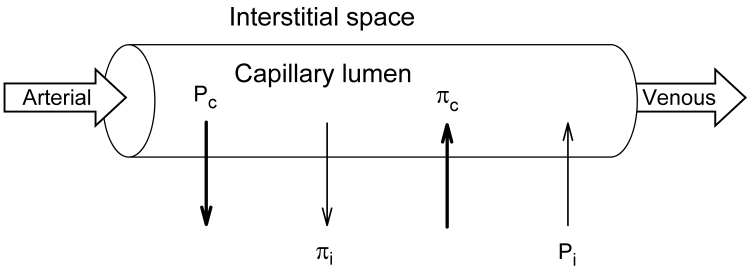

Figure 2. Fluid transport over the capillary membrane.
P_c = capillary hydrostatic pressure, P_i = interstitial hydrostatic pressure, π_c = capillary oncotic pressure, and π_i = interstitial oncotic pressure.

Unlike in the lungs and the kidneys, where reabsorption and filtration predominate, respectively, both filtration (initial capillary segment) and reabsorption (terminal capillary segment) occur at the capillary level in most other tissues. Generally, filtration exceeds reabsorption by 10-15%. This excess interstitial fluid is returned to the circulation together with leaked albumin and other proteins by the lymphatic system.

5.5 Transcapillary fluid transport during hemodialysis and ultrafiltration

The equilibrium existing at the capillary level before the start of UF in a dialysis patient is disturbed the moment that UF starts. Removal of water from the vascular compartment by UF reduces the systemic arterial and venous pressure. This will tend to lower the capillary hydrostatic pressure both from the arterial and the venous side of the capillaries (Figure 2). In addition, the sympathetic response to BV depletion [8] and stimulation of the renin-angiotensin system [9] will induce precapillary vasoconstriction, reducing the hydrostatic pressure even further. Simultaneously, removal of fluid from the intravascular compartment passively increases the intravascular colloid osmotic pressure [10]. Together, these changes may tip the balance of Starling forces in such a way that reabsorption gets to dominate filtration at the tissue level, resulting in net refill of fluid from the interstitial to the intravascular compartment. Refill will slow the decline in BV, but generally does not fully compensate for the UF-induced BV reduction, unless the patient is strongly fluid overloaded (see below).

Adding to the problem of the decline in BV is that the refill rate will fall as dialysis and UF proceed. In a dialysis patient with an overhydrated interstitial compartment, the interstitial hydrostatic pressure will be increased and the interstitial oncotic pressure reduced [10] at the start of a dialysis session, respectively. Together with the factors mentioned above, this will greatly facilitate refill at the capillary level in the early stages of dialysis. Indeed, Koomans *et al*. have shown that the hydration state of the interstitial compartment is a major determinant of refill from the interstitial compartment [11]. They measured the plasma and extracellular fluid volume as ^{131}I-labelled albumin and ^{82}Br-distribution volumes and calculated the interstitial fluid volume from these variables in 21 patients. Next, they subjected these patients to a standardized UF protocol (2 litres in 1 hour). They found a highly negative correlation ($r = -0.89$, $p < 0.0001$) between the initial interstitial fluid volume and the plasma volume reduction one hour after stopping UF. This indicated that the UF-induced plasma volume reduction was greatest in patients with a low interstitial fluid volume. As the UF challenge was the same in all patients, the authors concluded that the plasma refilling directly relates to the interstitial hydration state. Consequently, ongoing depletion of the interstitial compartment during continued UF will progressively reduce refill during the later stages of dialysis. In addition, it has been suggested that the intrinsic capillary permeability or the capillary surface area may decrease as dialysis and UF proceed [12], which may also contribute to a reduction of refill in the later stages of dialysis. The implications are that that the BV is likely to decline as dialysis and UF proceed. At present, however, very little is known about the dynamics of refill as hemodialysis and UF proceed.

5.6 Assessment of refill during hemodialysis

Removal of fluid from the intravascular compartment by UF will increase the hematocrit (Ht) and total protein concentration in the blood. Present day dialysis equipment allows the continues measurement of these variables during dialysis either by the optical absorbance of red light, reflecting the hemoglobin concentration [13] or the sound wave velocity in whole blood, reflecting the total protein concentration [14]. From the changes in Ht or protein concentration, the relative change in blood volume (rBV) can be calculated

by simple arithmetic: (e.g. Δ BV = (Ht_2-Ht_1/Ht_2) * 100%) and rBV curves can be plotted continuously on-line during dialysis. This is relevant because, as outlined below, rBV curves obtained during continuous UF contain information on refill.

At a fixed UF rate and a constant refill rate, the slope of the change in relative BV (as a rule negative because UF usually exceeds refill) will become linear (Figure 3). Any reduction in the refill rate at a constant UF rate will then increase the steepness of the rBV curve (modeled in Figure 4), and hence changes in the steepness of rBV curves reflect alterations in refill. In reality, however, rBV curves more often have a curvilinear, exponential and not a linear pattern [2] (Figure 5). At a constant UF rate, this implies that refill is not constant during dialysis, but is stimulated by the UF-induced fall in BV. The rBV curve not only becomes less steep, but sometimes even plateaus in some patients (Figure 6), indicating that the refill rate has become equal to the UF rate. In severely overhydrated patients, the relative BV curve may even be flat initially, indicating that refill equals the UF rate at the beginning of dialysis (Figure 7). Whenever the rBV curve is horizontal, it provides an absolute value of the refill rate; it then equals the UF rate in ml/min preset at the dialysis machine. In addition, relative BV curves as shown in Figure 6 can provide fascinating information on the dynamics of refill. In the initial 30 minutes of this particular dialysis session, rBV declines in a linear fashion, implying a constant refill rate. Suddenly, however, the curve flattens, implying a stepwise activation of the refill mechanism leading to a refill rate equal to the UF rate. After a short period, the compensation fails and rBV again starts to decline in a linear manner. This stepwise phenomenon is then repeated twice. It is intriguing to know which compensatory mechanism can bring about these sudden alterations in refill.

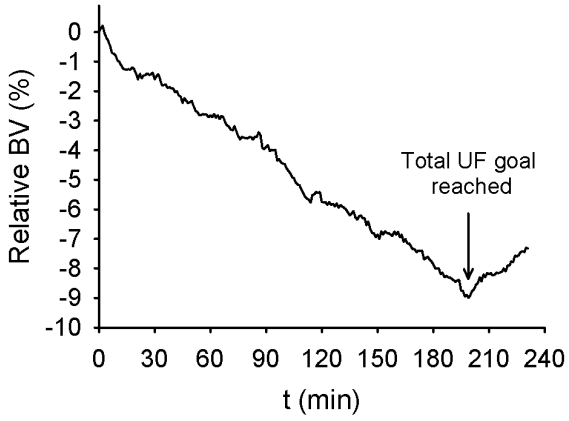

Figure 3. Linear relative BV reduction under a constant UF rate of 16 ml/min. Total UF goal was 3200 ml. It is of interest that after reaching the total UF goal (t = 200 min), the relative BV curve increases again despite the absence of ultrafiltration. This indicates that refill to the circulation was still present, and thus a normal volume of the interstitial compartment had most likely not been reached yet.

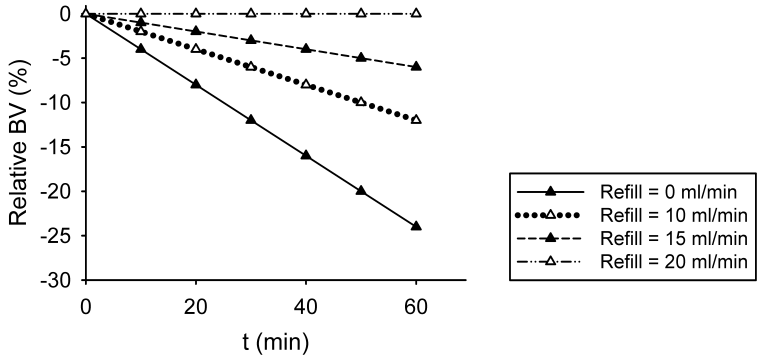

Figure 4. Refill rates determine the slope of relative BV curves during a constant UF rate.
BV = 5000 ml, UF rate = 20 ml/min. If refill is totally absent, a very quick and steep BV reduction ensues. Vice versa, a refill rate of 20 ml/min will maintain the relative BV on the initial value.

The recovery of the rBV after stopping UF at the end of a dialysis session can also be used as an indicator of refill, a flat line indicating the absence of refill, and a steep increase in rBV (e.g. Figure 3, arrow) suggesting considerable refill [15]. In this situation, an absolute refill value is obtained if the rBV does not increase after stopping UF, the refill rate then being equal to zero. In all other situations, simply assessing the changes in the rBV can not be used to measure the absolute refill rate (ml/min) but only provides information on directional changes in refill.

To calculate the absolute refill rate in ml/min from rBV values, the absolute blood volume at the start of dialysis is needed. Although the latter can be measured by isotope dilution techniques or the administration of dyes that bind to plasma proteins (e.g. Evans blue), BV estimates from algorithms [4] are commonly applied in clinical studies reporting absolute refill values. In this case, the estimate of the absolute refill rate depends on the accurateness of the BV estimate. The calculation of the absolute refill rate in period t_1-t_2 (ml/min) is straightforward:

(UF volume (ml) in t_1-t_2) - (change in BV (ml) in t_1-t_2) / time t_1-t_2 (min)

The absolute change in BV is calculated from the percent change rBV and the absolute BV (BV_abs) measured or estimated before dialysis: rBV_{t1} (%) * BV_{abs} (ml).

5.7 Patterns in relative BV changes to assess refill and dry weight

A number of papers have reported that the analysis of rBV curves during dialysis can be used to aid in determining dry weight at the end of a dialysis session and improve for instance blood pressure regulation or reduce the incidence of intradialytic hypotension [15-17]. In

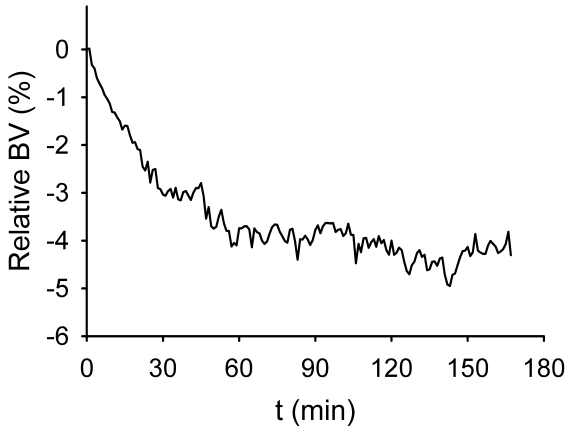

Figure 5. Under constant UF rate, a curvilinear relative BV pattern emerges after an initial steeper rBV reduction, indicating UF-induced refill to the circulation.
UF rate = 12.1 ml/min, total UF goal = 2000 ml.

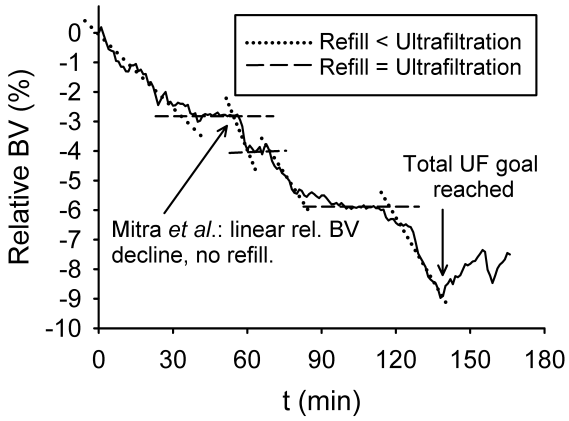

Figure 6. The balance between refill and ultrafiltration differs throughout the dialysis session. First, refill is smaller than ultrafiltration, leading to a negative BV slope (dotted line). Refill then increases and apparently is in equilibrium with the ultrafiltration rate: the BV slope becomes horizontal. This pattern repeats in this patient.
UF rate = 15.2 ml/min, total UF goal = 2600 ml.

general, patterns showing a steep, linear decline in rBV in the early stages of dialysis have been interpreted as a sign impending hypovolemia, whereas patients with flat rBV curves are

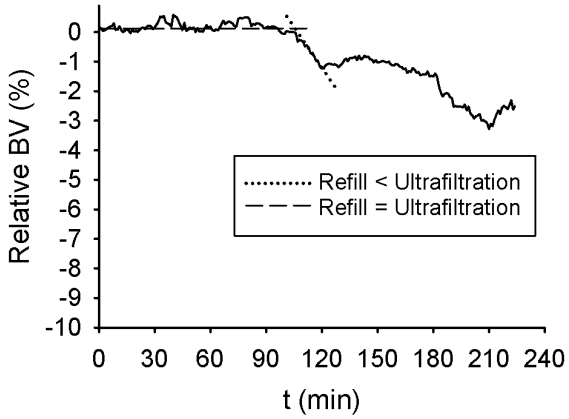

Figure 7. For the first 105 minutes, the relative BV does not drop despite constant ultrafiltration of 9.0 ml/min (total UF goal = 1900 ml). This indicates interstitial overhydration.

judged to be overhydrated. Patients with intermediate, curvilinear rBV pattern are thought to be in a transitional state approaching dry weight. Assuming that most dialysis centres apply constant, linear UF, these patterns in rBV are entirely determined by the dynamics of refill from the interstitial compartment, which in turn depends on the state of hydration of this compartment and the UF induced alterations in the capillary Starling forces. Nevertheless, the importance of refill in determining these patterns has remained unmentioned in many of these papers. Some studies, however, have specifically analyzed rBV patterns in the context of refill.

As an example, Rodriquez *et al*. studied rBV patterns during combined UF and hemodialysis, and 10 to 30 minutes after termination of the dialysis session [15]. Any change in rBV at this point, when UF had been stopped, is solely attributable to refill. They defined three patterns in rBV: 1. A strong reduction in rBV during dialysis (approximately 16%) without an increase in rBV after dialysis, implying poor or absent refill during dialysis and definite absence of refill after dialysis; 2. A less pronounced rBV reduction during dialysis (approximately 12%) and a marked increase in rBV after dialysis, suggesting better refill during dialysis and the definite presence of refill after dialysis; 3. A minor decrease in rBV during dialysis (6% reduction at the most), indicating a high refill rate that almost equaled the UF rate. There was virtually no change in rBV after dialysis, possibly due to the fact that a certain degree of BV reduction is needed to stimulate refill. Patients with pattern 1 were considered to be underhydrated and dry weight was increased by 0.5 kg. Patients with pattern 2 were considered to be mildly overhydrated and dry weight was reduced by 0.9 kg, whereas patients with pattern 3 were considered to be markedly overhydrated, tolerating a dry weight reduction of 5 kg. These patterns of changes in rBV can apparently be used successfully to adjust dry weight in dialysis patients.

Another example is the study by Mitra *et al*., who performed automated curve-fitting analysis on rBV curves obtained in 30 patients during three strong UF pulses and UF-free equilibration periods [2]. They found that approaching dry weight was best predicted by switching of the rBV curve during the UF pulses from a curvilinear to a linear pattern. They postulated that this phenomenon signifies the transition from a two-compartment model (the vascular compartment, from which fluid is removed at a constant rate by UF, and the interstitial compartment, from which fluid moves to the vascular compartment in a non-linear fashion) to a one-compartment model (the vascular compartment from which fluid is removed, while refill from the interstitial compartment is zero). They argued that this cessation of refill indicates that the interstitial compartment is depleted from readily mobilizable fluid and that the dry weight is approached. Although the authors submit that their model could be used as a tool to determine dry weight in dialysis patients, no clinical studies have been reported demonstrating the clinical usefulness of this interesting approach. Moreover, their method to determine dry weight involves a major adaptation of the dialysis procedure, involving pulsed UF, which makes it inconvenient to apply. Importantly, Figure 6 clearly shows that episodes of linear refill may occur early during dialysis with linear UF. If the hypothesis is correct that a change to a linear rBV pattern reflects a normal interstitial fluid volume, then dry weight would have been reached at the first arrow in this patient: refill becomes linear after an initial period of curvilinear decline. Consequently, according to Mitra *et al*., UF would have been stopped at that point. This would have been an incorrect decision since Figure 6 clearly shows that marked refill capacity was still present during the later stages of dialysis. In summary, the switching of rBV curves to linearity is not evidence of the absence of refill during standard dialysis and linear UF, but merely indicates that refill has become constant (and not necessarily zero) over a certain interval. Accordingly, a change to linearity of rBV curves during standard hemodialysis treatment can not be used as an index of dry weight.

5.8 Absolute measurements of refill during hemodialysis and ultrafiltration

Fauchald measured the absolute plasma volume before dialysis (^{125}I-albumin distribution space). He calculated refill from the UF volume and the UF induced change in plasma volume, the latter calculated from the initial plasma volume and the changes in the plasma albumin concentration or Ht. He reported a mean refill rate of 16.5 ml/min (range 11.2 - 20.7 ml/min), presumably obtained during 2 hours of isolated UF [10]. These observations agree very well with an earlier value of $\sim$ 17 ml/min reported during isolated UF by Rouby *et al*., who measured the absolute BV using ^{51}Cr-labeled erythrocytes [18]. Chaignon *et al*., who also measured the absolute plasma volume before dialysis directly (^{125}I-albumin distribution space), reported a lower value of 5.5 ml/min during combined hemodialysis and UF [19]. This is probably an average value obtained during 5 hour dialysis sessions, and it may be that the refill rates in the initial stages of dialysis were higher and similar to those reported by previous authors. The paper, however, does not provide enough detailed information on this issue. In general, these findings agree with the results of Hsu *et al*., who measured the plasma volume before dialysis by a radioisotope method and reported a total

refill volume of 1.3 liter / 3 hours of bicarbonate dialysis [20]. This implies a mean refill rate of $\sim$ 7ml/min for an entire dialysis session. As the plasma volume hardly decreased during the first 2 hours of dialysis at an UF rate of $\sim$ 10 ml/min, suggesting a refill rate close to this value, the refill rate must have been considerably lower in the third hour of dialysis. In addition, they showed that the type of dialysate buffer may affect refill (bicarbonate buffer: refill was 7.2 ml/min, acetate buffer: refill was 5.3 ml/min).

