

Prompt Thrombo-Inflammatory Response to Ischemia-Reperfusion Injury and Kidney Transplant Outcomes



Gabriel Strandberg¹, Carl M. Öberg², Anna M. Blom³, Oleg Slivca¹, David Berglund⁴, Mårten Segelmark², Bo Nilsson⁴ and Ali-Reza Biglarnia¹

¹Department of Surgery, Department of Clinical Sciences Malmö, Skåne University Hospital, Lund University, Malmö, Sweden;

²Department of Nephrology, Department of Clinical Sciences Lund, Skåne University Hospital, Lund University, Lund, Sweden;

³Department of Translational Medicine, Lund University, Malmö, Sweden; and ⁴Department of Immunology, Genetics, and Pathology (IGP), Rudbeck Laboratory C5:3, Uppsala University, Uppsala, Sweden

Introduction: In kidney transplantation (KT), the role of the intravascular innate immune system (IIIS) in response to ischemia-reperfusion injury (IRI) is not well-understood. Here, we studied parallel changes in the generation of key activation products of the proteolytic cascade systems of the IIIS following living donor (LD) and deceased donor (DD) transplantation and evaluated potential associations with clinical outcomes.

Methods: In a cohort study, 63 patients undergoing LD ($n = 26$) and DD ($n = 37$) transplantation were prospectively included. Fifteen DD kidneys were preserved with hypothermic machine perfusion (HMP), and the remaining were cold stored. Activation products of the kallikrein-kinin, coagulation, and complement systems were measured in blood samples obtained systemically at baseline and locally from the transplant renal vein at 1, 10, and 30 minutes after reperfusion.

Results: DD kidneys exhibited a prompt and interlinked activation of all 3 cascade systems of IIIS post-reperfusion, indicating a robust and local thrombo-inflammatory response to IRI. In this initial response, the complement activation product sC5b-9 exhibited a robust correlation with other IIIS activation markers and displayed a strong association with short-term and mid-term (24-month) graft dysfunction. In contrast, LD kidneys did not exhibit this thrombo-inflammatory response. The use of HMP was associated with reduced thromboinflammation and preserved mid-term kidney function.

Conclusion: Kidneys from DD are vulnerable to a prompt thrombo-inflammatory response to IRI, which adversely affects both short-term and long-term allograft function. Strategies aimed at minimizing graft immunogenicity prior to reperfusion are crucial to mitigate the intricate inflammatory response to IRI.

Kidney Int Rep (2023) 8, 2592–2602; <https://doi.org/10.1016/j.ekir.2023.09.025>

KEYWORDS: delayed graft function; intravascular innate immune system; ischemia-reperfusion injury; kidney transplantation; organ preservation; thromboinflammation

© 2023 International Society of Nephrology. Published by Elsevier Inc. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

The IIIS is a composite entity that consists of the proteolytic cascades of the complement, coagulation, kallikrein-kinin (contact system), and fibrinolytic systems, along with blood effector cells such as granulocytes, monocytes, and platelets in this crosstalk.¹ In contrast to the adaptive immune system, the IIIS can distinguish self from nonself at the species level but not at the individual level. This preset feature is not only relevant for defense against invasive pathogens

but also enables the IIIS to react to both autologous and allogeneic cells, which is essential for its role in medical conditions and interventions.²

Solid organ transplantation comprises a series of coordinated steps from donor management and procurement to organ preservation and engraftment. These sequential events require a temporary impairment and cessation of blood supply to organs, thus causing a deprivation of oxygen delivery, that is, ischemia, which is a major stress to the cells. In addition to the creation of an acidic environment and upregulation and downregulation of several genes, which results in a proinflammatory state, ischemic stress can alter the phenotype of cells by changing their membrane composition and protein expression.^{3–6} For example, the disintegration of the glycocalyx with subsequent

