ORIGINAL ARTICLE

Effects of immunoadsorption combined with membrane filtration on complement markers – results of a randomized, controlled, crossover study

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SUMMARY

The complement system has been implicated in several kidney diseases, such as antibody-mediated rejection after kidney transplantation. Antibody-depletion techniques allow successful ABO- and/or HLA-incompatible transplantation. Considering the IgG removal, the use of semi-selective immunoadsorption (IA) has been advocated. However, because of results on incomplete IgM depletion, the adjunctive use of membrane filtration (MF) has been proposed to enhance the removal of macromolecules and to interfere with complement activation. This secondary endpoint analysis of a recently published randomized, controlled, cross-over trial was designed to investigate the effect of combined treatment IA + MF compared to IA alone on complement depletion. Two treatment sequences, a single session of IA + MF followed by IA (and vice versa), were analyzed with regard to C5b-9, properdin, and mannose-binding lectin (MBL) levels. Neither IA alone nor IA + MF provoked complement activation as demonstrated by stable low levels of C5b-9 after the procedure as compared to the previous. The combined treatment substantially lowered properdin (77% vs. 26% reduction, $P < 0.0001$) as well as MBL concentrations (81% vs. 11% reduction, $P < 0.0001$). Recovery of properdin and MBL levels appears to be longer after IA alone compared to IA + MF. Depletion of properdin and MBL levels may have potential clinical implications in the setting of kidney transplantation.

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Key words
complement, immunoadsorption, kidney transplantation, mannose-binding lectin, membrane filtration, properdin

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Introduction

The complement system (CS) is a powerful cascade of soluble and cell-bound components, regulators, and receptors that work together in a delicate balance to drive responses to pathogens and host homeostasis. CS is initiated by classical (CP), lectin (LP), and alternative (AP) pathways that converge in the cleavage of the central C3 component into C3a and C3b fragments. Subsequent C5 conversion by C5 convertases ultimately results in formation of the terminal complement complex (also referred as C5b-9), causing the lysis of cells [1].

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Classical pathway is triggered by antigen–antibody immune complexes through binding of C1q; LP uses members of the collectin family, namely mannose-binding lectins (MBL) and ficolins, to identify and bind carbohydrate ligands on the pathogen surface. AP is constitutively active at low levels ("tick over") [1]. A number of regulatory factors, including both soluble and membrane-bound proteins, work to maintain the delicate balance between activation and inhibition. The only known positive regulator of complement activation is properdin, which slows down dissociation of the short-lived AP C3 convertase. Evidence also exists that properdin, in addition to serving as a stabilizer of AP C3 convertase, may act as a pattern recognition molecule to direct and trigger AP activation by interacting with surface AP C3 convertase or pathogen antigens [2]. Activation of complement is involved in a number of kidney diseases, such as antibody-mediated rejection (ABMR) in kidney transplantation reviewed in refs. [3,4]: LP has been shown to play a crucial role in multiple renal diseases as well as during renal replacement therapy [5]; properdin is detected in kidney biopsies and in samples from patients with various complement-mediated renal diseases [6].

An increasing number of people worldwide present with end-stage renal disease and need a kidney transplantation. Some have developed broad-spectrum anti-HLA alloantibodies, which render transplantation more difficult, that is, in some cases, it is almost impossible to find a suitable HLA-compatible donor. In such desperate situations, we can attempt to remove these anti-HLA alloantibodies before transplantation using semi-selective immunoadsorption (IA) combined with immunosuppression, based on tacrolimus, mycophenolic acid, steroids, and rituximab [7–10]. Semi-selective IA is an extracorporeal technique where patient blood is first separated into plasma and corpuscular elements via centrifugation. The plasma is then pumped over two immunoadsorption columns before being returned to the patient. The two columns work in tandem [11] and retain immunoglobulins (Ig) mostly of the IgG isotype via binding and capturing their Fc portion. Depending on the column type also IgM may be removed, but to a much lesser amount [12]. Recently, we have shown that the combination of IA with the innovative technique of membrane filtration (MF) allows for an efficient removal of large-sized effector molecules. MF is based on a porous membrane filter originally developed for double filtration plasmapheresis that can be placed in between a plasma separator and the immunoadsorption unit (IA + MF). In a randomized controlled crossover study we were able to demonstrate, that the addition of MF to IA not only significantly increased ABO-specific IgM elimination (69% vs. 25% reduction) and C1q (86% vs. 58% reduction) elimination, but also resulted in a reduction of classical complement pathway functionality when compared to IA alone [13]. The purpose of the present substudy was to evaluate the ability of IA + MF to remove additional macromolecular complement components, such as MBL and properdin. In order to rule out any effect of MF on complement activation, soluble C5b-9 (sC5b-9) was also evaluated.