Schneditz *et al*. reported absolute refill values during and after an UF pulse in the first hour of dialysis using an estimated BV value and changes in Ht [21]. During a 20 min UF period, the estimated absolute refill rate was $\sim$ 16 ml/min, which fell to $\sim$ 11 ml/min in a subsequent recovery phase of equal duration. Assuming that the transcapillary hydrostatic and the interstitial oncotic pressures did not change during the short UF period, the authors also calculated the capillary filtration coefficient'Lp'from the estimated BV, the UF rate and the change in plasma oncotic pressure induced by UF. The value of 5.6 ml/min*mmHg*50 kg lean body mass (LBM)), which did not change in the recovery phase, was in the range of the capillary filtration coefficient of skeletal muscle reported earlier. The authors studied refill and Lp only in the first hour of dialysis, and no information was provided on the refill profile in the course of dialysis treatment.

Schroeder *et al*. also derived the absolute refill rate from rBV changes at a constant UF rate [22]. They estimated the absolute plasma volume (PV) using an algorithm based on gender and dry weight. The plasma refill rate was then calculated from the difference between the UF rate and the absolute reduction in PV, the latter derived from the initial estimated PV and changes in rBV. In the early phase of dialysis, when patients were hemodynamically stable, the plasma refill rate was 1360 $\pm$ 550 ml/h ($\sim$ 22 ml/min). During recovery from an episode of intradialytic hypotension, however, this figure dropped to 242 $\pm$ 151 ml/h ($\sim$ 4 ml/min). In this study, the refill rate was measured only during a 15 min period in the first hour of dialysis, in which the blood pressure was stable, and during recovery from a hypotensive episode. Therefore, the dynamics of refill during an entire dialysis session, in particular refill values in the interesting period directly preceding hypotension, were not reported.

Extending the work of Schneditz *et al*. [21], several groups have estimated one of the factors that determines refill, namely the capillary filtration coefficient, during dialysis. Although this coefficient only provides information on the absolute refill rates if the transcapillary pressure gradients are known, changes in this coefficient do reflect changes in refill if one assumes that the net transcapillary pressures do not change appreciably during dialysis.

Using an approach similar to that of Schneditz, Tabei *et al*. [12] calculated the plasma refill coefficient (Kr), expressed in ml/h/mmHg of transcapillary pressure gradient. They estimated the absolute BV (7.7% of body weight) and made a large number of assumptions on transcapillary hydrostatic and oncotic pressure gradients, and the arterial and venous capillary resistance ratio. In essence, their calculations were based on the measurement of body weight, the UF rate, the total protein concentration at the start of dialysis, and hourly Ht values. From the latter, they estimated changes in the total protein concentration and capillary oncotic pressure, which as expected increased during dialysis. They determined Kr in 14 patients during hemodialysis at 1 hour intervals and reported a marked stepwise reduction from 405.3 $\pm$ 75.4 ml/mmHg/h after 1 hour of dialysis to 93.9

$\pm$ 14.3 ml/mmHg/h after 4 hours of treatment. These findings suggest that the capillary water permeability falls markedly during dialysis and UF, which would impact negatively on refill in the later stages of dialysis. It should be considered, however, that the calculated fall in Kr was artificial, due to input of variables into the equations that were based on incorrect assumptions. Alternatively, redistribution of blood flow to tissues with lower capillary filtration coefficients could also explain the dialysis-induced fall in Kr [21]. The authors, however, suggested that the drop in capillary permeability was due to falling atrial natriuretic peptide (ANP) concentrations during dialysis. In a separate study, they indeed observed a marked reduction in plasma ANP concentrations during dialysis and a strong correlation between the plasma ANP concentration and Kr [23].

Using similar methodology, Yashiro *et al*. investigated whether the capillary filtration coefficient, in their study designated as 'Lpst', could be used as a tool to assess dry weight in dialysis patients [24]. Based on post-dialysis plasma ANP concentrations, they stratified patients as being overhydrated (Hi; ANP > 43.2 pg/ml) or normohydrated (Lo; ANP < 43.2 pg/ml). Lpst was significantly lower in the Lo group than in the Hi group (0.83 $\pm$ 0.19 ml/mmHg/min vs. 2.64 $\pm$ 2.73), and plasma ANP concentrations and Lpst were significantly correlated. The latter confirms the observations of Iimura *et al*. [23], but in addition to considering a causative role of ANP in the change in capillary permeability, Yashiro *et al*. also considered that both the elevated ANP levels and the increased Lpst could be independent reflections of an overhydrated state. Lpst was also higher in patients with a dilated inferior vena cava compared to patients with inferior vena cava diameters in the normal range. Although the authors suggested that Lpst measurements can be used to assess volume status in dialysis patients, they did not demonstrate whether Lpst was superior to plasma ANP or inferior vena cava diameter measurements in this respect. In a later study, Yashiro *et al*. demonstrated that Lpst was reduced in patients with large UF volumes and marked reductions in relative BV [25]. This reduction was attributed to either reductions in ANP levels, redistribution of blood flow away from tissues with a high capillary permeability, or sympathetic overactivation which may reduce Lpst [26].

5.9 Conclusion and perspectives

Studies on the absolute refill rate during hemodialysis are still relatively scarce [10, 12, 18, 19, 21-25], and many of these studies do not directly measure absolute refill but report data on one of the determinants of refill, the capillary filtration coefficient [12, 21, 23-25]. The latter studies apply complex mathematics to calculate indices of the vascular permeability coefficient, using numerous assumptions that have not been validated. The absolute values for the BV at the start of dialysis needed to calculate the absolute refill or capillary filtration coefficient were not always measured directly, but were estimated from algorithms or indices of body composition, which affects the accuracy of the results. Moreover, measurements of the absolute refill rate have been performed mostly during isolated UF (which might affect refill) episodes of different duration in the early stages of dialysis, and therefore the sequential changes in refill in the course of regular dialysis sessions are unknown.

Values reported for the absolute refill rate in the early stages of dialysis agree remarkably: 16.5 ml/min [10], 17 ml/min [18], 16 ml/min [21], and 22 ml/min [22]. The value of 5.5 ml/min reported by Chaignon *et al*. [19] is much lower, but probably represents an average value during a 5 hour dialysis session. Combined with the much higher values reported in the early stages of dialysis, this suggests that refill declines as the dialysis procedure proceeds. Interestingly, the refill rate during recovery of a hypotensive episode, which usually occurs in the later stages of dialysis, was only $\sim$ 4 ml/min [22], confirming that the refill rate can indeed change markedly during dialysis. The fall in the capillary filtration coefficient reported by Tabei *et al*. [12] agrees with this notion.

Many of the uncertainties mentioned above could be clarified if the absolute refill rate could be measured continuously during hemodialysis using a reliable and precise method that is easy to apply repeatedly in large groups of patients. There are several interesting and relevant areas of research in which such continuous measurements of the absolute refill rate during dialysis could be applied. These include:

(1) Use of absolute refill measurements in determining dialysis duration
The tendency to develop hypotension during dialysis differs greatly between patients, which could at least in part be due to individual differences in refill capacity. The latter could for instance be related to inadequate precapillary vasoconstriction during BV reduction (e.g. in autonomic neuropathy). Provided that the dry weight was estimated correctly (vide infra), inherent slow refillers would benefit from longer dialysis duration. On-line, continuous assessment of the absolute refill rate could help to adapt the UF rate to the refill rate, thus determining the duration of the dialysis session.

(2) Use of absolute refill measurements to study the effect of interventions on refill
Refill often lags behind the UF rate during hemodialysis, resulting in a BV reduction and intradialytic hypotension. It is obviously of interest to study whether interventions can enhance refill during dialysis. In this respect, one can consider interventions that enhance lower limb tissue hydrostatic pressure (e.g. Medical Anti Shock Trousers), increase the plasma oncotic pressure (infusion of oncotic solutions), or induce precapillary vasoconstriction and possibly reduce the capillary hydrostatic pressure (e.g. midodrine). Sodium profiling probably replenishes the interstitial compartment from the intracellular space during dialysis and would therefore be expected to facilitate refill [27]. The steepness of rBV curves has indeed been shown to decrease during sodium profiling [28, 29], which supports the notion that this intervention increased refill. Only measurement of the absolute refill rate, however, can quantify such an effect on refill.

(3) Use of absolute refill measurements in determining the dry weight
Mitra *et al*. suggested that linearity of rBV curves during repeated, pulsed UF reflects depletion of the interstitial compartment, absence of refill, and possibly attaining dry weight. As discussed above, however, the change of rBV curves from an exponential to a linear pattern can not be used to demonstrate the absolute absence of refill during standard dialysis with continuous, linear UF. Therefore, solely direct assessment of the absolute refill rate can demonstrate the absence of refill.

A method to measure the absolute refill rate continuously during dialysis would be a very interesting tool to test the hypothesis that cessation of refill reflects a state of normal interstitial volume. Such a method could then be used to determine the dry weight in dialysis patients.

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Chapter 6

Continuous, online measurement of the absolute plasma refill rate during hemodialysis using feedback regulated ultrafiltration: preliminary results

Abstract Methods to continuously measure absolute refill during dialysis are not available. It would be useful to have such a method because it would allow investigating the mechanism of refill and study the effect of interventions. We designed a feedback algorithm that adjusts ultrafiltration rate (Q_{UF}) according to hemoglobin (Hb) concentration changes in such a way that relative blood volume (BV) remains constant within a narrow target range. In this situation, the generated Q_{UF} quantitatively reflects refill. Refill patterns were studied in five hypotension prone patients. In addition, on separate occasions, we studied the effect of antiembolism stockings (AES) and infusion of hydroxy-ethylated starch (HAES) on refill in these patients. Refill during the first hour fell significantly from 21 ± 3 ml/min to 9 ± 2 ml/min ($p < 0.05$). In the second hour, refill decreased further and became zero in four out of five patients. Neither AES nor HAES measurably affected refill. The marked and rapid fall in refill in the early stages of dialysis suggests untimely depletion of the interstitial compartment and underestimation of dry weight. We propose that continuous, online measurement of refill patterns may be of value for accurate estimation of dry weight in dialysis patients.

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6.1 Introduction

Hypotension is one of the major complications during combined ultrafiltration (UF) and hemodialysis. The reduction in blood pressure (BP) is caused by a decline in blood volume (BV) which results from an imbalance between external UF and internal refill from the interstitial space to the blood stream. Because of the great efficiency of current dialyzers, the time needed to remove low molecular weight uremic toxins by dialysis has been greatly reduced. Although this reduced time on dialysis seems to be an advantage to the patient, the drawback is that less time is available for UF [1]. The ensuing increase in ultrafiltration rate (Q_{UF}) enlarges the imbalance between refill and UF and thus increases the propensity to hypotension. In this respect, methods that increase refill from the interstitial space would be of great value. In addition, a continuous, direct refill measurement would facilitate a better understanding of refill-related hypotension, and would also allow detailed study of different interventions on refill. At present, no methods are available to directly measure absolute refill rates repeatedly and continuously.

A possible means to measure refill would be to create a situation in which Q_{UF} is continuously adapted by a feedback mechanism in such a way that the relative BV, measured by changes in hemoglobin (Hb) concentration, does not change. Under these conditions, the generated Q_{UF} equals the refill rate. We developed this method using feedback-regulated UF and performed pilot experiments to determine the magnitude and patterns of absolute refill rate during dialysis. In addition, we examined the effect on refill of two interventions: anti-embolism stockings (AES) and a hyperoncotic fluid, hydroxyethylated starch (HAES).

6.2 Materials and methods

A Gambro AK200 dialysis machine (Gambro AB, Lund, Sweden) with built-in BV sensor (BVS) was coupled to a computer controlled external pump (ISM444, Ismatec Laboratoriumstechnik GmbH, Wertheim-Mondfeld, Germany) and a customized connector on the dialysate compartment of an F80 dialyzer (Fresenius Medical Care AG, Bad Homburg, Germany). The BVS contained a sensor with optical components for transmitting light through the cuvette in the BL200B bloodline (Gambro Dasco S.p.A., Medolla, Italy). Continuously measured changes in Hb concentration were translated by the BVS unit

Table 1. Patient characteristics

Patient	Sex	Age (year)	Dry Weight (kg)	Height (m)	BMI (kg/m^2)	Primary Diagnosis	Months on HD	Target BV Range (%)	Target V_{UF} (%)	ACEi	BB	A2RB	CEB	Diuretic
1	M	69	72.5	1.77	23.1	Interstitial nephritis	9	-5.0 to -7.5	3.7	-	+	-	-	+
2	M	49	62.5	1.79	19.5	Membranous glomerulopathy	3	-4.0 to -6.5	3.2	+	-	+	-	-
3	M	59	71.1	1.74	23.5	Hypertensive nephropathy	7	-5.5 to -8.0	4.8	+	-	-	+	+
4	M	83	61.0	1.63	23.0	Unknown	21	-4.5 to -7.0	4.9	+	-	-	-	+
5	M	71	57.8	1.65	21.2	Hypertensive nephropathy	14	-5.0 to -7.5	4.7	+	-	-	-	-

Target V_{UF} (%) is V_{UF} expressed as percent of dry weight.
A2RB, angiotensin-2 receptor blocker; ACEi, angiotensin-converting enzyme inhibitor; BB, beta blocker; BMI, body mass index; BV, blood volume; CEB, calcium entry blocker; HD, hemodialysis; V_{UF}, ultrafiltration volume.

into percent changes in initial BV (sensor active for Hb $\geq$ 4.9 mmol/L and blood flow $\pm$ 180 ml/min). A combination of N751 dialysate concentrate (Gambro Dialysatoren GmbH, Hechingen, Germany) and BiCart bicarbonate cartridge (Gambro Lundia AB, Lund, Sweden) was used to achieve a dialysate concentration of Na^+ 140 mM, K^+ 2.00 mM, Ca^{2+} 1.50 mM, and glucose 11 mM. Dialysate flow was 500 ml/min, target blood flow was 300 ml/min but effectively varied between approximately 250 and 350 ml/min. Patient core temperature was measured three times directly before the start of dialysis with a FirstTemp Genius tympanic thermometer (Sherwood Medical, Crawley, UK). Dialysate temperature was adjusted to the average patient's predialysis core temperature and was not changed during dialysis. The Q_{UF} of the AK200 was set to zero and all UF was transferred to the external pump. The UF volume (V_{UF}) generated by this pump was collected in a container on an electronic scale (Tanita Europe GmbH, Sindelfingen, Germany) (Figure 1). Data were transferred at a frequency of 4 Hz from the data output ports of the AK200 and the scale to an Intel Pentium-S 133 MHz based computer running Poly 5.0 (Inspektor Research Systems, Amsterdam, The Netherlands). The BVS gave a BV value every 30 seconds. An algorithm was programmed in the software program (Poly) that adjusted pump speed every 30 seconds to maintain BV within a preset target range. This range was defined as either the lowest percent change in BV achieved during linear UF without hypotension plus 2.5% (Figure 2), or the percent change in BV at which hypotension occurred plus 2.5%. The initial Q_{UF} was set to 3 x linear Q_{UF} to activate the refill mechanism and quickly reach the BV target range ("induction phase"), allowing sufficient time for feedback-guided absolute refill measurements ("feedback phase"). During the dialysis sessions including refill measurements, the pump speed was adjusted linearly in 25 steps from maximum (= 3 x linear Q_{UF}) at the upper BV target level to zero at the lower BV target level (Figure 3). The range of 2.5% BV was chosen because of technical considerations. The BVS provided output every 30 seconds at BV steps of 0.1%. At a smaller range, e.g. 1% large oscillations in Q_{UF} and BV started to occur. This was because of the fact that at a range of 1% the pump speed could only be varied with 10 steps (1 step at each 0.1% BV change), which was apparently too crude to prevent oscillations from occurring. Expanding the range to 2.5% allowed more refined adjustments (in 25 steps of the pump speed) resulting in a smoother and more stable BV regulation. It is of note that close inspection of Figure 3 shows that the variations in BV were actually much smaller than 2.5%, effectively being $\pm$ 0.5%. The V_{UF} was determined by weighing the ultrafiltrate, correcting for the specific gravity of dialysate, and Q_{UF} was calculated from the weight change per min. At a constant BV, Q_{UF} represents the absolute refill rate.

Extensive safety measures were implemented to prevent unwanted UF, e.g. during insufficient blood flow to the dialyzer or failure of the BVS. A BP monitor (Ohmeda 2300 Finapres, BOC Health care, Englewood, CO) continuously monitored BP and heart rate. Because Finapres obtained BP is known to be dependent on body position and peripheral vasoconstriction, this measurement was validated every 30 minutes electronically with an Accutorr Plus BP monitor (Datascope, Paramus, NJ). When hypotension occurred, UF was stopped and the patient was placed in the head-down lying position. When hypotension was very severe, a 100 ml saline bolus infusion was given.

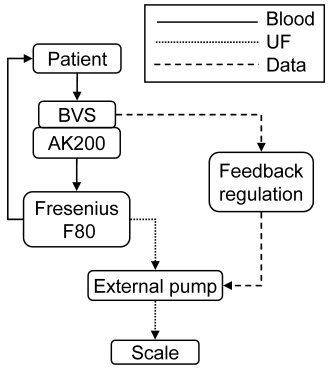

Figure 1. Schematic overview of the experimental setup. AK200, Gambro AK200 dialysis machine; BVS, blood volume sensor; UF, ultrafiltration.

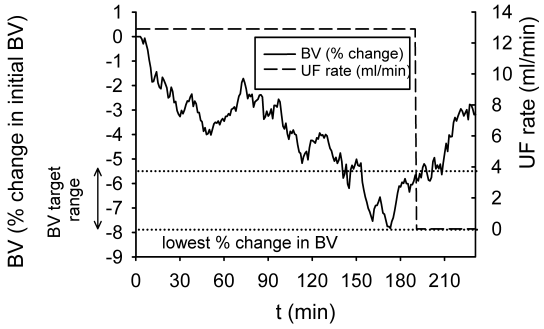

Figure 2. Determination of lowest percent BV change and BV target range during constant UF rate of 12.9 ml/min during a dialysis session without hypotension. The lowest percent BV change is -7.9% (= lower limit). Consequently, the upper BV target is set to be -5.4% (=-7.9% + 2.5%). BV, blood volume; UF, ultrafiltration.