Correspondence: Ali-Reza Biglarnia, Department of Surgery, Lund University Department of Clinical Sciences Malmö, Ruth Lundska gata 10, 205 02 Malmö, Sweden. E-mail: alireza.biglarnia@med.lu.se

Received 18 July 2023; revised 27 August 2023; accepted 18 September 2023; published online 24 September 2023

loss of antithrombotic and anti-inflammatory regulators on the endothelial cell surface is a critical event induced by ischemia that exposes the “naked” endothelial cells to attacks from the IIIS and further damage.⁴

The restoration of the blood supply, that is, reperfusion to organ grafts, is a necessary step. Reperfusion is the starting point for healing from hypoxic stress, thereby ensuring organ graft viability and function. However, depending on the extent of hypoxic injury, the direct interaction between altered cell lining and IIIS following reperfusion can initiate a response that misfires, causing an inadvertent response that causes strong activation of proteolytic cascades and, by extension, IRI. Similarly, when the IIIS reacts to altered autologous cells with different phenotypes than the native undamaged self, IRI can also occur in other medical conditions, such as stroke, myocardial infarction, or sepsis.⁴

In clinical KT, complement activation is traditionally considered the major effector response during IRI-induced inflammation. Evidence, most of which comes from animal studies, points to early complement activation following reperfusion that has been linked to early and late graft dysfunction.^{7–11} However, in clinical trials, the use of complement therapeutics to attenuate IRI-induced graft injury has not yielded strong conclusions.^{12–14}

Here, we assumed that in kidney allografts exposed to ischemia, the direct attack by IIIS could trigger the coactivation of multiple proteolytic cascades as a common system, resulting in a multifaceted inflammatory response to IRI. To assess this, we conducted a prospective cohort study that investigated simultaneous changes in key activation products of the kallikrein-kinin, coagulation, and complement systems in early samples obtained from the transplant renal vein during consecutive minutes after reperfusion. All patients were followed-up with for 24 months to evaluate short-term and mid-term consequences of the initial inflammatory response to IRI.

METHODS

Study Population

The study enrolled all 63 uremic patients (44 males and 19 females) who underwent sequential KT at the Department of Transplantation, Skåne University Hospital, Sweden, between August 2018 and June 2019. Twenty-six patients were recipients of LD kidneys, and 37 patients were recipients of DD kidneys, of which 22 kidneys were preserved with static cold storage (DD_{CS}) and 15 were preserved with HMP (DD_{HMP}) (LifePort Kidney Transporter, Itasca, Illinois). At our center, the

donation-after-circulatory-death modality was implemented after the study period; therefore, the DD population exclusively consisted of donation-after-brain-death donors. Per standard of care, 61 of 63 patients received induction treatment with basiliximab and methylprednisolone. Two patients (DD_{CS}) received induction with thymoglobulin due to human leukocyte antigen immunization. One patient (DD_{HMP}) received rituximab in combination with basiliximab on day 0, due to ABO-incompatibility. All patients received the same maintenance immunosuppressive regimen combining tacrolimus, mycophenolate mofetil, and prednisolone, following the widely implemented SYMPHONY protocol.¹⁵

Data on donor/recipient characteristics and outcome parameters (serum creatinine, estimated glomerular filtration rate [eGFR], delayed graft function (DGF), graft failure, *de novo* donor-specific antibodies, biopsy-proven acute rejections, and patient death) were retrieved from patient records, the ScandiaTransplant database and the local transplant registry for a total follow-up period of 24 months post-transplantation. Cold ischemic time (CIT) was defined as the duration between *in situ* (in DD) or *ex situ* (in LD) cold perfusion and established graft reperfusion in recipients. The eGFR (ml/min per 1.73 m²) was calculated using the Lund-Malmö revised formula.¹⁶ DGF was defined as the need for dialysis within the first week post-transplantation. The study was approved by the Regional and National Ethical Committee (DNR 2017-79), and written informed consent was obtained from all patients.