Patients and methods

Trial design

A randomized controlled open-label cross-over trial (http://clinicaltrials.gov identifier: NCT01698736) was conducted at the nephrology division of the medical University of Vienna (Austria) between August 2012 and March 2013. The study protocol was approved by the local ethics review board (EK1540/2012) and all participants gave their written informed consent in accordance with the Declaration of Helsinki. The primary objective of this study was to investigate whether adding a MF to the circuit during semi-selective IA enhanced the elimination of ABO reactivity. We previously shown that combined treatment enhanced the elimination of ABO-specific IgM (median 69%; IQR: 67–74%) compared to IA alone (median 25%; IQR: 21–29%; $P < 0.001$) [13]. Secondary objectives included the outcomes from standard coagulation assays during and after procedures [13,14], and the ability to remove complement components, such as MBL and properdin. Soluble C5b-9 was measured to rule out complement activation by MF.

Plasma was separated using centrifugation (COBE Spectra® Apheresis System; Terumo BCT, Tokio, Japan) and we processed 2.5 calculated patient plasma volumes during each session under regional and systemic anticoagulation with citrate and heparin (1000IE as iv bolus and then 1000IE per hour). A regenerative GAM-146-peptide-coated twin column system (Globaffin®; Fresenius Medical Care, Bad Homburg, Germany) was connected to the ADAsorb® device (Medicap GmbH, Ulrichstein, Germany) and plasma was pumped alternatively through the immunoadsorption columns. When applying IA + MF, a porous polysulfone membrane filter (MONET®; Fresenius Medical Care) was placed in between the plasma separator and immunoadsorption unit and after processing 4000 ml plasma, MF was stopped and IA was continued as a sole treatment.

Patients were randomized into two intervention sequences (Fig. 1): a single session of IA combined with MF (IA + MF: intervention A) followed by a single session of IA alone (IA: intervention B), or vice versa. Adult patients were treated with semi-selective IA at regular intervals for autoimmune disease. Only patients with blood groups A, B, or O, and detectable levels of flow-cytometric ABO-specific IgM (>1000 mean fluorescence intensity) were included. A minimum interval of 7 days was mandatory before intervention A or B was chosen for the washout phase to avoid carryover effects. Exclusion criteria for the trial were patients aged <18 years and those that were participating in another interventional study.

Complement testing

Soluble C5b-9 quantification was performed using a commercially available enzyme-linked immunosorbent assay (ELISA) for Terminal Complement Complex C5b-9 (Cloud-Clone Corp., Houston, TX, USA), according to the manufacturer's instructions.

Properdin was measured using an in-house sandwich ELISA. Briefly, microtiter plates were coated overnight at 4 °C with a polyclonal anti-properdin antibody (LSBIO LS-C195843, Seattle, WA 98121, USA, dilution 1/2500). The plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (w/v; PBS-T), and then blocked for 1 h at 37 °C with PBS-T containing 1% BSA (w/v). Serum samples and standard sera were added to the wells and incubated for 1 h at 37 °C. After washing, monoclonal anti-human properdin antibody IgG (Quidel A235, Quidel, San Diego, CA 92130, USA, diluted to 1/500) was added, and then heated for 1 h at 37 °C. After washing (as above), horseradish peroxidase (HRP)-conjugated goat polyclonal anti-mouse antibody (Dako P00447, Dako Denmark A/S, Glostrup DK-2600, Denmark; dilution 1/6700) was added and then heated for 1 h at 37 °C. After additional washing, enzymatic activity was revealed using tetramethyl-benzidine as the substrate. The patients' results were expressed as a percentage of the standard sera.

