Tailoring high-cut-off membranes and feasible application in sepsis-associated acute renal failure: *in vitro* studies

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Abstract

**Background.** As removal of pro-inflammatory cytokines is limited in conventional diffusive or convective extracorporeal therapies, we studied in two polysulphone membranes with an industrial albumin sieving coefficient of 0.05 (Type A) and 0.13 (Type B) cytokines (IL-6, IL-8, IL-1β, IL-1ra, TNF-α) and plasma protein (albumin, cystatin C, total proteins) permeability profiles. Based on the convective membrane permeability, we evaluated *in vitro* the dialytic modality that could provide an acceptable balance between high cytokine and low albumin clearances.

**Methods.** Cytokine and plasma protein sieving coefficient (SC) and clearance were studied in (i) post-dilutional haemofiltration mode at 20% fixed ultrafiltration rate; (ii) haemodialysis mode (dialysate flow rate of 3 and 5 l/h); and (iii) haemodiafiltration mode (dialysate flow rate of 3 or 5 l/h with 0.5 l/h of ultrafiltrate).

**Results.** In haemofiltration mode both Type A and Type B haemodialysers at QB 150 ml/min exhibited similar median SC nearly up to 1 for IL-1β and IL-1ra, at about 0.6 for IL-6, 0.4 for IL-8 and 0.7 for TNF-α, with clearance values ranging from 15 to 30 ml/min. SC were independent of blood flow and were stable throughout the whole experiment. Albumin SC was higher in Type B than in Type A and rapidly decreased from 0.2 to 0.02 and from 0.5 to 0.04 within 3 h for haemodialyser Types A and B, respectively. Cytokine SC was lower in haemodialysis than in haemodiafiltration mode, and by increasing dialysate flow from 3 up to 5 l/h in both haemodialysis and haemodiafiltration mode, SC for all tested cytokines decreased. However, at 5 l/h clearances were not different or were higher, since increased amounts of dialysate outlet compensated for the decreased SC. Albumin clearances in haemodialysis and haemodiafiltration mode after 360 min at 5 l/h were 0.81 and 0.91 ml/min, respectively.

**Conclusions.** Our studies show that a mixed convective and diffusive technique ensures high cytokine clearances with an acceptable loss of albumin.

**Keywords:** albumin permeability; cytokines; high-cut-off membrane; polysulphone; sepsis

Introduction

Increased levels of plasma pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukins (IL-1β, IL-6 and IL-8), interleukin-1 receptor antagonist (IL-1ra), soluble TNF receptors types I and II and lipid mediators, such as platelet-activating factor, are produced early in the course of human sepsis and are considered to be of pathogenetic relevance (for review see [1]). The close relationship between high levels of both pro- and anti-inflammatory cytokines in the plasma and severity and mortality of septic patients has suggested that intense activation of the inflammatory mediator network may play an important role in the development of organ dysfunction (for review see [2]).

Conventional continuous extracorporeal treatments, such as haemofiltration and haemodiafiltration, have failed to significantly reduce cytokine plasma levels [3,4], even if immunomodulatory substances are removed in the ultrafiltrate [5,6]. As removal rates and clearances of pro-inflammatory cytokines by conventional haemodialysis are hindered by limited diffusive or convective rates [7], an alternative approach to increasing mediator removal is the use of ‘open’ membranes coupled with sorbent technology [8].

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or without regeneration of ultrafiltrate. More than 10 years ago, Lee et al. [9] showed a remarkable improvement in survival in an experimental model of sepsis in the swine, using a 100 kDa polysulphone-like membrane. High-cut-off haemofiltration performed with haemofilters incorporating either triacetate cellulose or polyamide membranes has been described as a new approach to remove sepsis-associated mediators more effectively both in vitro [10,11] and in vivo [12]. The possibility to attain high clearances of cytokines in septic patients undoubtedly draws the interest of the clinical nephrologist. However, this is obtained at the expense of a high loss of albumin and plasma proteins. This poses the question of whether the high potential of this approach in the context of blood purification is really clinically feasible.