Sample Collection

Six milliliter whole-blood aliquots were drawn from the kidney vein into EDTA vacutainer tubes (K2E BD Vacutainer, Becton, Dickinson and Company) during the transplant procedure. An initial baseline sample was obtained preimplantation by venipuncture of the recipient's external iliac vein at the transplant site followed by 3 sequential samples drawn from the transplant vein at 1, 10, and 30 minutes post-reperfusion. To minimize the risk of unintentional activation of the proteolytic cascades, samples were drawn directly into EDTA-anticoagulated tubes by venipuncture with a butterfly needle system (21G BD Vacutainer UltraTouch, Becton, Dickinson and Company) and immediately cooled in ice-sludge. The transplant vein was punctured in its proximal portion with the needle tip directed toward the hilum to minimize contamination with systemic venous blood. Needles were extracted and disposed of after each venipuncture. Samples were centrifuged at 1900g for 10

minutes at 4 °C to separate the plasma portion. The plasma was extracted, aliquoted, and stored at −80 °C.

Sample Analyses

All samples were assigned numeric codes to ensure sample analysis in a blinded fashion. In addition, staff analyzing samples were kept unaware of the study design and purpose. The proteolytic cascade systems of the blood consist of proteases whose zymogens, for example, prothrombin and prekallikrein, are activated by an upstream protease. Many of their inhibitors are serpins, for example, C1-inhibitor (C1INH) and antithrombin (AT), which form complexes with the protease. This allows analyses of the activation of individual zymogens if the zymogen-serpin complex is quantified in blood plasma.¹⁷ The following activated proteases were measured in complexes with both serpins AT and C1INH: (i) kallikrein-kinin system: factor XIIa (FXIIa-AT and FXIIa-C1INH), kallikrein (KK-AT and KK-C1INH); (ii) coagulation system: factor XIa (FXIa-AT and FXIa-C1INH), thrombin (thrombin-AT and thrombin-C1INH); and (iii) complement system: mannan-binding lectin serine protease 1 (MASP1-AT and MASP1-C1INH), mannan-binding lectin serine protease 2 (MASP2-AT and MASP2-C1INH). In-house analyses were performed according to previously described methods^{17,18} using Luminex xMAP Technology (Merck Millipore) on a clinical diagnostics instrument (MAGPIX, Luminex Corporation). Soluble terminal complement activation fragment (sC5b-9) and complement protein C3a (C3a) were assessed using in-house ELISAs.¹⁹

Statistical Analysis

Continuous variables are expressed as medians (interquartile ranges), and categorical variables as frequencies (percentages). Kruskal–Wallis tests with Bonferroni correction or Mann–Whitney U and chi-square or Fisher exact tests were used to assess differences in characteristics, outcome parameters, and eGFR slopes. Multiple comparison analysis was performed using analysis of variance on aligned rank-transformed data. Simple linear regression was used to assess slopes for eGFR from 1 to 24 months post-transplantation. Associations of outcome parameters or CIT with IIS activation markers were performed using receiver operating characteristic curve analyses. Spearman's correlation analysis was employed to estimate correlations between IIS markers within and across cascade systems. A *P*-value of less than 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism 9.2.0, GraphPad Software, San Diego California USA; IBM SPSS Statistics 28, IBM Corp., Armonk, NY; and R version 4.1.1, for Mac, R Foundation for Statistical Computing, Vienna, Austria.

RESULTS

There were no differences in sex (referred to different biological characteristics of female and male), recipient/donor body mass index, preformed donor-specific antibodies, cause of kidney failure, and induction treatment between the DD-KT and LD-KT groups. In the DD-KT population, there was no difference in the kidney donor risk index between DD_{CS} and DD_{HMP}. Recipient/donor age and CIT were higher in DD-KT than in LD-KT. The overall time of pretransplant dialysis was shorter in LD-KT than in DD-KT.