Determination of MBL protein concentration was realized by ELISA, as described previously [15].

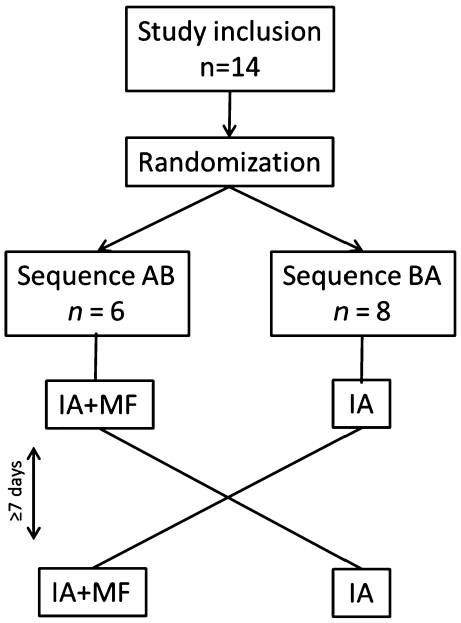

Figure 1 Trial flowchart. Fourteen patients were assigned to intervention group AB (sequence: IA + MF/IA) or BA (sequence: IA/IA + MF), respectively. IA, immunoadsorption; MF, membrane filtration.

Statistical analyses

Statistical analyses were assessed with GRAPHPAD PRISM 8.0 (Graph Pad Software, La Jolla, CA, USA) using the Wilcoxon signed-rank test to assess nonparametric paired differences between the two treatment modalities. A two-sided *P*-value of <0.05 was considered statistically significant.

Results

This secondary endpoint analysis of a recently published randomized, controlled, cross-over study was designed to investigate the addition of MF to semi-selective IA compared to IA alone on complement depletion. We examined the impact of semi-selective immunoadsorption on global complement functional activity (sC5b-9), and properdin and MBL concentrations. Patient demographics of the overall study population are provided in Table 1.

Impact of apheresis on complement activation

In order to evaluate the impact of apheresis modalities on complement activation, levels of sC5b-9 were determined. No difference was found between the two types of intervention regarding pretreatment and post-treatment levels ($P = 0.79$ and $P = 0.90$, respectively; Table 2).

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Table 1. Patient demographics.

Characteristics	*N* = 14
Age (years), median (IQR)	43.5 (35.0–60.6)
Female sex, *n* (%)	10 (71)
Indication for IA therapy, *n* (%)	
Myasthenia gravis	6 (42)
Systemic lupus erythematosus	4 (29)
Other*	4 (29)
Immunosuppressive therapy†, *n* (%)	
MMF	2 (14)
Azathioprine	7 (50)
Steroids	8 (57)
Other treatment‡	3 (21)
BMI, median (IQR)	25.9 (22.6–28.7)
Processed plasma (L), median (IQR)	8.5 (7.6–10.0)

BMI, body mass index; IQR, interquartile range; MMF, mycophenolate mofetil.

*Other: multiple sclerosis (*n* = 1), dermatomyositis (*n* = 1), mixed connective tissue disease (*n* = 1), chronic inflammatory demyelinating polyneuropathy (*n* = 1).

†Twelve patients received chronic treatment with immunosuppressive therapy.

‡Other treatment with: tacrolimus (*n* = 1), methotrexate (*n* = 1), cyclophosphamide (*n* = 1).