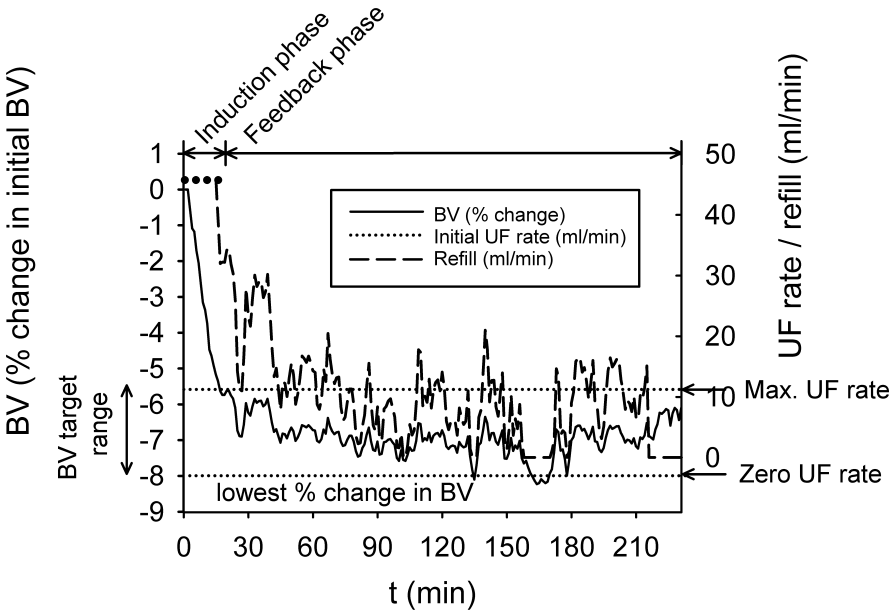

Figure 3. Determination of refill in BV target range during virtually constant BV, in which continuously adjusted UF rate equals refill. Initial UF rate of 45.6 ml/min. BV, blood volume; UF, ultrafiltration.

6.2.1 Statistical analysis

All results are expressed as mean ± SD, except Figure 6, which is expressed as mean ± SEM. All comparisons were performed using SigmaStat version 3.10 (Systat Software Inc., San Jose, CA, 2004). Induction phase duration and induction phase V_{UF} were compared between groups using one-way repeated measures analysis of variance (RM-ANOVA) where appropriate followed by a Student-Newman-Keuls (SNK) post hoc test. Refill was compared within each group and between groups using two-way RM-ANOVA where appropriate followed by a SNK post hoc test. A $p < 0.05$ was considered significant.

6.2.2 Patients

Five male patients regularly suffering from dialysis hypotension were selected from our center (Table 1) and informed consent was obtained. Patients served as their own control and were studied in the semirecumbent position. This position was maintained and patients were asked to refrain from eating and drinking because these factors can have a direct influence on BV [2, 3]. The target V_{UF} (= body weight - target dry weight) was virtually the same for each measurement within a single patient (Table 2). Symptomatic hypotension was defined as a systolic BP $\leq$ 100 mm Hg accompanied by vagal signs and symptoms.

6.2.3 Pilot setup

The BV target range was determined individually during a separate dialysis session using linear Q_{UF}. In another session, refill was measured using the previously mentioned method. On separate occasions, refill measurements were repeated during two interventions:

1) application of AES (Comprinet pro, BSN-Jobst Inc., Rutherford College, NC), and 2) infusion of 100 ml of a hyperoncotic 10% HAES solution (HAES-10%) (Fresenius Kabi BV, 's-Hertogenbosch, the Netherlands; MW 200 kDa).

The stockings were fit individually and applied directly before the start of dialysis. We assumed that external compression induced by AES would increase the interstitial hydrostatic pressure and thus increase refill from the legs. Controlled HAES infusion by

Table 2. Mean target V_{UF} and mean actual V_{UF} during linear, control, AES and HAES measurements

Measurement	Mean Target V_{UF} (ml)	Mean Total V_{UF} (ml)
Linear	2660 ± 573	2164 ± 615 (N.A.)
Control	2729 ± 789	2680 ± 800 (73 ± 31%)
AES	2442 ± 740	2231 ± 905 (80 ± 19%)
HAES	2510 ± 753*	2330 ± 845 (97 ± 3%)

In parentheses the percent UF removed during the feedback-phase.
* Target value includes 100 ml infused HAES.
AES, antiembolism stockings; BV, blood volume; HAES, 10% hydroxy-ethylated starch; V_{UF}, ultrafiltration volume.

an IVAC 560 pump (IVAC Corporation, San Diego, CA) on the venous side of the tubing system was commenced directly after starting UF and continued until the end of the dialysis session. Gradual infusion of HAES leads to a maximum oncotic gradient at the end of dialysis, when refill is likely to be compromised most.

6.3 Results

Refill was estimated in the feedback phase over 15 minute intervals after reaching the BV target range. The mean induction phase was 10 $\pm$ 4 min in control, 12 $\pm$ 9 in AES, and 13 $\pm$ 6 in HAES (NS). The V_{UF} at this time was 464 $\pm$ 233 ml in control, 464 $\pm$ 239 in AES, and 527 $\pm$ 267 in HAES (NS), or 17 $\pm$ 6%, 20 $\pm$ 12%, and 24 $\pm$ 14% of the total V_{UF} target, based on the respective estimated dry weights. During the first 15 minutes of the feedback phase, refill was 21 $\pm$ 3 ml/min in control, 21 $\pm$ 2 in AES, and 22 $\pm$ 3 in HAES. From t = 45-60 minutes, refill had fallen to 9 $\pm$ 2 ml/min in control, 6 $\pm$ 1 in AES, and 9 $\pm$ 1 in HAES (Figure 4). Within each group, the reduction in refill was significant during the first hour ($p < 0.05$). There were no statistical differences when comparing groups for any of these variables. From t = 60-120 minutes, refill further decreased to values close to zero (four out of five persons for all sessions, Figure 5), usually before the UF goal had been reached. Feedback-regulated UF was then stopped and BV was allowed to climb well above the lower limit of the BV target range, after which an attempt was made to remove the remaining V_{UF} using linear UF. Blood pressures during dialysis were not statistically different between groups (Figure 6).

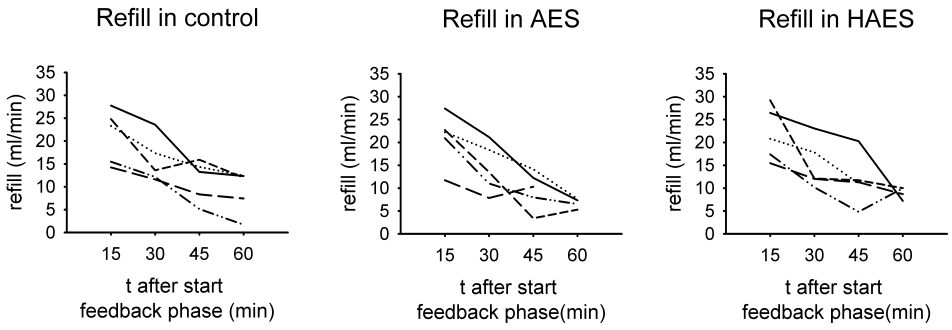

Figure 4. Refill in each patient per 15 min during first hour of feedback phase in control, AES and HAES group. AES, antiembolism stockings; HAES, 10% hydroxy-ethylated starch.

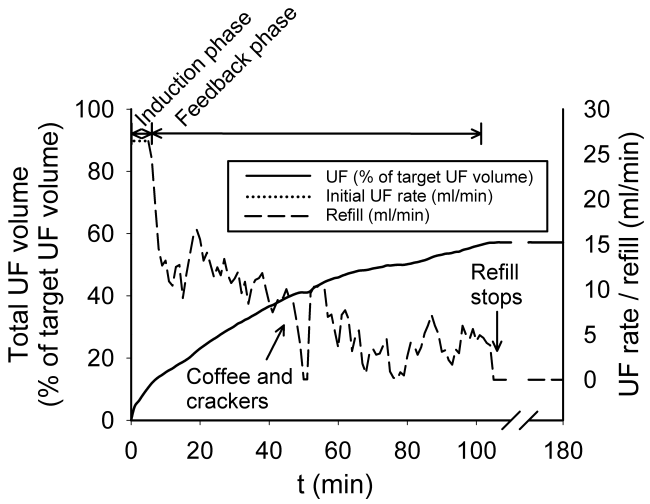

Figure 5. Typical example of failing refill during the second hour of the feedback phase. In the second half of dialysis, 105 min into the feedback phase, refill during constant BV becomes zero when only 57% of the target UF volume has been removed (second arrow). The temporary sudden decline in refill at 45 min follows after the ingestion of a cup of coffee and two cream crackers (first arrow). For the sake of clarity, BV values are not shown. BV, blood volume; UF, ultrafiltration.

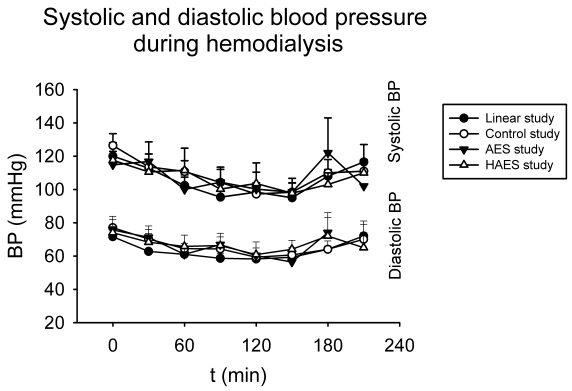

Figure 6. Mean intradialytic blood pressures per 0.5 h.

The number of symptomatic hypotensive episodes was four in the linear group and two in the control, AES, as well as the HAES group. Of these six hypotensive episodes during feedback-regulated measurements, two occurred after the feedback phase had ended. Refill had declined to virtually zero directly before hypotension in two of the four episodes that occurred in the feedback phase.

6.4 Discussion

The method we developed is the first that allows continuous, online measurement of refill. Using this method, it was possible to quantitatively measure refill and to study the pattern of absolute refill during combined hemodialysis and UF. During the first hour of feedback-regulated UF, refill declined from initial values of approximately 20 ml/min to approximately 9 ml/min. In four of the five hypotension-prone patients, refill approached zero halfway during dialysis. This fall in refill rate is most probably caused by UF-induced depletion of the interstitial space and the ensuing lower interstitial hydrostatic pressure. Our findings correspond well with those of Schroeder *et al*. [4], who calculated plasma refill rates from plasma volume estimated by a nomogram-derived equation and changes in hematocrit during linear UF. In their study, the mean refill was estimated to be 1360 ml during the first hour of dialysis, corresponding with a refill rate of approximately 23 ml/min. During hypotensive episodes, the estimated refill fell to approximately 4 ml/min, which agrees with the notion that the imbalance between Q_{UF} and refill is of major importance in the genesis of dialysis-related hypotension. It would be possible to implement an online refill measurement based on the method of Schroeder *et al*., but the accuracy of this method is limited by the fact that BV, and thus refill, is estimated and not measured directly.

Our method implies rapid (approximately 10 minutes) removal of approximately 20% of the V_{UF} before starting the actual refill measurement by applying a high initial Q_{UF} ("induction phase"). This is done to rapidly lower the BV, activate the refill mechanism and allow sufficient time during dialysis for feedback regulation and refill measurement ("feedback phase"). It is conceivable that the procedure itself, in particular the induction phase, may affect the refill rate. On the one hand, fluid removal in the induction phase will deplete the interstitial compartment and reduce the interstitial hydrostatic pressure, which would negatively affect refill. On the other hand, rapid fluid removal will also increase the plasma oncotic pressure, which favors refill. In addition, a rapid BV reduction will activate the sympathetic nervous system, which, through precapillary vasoconstriction, reduces intracapillary hydrostatic pressure and enhances refill. It is impossible to predict which of these factors predominates. Additional studies will have to be performed to assess the effect of varying initial Q_{UF} and BV target ranges on refill.

The BVS used in the current study translates optical absorbance differences into changes in Hb concentration [5], which in turn reflect changes in relative BV. When applying this technique, one assumes that blood is uniformly mixed throughout the vascular space. Recently, however, it was reported that the F-cell ratio in the central blood compartment decreases during UF [6]. The authors hypothesized that this decrease was because of a shift of blood from the microcirculation, which has a relatively low Hb concentration, to the central blood compartment. We should therefore consider the possibility that the blood Hb concentration during UF was not kept constant in our study by refill alone, but that the addition of blood from the microcirculation with a relatively low Hb concentration contributed to this. This would imply that our method overestimated the refill from the interstitium to some extent. However, the fall in F-cell ratio occurred predominantly in the later stages of dialysis, when refill was already very low or absent in our patients. This would imply that the values measured in the early stages of dialysis in our study are a close estimate of the true refill.

The interventions applied in our study did not enhance refill. This could partly be explained by the limited number of patients enrolled in the study. Antiembolism stockings can theoretically improve refill, either at the capillary level or by increasing lymphatic return. The fact that we did not find any effect on refill may be explained by insufficient, non-standardized pressure increases in the lower limbs. Applying higher pressures, e.g. using the medical antishock trousers (MAST suit [7]), may be more effective. The continuous infusion of HAES was intended to gradually increase the plasma oncotic pressure during dialysis, as refill is compromised most when the interstitial compartment is depleted at the end of dialysis. Hence it is not surprising that we did not find an effect on refill in the initial phase of dialysis. Because the absolute refill rates were very low or zero during the final stages of dialysis in most patients, it was difficult to demonstrate an effect on absolute refill rates. Alternatively, it is also plausible that the amount of HAES infused was too small to effectively affect refill. It is possible that the beneficial effect of HAES on refill could have been shown during the first phase of dialysis if more HAES or a HAES bolus had been used. However, further tests with HAES infusion will not be performed because of the risk of side effects such as pruritus, anaphylaxis, and platelet dysfunction [8-10]. As none of the interventions previously mentioned affected refill in our study, it was apparent that the patterns and values of absolute refill were quite consistent within each subject (Figure 4). This suggests a high methodological reproducibility, although this still has to be validated formally.

Although currently used short dialysis protocols trigger dialysis hypotension because of an imbalance between high Q_{UF} and inadequate refill, incorrect determination of dry weight may also play a role. Dry weight is generally estimated on imprecise clinical grounds [11], and more precise methods to determine dry weight are difficult to apply on a regular basis in clinical practice [12, 13]. We suggest that studying the refill pattern during dialysis may provide very useful information on the hydration status of individual patients. In agreement with Mitra *et al*. [14], we also hypothesize that the moment refill ceases identifies the moment of depletion of the interstitial compartment and reflects attainment of the dry weight. Studies are underway to test this hypothesis.

6.5 References

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Chapter 7

Sodium profiling, but not cool dialysate, increases the absolute plasma refill rate during hemodialysis

Abstract Intradialytic hypotension is often caused by a discrepancy between ultrafiltration and plasma refilling. Increasing the plasma refill rate could therefore reduce intradialytic hypotension. We used a recently developed method to measure the effect of cool dialysate and sodium (Na) profiling on refill during hemodialysis (HD). Using a Gambro AK200 with blood volume (BV) sensor plus computer-guided external pump, a high ultrafiltration rate quickly induced a preset BV reduction. A software feedback mechanism subsequently adjusted the ultrafiltration rate continuously to maintain BV between very narrow preset boundaries. The continuously changing, software-generated ultrafiltration rate then quantitatively equalled refill. Absolute plasma refill rate was measured in six stable patients without intradialytic hypotension, undergoing HD without intervention, with cool dialysate (1°C below core temperature), and with Na profiling (gradually declining from 150 to 140 mmol/l). Baseline refill rate was 20.1 ± 4.0 ml/min (mean ± SD). Although cool dialysate did not affect refill (22.2 ± 4.1 ml/min, $p = 0.27$ vs. baseline), Na profiling induced a significant improvement (26.8 ± 3.7 ml/min, $p = 0.006$ vs. baseline). Using our method to measure absolute plasma refill rate during HD, we demonstrated that Na profiling indeed improves the plasma refill rate. A potential effect of cool dialysate could not be established.

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7.1 Introduction

During hemodialysis (HD), the excess sodium (Na) and water that accumulates in the interdialytic interval has to be removed by ultrafiltration. Most excess fluid has accumulated outside the blood compartment in the interstitial space [1]. During dialysis, however, fluid is primarily removed from the blood compartment by ultrafiltration, resulting in a reduction of the circulating blood volume (BV). This BV decline in turn causes interstitial fluid to shift toward the circulation. Consequently, the net BV change is a function of the ultrafiltration rate and the internal plasma refill rate. Because the plasma refill rate during dialysis is usually smaller than the ultrafiltration rate, refill only partially compensates the ultrafiltration-induced BV reduction [2]. Marked reductions in BV predispose to intradialytic hypotension, and interventions that stimulate the plasma refill rate could help to prevent this.

Fluid transport between the circulation and the interstitial space is dictated at the capillary level by the Starling forces [3], meaning that fluid moves between the capillary and the interstitial space under influence of hydrostatic and oncotic forces in both compartments. Interventions that improve blood pressure stability during HD, such as reducing the dialysate temperature and Na profiling [4, 5], could do so, at least partly, by enhancing refill. Reducing the dialysate temperature activates the sympathetic nervous system and increases the peripheral resistance via precapillary vasoconstriction [5]. Besides stabilizing the blood pressure directly, temperature reduction could also enhance refill at the capillary level by lowering the capillary hydrostatic pressure. Whether reducing the dialysate temperature indeed enhances refill has not been studied. The increased dialysate Na concentration during Na profiling increases the extracellular fluid osmolarity, which causes intracellular water to shift to the interstitial space [4]. Because the volume of the interstitial compartment is an important determinant of refill to the intravascular compartment [6], it has indeed been postulated that Na profiling improves blood pressure stability by enhancing refill [4].

Until recently, it was not possible to measure refill quantitatively. This made objective assessment of interventions that could affect refill difficult. We recently developed a method to measure the absolute refill rate during HD [7] and used this method to test whether reducing the dialysate temperature or applying Na profiling would enhance refill during HD.

7.2 Materials and methods

7.2.1 Patients

The study was performed in six patients not prone to intradialytic hypotension who had good vascular access and stable weight gain between treatment sessions (Tables 1 and 2). Informed consent was obtained.

7.2.2 Study protocol

The study protocol is depicted in Table 3. Each patient served as his own control. Patients were studied in the semirecumbent position and were asked to refrain from eating and

Table 1. Patient characteristics

Patient	Primary Disease	Sex	Age (year)	Dry Weight (kg)	BMI (kg/m^2)	Months on HD	Diabetes	Cardiovascular Medication	Treatment Time (h)
1	Diabetic nephropathy	M	36	92.5	34.0	39	+	ACEi	4
2	Unknown	M	82	78.0	24.6	11	-	BB, IMN	3
3	Hypertension	M	66	78.5	25.1	7	-	ACEi	3
4	Unknown	M	77	67.0	19.8	21	-	IMN	3
5	Hypertension	M	78	72.5	25.1	35	-	-	3
6	Unknown	M	79	75.5	26.1	42	-	IMN	3

ACEi, angiotensin-converting enzyme inhibitor; BB, beta blocker; BMI, body mass index; HD, hemodialysis; IMN, isosorbide mononitrate

drinking during dialysis. After determining the appropriate BV range (see below for explanation) during a separate dialysis session (study 1), the plasma refill rate was measured during another dialysis treatment using a dialysate temperature of 37°C and a constant dialysate Na concentration of 140 mmol/l (study 2). During two other dialysis treatments (studies 3 and 4, respectively), the effect on plasma refill rate of reducing the dialysate temperature (1°C below body core temperature) or Na profiling (dialysate Na concentration starting at 150 mmol/l, declining exponentially to 145 mmol/l at 80% of the treatment time, and 140 mmol/l at the end of dialysis) was determined. To avoid the potential effect of the degree of predialysis volume overload on the refill rate during HD, we selected dialysis sessions for the individual patients in which the total ultrafiltration goal was virtually the same (Table 2).