The overall median CIT in DD-KT was restricted to 692 minutes (interquartile range [IQR] 547–902). Between the DD_{CS}-KT and DD_{HMP}-KT subgroups, there were no differences in median CIT (684 [IQR 547–858] vs. 781 [IQR 504–1029] minutes, *P* = 0.53) and kidney donor risk index (1.56 [IQR 1.28–1.97] vs. 1.50 [IQR 1.28–1.97], *P* = 0.92) (Table 1). There was one case of patient death (with a functioning graft) in DD_{CS}-KT on day 64 post-transplantation due to tissue-invasive cytomegalovirus disease. There were 2 graft failures post-DD_{CS}-KT (due to acute antibody-mediated rejection) and DD_{HMP}-KT (recurrence of thrombotic microangiopathy) on days 151 and 396, respectively. In LD-KT, 1 graft failure occurred due to recurrence of focal segmental glomerulosclerosis on day 469 post-transplantation. At the last follow-up, no differences were observed in overall transplant function, the incidence of biopsy-proven acute rejections, and both graft and patient survival (Table 2). One LD-KT patient was lost to follow-up on day 69 due to emigration. There were no surgical complications related to intraoperative venipuncture of the kidney allograft.

Generation of Key Activation Markers of IIS During IRI

The relationships between IIS activation markers and the 2 independent variables, time (baseline and 1, 10, and 30 minutes after reperfusion) and modality (LD-KT, DD_{CS}-KT, and DD_{HMP}-KT), and their interaction were analyzed by an analysis of variance on aligned rank-transformed data. To maintain statistical power, the various parameters were not normalized to baseline values. This decision was made considering that repeated measures analysis of variance already accounts for baseline values. Factors representing activation of complement (sC5b-9), coagulation (FXIa-AT and FXIa-C1INH), and kallikrein-kinin systems (KK-AT, KK-C1INH, and FXIIa-C1INH) were dependent on time and modality. Furthermore, the combined effect of time and modality on the activation of these markers was indicated by significant interactions. The downstream activation of the coagulation system indicated by thrombin-AT and thrombin-C1INH complexes

Table 1. Baseline characteristics of the study population separated by KT subgroups LD, cold stored DD (DD_{CS}), and hypothermic machine perfused DD (DD_{HMP})

Baseline characteristics-median (1st–3rd quartile) or <i>n</i> (%)	Transplant modality (63)			<i>P</i> value
	LD (26)	DD (37)		
		DD _{CS} (22)	DD _{HMP} (15)	
Donor age	51.0 (46.0–59.0)	56.5 (49.8–67.8)	62.0 (46.0–68.0)	0.036 ^a
Donor BMI	26.4 (23.5–29.3)	24.8 (22.2–29.5)	23.6 (22.2–29.7)	ns
Donor anoxic brain injury (prior cardiac arrest)	-	6 (27.2)	7 (46.7)	ns
Kidney donor risk index	-	1.56 (1.28–1.97)	1.50 (1.28–1.97)	ns
Cold ischemic time in minutes	116.5 (95.0–154.8)	684.0 (546.8–858.0)	781.0 (504.0–1029.0)	<0.001 ^{a,b,c}
Recipient sex–male	18 (69.2)	14 (63.6)	12 (80.0)	ns
Recipient age	45.5 (35.8–54.3)	53.5 (46.0–60.5)	60.0 (45.0–66.0)	0.011 ^a , 0.037 ^c
Recipient BMI	25.9 (22.5–28.0)	26.0 (23.8–28.5)	24.9 (23.1–26.9)	ns
Preformed DSA	4 (15.4)	4 (18.2)	2 (13.3)	ns
Induction treatment				
Basiliximab	26 (100.0)	20 (90.9)	14 (93.3)	ns
Basiliximab + Rituximab	0 (0.0)	0 (0.0)	1 (6.7)	ns
Thymoglobulin	0 (0.0)	2 (9.1)	0 (0.0)	ns
Pretransplant dialysis modality				
Predialytic	11 (42.3)	3 (13.6)	1 (6.7)	0.006 ^a , 0.03 ^c
Hemodialysis	9 (34.6)	15 (68.2)	9 (60.0)	0.023 ^a , 0.04 ^b
Peritoneal dialysis	6 (23.1)	4 (18.2)	5 (33.3)	ns
Days of pretransplant dialysis	392 (182–730)	1529 (683–1924)	785 (539–1354)	<0.001 ^a , 0.001 ^b
Cause of kidney failure				
Glomerulonephritis	14 (53.8)	5 (22.7)	6 (40.0)	0.027 ^b
Diabetic nephropathy	3 (11.5)	5 (22.7)	1 (6.7)	ns
Polycystic kidney disease	3 (11.5)	3 (13.6)	1 (6.7)	ns
Hypertensive nephrosclerosis	3 (11.5)	2 (9.1)	3 (20.0)	ns
Alport syndrome	1 (3.8)	3 (13.6)	0 (0.0)	ns
Unknown	0 (0.0)	1 (4.5)	3 (20.0)	0.043 ^c
Other	2 (7.7)	3 (13.6)	1 (6.7)	ns