Effect of IA + MF versus IA on properdin concentration

We then evaluated the effect of apheresis modalities on serum properdin concentration (Table 3, Fig. 2). Although there was no difference between the two interventions regarding baseline levels (i.e., IA: 67.0%; IA + MF: 77.5%; $P = 0.39$; Table 3), IA + MF significantly enhanced properdin elimination: median post-treatment reductions of initial serum properdin were 26% (IA) and 77% (IA + MF), respectively ($P < 0.0001$; Fig. 2); median properdin concentration was 49.5% (IA) and 17.0% (IA + MF) of reference values after treatment ($P < 0.0001$; Table 3); Fig. 3 shows the dynamics of properdin concentration in the two randomization arms: as illustrated in Fig. 3a, at ≥7 days after IA + MF apheresis, properdin had returned to levels similar to those detected before the first apheresis session. In contrast, IA alone impaired the restoration of properdin concentration (Fig. 3b).

Effect of IA + MF versus IA on MBL concentration

We next evaluated the antigen levels of MBL. No difference was found between the two sequences regarding pre-treatment levels (IA: 1000 µg/l; IA + MF: 850.0 µg/l; $P = 0.35$; Table 4). The combined treatment of IA + MF led to more pronounced depletion of MBL compared to IA alone [reduction of baseline levels: 11% (IA) vs. 81% (IA + MF); $P < 0.0001$; Fig. 4]; median MBL concentration was 825 µg/l (IA) and 160 µg/l (IA + MF) after treatment ($P < 0.001$; Table 4). After ≥7 days with IA + MF, MBL had returned to levels similar to those measured before the first apheresis session (Fig. 5a). In contrast, following IA and before the second treatment sequence, all four patients showed MBL concentrations below those measured before the first apheresis session (Fig. 5b). Five patients were excluded from the statistical analyses because of MBL deficiency (i.e., MBL concentration at baseline below the reference values).

Discussion

In this study, we have demonstrated that neither IA nor IA + MF activated complement as assessed by sC5b-9; conversely, we have shown that combined treatment IA + MF was associated with the significant removal of properdin and MBL as compared to IA.

Interfering with complement activation represents an effective strategy to counteract humoral rejection in renal transplantation. We have previously shown that combined semi-specific IA + MF treatment substantially impaired C1q concentrations (86% vs. 58% reduction, $P < 0.001$) and the functionality of patients' sera to

Table 2. Impact of IA with or without MF on sC5b-9 levels.

	IA (*n* = 14)		IA + MF (*n* = 14)			
	Pretreatment	Post-treatment	Pretreatment	Post-treatment	P1	P2
sC5b-9 (ng/ml)	2.7 (1.9–3.1)	2.1 (1.3–3.3)	2.2 (1.6–3.3)	1.6 (1.2–2.4)	0.79	0.90

IA, immunoadsorption; MF, membrane filtration.

Values are the median and interquartile range. P1 denotes the change between pretreatment levels; P2 denotes the changes between post-treatment levels.

Table 3. Impact of IA with or without MF on properdin levels.

	IA (n = 14)		IA + MF (n = 14)			
	Pretreatment	Post-treatment	Pretreatment	Post-treatment	P1	P2
Properdin, % of RV*	67.0 (52.0–91.0)	49.5 (42.5–58.5)	77.5 (58.5–88.5)	17.0 (13.5–21.0)	0.39	<0.0001

IA, immunoadsorption; MF, membrane filtration.

Values are the median and interquartile range. P1 denotes the change between pretreatment levels; P2 denotes the changes between post-treatment levels.

*RV, reference values: 70–130% of standard sera.

restore classical complement activity, as reflected by the significant reduction of C3d deposition that exceeded IA alone [13]. In the setting of specific IA (i.e., against isoagglutinins) it has been demonstrated that C3a and the C3a/C3 ratio declined when IA treatment was started, and that this decline was maintained postoperatively. In addition, C1q declined from day −30 to a lower value on the day before transplantation, without an increase in sC5b-9 levels during the entire follow-up period [16]. Accordingly, none of the techniques increased the terminal complement complex in our study, i.e., sC5b-9.