In these studies we first studied the sieving coefficients (SC) and clearances of different cytokines (TNF-α, IL-1β, IL-6, IL-8 and IL-1ra) and the protein permeability profile (albumin, cystatin C and total proteins) of a newly designed high-cut-off polysulphone membrane in an in vitro haemofiltration circuit. Based on the membrane permeability that could provide an acceptable balance between high cytokine and low albumin clearances, we evaluated the haemodialytic modality (in vitro haemodialysis and haemodiafiltration) that could best express the property of this membrane.

Methods

In vitro sepsis model

We used an in vitro sepsis model that allowed the blood production of different cytokines in high concentrations from human whole blood exposed to bacterial lipopolysaccharide (LPS). Three hundred millilitres of fresh blood from the local blood bank was drawn into sterile blood standard ACD-containing collection bags. Blood was heparinized (5000 U heparin, Liquemin®; Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany), recalcified (calcium chloride 10%, 44 µl/ml blood) and diluted with 300 ml isotonic saline solution (Baxter Italia, Rome, Italy). After addition of 1 mg LPS (from Pseudomonas aeruginosa serotype 0; Sigma Chemical Company, St Louis, MO, USA), blood was incubated for 4 h in a water bath at 37°C and then overnight at room temperature.

In vitro haemofiltration

All experiments were performed with a roller blood pump (Fresenius Medical Care GmbH, Bad Homburg, Germany). The hollow-fibre haemodialysers (Cytoflux; Fresenius Medical Care, Bad Homburg, Germany; inside diameter: 220 µm, wall thickness: 35 µm, effective surface area: 1.4 m²) used in these studies incorporated two newly designed polysulphone membranes (Figure 1) with industrial albumin SC of 0.05 (Type A) and 0.13 (Type B).

Fig. 1. Scanning electron microscopy electron micrographs of a Type A polysulphone membrane (with an industrial permeability of 0.05). (A) Cross section of a hollow fibre (magnification: ×1000); (B) separation layer (magnification: ×20000); (C) internal side (magnification: ×20000) and (D) external side (magnification: ×5000).
The extracorporeal circuit used for the in vitro studies was a closed loop, post-dilutional haemofiltration circuit with three sampling sites (arterial site: before the haemodialyser; venous site: immediately after the haemodialyser and before the ultrafiltrate reinfusion; and ultrafiltrate site: on the ultrafiltrate line). Experiments were performed with Type A and B haemodialysers at three different blood flow rates (QB) (100, 150 or 200 ml/min) and with a fixed (20%) ultrafiltration rate (UFR: 1.2, 1.8 and 2.4 l/h, respectively). The circuit operated at zero balance. After establishing the desired blood flow rate (100, 150 or 200 ml/min) within the circuit, baseline blood samples were taken from the arterial port at the filter inlet and the ultrafiltration was started. Blood flow rates and UFR were maintained across the circuit throughout the whole experiment. Samples were taken from arterial, venous and ultrafiltrate sites at 10, 30, 60, 120, 240, 360 and 480 min. Blood samples were collected in tubes containing ethylenediaminetetraacetaate as anticoagulant, centrifuged at 1200 g for 5 min and the supernatants removed. All samples were stored at −70°C until assayed.

In vitro haemodialysis and haemodiafiltration

On the basis of the ex vivo results on haemofiltration (see ‘Results’), only Type A haemodialysers at a blood flow rate of 150 ml/min were used. After establishing blood flow within the circuit, baseline blood samples were taken from the arterial line and the experiment started with haemodialysis at a dialysate flow rate of 3 l/h for 10 min; the spent dialysate was recovered in a bag and samples were taken from the arterial site, the venous site and the dialysate bag. The dialysate flow was then switched to 5 l/h for 10 min and again blood samples were taken from the arterial site, the venous site and the recovered dialysate bag. The above-described sequence of 3 and 5 l/h haemodialysis protocol and sampling was repeated at 60, 120, 240 and 360 min. During the interval times between the sequential samples, blood and dialysate flows were maintained at 150 ml/min and 3 l/h, respectively.

In the experiments performed in the haemodiafiltration mode at 3 and 5 l/h, we used the same sequence of dialysate flow rates and samplings. Haemodiafiltration was performed with a fixed UFR (0.5 l/h), so that the outlet dialysate flows of 3 and 5 l/h included 0.5 l/h ultrafiltrate + 2.5 l/h inlet dialysate or 0.5 l/h ultrafiltrate + 4.5 l/h inlet dialysate, respectively. During the interval times between the sequential samples, blood and dialysate flows were maintained at 150 ml/min and 3 l/h, respectively.