BMI, body mass index; DD, deceased donor; DD_{CS}, deceased donor kidney preserved with static cold storage; DD_{HMP}, deceased donor kidney preserved with hypothermic machine perfusion; DSA, donor-specific antibodies; LD, living donor.

Data are presented as frequencies (column percentages) and medians (1st–3rd quartile). P-values are expressed for specified comparisons:

^aLD vs. DD.

^bLD vs. DD_{CS}.

^cLD vs. DD_{HMP}.

ns = not significant.

showed a relationship with time and an interaction effect of time and modality for thrombin-C1INH. FXIIa-AT levels were associated with time and modality, but no interaction effect was observed. Figure 1 shows

activation markers of IIS with a significant interaction in either or both of their respective protease-serpin complexes. The complete dataset is presented in Supplementary Table S1.

Table 2. Outcome parameters of the study population separated by KT subgroups LD, cold stored DD (DD_{CS}), and hypothermic machine perfused DD (DD_{HMP})

Outcome at 24 months median (1st–3rd quartile) or <i>n</i> (percent)	Transplant modality (63)			<i>P</i> -value
	LD (26)	DD (37)		
		DD _{CS} (22)	DD _{HMP} (15)	
s-Creatinine μmol/l	103.0 (93.8–138.5)	117.5 (96.3–149.0)	130.0 (103.0–155.0)	ns
eGFR ml/min per 1.73 m ²	62.0 (49.0–69.8)	56.5 (34.3–68.0)	45.5 (34.8–64.8)	ns
Graft failure	1 (3.8%)	2 (9.1%)	1 (6.7%)	ns
Delayed graft function	0 (0.0%)	4 (18.2%)	2 (13.3%)	0.038 ^{a,b}
Patient death	0 (0.0%)	1 (4.5%)	0 (0.0%)	ns
Biopsy-proven acute rejection	7 (26.9%)	6 (27.3%)	5 (33.3%)	ns
De novo DSA	3 (11.5%)	3 (13.6%)	0 (0.0%)	ns

DD, deceased donor; DSA, donor-specific antibodies; DD_{CS}, deceased donor kidney preserved with static cold storage; DD_{HMP}, deceased donor kidney preserved with hypothermic machine perfusion; LD, living donor.

Data are presented as frequencies (column percentages) and medians (1st–3rd quartile). P-values are expressed for specified comparisons:

^aLD vs. DD.

^bLD vs. DD_{CS}.

ns = not significant.