Properdin is a plasma glycoprotein mainly produced by leukocytes that stabilizes AP C3 convertase and prolongs its half-life by 5–10 times [2]. Historically, properdin deficiency has been strongly associated with an increased risk for meningococcal infections. It has been reported that properdin can also bind to target cells and microbes, it can provide a platform for convertase assembly, and can function and promote target phagocytosis. The role of properdin as a pattern-recognition molecule and initiator of the AP has been extensively discussed: it seems that binding of properdin to complement activating surfaces depends on the initial C3b deposition [17]. As the only known positive regulator of the CS, it has not been established whether properdin plays a role in complement-mediated renal diseases, or whether properdin could be a therapeutic target [6,18]. Properdin seems to be implicated in complement-mediated diseases, such as in renal ischemia/reperfusion injury (IRI) lesions [19]. In a mouse model [DAF(−/−) CD59(−/−)] it was shown that the function-blocking anti-mouse properdin monoclonal antibody ameliorated renal IRI lesions when given to mice 24 h before, but not at 4 or 8 h after ischemia/reperfusion [20].

In the present study, we have demonstrated that properdin levels were significantly decreased by

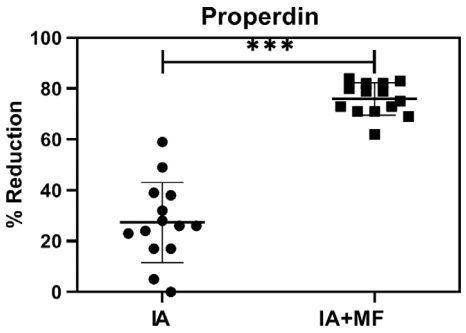

Figure 2 Impact of IA + MF versus IA apheresis on properdin antigen levels. Results are given as percent reduction from pretreatment levels. IA, immunoadsorption; MF, membrane filtration.
***P < 0.0001.

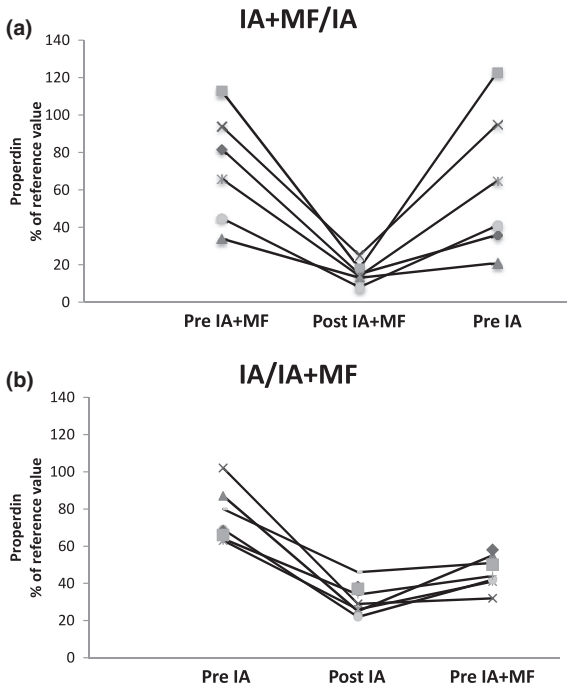

Figure 3 Course of serum-properdin levels according to the randomization arm. The values were obtained immediately before and after the first session of treatment and before the second treatment session for (a) patients randomized to receive IA + MF followed by IA (n = 6) and (b) patients received IA followed by IA + MF (n = 8). IA, immunoadsorption; MF, membrane filtration.

Table 4. Impact of IA with or without MF on mannose-binding lectin (MBL) levels.

	IA (n = 9)		IA + MF (n = 9)			
	Pretreatment	Post-treatment	Pretreatment	Post-treatment	P1	P2
MBL (μg/l)	1000 (725–1175)	825 (645–1175)	850 (662–1200)	160 (120–239)	0.35	0.001

IA, immunoadsorption; MF, membrane filtration.

Values are the median and interquartile range. P1 denotes the change between pretreatment levels; P2 denotes the changes between post-treatment levels.

Reference values: 30–3000 μg/l.