7.2.3 Clinical parameters

Patient core temperature was measured directly before and at the end of HD sessions (average value of three measurements) with a FirstTemp Genius tympanic thermometer (Sherwood Medical, Crawley, UK). The plasma Na concentration was measured before and after dialysis by a Vitros 950 Chemistry system (Ortho-Clinical Diagnostics, Raritan, NJ). Blood pressure before and after HD was measured with an Accutorr Plus blood pressure monitor (Datascope, Paramus, NJ). During HD, blood pressure was determined by an Ohmeda 2300 Finapres blood pressure monitor (BOC Health Care, Englewood, CO).

7.2.4 Absolute plasma refill rate

Absolute plasma refill rate, measured by computer-guided, algorithm-controlled ultrafiltration described previously [7], was measured as follows: a HD machine (Gambro AK200; Gambro AB, Lund, Sweden) with a high-flux dialyzer (Fresenius HF80S; Fresenius Medical Care AG, Bad Homburg, Germany) was coupled to an external computer running Poly 5.0 (Inspektor Research Systems, Amsterdam, The Netherlands). The AK200 contained a BV sensor that optically measured ultrafiltrationinduced hemoconcentration during HD, enabling calculation of the percent change in BV. A combination of N751 dialysate concentrate (Gambro Dialysatoren GmbH, Hechingen, Germany) and BiCart bicarbonate cartridge (Gambro Lundia AB, Lund, Sweden) was used to achieve a dialysate concentration

Table 2. Hemodialysis parameters

Study	Ultrafiltration Goal (ml)	Ultrafiltration Rate in Induction Phase (ml/min)*	Duration of Refill Measurement (min)	Mean Arterial Pressure Before Hemodialysis (mmHg)	Mean Arterial Pressure After Hemodialysis (mmHg)
1. Standard hemodialysis	2533 ± 234	14.3 ± 1.8	-	93 ± 12	79 ± 11
2. Baseline	2550 ± 619	33.9 ± 3.6	107 ± 51	99 ± 19	92 ± 21
3. Cool dialysate	2633 ± 695	35.4 ± 6.4	84 ± 37	97 ± 14	92 ± 13
4. Na profiling	2517 ± 705	33.3 ± 4.5	62 ± 48	97 ± 18	98 ± 15

* Ultrafiltration rate was constant during standard hemodialysis.
Na profiling, sodium profiling.

of Na^+ 140 mM, K^+ 2.00 mM, Ca^{2+} 1.50 mM, and glucose 11 mM. Dialysate flow was 500 ml/min, and target blood flow was 300 ml/min but effectively varied between 250 and 350 ml/min. This information was relayed to the computer that controlled an external pump (ISM444, Ismatec Laboratoriumstechnik, GmbH, Wertheim-Mondfeld, Germany), which was connected to the dialysate outlet of the dialyzer. After setting the ultrafiltration rate of the dialysis machine to zero, this external BV-guided pump directly controlled the ultrafiltration rate. Computer software was programmed to keep the relative BV within a preset range by varying the ultrafiltration rate. The BV range was defined as either the lowest percent change in BV achieved during standard HD without hypotension plus 2.5% or the percent change in BV at which hypotension occurred plus 2.5% (Table 3; study 1). A high initial ultrafiltration rate, 2.5 times the linear ultrafiltration rate needed to achieve the dry weight at the end of dialysis, was applied to quickly reach the upper limit of the preset BV range ("induction phase"). After reaching the upper BV limit, algorithm-controlled ultrafiltration started via the computer system and the external pump ("refill measurement phase"). The pump speed was continuously adapted to maintain the relative BV virtually constant within the predefined range. In this situation of a constant BV, the ultrafiltration rate generated by the algorithm quantitatively equals the plasma refill rate (Table 3; study 2). The ultrafiltration rate was adjusted every 30 seconds in the refill measurement phase. Refill was then calculated per 15 minutes as the average of 30 ultrafiltration rate values.

7.2.5 Indicators of refill in the induction phase

A consequence of our method is that the absolute refill rate can only be measured after an induction phase in which a certain amount of the volume excess has been removed to reach the upper limit of the relative BV range. However, the design of our study was such that the upper limit of the relative BV was the same for each study setting. In addition, the initial ultrafiltration rates were similar during the baseline study and the two interventions. Hence, changes in the induction phase duration and the volume removed in this phase reflect changes in refill.

Our system did not allow direct measurement of the absolute refill rate in the induction phase (see above). However, refill can be estimated from changes in the absolute BV in this phase of constant ultrafiltration according to the following equation:

$$\text{Refill (ml/min)} = \frac{\text{UF in induction phase (ml) - (estimated absolute BV (ml)*upper relative BV limit (\%))}}{\text{Induction phase duration (min)}}$$

Although the absolute BV was not measured in our study, it can be estimated using an algorithm [8].

Table 3. Study protocol

Study	Ultrafiltration Rate	Dialysate Na Concentration (mmol/L)	Dialysate Temperature (°C)	Aim
1. Standard hemodialysis	Constant	140	37.0	Detect lowest achievable BV reduction without hypotension occurring
2. Baseline	2.5x linear ultrafiltration rate (pulse), then computer-guided ultrafiltration	140	37.0	Obtain baseline absolute refill rate and indirect indicators of refill (= induction phase duration and induction phase ultrafiltration volume)
3. Cool dialysate	Same as baseline	140	1 °C below core temperature	Investigate the influence of cool dialysate on refill and on indirect indicators of refill
4. Na profiling	Same as baseline	150 to 140*	37.0	Investigate the influence of Na profiling on refill and on indirect indicators of refill

* Na concentration declined exponentially, reaching 145 mmol/L at t = 0.8 x treatment time.
BV, blood volume; Na profiling, sodium profiling.

7.2.6 Statistical analysis

All results are expressed as mean ± standard deviation (SD). All comparisons were performed using SigmaStat version 3.10 (Systat Software Inc., San Jose, CA, 2004). The differences in the various outcome measurements were tested for statistical significance by one-way analysis of variance for repeated measures (RM-ANOVA) where appropriate followed by a Student-Newman-Keuls post hoc test. A p value of < 0.05 was considered significant.

7.3 Results

7.3.1 Clinical parameters

The blood pressure and the incidence of hypotensive episodes (data not shown) was not affected by the interventions applied in this study (Table 2). Interestingly, the postdialysis blood pressure value in the standard dialysis was markedly lower than in the baseline and intervention studies ($p = 0.077$ between treatments). Reducing the dialysate temperature to 1°C below the predialysis body core temperature (study 3) reduced the core body temperature with 0.5°C ± 0.2°C at the end of dialysis. The plasma Na concentration before and after the baseline study was 138 ± 2 and 139 ± 1 mmol/l, respectively. Similar values were obtained in the cool dialysate study. Under Na profiling, the postdialysis value was 141 ± 2 mmol/l. The mean upper and lower limits for the BV range determined in the

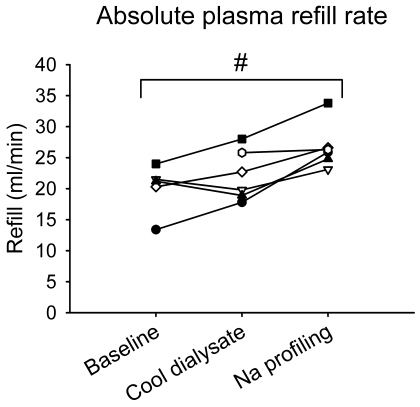

Figure 1. Absolute plasma refill rates for each study group.
p = 0.006 vs. baseline.

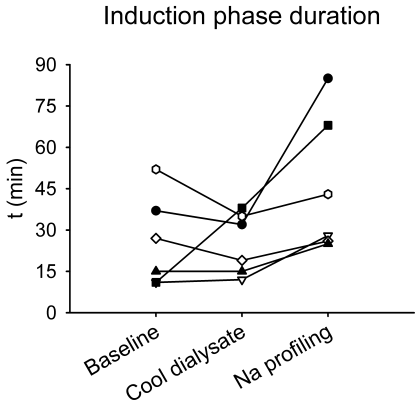

Figure 2. Duration of the induction phase.

individual patients were -6.3% ± 2.0% and -8.8% ± 2.0%. The mean estimated absolute BV was 5188 ± 225 ml.

7.3.2 Absolute plasma refill rate

During the Na profiling study, the refill phase lasted beyond 15 minutes in only three patients. Therefore, comparison between refill values could only be performed for the first 15 minutes of refill measurement for all patients. The baseline plasma refill rate (measurement duration 107 ± 51 minutes) was 20.1 ± 4.0 ml/min (Figure 1). Reduction of the dialysate temperature did not significantly affect refill (22.2 ± 4.1 ml/min, nonsignificant (NS) compared to baseline; duration refill phase 84 ± 37 minutes). However, Na profiling significantly increased refill to 26.8 ± 3.7 ml/min (p = 0.006 vs. baseline; duration refill phase 62 ± 48 minutes), which is an increase of 6.8 ± 4.4 ml/min (or 38% ± 34%) versus baseline. One patient had already reached his total ultrafiltration goal in the induction phase of the baseline study. Consequently, the absolute baseline refill could not be measured in this patient.

7.3.3 Indicators of refill in the induction phase

Induction Phase Duration. The duration of the induction phase in the baseline study was 25.5 ± 16.5 minutes and 25.2 ± 11.1 minutes during reduction of the dialysate temperature (Figure 2). During Na profiling, the induction phase increased markedly to 45.8 ± 25.2 minutes. However, this change just failed to reach statistical significance (p value between treatments: 0.052).
Induction Phase Volume. The total induction phase ultrafiltration volume was 830 ± 480 ml (39% ± 33% of the total ultrafiltration goal) (Figures 3 and 4). During reduction of the dialysate temperature, the ultrafiltration volume was similar (891 ± 409 ml or 35% ± 19%

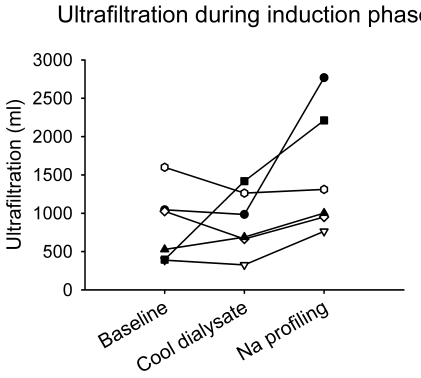

Figure 3. Ultrafiltration in milliliter within each study group during the induction phase.

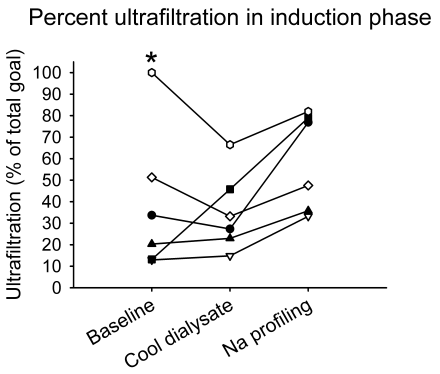

Figure 4. Ultrafiltration during the induction phase in percent of total ultrafiltration goal. *During this measurement, total ultrafiltration goal had been reached before the refill measurement phase.

of the total ultrafiltration goal). During Na profiling, however, this value had increased to 1501 $\pm$ 805 ml or 59% $\pm$ 23% of the total ultrafiltration goal. Again, this change failed to reach statistical significance between treatments (ultrafiltration volume in milliliter: p = 0.074; ultrafiltration volume in %: p = 0.064).

7.3.4 Estimated refill rate

The estimated plasma refill rate during the induction phase was 18.4 $\pm$ 7.1 ml/min in baseline, 21.0 $\pm$ 9.4 ml/min during cool dialysate, and 25.2 $\pm$ 4.5 ml/min under Na profiling (NS).

7.4 Discussion

In this study, we investigated whether two interventions that improve blood pressure stability during HD, namely reducing the dialysate temperature and increasing the dialysate Na concentration, affect refill from the interstitial to the vascular compartment. The beneficial effect of reducing the dialysate temperature is generally attributed to an increased total arterial peripheral resistance caused by constriction of the resistance vessels [9]. We hypothesize, however, that the blood pressure stabilizing effect of this intervention could partly be due to an effect on the forces that determine refill at the capillary level. Precapillary vasoconstriction could reduce the capillary hydrostatic pressure, which would facilitate refill to the capillary lumen. The use of a high Na dialysate concentration is postulated to enhance refill by increased filling of the interstitial compartment [4], which has been shown to be an important determinant of refill [6]. We used a recently developed method to measure the absolute refill rate during dialysis to establish the effect of the interventions mentioned

above on refill of the vascular compartment during HD [7].

Using this method, we found that increasing the Na concentration during dialysis increased the absolute refill rate by 6.8 ml/min (38%). This is a clinically relevant increase if one considers that the mean estimated refill rate in a typical dialysis patient is approximately 10 ml/min (BV of 5000 ml, 10% BV reduction after 4 hours of dialysis, ultrafiltration goal 2900 ml). This marked increase in refill under Na profiling is particularly impressive because more ultrafiltration had been achieved during the induction phase: 60% of the total ultrafiltration goal under Na profiling and 39% in baseline. This implies that Na profiling had intensely replenished the interstitial compartment by recruiting intracellular fluid, thereby facilitating vascular refilling. Indirect refill indicators, such as induction phase duration and volume, also tended to improve under Na profiling. This suggests that refill was also increased in the early stage of dialysis, when quantitative refill measurements could not be made due to the characteristics of our method. The estimated refill rate during this phase also supports this assumption.

The estimated refill values in the induction phase were consistently lower than the absolute refill values found by our method, although the same trend between treatments was observed. This underestimation of refill suggests an overestimate of the total BV by the BV algorithm. This could be due to the fact that this algorithm was determined in a healthy population and not in dialysis patients.

Our findings regarding Na profiling are in agreement with studies that showed less BV reduction at the end of dialysis during Na profiling and constant ultrafiltration rate, suggesting more refill [10, 11]. They are also in accordance with the bioimpedance study of de Vries *et al*., who found that a high Na dialysate concentration caused a fluid shift from the intracellular to the extracellular compartment. This was accompanied by better BV preservation, indicating that the transcellular fluid shift stimulated refill [4]. As discussed elsewhere, however, Na profiling may have detrimental effects on the long-term volume and blood pressure homeostasis. Increased Na load induces thirst and increases fluid intake, leading to hypertension in the interdialytic interval. Hence, it may be useful only in selected patients [12].

We could not demonstrate an effect of reducing the dialysate temperature by 1°C below core temperature on refill. Because the core temperature declines slowly when using a reduced dialysate temperature, the lack of an effect on the indirect refill parameters in the early, induction phase of dialysis was no surprise. However, the absolute refill measurement after the induction phase also did not differ from the baseline study. This suggests that the degree of precapillary vasoconstriction induced by the decrease in dialysate temperature, as applied in this study, was too small to induce a capillary hydrostatic pressure reduction great enough to measurably increase refill. Alternatively, constriction of the capacitance vessels induced by the use of cool dialysate could have led to a retrograde elevation of the capillary hydrostatic pressure [13]. This would counteract any reducing effect of precapillary vasoconstriction on the capillary hydrostatic pressure. It is conceivable that a further decrease in dialysate temperature could lead to more precapillary vasoconstriction and a significant increase in refill. However, most patients on cool dialysate in our study experienced discomfort and reported feeling cold, making a further decrease of the dialysate temperature impractical.

Given the small number of patients in our study, it is interesting to see that the refill measurement procedure itself (implying a high initial ultrafiltration rate) tended to preserve the blood pressure after dialysis, compared with the standard dialysis. One possible explanation is that our refill protocol resembles a step-down ultrafiltration profile; previous studies have shown that such profiles, in particular when combined with Na profiling, have a beneficial effect on vascular stability during HD [14, 15].

In our study, we assume that changes in relative BV as perceived by the BV sensor are exclusively the net result of changes in ultrafiltration and refill rate. This assumption may not be entirely correct. First, it has been reported that the F-cell ratio (the ratio between the whole body hematocrit (Ht) and the arterial or venous Ht) increases during HD and linear ultrafiltration [16]. This is probably caused by a translocation of microcirculatory blood with low Ht to the central circulation. A changing F-cell ratio would imply that the Ht is affected by other factors than ultrafiltration and refill. In our study, substantial excess volume is removed in the first 30-60 minutes of the study ("induction phase"). It can therefore be expected that the major part of the F-cell ratio increase occurred before the absolute refill measurement was started. However, we cannot exclude that some increase in F-cell ratio still occurred in the refill phase. Any decrease in Ht by a change in F-cell ratio (interpreted by the algorithm as an increase in relative BV) could cause some increase in ultrafiltration rate, potentially leading to some overestimation of refill by our method. Second, Mercadal *et al*. [17] have shown that an increase in plasma Na may affect the hemoglobin-derived BV measurement, causing an apparent BV reduction. We assume that such an effect during Na profiling was maximal in the induction phase, in which the dialysate Na concentration was at its peak. The consequence of this would be that the upper BV limit is reached earlier than in the baseline study. If Na profiling would not affect the plasma refill rate at all, this would lead to shortening of the induction phase. We actually found that the induction phase increased during Na profiling, providing direct evidence for enhanced refill. If the BV measurement error would still increase during the refill phase, the result would be that the lower BV limit is also reached prematurely. In this case, ultrafiltration would stop earlier than would be dictated by the true BV reduction, and the observed increase in refill during Na profiling would be underestimated. We cannot establish the net effect of the opposing phenomena mentioned above on the absolute refill values during Na profiling, but we submit that the actual absolute refill measurements were relatively unaffected.