Laboratory assays

IL-6, IL-8, IL-1β, IL1-ra and TNF-α concentrations in plasma and ultrafiltrate were measured using an enzyme-linked immunosorbent assay (ELISA) (Quantikine; R&D Systems, Inc., Minneapolis MN, USA) according to the manufacturer’s suggestion. All samples were tested in duplicate. The lowest concentration of detectable cytokines in the sample from the zero value were IL-6 3.0 pg/ml, IL-8 16 pg/ml, IL-1β 4.0 pg/ml, IL-1ra 12 pg/ml and TNF-α 8.0 pg/ml. A single calibration curve for each type of sample (plasma and ultrafiltrate) was constructed. The intra-assay variabilities of the assays were <5% for IL-1β, IL-1ra and IL-8, 5% for IL-6 and 3% for TNF-α. The interassay variability was ~6% for all tested cytokines.

In the case of TNF-α, and in selected experiments, we also used a BioLisa kit (Human TNF-α BioLisa; Bender MedSystems GmbH, Vienna, Austria). This test differs from a conventional ELISA because recombinant TNF-receptor is adsorbed onto microwells and active TNF-α present in the sample or standard binds to the receptors. A biotin-conjugated monoclonal anti-TNF is added and binds to TNF-α captured by the receptors adsorbed to the microwells. This assay combines the advantage of the highly specific measurement of an ELISA with the functionally relevant result of a bioassay. The lowest concentration of detectable TNF-α by BioLisa was 11 pg/ml. The intra-assay and interassay variabilities were 3.3% and 7%, respectively.

Cystatin C (MW: 13 kDa) and albumin (MW: 69 kDa) were measured in plasma and ultrafiltrate with the immunoturbidimetric method (BN Method; Dade Behring, Marburg, Germany). Total proteins were measured with the biuret method (Beckmann Instruments, Inc., Brea, CA, USA). The intra- and interassay coefficients of variation were 3.0% and 2.0%, respectively.

Calculations

The SC and clearance of each cytokine were, respectively, calculated as follows:

\[
SC = \frac{C_{uf}}{C_i + C_{o}}
\]

\[
CI = SC \times Q_{uf}
\]

where \(C_i\) is the concentration in the inlet plasma (pg/ml), \(C_o\) is the concentration in outlet plasma (pg/ml), \(C_{uf}\) is the concentration in ultrafiltrate (pg/ml) and \(Q_{uf}\) is the ultrafiltrate flow rate (ml/min).

Statistical analysis

The data were expressed as means±SD. The data were evaluated by analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison test. For significance analysis between multiple groups, SC or clearances were used as the dependent variable and type of filter (A or B), blood flow rate (100, 150 or 200 ml/min), type of treatment (haemodialysis or haemodiafiltration) and dialysate flow (3 or 5 l/h) as independent variables (Tables 1–4 and Figures 2 and 3). For significance analysis of a variable over time, one-way ANOVA for repeated measures was used (Figures 2 and 3).

\(P\)-values of <0.05 were considered statistically significant.

The statistical analysis was carried out with software package Statistica ’99 Edition (Statsoft, Tulsa, OK, USA).

Results

In vitro sepsis model

The baseline median plasma concentrations of cytokines in the blood circuit were 40.69 ± 31.58 ng/ml for IL-6, 45.70 ± 35.72 ng/ml for IL-8, 10.48 ± 6.43 ng/ml for IL1-β, 34.71 ± 19.01 ng/ml for IL1-α and 6.63 ± 4.52 ng/ml for TNF-α.
Type A and B haemofiltration was performed at a fixed 20% UFR. The data are given as means ± SD of three different experiments.