IA + MF as compared to IA alone. However, ≥7 days after IA, recovery of properdin levels was much more impaired compared to after IA + MF. This suggests that IA has a significant impact on properdin level and this could translate into greater susceptibility to some bacterial infections. However, having low levels of properdin at kidney transplantation and after IA + MF may decrease the risk of IRI lesions.

Mannose-binding lectin is a soluble pattern-recognition molecule that is mainly synthesized by the liver and is involved in LP activation. Complement, and particularly MBL, has been shown to have an important role in antibody-mediated graft loss. However, conflicting data exist on the deleterious versus protective role of LP activation in graft rejection. A study by Berger *et al.* [21] included 266 kidney-transplant patients who had higher MBL levels that seemed to be associated with a more severe form of rejection that led to treatment failure and graft loss. A limitation of this study was that it was not shown if the patients were HLA-immunized. In another prospective study of 544 kidney-transplant patients, Bay *et al.* [22] reported that low levels of MBL at the time of transplantation were significantly associated with decreased 5-year death-censored graft survival, but there was no significant association with MBL levels and graft survival in HLA-immunized patients. Interestingly, Berger *et al.* [23] related an emerging role of the lectin pathway in solid-organ transplantation, with the contribution of MBL to immunoglobulin-mediated complement activation in both ischemia-reperfusion and rejection. The interaction of MBL with IgM may be of importance in the setting. In this review, the authors attempted to explain some of the conflicting results on the beneficial and harmful effects of the LP.

The MBL baseline value in our patients was ~1000 ng/ml: this significantly decreased after IA + MF.

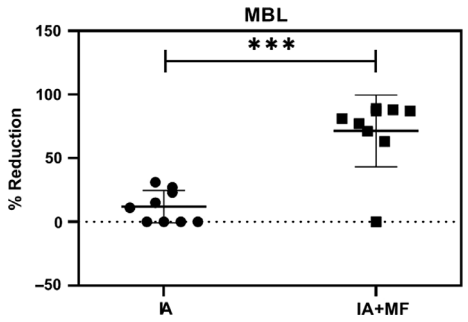

Figure 4 Impact of IA + MF versus IA apheresis on mannose-binding lectin (MBL) antigen levels. Results are given as the percent reduction from pretreatment levels. IA, immunoadsorption; MF, membrane filtration. ***P < 0.0001.

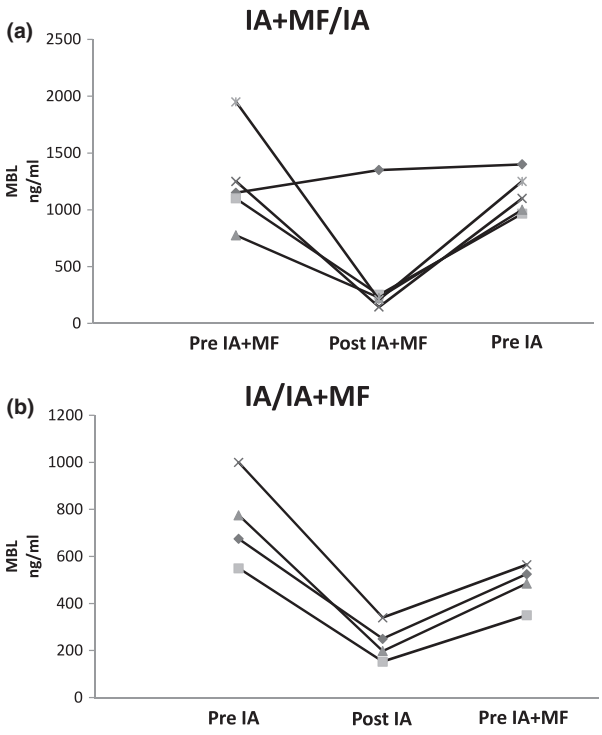

Figure 5 Dynamics of serum mannose-binding lectin (MBL) levels according to the randomization arm. Values were obtained immediately before and after the first session of treatment and before the second treatment session for (a) patients randomized to receive IA + MF followed by IA (n = 5) and (b) patients received IA followed by IA + MF (n = 4). IA, immunoadsorption; MF, membrane filtration.