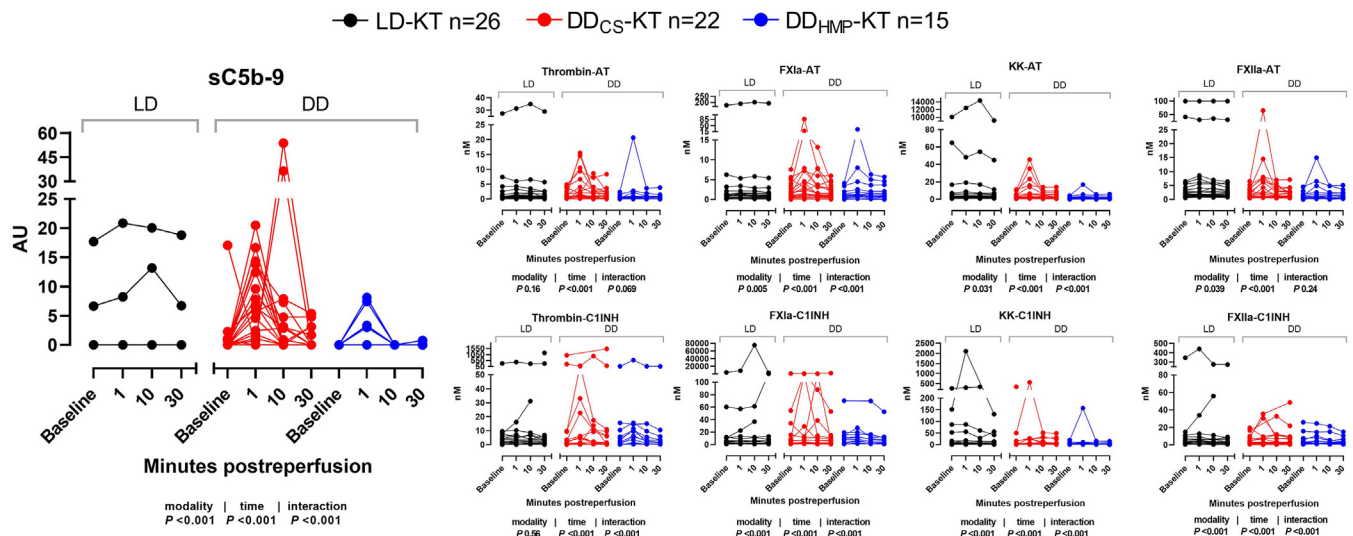


Figure 1. Levels of activation markers of the IIIS stratified by modality and time for the study population ($n = 63$). Connected scatter plots are color-coded for the specified modalities; black = LD-KT, red = DD_{CS}-KT and blue = DD_{HMP}-KT. P -values for differences from analysis of variance on aligned rank-transformed data by modality (LD-KT [$n = 26$], DD_{CS}-KT [$n = 22$] and DD_{HMP}-KT [$n = 15$]), time (baseline, 1, 10, and 30 minutes postreperfusion), and the interaction (between time and modality) are presented below each graph (ns = not significant). DD_{CS}, deceased donor kidney preserved with static cold storage; DD_{HMP}, deceased donor kidney preserved with hypothermic machine perfusion; LD, living donor.

Correlations of Markers Within and Between the Proteolytic Cascades of IIIS

Spearman's correlation analysis was performed to assess the relationship between IIIS activation markers within and across the proteolytic cascades. Apart from strong

overall correlations within each cascade system, the pattern of cross-cascade correlations differed between modalities (LD-KT, DD_{CS}-KT, and DD_{HMP}-KT) and/or time (Figure 2). This difference was most evident in the generation of the complement activation product sC5b-