7.5 Conclusion

We applied a recently developed method to measure refill during HD to study the effect on refill of two interventions commonly used to improve blood pressure stability during dialysis. We confirmed that increasing the dialysate Na concentration markedly improves refill during dialysis, presumably by increasing the filling of the interstitial compartment at the expense of the intracellular compartment. In contrast, reducing the temperature of the dialysate below core temperature had no effect on refill of the vascular compartment from the interstitial tissues. Our method can be used in further research to study the effect of interventions on refill. In addition, as refill depends strongly on the volume status of the interstitial compartment, refill patterns may be used to determine interstitial filling and aid

in determining dry weight. Further studies are underway to investigate this hypothesis.

7.6 References

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Chapter 8

Refill capacity during hemodialysis as a novel indicator of dry weight in dialysis patients

Abstract The consequence of incorrect dry weight estimation in the hemodialysis patient is either intradialytic hypotension due to hypovolemia, or sustained hypertension due to excess fluid in the interstitial space. Nowadays, several methods are available to analyze the volume status of patients on dialysis. However, appropriate dry weight estimation remains problematic since none of these methods is the gold standard. Therefore, novel methods to assess dry weight in these patients are still needed. We previously reported a method to determine the absolute rate of refill from the interstitial space to the circulation during combined hemodialysis and ultrafiltration. Using this method, we determined absolute plasma refill rates and observed the disappearance of refill during dialysis in hypotension-prone patients. In this study, we therefore investigated the hypothesis that a normal interstitial fluid volume, and thus dry weight, can be determined by the moment that refill disappears during dialysis. This method was applied in 10 patients. Several methods to assess volume status (e.g. bio-impedance analysis, ultrasonography of the inferior vena cava, and plasma brain natriuretic peptide) were used as reference tests. The dry weight adjustment on the basis of refill cessation during dialysis was well tolerated in all patients. There was no clear agreement between the volume status, defined by the amount of dry weight adjustment by our method, and methods such as bio-impedance and ambulatory blood pressure. Dry weight adjustment did not shift the results of reference tests toward euvolemia. Changes in the extracellular water corrected for height, and total body water, measured by bio-impedance, correlated significantly with the amount of dry weight change. Other reference tests failed to show a significant correlation. Determination of dry weight based on refill patterns is a feasible method that is tolerated by patients on dialysis.

Brummelhuis WJ, Boer WH.

In progress

8.1 Introduction

Despite ongoing improvements in hemodialysis (HD) treatment in recent years [1], accurate dry weight estimation remains a problem unsolved [2]. If dry weight is underestimated, too much fluid is removed from the extracellular space during dialysis, leading to hypovolemia and hypotension during dialysis or in the interdialytic interval. If the dry weight is set too high, the ensuing sustained fluid overload creates hypertension and increases the cardiovascular burden.

Unfortunately, there is no gold standard for determining the dry weight in dialysis patients. Most clinicians rely on clinical parameters (blood pressure, edema) and chest radiography to estimate dry weight, using a trial and error strategy [3]. Many tests to determine the volume status more correctly, such as the use of bio-impedance techniques [4], measurements of the inferior vena cava diameter [5], or changes in natriuretic peptide levels have been proposed [6, 7], but their clinical relevance and applicability is as of yet unclear [8].

We recently developed a method to determine the absolute rate of refill from the interstitial space to the vasculature during HD and ultrafiltration. Initially, we used this method to test the effect of interventions that might enhance refill during dialysis [9, 10]. During this research, several hypotension-prone patients showed marked reductions in or even complete cessation of refill long before the estimated dry weight had been achieved (Figure 1). As the hydration state of the interstitial compartment is an important determinant of refill [11], our findings suggested that the interstitial fluid compartment was depleted and that the dry weight had been set too low. This led to the hypothesis that a marked reduction in refill during combined HD and ultrafiltration may indicate that the interstitial compartment has been reduced to its normal volume, and that the weight attained at that moment is the appropriate dry weight.

In the present study we adjusted the dry weight of dialysis patients according to the point of refill cessation during dialysis. To indirectly test whether this approach helped to attain a state approaching euvolemia (i.e. normal blood volume in conjunction with normal interstitial volume) or not, we applied an extensive panel of volume status markers before and after adjusting the dry weight, according to the refill method.

8.2 Materials and methods

8.2.1 Patients

The study was performed in ten patients who had patent vascular access and stable weight gain between treatment sessions (Table 1). The recruitment of patients and the study protocol were conducted in compliance with the Declaration of Helsinki, and approved by the University Medical Center Utrecht's Medical Ethics committee.

8.2.2 Study protocol

First, during and after standard dialysis session, the volume status of the patient was determined by a panel of markers (Figure 2). These consisted of blood pressure (BP) directly

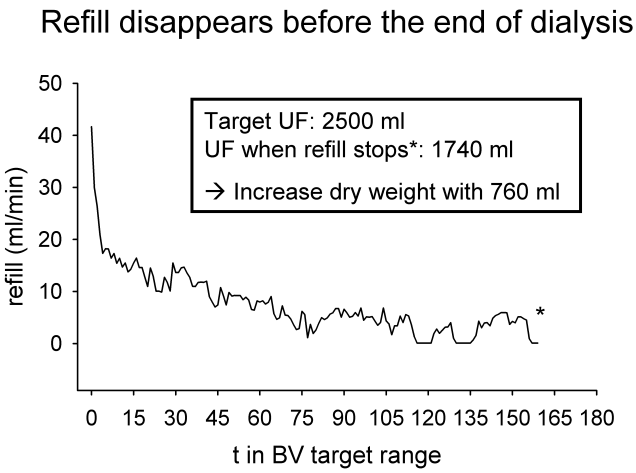


Figure 1. Before the total ultrafiltration goal of 2500 ml was achieved, refill was reduced to zero twice between 120 and 135 min in the refill measurement. From 155 min onwards, refill was completely absent (*). According to our hypothesis, this indicated premature interstitial emptying due to an incorrect dry weight. Consequently, dry weight should be increased by 760 ml.
Abbreviations: BV, blood volume; UF, ultrafiltration

before, during, and after HD, brain natriuretic peptide (BNP) directly before and after HD, inferior vena cava index and bio-impedance analysis 30 min after HD, 24 hour interdialytic ambulatory blood after HD, as well as a chest X-ray and quality of life-form the day after HD.

In the next dialysis session, the absolute plasma refill rate was determined using our method of initial quick relative blood volume (rBV) reduction followed by computer-guided ultrafiltration [10]. The latter allowed us to determine quantitative refill rate values. In short, a standardized initial rBV reduction of -7.5% (measured by the hemoglobin sensor integrated in the dialysis machine) was achieved by an UF rate three times higher than the UF rate needed to achieve dry weight within the dialysis time. On average, this phase lasted for 20-30 min. After achieving the rBV value of -7.5%, a computer-controlled algorithm started to adjust the UFR in 25 steps between the maximum value (= 3x the regular UFR-value at rBV = -7.5%) and zero (= 0 ml/min at rBV = -10%). In this way, the algorithm kept the rBV constant between the values of -7.5% and -10%, with an effective rBV variation of $\pm$ 0.5%. In this situation of constant rBV, the ultrafiltration rate generated by the algorithm equalled the plasma refill rate. This setup allowed us to quantitatively measure the plasma refill rate in ml/min.

There were three possibilities during this refill measurement, each requiring a different action (Figure 2): 1. refill stopped before the total ultrafiltration goal was reached. According to our hypothesis, this meant that the interstitial space was depleted of readily movable water, and thus no further ultrafiltration was needed. The target dry weight for the next hemodialysis sessions was increased with the amount of fluid not removed; 2. refill

Table 1. Patient characteristics

Patient	Sex	Age (year)	BMI (kg/m^2)	Initial DryWt (kg)	DryWt after Adjustment (kg)	DryWt Change (kg)	DryWt Change (%/Initial DryWt)	Months on HD	Cardiovascular Medication	Treatment Time (h)
1	M	73	23.7	76	75.2	- 0.8	- 1.1	52	ACEi, IDN	4
2	M	67	26.4	85.5	85.7	+ 0.2	+ 0.2	9	BB, diuretic	4
3	F	73	22.3	67.5	68.2	+ 0.7	+ 1.0	18	A2RB, diuretic	3
4	F	88	24.5	72.5	72.7	+ 0.2	- 0.3	5	Diuretic	3
5	F	79	18.1	48	47.7	- 0.3	- 0.6	8	ACEi, BB, diuretic, CEB, IDN	3
6	M	59	16.5	61	58.7	- 2.3	- 3.8	37	ACEi	4
7	F	62	30.7	78.5	78.9	+ 0.4	+ 0.5	17	ACEi, BB, CEB, diuretic	3
8	M	55	29.0	100.5	97.4	- 3.1	- 3.1	1	ACEi	3
9	M	80	24.2	80	81.9	+ 1.9	+ 2.4	9	BB, IDN	3.5
10	M	80	26.9	82.5	82.1	- 0.4	- 0.5	2	ACEi, A2RB, diuretic	3
Mean ± SD		70 ± 10	24.2 ± 4.4	75.2 ± 14.3	74.9 ± 14.1	- 0.3 ± 1.4	- 0.5 ± 1.8	16 ± 16		

A2RB, angiotensin-2 receptor blocker; ACEi, angiotensin-converting enzyme inhibitor; BB, beta blocker; BMI, body mass index; CEB, calcium entry blocker; DryWt, dry weight; HD, hemodialysis; IDN, isosorbide dinonitrate

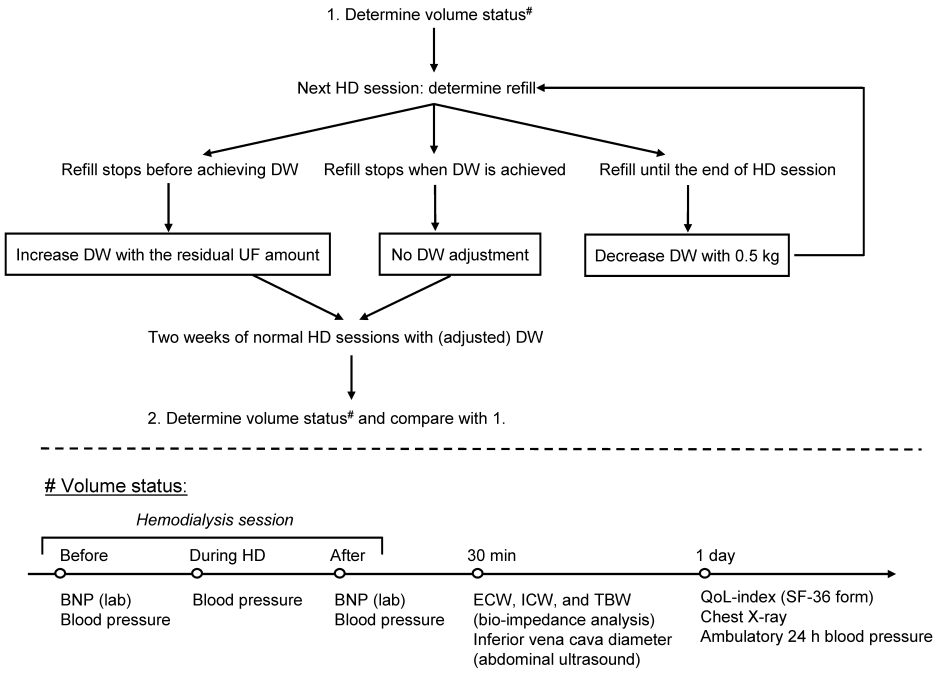


Figure 2. Schematic representation of the study protocol. On the basis of refill measurements dry weight is adjusted. The volume status of the patient is determined using a panel of accepted methods beforehand and after two weeks of standard hemodialysis procedure with the new dry weight.
Abbreviations: BNP, brain natriuretic peptide; BV, blood volume; DW, dry weight; ECW, extracellular water; TBW, total body water; HD, hemodialysis; QoL, quality of life; VCI, vena cava index.

stopped exactly when total ultrafiltration goal was achieved. This required no adjustment of dry weight; 3. refill continued until the end of dialysis. The latter indicated excess volume still being present in the interstitial space. The target dry weight was reduced by 0.5 kg and the refill measurement was repeated in the next dialysis session. This procedure was iterated until situation 1 or 2 was attained.

The patient was then dialyzed for two weeks with the adjusted dry weight using standard hemodialysis equipment and procedures. After this two-week period, the volume status was determined again using the panel of markers mentioned above.

8.2.3 Methods to assess volume status

The patients' volume status (either hypovolemic, euvolemic, or hypervolemic) can be regarded in several ways. First, on the basis of the volume status markers described below. Second, by the amount of dry weight reduction on the basis of our refill method:

1. Hypervolemic: more than 0.5% dry weight reduction (solid line in the figures below)
2. Euvolemic: -0.5 to +0.5% change in dry weight (dashed line)
3. Hypovolemic: more than 0.5% dry weight increase (dotted line)

Figure 3 shows the (hypothetical) ideal situation where both methods of volume status identification are in agreement with each other: all three volume states can be identified as separate groups at baseline. If our hypothesis is correct, then after dry weight adjustment both hypovolemic and hypervolemic patients will shift toward euvolemic values. In this case, the mean value of the volume status marker at baseline and after two weeks will not change very much, whereas the standard deviation will decrease considerably. In other words, the variance ratio will decrease.

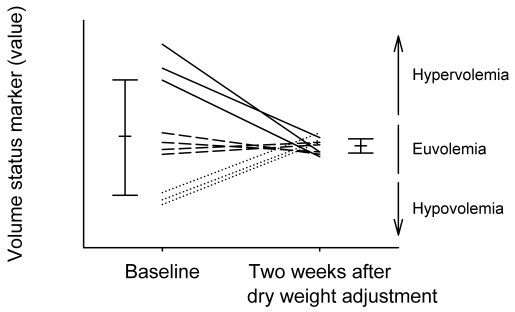


Figure 3. Schematic representation of the situation where both methods of volume status identification (based on volume status markers, and according to our refill method) are in agreement with each other. Three separate volemic states can be identified at baseline. After adjusting the dry weight according to the refill method, hypovolemic and hypervolemic patients would be expected to shift toward euvolemic values. The mean values of the volume marker at baseline and after two weeks will not change considerably as individual values increase and decrease, whereas the standard deviation (and variance ratio) will decrease.

Intradialytic blood pressure
Blood pressure was measured (Ohmeda 2300 Finapres blood pressure monitor; BOC Health care, Englewood, CO) before and after dialysis in the standing position. In patients who were not able to stand, BP was measured after the patient had transferred from the dialysis chair to his wheelchair.

Ambulatory blood pressure (ABPM)
Ambulatory blood pressure was recorded for 24 h after dialysis (Mobil-o-graph®, Industrielle Entwicklung Medizintechnik GmbH, Stolberg, Germany).

Vena cava ultrasonography
Thirty min after hemodialysis, we measured the (inspiratory) minimum IVC (= IVC_{min}) and the (expiratory) maximum IVC (= IVC_{max}) in M-Mode (SonoSite Titan; SonoSite Inc., Bothell, WA). Indexed IVC diameter (VCDi) was defined as the IVC_{max} divided by the body surface area (BSA). The inferior vena cava collapsibility index (IVCCI) was calculated as (IVC_{max}-IVC_{min}) / IVC_{max} x 100. Values for both VCDi and IVCCI were classified as hypovolemic, euvolemic, or hypervolemic on the basis of Brennan *et al*. [5].

Brain natriuretic peptide (BNP)
We analyzed BNP before and after hemodialysis (UniCel^{TM}DxI 800 immunoassay analyzer; Beckman Coulter GmbH, Krefeld, Germany). Hypervolemia was indicated by BNP values above 67 (pmol/l) [12].

Bio-impedance analysis (BIA)
Estimated extracellular, and total body water was obtained by BIA (measured three times) approximately 30 min after dialysis (Body Composition Monitor; Fresenius Medical Care, Bad Homburg, Germany). ECW was divided by height, the normovolemic range being 9.7 $\pm$ 1.3 (l/m) and the hypervolemic range 11.8 $\pm$ 2.0 (l/m) [13]. In addition, ECW corrected for total body water was analyzed (hypervolemia: 50 $\pm$ 9.6% [12]).

Chest X-ray
The day after dialysis, patients returned to the hospital for an anterior-posterior chest X-ray. The cardiothoracic ratio was evaluated (= the widest horizontal distance of the heart divided by the widest horizontal distance of the inner rib margins), with normovolemia defined as a ratio of less than 0.5. In addition, signs of overhydration such as pleural effusions and pulmonary edema were evaluated.

Quality of life Short Form 36 (SF-36) Health Survey questionnaire
The patients completed the SF-36 questionnaire at home, after the dialysis. Data were analyzed in SPSS 15.0.1 (SPSS Inc. Headquarters, Chicago, Illinois) and expressed as Physical Composition Score and Mental Composition Score. Higher scores indicate better well-being.

8.2.4 Dialysis technique

All dialysis sessions were performed on an AK200 (Gambro AB, Lund, Sweden). A dialysate concentration of Na^+ 140 mM, K^+ 2.00 mM, Ca^2+ 1.50 mM, and glucose 11 mM was achieved using N751 concentrate (Gambro Dialysatoren GmbH, Hechingen, Germany) and a BiCart bicarbonate cartridge (Gambro Lundia AB, Lund, Sweden). In those patients with a high plasma potassium level, N292 concentrate was used to achieve concentrations of Na^+ 140 mM, K^+ 1.00 mM, Ca^{2+} 1.70 mM, and glucose 5.5 mM. The dialysate flow was 500 ml/min, and the target blood flow was 300 ml/min but effectively varied between 250 and 350 ml/min. Dialysis time varied between 3 and 4 hours.

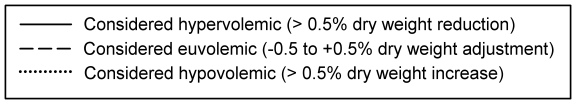
8.2.5 Statistical analysis

All results are expressed as mean ± SD. All comparisons were performed using SigmaStat version 3.10 (Systat Software Inc., San Jose, CA, 2004). Correlations between changes in dry weight (based on the refill measurements) and the methods to assess volume status were determined using Spearman Rank Order (r_s). In those volume status markers where a shift toward more euvolemic state after dry weight adjusment was expected, a variance ratio test was performed. A p value < 0.05 was considered statistically significant.

8.3 Results

Dry weight adjustments
Patient characteristics and dry weight adjustments guided by the refill measurements are shown in Table 1. In five patients, refill disappeared before the end of dialysis. The dry weight was therefore increased (0.7 ± 0.7 kg; or 0.9 ± 0.9% of the initial dry weight). In the remaining 5 patients, refill continued until the end of dialysis. The dry weight was then reduced by 0.5 kg, sometimes several times, until a refill measurement occurred were refill stopped at the end or during dialysis. This resulted in a mean dry weight reduction of 1.4 ± 1.3 kg, or 1.8 ± 1.5 % of the initial dry weight.