However, Riwes *et al.* [24] recently reported that, in the setting of hematologic malignancy, patients that received myelosuppressive chemotherapy or allogeneic hematopoietic stem-cell transplantation had MBL levels that were <1000 ng/ml, and this was not associated with an increased risk of developing invasive fungal disease and had no impact upon overall survival.

As observed for properdin, recovery of MBL levels took significantly longer after IA alone compared to IA + MF. We do not have an explanation for the observed delayed recovery for properdin and MBL; however, we cannot exclude a carry-over effect across treatments even after a washout phase of 7 days. This is in contrast with earlier results obtained for fibrinogen that increased to levels that were similar to those detected before the first apheresis session for both treatments [14].

MBL2 gene polymorphisms produce intermediate/low/null or normal MBL serum levels (MBL-deficient or MBL-sufficient phenotypes, respectively). Single nucleotide polymorphisms (SNPs) in the *MBL2* gene have been associated with susceptibility to infection [25], although data from solid-organ transplant recipients remain inconclusive. Recently, Lombardi-Quezada *et al.* [26] reported that liver-transplant recipients of MBL-deficient livers had a higher risk of bacterial infection. The 1-year bacterial infection-related mortality was higher in recipients of MBL-deficient versus MBL-sufficient livers (65.8% vs. 56.1%, respectively; $P = 0.0097$). The incidence of rejection, viral, or fungal infection was similar in both groups. Conversely, recipients with the *MBL2* genotype did not have a significantly increased risk of bacterial infection [26].

Fernández-Ruiz *et al.* [27] performed a meta-analysis (11 studies comprising 1858 patients, with liver-transplant recipients accounting for 80.4% of the pooled population) with the aim of investigating the association between post-transplant bacterial and fungal infections and the variant alleles of the *MBL2* gene SNPs in the promoter/5′ untranslated region and exon 1. Cytomegalovirus (CMV) infection and/or disease were considered secondary outcomes. They found that, compared to high-MBL expression haplotypes (YA/YA, YA/XA), any MBL-deficient haplotype was associated with an increased risk of post-transplant bacterial and fungal infections. Low/null-MBL expression haplotypes (XA/O, O/O) also increased the risk of primary outcome and CMV events. No effect was observed for individual promoter SNPs [27].

The levels of MBL at the time of kidney transplantation have been evaluated with regard to post-transplant outcomes. Golshayan *et al.* [28] have shown that low MBL serum levels and deficient *MBL2* diplotypes at the time of kidney transplantation were associated with a higher incidence of acute cellular rejection during the first year, in particular in recipients of a deceased-donor kidney. In contrast, there was no significant association between rates of antibody-mediated rejection, patient mortality, or early graft dysfunction or loss. Because we found that IA + MF could profoundly and consistently decrease MBL levels, this implies a risk of either acute cellular rejection and/or more infectious complications within the post-transplant period. A limitation of this study was the small number of patients and that these patients were not kidney-transplant candidates, that is, they were not uremic. This may limit our findings when applying IA + MF to highly sensitized donor-specific antibody positive transplant candidates that undergo peritransplant crossmatch conversion or desensitization for transplantation. When compared to plasma exchange (PLEX) in the peritransplant setting, besides IA + MF being more cost intensive than PLEX, IA + MF would have the advantage of enabling the processing of much higher plasma volumes than with PLEX but without the undesirable effect of restoring complement effector molecules such as C1q by the administration of fresh frozen plasma units during PLEX.

We conclude that combined treatment of IA + MF profoundly decreased the levels of properdin and MBL as compared to IA alone, which may have potential clinical implications.

Authorship

FD, CDP, LR: designed the study. FD, CDP: performed research. FE, GB: contributed important reagent. FD: collected and analyzed data. FD, CDP, LR: wrote paper. FE, GB, PM, JYC: final approval the version to be published.

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Conflict of interest

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