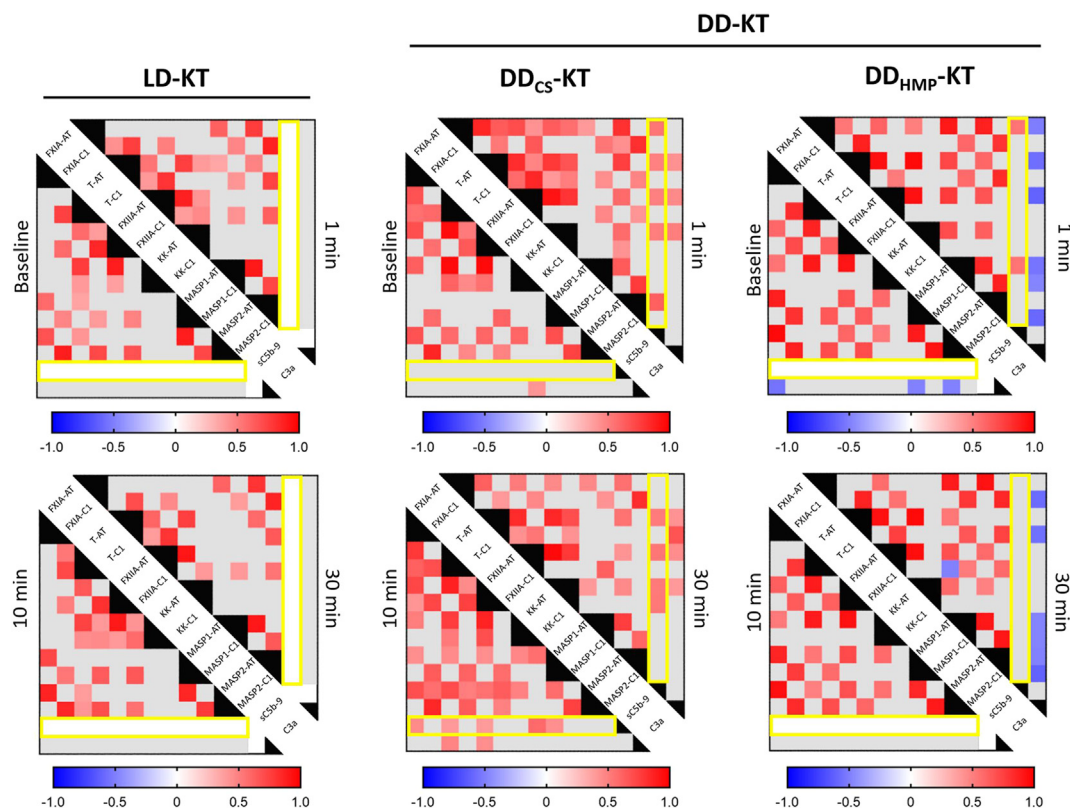


Figure 2. Spearman correlation matrices by modality (DD separated by preservation method) from baseline to 30 minutes postreperfusion. The red gradient denotes significant positive ρ coefficients, and blue denotes significant negative ρ coefficients. Gray squares denote nonsignificant correlations. White squares indicate incomputable correlations due to non or insufficient generation of sC5b-9. A yellow outline highlights sC5b-9. DD_{CS}, deceased donor kidney preserved with static cold storage; DD_{HMP}, deceased donor kidney preserved with hypothermic machine perfusion; LD, living donor.

9. In LD kidneys, sC5b-9 generation was absent in 24 out of 26 cases (92.3%) throughout the sampling time. Consequently, calculations on sC5b-9 correlations were not performed in LD-KT. Furthermore, correlations within and between the proteolytic cascades remained largely unchanged in LD-KT postreperfusion. Notably, sC5b-9 generation was detected at baseline and throughout the sampling period in only 2 out of 26 LD-KT cases. Therefore, the confounding effect of these 2 individuals was deemed to be minimal.