Mean arterial pressure (MAP) before and after hemodialysis
NB. The lines in the figures below are as follows:



MAP before dialysis was 100 ± 22 before dry weight adjustment and 97 ± 22 mmHg afterwards (NS). Three patients could be classified as hypertensive by both volume status according to MAP, and the amount of dry weight adjustment on the basis of refill measurements. This was also the case for one patient considered hypovolemic, but not for one hypovolemic, one hypertensive, and four euvolemic patients. There was no convergence of MAP toward euvolemia after dry weight adjustment. The correlation with the dry weight change was not significant (r_s = 0.21; p = 0.5).

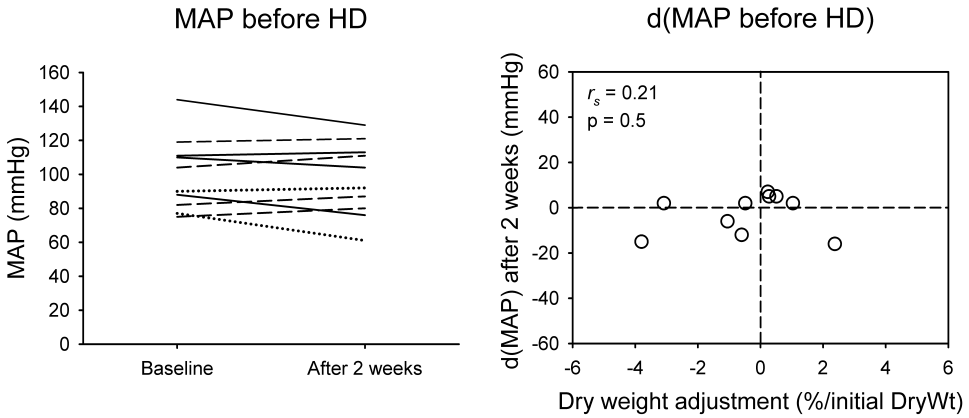


Figure 4. MAP before hemodialysis and the correlation with the amount of dry weight adjustment.

MAP values after dialysis were 88 $\pm$ 31 and 86 $\pm$ 23 mmHg (NS). The convergence toward normotensive values after two weeks was somewhat better, which is reflected in a smaller SD at similar mean MAP values (variance ratio test: $p = 0.19$). The correlation with dry weight change was not significant ($r_s = 0.20$; $p = 0.6$).

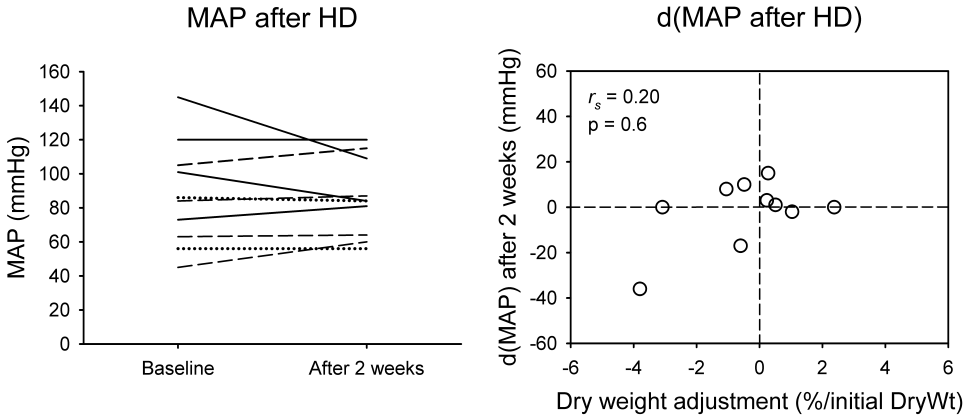


Figure 5. MAP after hemodialysis and the correlation with the amount of dry weight adjustment.

Ambulatory blood pressure (ABPM)

24 Hour blood pressure during the daytime, measured the day after dialysis, did not change (MAP value 88 $\pm$ 21 to 87 $\pm$ 20 mmHg). There was an acceptable concordance between classification on the basis of ABPM and dry weight change (for two hypervolemic, two euvolemic, and two hypovolemic-indexed patients). Convergence of the values after two weeks was not present. The correlation with dry weight change was absent (r_s = -0.10; p = 0.8).

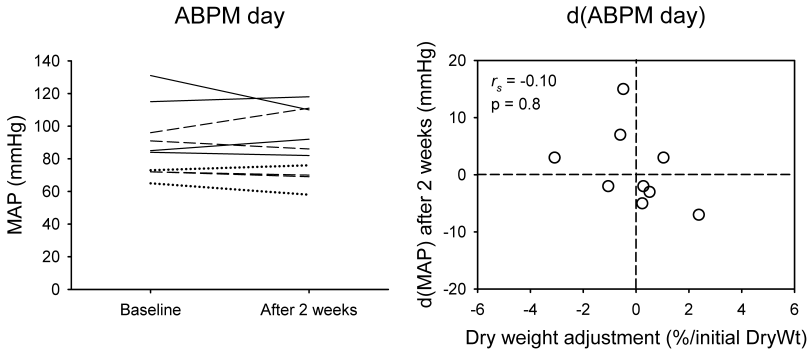


Figure 6. Ambulatory blood pressure during the day and the correlation with the amount of dry weight adjustment.

During the night, both methods of volume status stratification were not as well in agreement. MAP values were lower, with no correlation with dry weight change (mean MAP 83 $\pm$ 19 and 81 $\pm$ 19 mmHg; r_s = -0.22; p = 0.5, respectively).

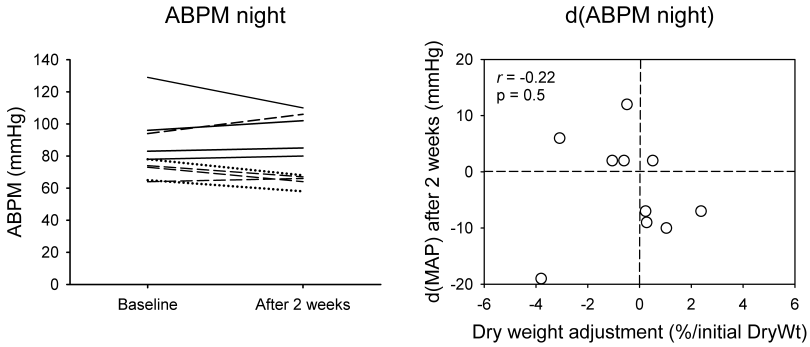


Figure 7. Ambulatory blood pressure during the day and the correlation with the amount of dry weight adjustment.

Vena cava ultrasonography
In one patient, the inferior vena cava could not be visualized by ultrasonography in the second measurement.

Indexed inferior vena cava diameter (VCDi)
There was no change in mean VDCi (baseline: 8.6 ± 2.4, after dry weight adjustment 8.2 ± 2.0 mm/m^2). Based on reference values provided by Brennan *et al.* [5], five patients were classified as euvolemic before dry weight adjustment, and four were hypovolemic. The agreement of the classification of the patients according to the refill method was poor. For instance, none of the patients classified as hypervolemic by the latter method was classified as such by ultrasonography. Of the four euvolemic patients, one shifted toward a more euvolemic state within the reference range, two remained at the same VDCi value, and one went to a hypovolemic value. The four hypovolemic patients remained hypovolemic with the exception of one who became euvolemic. In summary, three patients showed convergence, with one overshooting toward hypovolemia. The relation with the volume status stratification according to the refill method was not evident.

Inferior vena cava collapsibility index (IVCCI)
The values of IVCCI were not different between baseline and after dry weigth adjustment (46 ± 25 and 52 ± 26%, respectively). When the patients were classified according to IVCCI, the agreement with the classification according to the refill method was again relatively poor. With the exception of one hypervolemic patient according to the refill method, some convergence of the values toward the euvolemic range was visible.

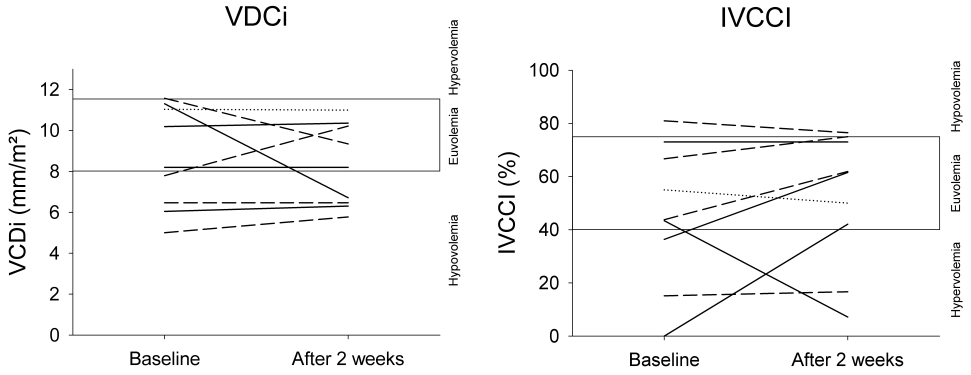


Figure 8. Indexed inferior vena cava diameter, Inferior vena cava collapsibility index, and the correlations with the amount of dry weight adjustment.

Brain natriuretic peptide (BNP)
BNP levels before dialysis were 98 ± 61 and 85 ± 47 pmol/l two weeks after dry weight adjustment (NS). Three hypervolemic patients on the basis of BNP (> 67 pmol/l)

were also classified as such by the refill method. Three patients showed agreement with (hypo/)euvolemic values. For the other patients, this agreement was not present. Correlation with dry weight adjustment was absent ($r_s = 0.22$; $p = 0.5$).

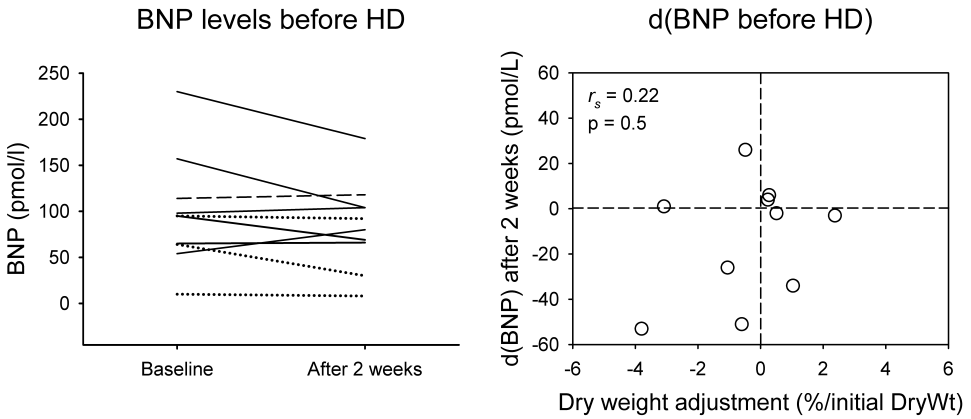


Figure 9. Brain natriuretic peptide before hemodialysis and the correlation with the amount of dry weight adjustment.

The post-dialysis values were 85 ± 47 and 77 ± 42 pmol/l (NS). A virtually equal pattern to that in the levels before dialysis was observed. The correlation with dry weight adjustment was somewhat stronger, although not quite significant ($r_s = 0.53$; $p = 0.1$).

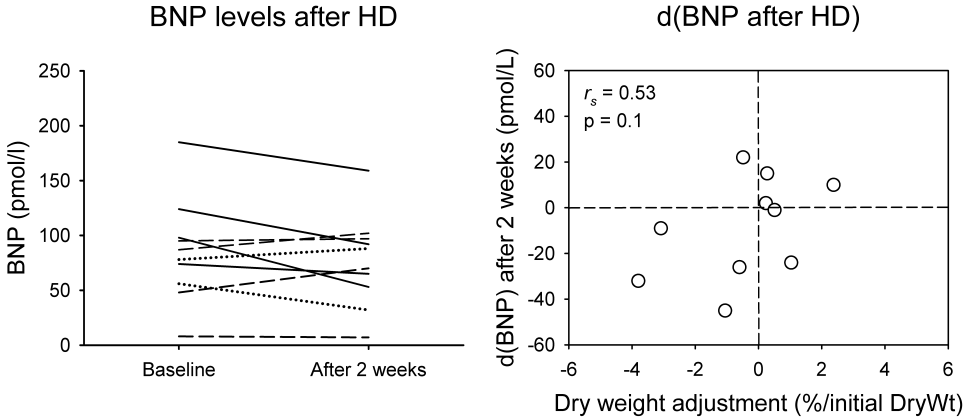


Figure 10. Brain natriuretic peptide before hemodialysis and the correlation with the amount of dry weight adjustment.

Bio-impedance analysis (BIA)
Bioimpedance measurements were obtained in 9 patients.

Mean TBW remained unchanged (35.1 $\pm$ 7.2 to 34.9 $\pm$ 6.8 l). Volume status classification values were not uniformly distributed compared to indexation on the basis of dry weight change. The correlation with the dry weight adjustment was significant (r_s = 0.82; p = 0.004).

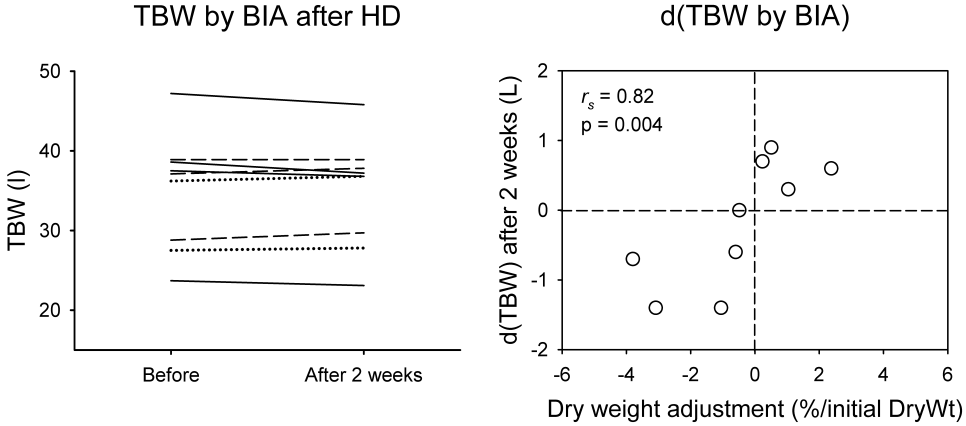


Figure 11. Total body water by bio-impedance and the correlation with the amount of dry weight adjustment.

Uncorrected ECW did not change (16.9 $\pm$ 3.7 to 16.7 $\pm$ 3.7 l). The classification of the volume status according to ECW did not agree with the classification according to the refill method. As an example, values of the four patients judged to be hypervolemic by the latter method were scattered in the ECW plot. The correlation with dry weight adjustment was significant (r_s = 0.75; p = 0.02).

Values of ECW corrected for height [13] were 9.5 $\pm$ 1.8 (baseline) and 9.4 $\pm$ 1.9 l. Again, the classification of the volume status according to the BIA method did not agree with the classification based on the refill method. For example, the four patients classified using the refill method as hypervolemic (solid line) were either hypervolemic (n = 1), euvolemic (n = 2) or hypovolemic according to the BIA analysis. No tendency of the values to converge to the euvolemic range was observed. The correlation with dry weight adjustment was significant (r_s = 0.75; p = 0.02).

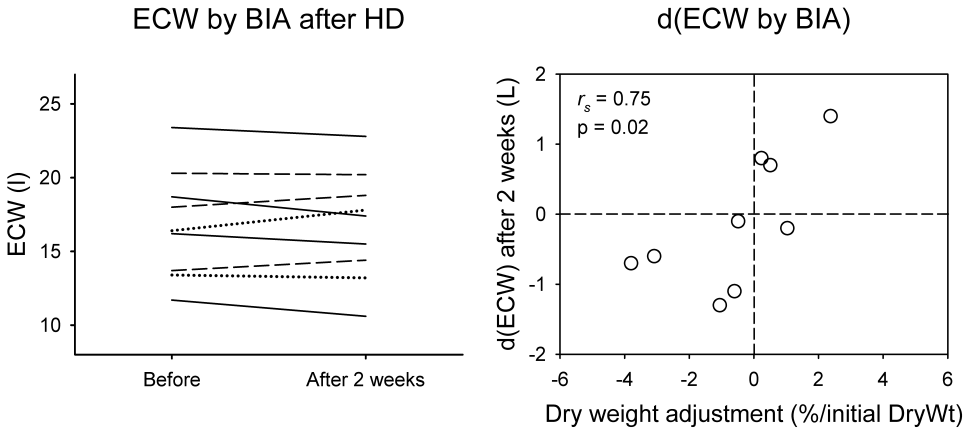


Figure 12. Uncorrected extracellular water by bio-impedance and the correlation with the amount of dry weight adjustment.

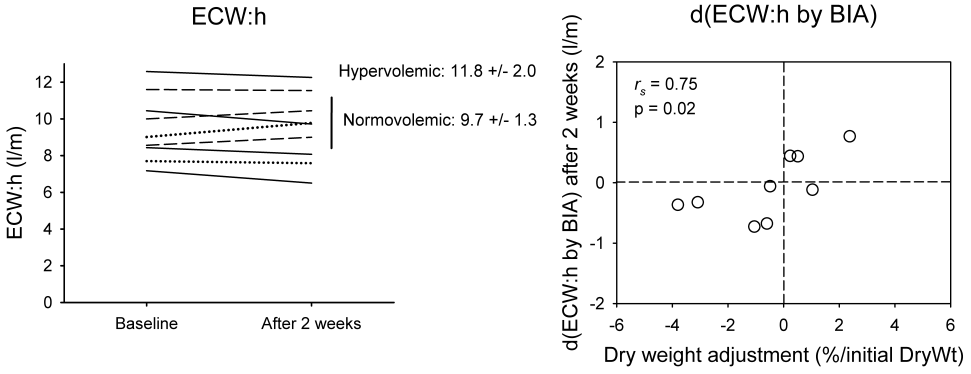


Figure 13. Extracellular water by bio-impedance, corrected for height, and the correlation with the amount of dry weight adjustment.

Cardiothoracic ratio (CTR)

There were no signs of overhydration on chest X-ray before or after dry weight reduction. A significant change in CTR could not be observed (49 $\pm$ 4 to 48 $\pm$ 4 mm), although one patient showed a clear reduction (CTR = 54 to 45 mm) after a dry weight reduction of 3.8% (2.3 kg) of the initial dry weight. Three patients (one classified as hypervolemic, one as hypovolemic, and one as euvolemic) showed a decrease in CTR. A general correlation between volume classification methods was not present. There were no significant correlations with dry weight adjustment ($r_s = 0.21$; $p = 0.5$).