### Table 1. SC of IL-6, IL-8, IL-1β, IL-1ra and TNF-α in haemofiltration

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<td>0.62 ± 0.33</td>
<td>0.63 ± 0.30</td>
<td>0.90 ± 0.48</td>
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<td>IL-1ra</td>
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<td>0.57 ± 0.14</td>
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<td>0.91 ± 0.77</td>
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<td>TNF-α</td>
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<td>0.63 ± 0.26</td>
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### Sieving coefficients in haemofiltration

As shown in Table 1, both Type A and Type B haemodialysers exhibited median SC nearly up to 1 for IL-1β and IL-1ra, at about 0.6 for IL-6, 0.4 for IL-8 and 0.7 for TNF-α. Of interest, SC of any of the tested cytokines (with the exception of IL-6 SC at 360 min, QB 100 vs QB 150) were significantly independent from blood flow rates with both haemodialysers (Table 1). Cytokine SC minimal square curves of Type A and B filters were overlapping and relatively stable (with the exception of TNF-α) throughout the whole experiment (480 min) and no significant difference was found between the two haemodialysers (Table 1 and Figure 2).

We observed that using ELISA, TNF-α had a higher SC than smaller cytokines, such as IL-6. To determine the SC for the biologically active form of TNF-α we applied in selected experiments a BioLisa, an ELISA for quantitative detection of active human TNF-α (see ‘Subjects and methods’). As shown in Figure 2, SC were 0.68 (at 5 min) and 0.43 (at 480 min) for Type A haemodialysers and 0.70 (at 5 min) and 0.49 (at 480 min) for Type B haemodialysers.

### Clearances in haemofiltration

Different tested cytokines showed clearance values of about 15 to 30 ml/min. These values were stable over time (up to 480 min) and showed no significant difference between Type A and B haemodialysers (Table 2).

On the contrary, albumin clearance declined abruptly in the first 60 min, was significantly lower in Type A than in Type B haemodialysers in early times and, of interest, tended to be reduced without statistical
significance when blood flow rate increased from 150 to 200 ml/min (Table 3).

**Type A haemodialyser sieving coefficients and clearances in haemodialysis and haemodiafiltration**

No significant differences for SC (Figures 4 and 5, left side panels) and clearances (Figures 4 and 5, right side panels) of all cytokines were found between haemodialysis and haemodiafiltration at the two different dialysate flow rates 3 and 5 l/h. In particular, at 3 l/h IL-6 and IL-8 SC in haemodiafiltration (Figure 4, top and middle panels in left side) were overlapping those obtained in haemofiltration (Figure 2), while IL-1β (Figure 4, bottom panel in left side), IL-1-ra and TNF-α (Figure 5, panels in left side) SC in haemodiafiltration were only about half of those obtained in haemofiltration (Figures 2 and 3). In haemodialysis and haemodiafiltration, by increasing dialysate flow from 3 up to 5 l/h at a constant blood flow of 150 ml/min, a decreased or maintained SC for all tested cytokines was observed (Figures 4 and 5, panels in left side).

By increasing dialysate flow from 3 up to 5 l/h, cytokine clearances in haemodialysis and haemodiafiltration were not significantly different or were higher, since the increased amount of dialysate outlet compensated for the decreased SC (Figures 4 and 5, panels in right side). Albumin clearances in haemodialysis and haemodiafiltration after 360 min at 3 and 5 l/h were lower than 1 ml/min, with no significant difference between the two modalities (Table 4).

**Discussion**

In the present studies we used two types of a newly designed high-cut-off polysulphone membrane differing in its SC to albumin. Using an in vitro sepsis model and an extracorporeal circuit, we provide evidence that both types of the high-cut-off polysulphone membrane could yield high clearances for different cytokines that are physiologically generated in response to LPS.

Firstly, we evaluated Type A and B haemodialysers in post-dilution haemodiafiltration. Clearances of
cytokines ranged from 15 to 30 ml/min. As shown in Tables 1 and 2, there was no significant difference in cytokine SC and clearances between Type A (albumin SC: 0.05) and Type B (albumin SC: 0.13) haemodialysers. SC were expectedly high for IL-1β, IL-1ra and TNF-α, whereas this was not the case for IL-8, possibly in relationship to its high cationicity and its consequent binding to heparin [13]. An SC (at 120 min) of 0.6 for IL-6 (MW: trimeric form: ~30 kDa; monomeric form: ~17 kDa) was unexpected since the cytokine with the highest molecular weight had a higher SC. The ELISA could measure split products of trimeric TNF-α. On the other hand, when using a BioLisa that specifically detects biologically active TNF-α, only a reduction of ~30% in SC could be observed (SC: 0.5 at 120 min). In addition, SC and clearances for IL-6 appeared to be without significant variation over 480 min and at different blood flow rates. Stability over time and blood flow independence of cytokine SC in convective transport are two clinically interesting characteristics of this new polysulphone membrane.

Important information resulted from studies on the permeability profile using plasma protein markers of different molecular weight, such as albumin, total proteins and cystatin C. SC for cystatin C was lower in the Type B compared with the Type A haemofilter. This could be due to the interference of various factors, such as pore size, available filtering surface and density of pores. A major difference occurred in SC and clearance of albumin between Type A and B haemodialysers. Albumin SC decreased in the first 60 min and then remained stable but significantly different between the two types of haemodialysers. As a matter of fact, albumin clearance is a key point of studying high-permeability dialysis membranes [14]. After 4 h, by using a Type A haemodialyser at a blood flow rate of 150 ml/min (UFR: 1.8 l/h), albumin clearance was 1.34 ml/min (Table 3), a value overlapping that described by Morgera et al. [11] at a blood flow rate 150 ml/min and with an UFR of 1 l/h. This value means a 24 h clearance of 2 l. Considering an albumin plasma concentration of 25 g/l, ~50 g/day of albumin would be lost. With a Type B haemodialyser and a clearance of 3.55 ml/min (Table 3), we would expect a daily loss of ~125 g albumin, a value not sustainable in clinical settings. The highest loss of albumin with a Type B vs a Type A haemodialyser was not accompanied with

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a higher SC or clearance for any of the investigated cytokines. Finally, at a blood flow rate of 200 ml/min, we observed for the Type A haemodialyser values of albumin SC and clearance lower than at 150 ml/min.

Although the clearance rates of albumin (MW C24 60 kDa) were much higher in the Type B haemofilter, no differences were found for the tested cytokines. For this reason, we chose the permeability of the Type A haemodialyser for subsequent studies aiming at defining the extracorporeal modality.

In tailoring haemodiafiltration and haemodialysis with the Type A haemodialyser, we took into account current clinical evidence concerning the substitutive treatment of sepsis-associated acute renal failure. Recent studies indicate that acute renal failure is per se a death risk factor and that daily ultrafiltrate exchange should reach ≥60 l in order to obtain improved survival of patients by efficient small molecule clearance [15]. Haemofiltration is not efficient in removing small molecules, because removal capacity in haemofiltration is due only to ultrafiltration volume, which is dependent on blood flow. Furthermore, in septic shock patients blood flow availability could be limited, because of low cardiac output or catheter malfunctioning. For all these reasons, in septic patients with associated acute renal failure it may be useful to use mixed convective–diffusive (or pure diffusive) dialysis modalities [16].

In agreement with others studies [11,12], we confirm that a diffusive modality could, in principle, lead to a lower albumin loss than pure convection while maintaining high clearances for cytokines. Indeed, we observed that in haemodialysis albumin loss was minimal (clearance: < 0.75 ml/min at 3 l/h after 4 h) and that increasing the ultrafiltration volume from 3 to 5 l/h led to a slight, not significant increase in albumin loss, with a clearance of 1 ml/min (Table 4).

Furthermore, similar behaviour in albumin loss at a dialysate flow of 5 l/h in respect to 3 l/h was also observed in haemodiafiltration, in which we maintained a fixed 0.5 l/h of ultrafiltration. Our studies provide evidence that a Type A haemodialyser can, in fact, maintain high clearances of cytokines while the loss of albumin remains remarkably low.