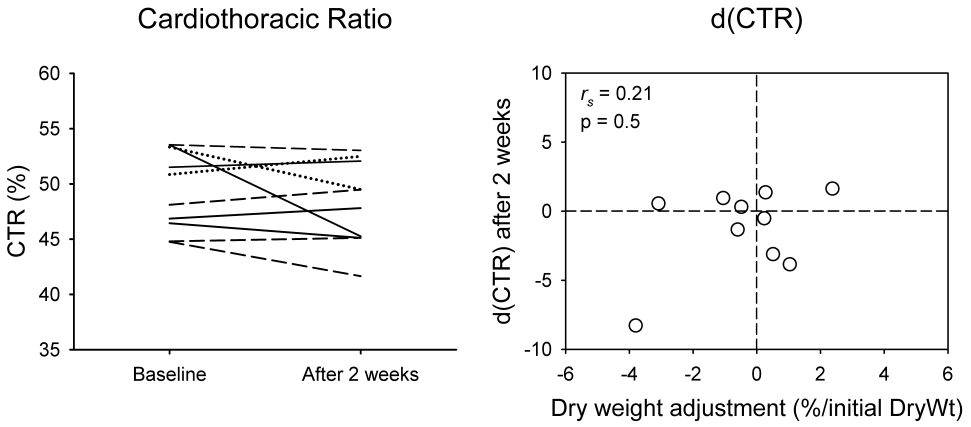


Figure 14. Cardiothoracic ratio and the correlation with the amount of dry weight adjustment.

Quality of life Short Form 36 (SF-36) Health Survey questionnaire

Both Physical Composition Score and Mental Composition Score remained unchanged (PCS: 30 $\pm$ 7 to 31 $\pm$ 7; MCS 48 $\pm$12 to 48 $\pm$ 9). The scores were not uniformly in agreement with volemia classification (by the amount of dry weight change). There were no correlations with the dry weight change (PCS: r_s = 0.32; p = 0.3; MCS: r_s = -0.042; p = 0.9).

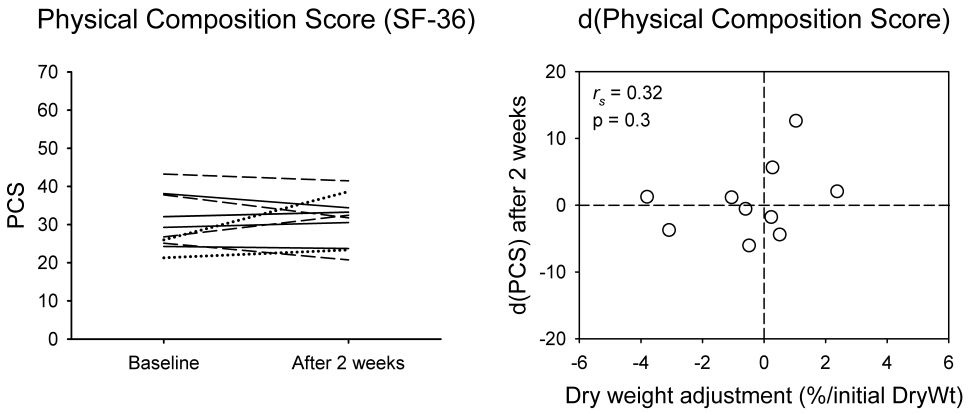


Figure 15. Physical Composition Score and the correlation with the amount of dry weight adjustment.

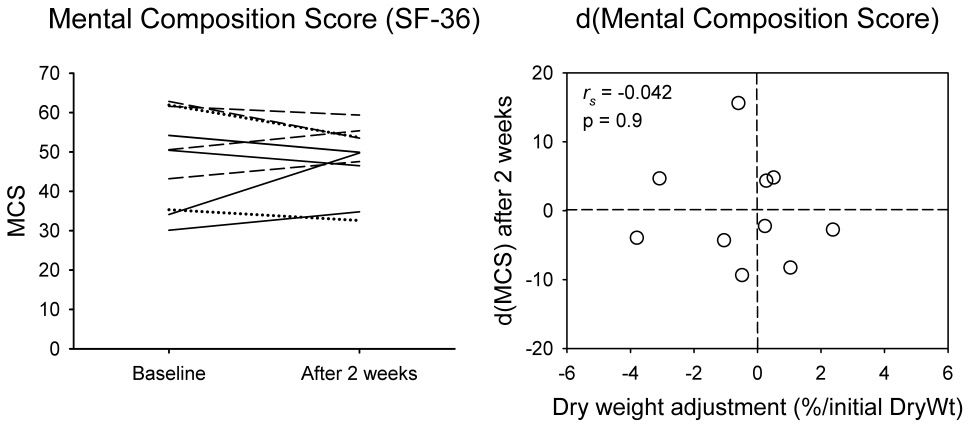


Figure 16. Mental Physical Composition Score and the correlation with the amount of dry weight adjustment.

8.4 Discussion

In the current study, we investigated whether cessation of refill from the interstitial to the vascular compartment can be used to determine dry weight in patients treated by hemodialysis. This hypothesis was based on the idea that the hydration state of the interstitial compartment is a major determinant of refill [11], and that cessation of refill reflects a state of normal volume of the interstitial compartment [14]. To do so, we applied a recently developed method that allows continuous, on-line measurement of the absolute plasma refill during hemodialysis and ultrafiltration [9, 10].

We adjusted the dry weight guided by the refill method in ten dialysis patients that were judged to be euvolemic on clinical grounds. According to this method, the patients were classified as hypovolemic (more than 0.5% dry weight increase), euvolemic (dry weight change between -0.5 and +0.5%), or hypervolemic (more than 0.5% dry weight decrease). It is of note that this change in dry weight was tolerated excellently by the patients: none of the patients with a marked upward or downward adjustment showed clinical signs of overhydration or dehydration, respectively. However, objective assessment of ambulatory blood pressure and quality of life did not show a beneficial effect of dry weight adjustment by our method.

To validate the changes in dry weight, as determined by the refill method more objectively, we also classified the volume status prior to adjusting the dry weight, using a panel of other volume status markers. As discussed in detail in the results section, the post hoc classification of the volume status by the refill method was only rarely in accordance with the a priori classification according to these other methods. As an example, the figures of bio-impedance analysis show that patients judged to be overhydrated by the refill method were classified as either normovolemic, hypervolemic or hypovolemic by the bioimpedance method.

We reassessed the volume status by the panel of volume markers two weeks after the dry weight adjustment according to the refill method. As the patients were classified as either hypervolemic (downward adjustment of dry weight), euvolemic (minor adjustments of dry weight) or hypovolemic (upward adjustment of dry weight) by the refill method, one would not expect the mean values of the results of these assessment to change importantly. One would, however, expect convergence of the values toward the euvolemic range for each of the methods, which should tend to reduce the standard deviation of the mean of these measurements (see Figure 3). We observed such a tendency only for the blood pressure after dialysis. Because blood pressure variability is associated with cardiovascular risk [15, 16], such reductions may convey long-term benefit.

The discrepancy between the results of the other methods to assess the volume status in dialysis patients and the refill method makes it difficult to draw firm conclusions from our study. However, none of the other methods to assess the volume status can be considered as the gold standard [8], as they have all been validated against other non-gold standard test. Moreover, there is also marked disagreement between the results of the individual markers of volume status in our own data set. For instance, patients considered to be euvolemic by the refill method were either classified as hypervolemic (according to BNP concentrations) or hypovolemic (according to inferior vena cava diameter assessment). Consequently, in our hands application of either of these tests would lead to a completely opposite adjustment in dry weight. The strength of our approach is that it is based on the functional assessment of the interstitial hydration state that can be obtained online and continuously during hemodialysis. A limitation of our method is that it can only be used to assess the volume status in patients treated by hemodialysis, and not in patients on peritoneal dialysis or in the predialysis phase. However, large volume swings are not expected in the latter conditions.

Accurate dry weight estimation in hemodialysis patients remains a difficult problem [8], and our study does not provide a definite solution. We submit that our direct, functional approach of assessing refill could be an interesting contribution to this field.

8.5 Acknowledgements

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Chapter 9

Summary and perspectives / Samenvatting en toekomstverwachtingen

9.1 Summary

The first part of this thesis, 'Removing substances', describes a novel immunoadsorption technique that uses variable domains of heavy chain llama antibodies (VHH) to remove a toxic agent, Toxic Shock Syndrome Toxin-1 (TSST-1) from the circulation. This study was designed as a proof-of-principle that immunoadsorption of substances by VHH is a feasible, effective technique. We chose the removal of TSST-1 from a porcine sepsis model because in this system, there was a direct relation between the (one) substance (TSST-1) and the disease (sepsis).

In *Chapter 2*, we showed that VHH against TSST-1 can be selected and produced in large quantities. Columns containing sepharose-coupled anti-TSST-1 VHH were shown to be very effective in reducing pathologically relevant levels of TSST-1 from both human and pig plasma *in vitro*: more than 96% of the toxin was depleted after one pass over the column. Thus, immunoadsorption of sepsis-inducing TSST-1 from plasma was feasible using VHH *in vitro*.

In *Chapter 3*, we investigated whether immunoadsorption of TSST-1 using VHH was possible *in vivo* by applying an anti-TSST-1 VHH filter in a porcine model of TSST-1-induced sepsis. Instead of sepharose-bound VHH, we used VHH coupled directly to the fibers of a plasma separation filter. After successful *in vitro* testing of these fibers comparable to the work in *Chapter 2*, the filter was incorporated in an extracorporeal circulation in the porcine sepsis model. Unfortunately, we were unable to show a beneficial effect of the filter on the clinical course and outcome of the sepsis syndrome. Unexpectedly, plasma toxin levels were not affected by the filter. In addition, the recovered TSST-1 plasma levels were much lower than anticipated from the amount of toxin infused. These findings have two implications; first, that the filter was much less efficient *in vivo*, for which several explanations can be proposed, for instance fouling of the filter with sepsis factors (e.g. cytokines and complement). Second, the low plasma levels of TSST-1 suggested that the toxin had moved into a space not accessible to our technique. This problem had not been foreseen, and to our knowledge, this property of TSST-1 had not been recognized in the literature.

Chapter 4 takes this finding as the basis for a review on the difficulties when trying to remove TSST-1 in sepsis. Although much is known about e.g. the physical properties of TSST-1 and its binding to various cell types, very little is known about the actual distribution of TSST-1 within body compartments. In addition, knowledge on the kinetics of TSST-1

are completely lacking in current literature. Several studies have been performed using experimental techniques (e.g. plasmapheresis, aspecific immunoadsorption) to remove TSST-1 in sepsis. None of these studies has convincingly shown a beneficial effect on the course and outcome of sepsis. Thus, better techniques to remove sepsis-causing agents such as TSST-1 are still needed. For future research, we proposed a set of conditions that are of importance when developing immunoadsorption techniques. These conditions for effective immunoadsorption can be extrapolated to other toxins, such as those present in the uremic syndrome.

To return to the issue addressed in the introduction of the thesis, namely that current dialysis technology is unable to remove sufficient quantities of middle molecules, the investigation using highly-selective llama antibody fragments has taught us the following: first, selection and production of large amounts of the variable domains of llama heavy-chain antibodies (VHH) against Toxic Shock Syndrome-Toxin-1 is feasible. Second, after coupling one such VHH to sepharose beads, TSST-1 spiked plasma was very effectively removed by running the toxin-laden plasma over a column of these beads. Finally, VHH coupled to plasma-separation fibers could remove TSST-1 from plasma *in vitro* in a similar setup as in the sepharose column tests. The *in vivo* testing of these fibers was not as effective as the *in vitro* tests. This is most likely more due to the properties of the model (namely the toxin itself disappearing from the circulation, and fouling of the filter by sepsis components) than to the VHH technique as such. Thus, immunocapturing with VHH is a technique that should be regarded as being able to remove substances of the uremic syndrome. The logical next step would be to target one component of the uremic syndrome, such as leptin, a toxic middle molecule. The considerations in *Chapter 4* should be taken into account when such future tests are planned.

The second part of this thesis, 'Removing excess volume', describes a novel method to measure absolute plasma refill rates during hemodialysis, using a feedback-regulated ultrafiltration system coupled to a dialysis machine. We designed this system because we wanted to be able to non-invasively measure absolute plasma refill rates continuously during dialysis. This system is the first to do so, and, using the method, we investigated the influence of blood-pressure stabilizing interventions on refill. Finally, the application of the method as a novel determinant of the patient's dry weight was investigated.

We have summarized the current knowledge about refill in *Chapter 5*. At present, few studies have been performed on the absolute plasma refill rate and its dynamics during hemodialysis. The only way to obtain absolute refill values is by the invasive and cumbersome method of radioactive substance infusion. Indirect ways to measure refill rely on various assumptions and algorithm-derived parameters (e.g. the absolute blood volume estimated from height and weight). Some groups also investigated the capillary filtration coefficient, one of the determinants of refill. Reviewing the literature revealed that a non-invasive, continuous refill measurement is needed to elucidate the properties of refill during hemodialysis. We therefore designed a method to measure the absolute plasma rate in ml/min continuously during hemodialysis.

This novel method is described in *Chapter 6*. We found refill values that were similar to the values reported in the literature as reviewed in *Chapter 5*. In this study, we also tested whether two blood pressure-stabilizing interventions (anti-embolism stockings and infusion

of a hyperoncotic solution) would improve, thereby explaining the beneficial effects of these interventions on blood pressure. Absolute refill values, obtained by our method, were not enhanced by these interventions. In *Chapter 7*, we expanded this research and investigated the effect on refill of two other blood-pressure stabilizing methods, namely sodium profiling and cool dialysate. We did find a significant improvement of refill by sodium profiling, whereas no effect was present using cool dialysate. The findings in *Chapter 6* and *Chapter 7* indicated that the blood-pressure stabilizing effects of commonly-used interventions can not always be attributed to an increase in refill.

During the research of *Chapter 6* and *Chapter 7*, we noticed that in several hypotension-prone patients refill disappeared before the end of dialysis. This led to the hypothesis that cessation of refill indicated normal volume of the interstitial space, and thus refill capacity could be used as a novel indicator of dry weight. To test this, we adjusted the dry weight in 10 patients on the basis of the moment that refill disappeared (*Chapter 8*). A panel of traditional volume status markers was applied as reference test to determine whether the change of dry weight would lead to euvolemic states of these markers. Finally, correlations between dry weight adjustment and the change in volume status markers were analyzed. Based on the analysis of the traditional volume markers, we were unable to prove that adjustment of the dry weight on the basis of refill measurement led to euvolemia. However, the sometimes major upward and downward adjustments in dry weight were tolerated well by the patients without causing clinical signs of overhydration or hypovolemia. The conflicting findings of the traditional volume status markers suggest that these markers may be far from perfect in determining the volume status in diaysis patients.

In conclusion, the second part of the thesis reports a novel method to continuously assess the absolute plasma refill rate during hemodialysis. The studies performed using this method gave a detailed insight into the dynamics of refill during dialysis, confirming findings by others. In addition, it allowed us to investigate the physiological mechanism of several blood-pressure stabilizing interventions. Whether our refill method can be used to determine dry weight of a dialysis patient remains to be established.

9.2 Perspectives

The novel method of highly-selective variable domains of llama heavy-chain antibodies (VHH) is a promising technique. The main advantage of these VHH is that, unlike conventional antibodies, they can be selected against virtually any target, can be easily produced in large quantities, have good stability, and can be coupled to various carrier matrices. These properties make them very suitable for the application in diseases where there is a single causative factor, e.g. Goodpasture syndrome, myasthenia gravis, hypercholesterolemia, acute humoral rejection after transplantation, etc. These are obvious targets since some of these diseases are currently treated by other immunocapturing techniques (e.g. by mobilized anti-Ig antibodies or staphylococcal protein A).

In view of the original problem of substance removal during hemodialysis, immunoadsorption is a promising technology. If we can remove one substance with this technique, and VHH can be selected against virtually any target, then coating a hemodialysis membrane with 10, 100, or even more VHH against components of the uremic syndrome would facilitate new treatment options for the hemodialysis patient. Such an approach is not purely hypothetical: recently, the development of the wearable artificial kidney has started. This device will continuously remove for instance protein-bound substances from the circulation by novel immunoadsorption methods such as nanoclays.

Another option would be to directly inject VHH into the circulation with subsequent VHH-antigen complex formation. This would then be followed by hepatic clearance or renal excretion of these complexes. These antigens could be for instance (parts of) viral surfaces or disease causing substances (e.g. in autointoxication).

The novel method of measuring the absolute plasma refill rate is the first that allows the continuous, online assessment of absolute refill values during hemodialysis. This method can be used to investigate interventions and actions that influence blood pressure during dialysis, e.g. the effect of body position and food intake on refill during dialysis. We postulated that patterns in refill, and more specific the moment that refill disappears, can be used to determine the dry weight of the dialysis patient. Although the results are not conclusive yet, it is conceivable that this method could be applied in everyday hemodialysis practice. Many modern day hemodialysis machines have advanced options and control mechanisms. For example, it is possible to set a relative blood volume reduction target that has to be achieved at the end of dialysis. The machine then adjusts the ultrafiltration rate to obtain this relative blood volume within the treatment time. At the same time, the blood pressure is monitored, and ultrafiltration is stopped if hypotension begins to develop. Such autoregulatory mechanisms are now common practice in modern hemodialysis treatment. It would therefore be relatively easy to adjust the available dialysis machines to operate in a similar fashion as our system. Online refill measurement could thus be a standard option on future dialysis machines. This method could then be applied regularly during dialysis to objectively assess the (patterns in) refill and to perhaps adjust the dry weight of the hemodialysis patient. Alternatively, it is conceivable that the dialysis duration will be automatically adjusted to the refill rate in order to prevent intradialytic hypotension. Either way, the co-morbidity and cardiovascular risk of blood pressure variation could be strongly reduced by refill assessment. Finally, patients could perhaps be classified as 'refillers' and 'non-refillers', which e.g. could help the nursing staff in anticipating (hypotensive) problems during dialysis. Another application of such a distinction would be to provide food and drink during dialysis (which is known to suddenly reduce the blood volume in some patients, with ensuing symptomatic hypotension) to 'refillers' only.

The findings in this thesis show that shortcomings of present hemodialysis treatment, namely the inadequate removal of substances, and the lack of methods to measure refill during hemodialysis, can be adequately addressed by novel techniques.