The use of high-cut-off membrane haemodialysers (100 kDa nominal cut-off point) was first suggested by

![Fig. 2. Minimal square curves of cytokine and protein marker SC in haemofiltration. Type A and B filters SC for IL-6, IL-8, IL-1ra and TNF-α obtained at different times and at blood flows of 100, 150 and 200 ml/min with fixed 20% UFR were pooled and the calculated mean SC (n = 9 experiments for each time and filter type) were compared. Calculated mean SC of Type A and B filters were also shown as minimal square fitting curves of permeability. TNF-α was assayed by both ELISA (that measures immunoreactive TNF-α) and BioLISA (that measures active TNF-α bound to its receptor). Two-way ANOVA followed by Student–Newman–Keuls multiple comparison test was performed with SC as the dependent variable and filter type as the independent variable. *P < 0.05 Type A vs Type B filter. One-way ANOVA for repeated measures followed by Student–Newman–Keuls multiple comparison test was conducted to analyse SC over time. IL-6: filters A and B: P > 0.05 for all times. IL-8: filters A and B: P < 0.05 only for 10 min vs other times (other comparisons are P > 0.05). IL-1ra: filters A and B: P > 0.05 for all times. TNF-α: filter A: P < 0.05 only for 10 min vs 180, 240, 360 and 480 min (other comparisons are P > 0.05); filter B: P > 0.05 for all times.](https://academic.oup.com/ndt/article-abstract/20/6/1116/1818937)
Uchino et al. [17] and later confirmed in a pilot clinical study performed by Morgera et al. [18]. Using a 60 kDa cut-off haemofilter in vivo, Morgera et al. showed that (blood flow rate: 150 ml/min; UFR: 1 l/h) a substantial filtration of IL-6 (SC: 0.9 and 0.7 at 4 and 12 h, respectively) and a remarkable plasma reduction in IL-6 concentrations occurred. Loss of albumin was nearly 5 g/12 h treatment and replacement with exogenous plasma proteins was necessary (fresh frozen plasma or albumin 20%) [18]. However, by analysing the interaction between the extracorporeal modality (haemofiltration and haemodialysis) and applied volume, these authors showed a clear-cut advantage of haemodialysis in respect of transmembrane protein and albumin loss. In addition, there was no adverse effect on coagulation, as factors such as antithrombin III, protein C, protein S and others remained unchanged [18]. However, in both these two studies TNF-α was very poorly eliminated [17,18]. An ‘open’ membrane with a nominal cut-off of 100 kDa and different surfaces (1.1–2.2 m²) was then tested, thus, rendering comparison among studies more difficult [19,20]. Uchino et al. [19] used a polyamide 100 kDa cut-off haemodialyser with a different surface to that used in the study by Morgera et al. [18]. Lee et al. [20] clearly showed that the use of high dialysate flows (200–500 ml/min) in purely diffusive techniques can attain the highest clearances of different cytokines ever to be observed. The highest median clearance of IL-1b was up to 106 ml/min, IL-6 clearance up to 67 ml/min, IL-8 clearance close to 62 ml/min and TNF-α clearance was 36 ml/min. However, purely diffusive techniques may not always be adequate in critically ill, overhydrated patients who may need convection to reach a net weight loss.

Cytokine SC and clearance studies as well as plasma protein permeability profile evaluation have led us to the tailoring of a newly designed polysulphone membrane having an industrial SC of 0.05 for albumin. Our studies at 3 l/h, that is 721/day, provide evidence
that a mixed convective and diffusive technique could ensure high clearances for the different cytokines with an acceptable loss of albumin, in experimental conditions, chosen as being close to the clinical application. However, how much cytokine clearance may occur during a treatment (whether 4 h as in intermittent haemodialysis or 24 h as in continuous renal replacement) should be assessed in in vivo studies in septic patients. Indeed, daily exchange of ≥60 l has been considered the volume necessary to obtain efficient small molecule clearance and a positive impact on patient survival [15].

Whether the use of high-cut-off membranes may be relevant in blood purification or even whether blood purification has a role in improving the survival of septic patients with multiorgan failure is still unknown. In the past, extracorporeal strategies for unselective cytokine removal were suggested. However, it has never been demonstrated in experimental models or in the clinical setting that plasma cytokine reduction per se leads to haemodynamic improvement or, even more importantly, to survival benefit. As a matter of fact, this does not contradict the potential interest in using high-cut-off membranes. Even if cytokines...
may be not appropriate, they may behave as markers for as yet undetected more relevant biologically active mediators.

Future studies will have to assess the significance and the safety of high-cut-off membranes in the treatment of acute renal failure in the context of septic multiorgan failure.

Conflict of interest statement. R.P., C.T. and J.P.-D. were full-time employees of Fresenius Medical Care at the time of this study.

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