9.3 Samenvatting

Het eerste gedeelte van dit proefschrift, 'Removing substances', beschrijft een nieuwe immunoadsorptie techniek om de gifstof Toxic Shock Syndrome Toxin-1 (TSST-1) uit de bloedsomloop te verwijderen. Deze techniek omvat antilichaam fragmenten afkomstig van lama's, meer specifiek het variabele domein van zware keten lama antilichamen (in het Engels: variable domains of heavy chain llama antibodies, afgekort VHH). De studies beschreven in dit gedeelte zijn ontworpen om aan te tonen dat immunoadsorptie met VHH mogelijk en effectief is, en gebruikt zou kunnen worden om stoffen te verwijderen die door hemodialyse niet verwijderd kunnen worden. Lama VHH hebben enkele voordelen ten opzichte van conventionele (menselijke) antilichamen, o.a. de relatief eenvoudige productie op grote schaal en de makkelijke koppeling aan dragermaterialen. De gifstof TSST-1 is gekozen als te verwijderen stof omdat er een direct verband is tussen de stof (TSST-1) en de veroorzaakte ziekte (bloedvergiftiging, ook wel sepsis genaamd).

In *Hoofdstuk* 2 tonen wij aan dat VHH gericht tegen TSST-1 (anti-TSST-1 VHH) geselecteerd en geproduceerd kunnen worden in grote hoeveelheden. Het anti-TSST-1 VHH is vervolgens gekoppeld aan een dragerstof, sepharose, waarna hiermee een kleine kolom gevuld werd. Plasma (= het vloeibare gedeelte van bloed) met daarin een ziekteverwekkende hoeveelheid gifstof (TSST-1) werd over deze kolom geleid, waarna meer dan 96% van de gifstof verwijderd was na een passage. Dit was zowel het geval bij menselijk plasma als bij plasma afkomstig van varkens. De conclusie van dit *Hoofdstuk* is dat in laboratoriumproeven het TSST-1 uit plasma verwijderd kan worden met behulp van VHH immunoadsorptie.

In *Hoofdstuk* 3 werd er getracht om (ingespoten) TSST-1 te verwijderen uit de bloedsomloop van varkens met behulp van VHH. In plaats van VHH gekoppeld aan sepharose, zoals in *Hoofdstuk* 2, werden de VHH gekoppeld aan de vezels van een filter dat plasma van bloed scheidt. Deze gemodificeerde vezels konden in het laboratorium zeer goed TSST-1 uit varkensplasma verwijderen (resultaten vergelijkbaar met die van *Hoofdstuk* 2). Helaas was de toepassing in een varkens TSST-1 bloedvergiftigingsmodel niet zo succesvol: het klinische beloop en de sterfte werd niet beter door het filter. Bovendien vonden wij dat de TSST-1 concentratie in het plasma vele malen lager was dan we verwachtten op basis van de ingespoten hoeveelheid. Deze twee bevindingen kunnen op de volgende manier uitgelegd worden. Ten eerste, dat het filter in de proefdieropstelling niet zo goed gepresteerd heeft als in de laboratoriumtest. Een mogelijke oorzaak hiervoor is vervuiling van het filter met stoffen die in bloedvergiftiging een rol spelen, zoals cytokines en complement. Ten tweede, dat het ingespoten TSST-1 verdwenen is uit de bloedbaan naar een ruimte in het lichaam die niet toegankelijk was voor onze verwijderingstechniek. Dit probleem was van tevoren niet voorzien en is, bij ons weten, niet bekend in de beschikbare literatuur over TSST-1.

Deze resultaten vormen de basis voor een literatuurbespreking van de problemen die men tegen kan komen wanneer men TSST-1 wil verwijderen bij bloedvergiftiging. Alhoewel er veel bekend is over bijvoorbeeld de fysieke eigenschappen en de binding aan verschillende celtypes, is er nog weinig bekend over de verspreiding van TSST-1 over de verschillende gedeeltes van lichaam. Sterker nog, over de farmacokinetiek (dit omvat o.a. de opname, verdeling, afbraak en uitscheiding van een stof) is helemaal niets bekend in de literatuur. Er zijn verschillende studies uitgevoerd waarin getracht werd

met experimentele technieken (bijvoorbeeld plasmaferese, aspecifieke immunoadsorptie) TSST-1 te verwijderen in bloedvergiftiging. Geen van deze studies heeft overtuigend aangetoond dat de geteste techniek een bevorderend effect op het beloop en de uitkomst van bloedvergiftiging heeft. Betere technieken om bloedvergiftiging veroorzakende stoffen, zoals TSST-1, te verwijderen zijn daarom nog steeds nodig. Voor toekomstig onderzoek hebben we een aantal randvoorwaarden beschreven die belangrijk kunnen zijn wanneer men dergelijke immunoadsorptie technieken wil ontwikkelen. Deze randvoorwaarden zijn ook van toepassing voor andere gifstoffen dan TSST-1, zoals bijvoorbeeld de uremische toxines die in eindstadium nierfalen een rol spelen.

Om terug te komen op de boodschap van de introductie van het proefschrift (namelijk dat de huidige dialysetechnieken niet alle stoffen uit het bloed kunnen verwijderen waardoor zogenaamde giftige middel-moleculen in het lichaam achterblijven na dialyse), heeft het hierboven beschreven onderzoek ons het volgende geleerd: ten eerste, dat lama antilichaam fragmenten (het variabele domein van zware keten lama antilichamen, of variable domains of heavy chain llama antibodies, afgekort VHH) gericht tegen de gifstof Toxic Shock Syndrome Toxin-1 (TSST-1) op grote schaal geproduceerd kunnen worden in het laboratorium. Ten tweede, dat deze VHH gekoppeld kunnen worden aan de dragerstof sepharose, waarmee vervolgens TSST-1 zeer succesvol uit plasma verwijderd kan worden in een laboratoriumexperiment. Ten slotte, herhaling van dit labexperiment met VHH gekoppeld aan vezels die plasma van bloed scheiden gaf gelijke resultaten. Bij toepassing van deze vezels in een dierproef met varkens, die bloedvergiftiging kregen door TSST-1, waren de met anti-TSST-1 VHH gecoate vezels echter niet effectief. Dit komt waarschijnlijk door de eigenschappen van het diermodel: enerzijds is het filter mogelijk vervuild tijdens de proef, en anderzijds verdween het TSST-1 uit de bloedbaan waardoor het niet meer verwijderd kon worden met onze toepassing. Dit doet niets af aan de mogelijkheden van VHH als potentiële techniek om stoffen te verwijderen die na de huidige dialysebehandeling achterblijven in de bloedbaan en het lichaam. Een bekend voorbeeld van zo'n stof is leptine; bij vervolgproeven zou dit een voor de hand liggend doelwit zijn. Het verdiend aanbeveling om de suggesties die gedaan zijn in *Hoofdstuk* 4 te betrekken in het opzetten van dergelijke vervolgproeven.

Het tweede gedeelte van dit proefschrift, 'Removing excess volume', behandelt een nieuwe methode waarmee continu de zogenaamde plasma refill rate tijdens hemodialyse gemeten kan worden. Plasma refilling is de vochtverplaatsing binnen het lichaam tijdens de dialyse. Deze vochtverplaatsing vindt plaats vanuit de interstitiële ruimte (waar het meeste overtollige vocht bij dialysepatiënten opgeslagen is) richting de bloedbaan. De hoeveelheid circulerend bloedvolume tijdens hemodialyse wordt voor een groot deel bepaald door de verhouding tussen het vocht onttrekken door de dialysemachine, en de interne aanvulling in het lichaam door refill. Een disbalans tussen refill en het onttrekken van vocht uit de bloedbaan leidt vaak tot een sterke daling van het bloedvolume met lage bloeddrukken. Anderzijds, als de hoeveelheid te onttrekken vocht te laag wordt ingeschat, en het bloedvolume dus structureel te weinig afneemt tijdens dialyse, dan ontstaat er overvulling, een hoge bloeddruk, en uiteindelijk o.a. hart- en vaatschade. De onttrekkingssnelheid van vocht door de dialysemachine is een bekend gegeven tijdens dialyse. Het meten van refill is echter niet eenvoudig: tot nu toe is dit alleen mogelijk geweest met behulp van invasieve

technieken, zoals radioactieve stoffen.

In *Hoofdstuk* 5 hebben we de huidige kennis over refill samengevat. Op dit moment zijn er maar een paar studies die daadwerkelijk refill gemeten hebben in milliliters per minuut (dat wil zeggen, zonder aannames te doen of een afgeleide waarde van refill te meten, zoals de capillaire filtratie coëfficiënt). De enige methode om dit wel te kunnen doen is met behulp van invasieve technieken. Tijdens het bestuderen van de beschikbare literatuur werd duidelijk dat er geen non-invasieve methode beschikbaar is om continu de refill tijdens hemodialyse te meten. Wij hebben daarom een methode ontwikkeld waarmee dat wel kan.

Deze methode wordt beschreven in *Hoofdstuk* 6. In het kort werkt dit als volgt: in plaats van vochtonttrekking door de hemodialysemachine wordt vocht uit de bloedbaan onttrokken door middel van een computer gestuurd systeem. De data van de gemeten bloedvolumewaarden tijdens hemodialyse worden hierbij gebruikt om, middels een terugkoppel mechanisme, de onttrekkingssnelheid van vocht zo te sturen dat het bloedvolume op elke gewenste hoeveelheid kan worden gehouden. In deze situatie geldt dan, dat de netto per tijdseenhoud hoeveelheid onttokken vocht (door het systeem) gelijk is aan de vochtaanvulling richting de bloedbaan binnen in het lichaam. Dit laatste proces wordt plasma refilling, of refill, genoemd. Met dit systeem vonden we waarden voor refill die overeenkwamen met de waarden die andere groepen ook gevonden hebben in *Hoofdstuk* 5. Tijdens onze studie hebben we ook bepaald wat de invloed was op refill van twee ingrepen die de bloeddruk stabiliseren tijdens hemodialyse (steunkousen en het geven van een zogenaamde plasma-expander). De refill werd echter niet verhoogd door deze ingrepen. In *Hoofdstuk* 7 hebben we dit onderzoek uitgebreid en gekeken naar de invloed van twee andere bloeddruk stabiliserende ingrepen: het gebruik van spoelvloeistof met een hoge zoutconcentratie en het koelen van de spoelvloeistof. Van deze twee gaf alleen de hogere zoutconcentratie een statistisch significante verhoging van de refill. De resultaten van *Hoofdstuk* 6 en *Hoofdstuk* 7 geven aan dat het bloeddruk stabiliserende effect van in de praktijk veel gebruikte ingrepen niet altijd het gevolg is van een verhoogde refill ten gevolge van de ingreep.

Tijdens het onderzoek van Hoofstuk 6 en *Hoofdstuk* 7 zagen we de refill tijdens de dialyse ophouden bij enkele patiënten die vaak lage bloeddrukken kregen. Dit leidde tot de gedachte dat patiënten, waarbij de refill verdwijnt, waarschijnlijk op dat moment hun streefgewicht bereikt hebben. (Het streefgewicht is het gewicht waarop de patiënt geen overtollig vocht meer in het lichaam heeft. Dit dient aan het einde van de dialyse bereikt te zijn. Het streefgewicht is vaak moeilijk in te schatten, en er zijn hiervoor geen gestandaardiseerde methoden.) Dit zou dus kunnen betekenen dat onze methode gebruikt zou kunnen worden om het streefgewicht in te schatten. Dit hebben wij onderzocht in *Hoofdstuk* 8. In 10 hemodialyse patiënten hebben we allereerst de vochtstatus zo goed mogelijk vastgelegd met een combinatie van hiervoor gebruikte methoden (o.a. 24 uurs bloeddrukmeting, röntgenfoto van de borstkas, en bloeddruk voor en na dialyse). Hierna hebben we onze refill methode toegepast; het moment waarop refill verdween bepaalde hoeveel vocht er in de volgende dialyses onttrokken moest worden (oftewel, het streefgewicht werd hierop aangepast). Na twee weken dialyseren met het nieuwe streefgewicht werden de uitgangsbepalingen herhaald om te analyseren of onze methode tot een betere vochtstatus had geleid. Dit bleek helaas niet het geval te zijn; er bestond geen goede overeenkomst tussen de volumestatus op basis van de klassieke methoden en

onze refill metingen. Aan de andere kant werden de wijzigingen in het streefgewicht goed getolereerd door de patiënten; ze kregen geen last van over- of ondervulling door onze methode. Bovendien waren de uitslagen van de klassieke methoden lang niet altijd in overeenstemming met elkaar.

Samenvattend hebben wij in het tweede gedeelte van dit proefschrift een methode ontwikkeld waarmee de werkelijke plasma refill rate continu gemeten kan worden tijdens hemodialyse. De studies naar refill die we uitgevoerd hebben gaven een verdieping van de kennis over refill tijdens dialyse en bevestigden bevindingen van anderen. Daarnaast stelde deze methode ons in de gelegenheid het onderliggende mechanisme van enkele bloeddruk stabiliserende ingrepen tijdens dialyse te bestuderen. Of onze methode gebruikt kan worden om het streefgewicht bij patiënten beter in te schatten is nog onduidelijk.

9.4 Toekomstverwachtingen

De antilichaam fragmenten afkomstig van lama's, meer specifiek het variabele domein van zware keten lama antilichamen (in het Engels: variable domains of heavy chain llama antibodies, afgekort VHH) zijn een veelbelovende techniek. In principe kan tegen elke stof een VHH geproduceerd worden. Het grote voordeel van de VHH is, dat ze, in tegenstelling tot klassieke antilichamen, gemakkelijk in grote hoeveelheden geproduceerd kunnen worden. Daarnaast zijn ze erg stabiel en kunnen ze gemakkelijk gekoppeld worden aan verschillende dragermaterialen. Door deze eigenschappen zijn de VHH zeer geschikt om toe te passen in ziekten waar er een enkele verwekkende stof is (bijvoorbeeld Goodpasture syndroom, myasthenia gravis, hypercholesterolemie, acute afstotingsreactie na orgaantransplantatie, etc.). Dit zijn voor de hand liggende voorbeelden; deze ziekten worden al gedeeltelijk behandeld met technieken die vergelijkbaar zijn met het principe van de VHH.

De VHH kunnen ook een veelbelovende techniek zijn als het gaat om problemen als het uremisch syndroom, zoals in de inleiding beschreven. Als we namelijk een stof heel efficiënt kunnen verwijderen met behulp van VHH, en er tegen vrijwel elke stof VHH geselecteerd kunnen worden, dan is het in theorie bijvoorbeeld mogelijk om hemodialysefilters te bekleden met VHH tegen vele componenten van het uremisch syndroom. Zo'n aanpak is niet puur hypothetisch; op dit moment wordt er gewerkt aan de draagbare kunstnier, waarin meerdere stoffen continu verwijderd zullen worden met behulp van immunoadsorptie.

Als laatste mogelijke toepassing moet men denken aan binding van in het lichaam van ingespoten VHH aan bijvoorbeeld virusdeeltjes of ziekteverwekkende stoffen. Dit kan dan gevolgd worden door klaring van de ontstane complexen door de lever of uitscheiding door de nieren.

Onze plasma refill rate bepaling is de eerste methode die dit continu tijdens de dialyse kan doen. Deze methode kan gebruikt worden om het effect van verschillende ingrepen (bijvoorbeeld het gaan liggen van de patiënt) en handelingen (zoals het eten en drinken tijdens dialyse) op refill te bepalen. Onze theorie, dat veranderingen in refill het streefgewicht kunnen helpen bepalen, is nog niet eenduidig bewezen. Desalniettemin is het zeer wel mogelijk dat onze methode om refill te meten ingebouwd wordt in normale

dialyse apparatuur. De huidige dialyse machines kennen bijna allemaal een of andere vorm van gestuurd vocht onttrekken. Meestal wordt van tevoren een bloedvolume daling ingesteld die de machine bereikt moet hebben op het einde van de dialyse. De snelheid van vocht onttrekken wordt hierop dan aangepast. Het zou maar een kleine stap zijn om de huidige apparatuur zo aan te passen, dat deze functioneert als ons systeem. Het meten van refill, en het eventuele gebruik hiervan om het streefgewicht aan te passen, zou op die manier een routinebepaling voor de dialysepatiënt kunnen worden. Een andere toepassing is het aanpassen van de dialyseduur op basis van veranderingen in refill, met als doel het voorkomen van lage bloeddrukken door (o.a.) grote dalingen in het bloedvolume. Beide toepassingen zouden de bijwerkingen van dialyse en het ontstaan van hart- en vaatschade kunnen verminderen. Ten slotte zouden met behulp van refill metingen patiënten ingedeeld kunnen worden als 'refillers' en 'niet-refillers', wat de verpleegkundigen enorm zou helpen bij hun werk omdat 'niet-refillers' eerder lage bloeddrukken zullen krijgen. Om dezelfde reden zou het geven van eten tijdens de dialyse (= bloeddruk verlagend) bij 'niet-refillers' beperkt moeten worden.

De bevindingen in dit proefschrift tonen aan dat nieuwe technieken gebruikt kunnen worden om de tekortkomingen van de huidige dialyseapparatuur, namelijk het niet kunnen verwijderen van giftige stoffen die achterblijven na dialyse, en het gebrek aan methoden om de plasma refill rate te kunnen meten tijdens dialyse, deels op te lossen.

Chapter 10

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Chapter 11

Over de auteur

De auteur is geboren op 12 maart 1980 te Nijmegen. Na afronding van de middelbare school in Winterswijk heeft hij een jaar Chemische Technologie gestudeerd aan de Universiteit Twente. Daarna werd de studie Geneeskunde aan de Universiteit Utrecht gevolgd en afgerond. Tijdens deze studie heeft hij al zijn onderwijsstages op de afdeling nefrologie van het Universitair Medisch Centrum Utrecht gevolgd, waarbij het accent lag op het onderzoeken van de plasma refill rate. Sinds 2005 was hij werkzaam als arts-onderzoeker op deze afdeling, waarvan dit proefschrift het resultaat is. In 2009 werd subsidie verkregen van de Nierstichting Nederland voor het onderzoel beschreven in *Hoofdstuk 8*. Per mei 2010 is de auteur in opleiding tot internist in het Gelre ziekenhuis te Apeldoorn. In zijn vrije tijd houdt hij zich het liefst bezig met muziek (luisteren, zelf maken op gitaar en hobo), fietsen op de racefiets en lekker huiselijk een beetje aanrommelen in het bijzijn van zijn lief Wolfje en kat Figaro.