Although absent at baseline, the generation of sC5b-9 in DD_{CS} kidneys was promptly accompanied by activation of MASP-2 (ρ 0.65, $P = 0.001$), FXIa (ρ 0.57, $P = 0.005$), thrombin (ρ 0.53, $P = 0.012$), FXIIa (ρ 0.54, $P = 0.01$), and kallikrein (ρ 0.54, $P = 0.009$) at 1-minute postreperfusion, which indicated prompt coactivation of the complement, coagulation, and kallikrein-kinin systems and, by extension, a thrombo-inflammatory response that was retained throughout the sampling period. In DD_{HMP} kidneys, sC5b-9 generation was correlated with the activation of FXIa (ρ 0.58, $P = 0.024$) and MASP-1 (ρ 0.58, $P = 0.023$) at 1-minute postreperfusion; however, these correlations were not retained throughout the sampling time. This indicated a marked attenuation of the thrombo-inflammatory response by HMP. A complete correlation dataset is provided in [Supplementary Table S2A–4D](#).

Associations Between sC5b-9 Generation, CIT, DGF, and Mid-Term Graft Function

Soluble C5b-9 was considered as a response marker for IIIS activation based on the following 2 observations: (i) concurrent generation of sC5b-9 and other IIIS activation markers in response to IRI and (ii) attenuation of IIIS activation in DD_{HMP} kidneys corresponded to a reduction in sC5b-9 generation. This consideration was also relevant to prevent spurious correlations that would have occurred when testing for various markers of IIIS individually.

To evaluate the impact of IIIS activation on DGF and mid-term kidney function, we tested for associations when accounting for sC5b-9 generation at different time points and for the “area under the curve” (AUC) value of sC5b-9 from baseline to 30 minutes (AUC_{b–30}) for all patients. The eGFR slope was calculated to assess the mid-term (24 months) transplant function for all patients.

The sC5b-9 AUC_{b–30} showed a significant association with DGF (receiver operating characteristic AUC 0.81 [95% CI 0.61–1.00, $P = 0.012$]). Considering the impact of HMP on retained IIIS activation in Spearman’s correlation analysis, a stratification for retained IIIS response (defined as sC5b-9 generation at 30 minutes postreperfusion) showed that the subgroup of patients

with retained sC5b-9 activity (LD-KT 2/26, DD_{CS}-KT 6/22 and DD_{HMP}-KT 1/15) had lower eGFR levels throughout the 24-month follow-up period with median eGFR (IQR) differences between those with retained sC5b-9 and those without retained sC5b-9 generation at 1 month: 40.0 (20.0–49.0) versus 54.0 (40.0–62.5) $P = 0.020$; 3 months: 33.8 (27.2–49.4) versus 54.7 (42.6–64.9) $P = 0.041$; 6 months: 31.1 (24.0–40.6) versus 54.3 (40.8–66.0) $P = 0.003$; 12 months: 29.0 (22.2–37.5) versus 58.0 (39.2–70.5) $P = 0.004$; and 24 months: 26.0 (17.0–35.0) versus 56.5 (44.0–69.5) $P = 0.002$ ([Figure 3a](#)). Furthermore, the eGFR slope in this subgroup differed considerably compared to those without retained sC5b-9 generation (–0.31, [IQR –0.56 to –0.20] and 0.19 [IQR –0.13 to 0.39], $P = 0.007$). Two patients had missing data on 30-minute sC5b-9 levels and were excluded from the retained sC5b-9 analyses (both LD-KT). Cases with graft failure were assigned an eGFR value of 10 ml/min per 1.73 m² for the remaining follow-up. Two patients with retained sC5b-9 generation were excluded from the slope analyses due to patient death (DD_{CS}-KT, day 64) and loss to follow-up due to emigration (LD-KT, day 69).

To assess the clinical impact of HMP, the eGFR slopes within DD-KT were also compared ([Figure 3b](#)). Here, the eGFR slope from 1 to 24 months differed between DD_{CS}-KT cases with and without retained sC5b-9 generation (–0.48 [IQR –0.56 to –0.31] vs. 0.13 [IQR –0.09 to 0.51], $P = 0.014$). However, there was no difference in eGFR slopes when comparing DD_{HMP}-KT to DD_{CS}-KT with retained sC5b-9 generation (0.15 [IQR –0.36 to 0.37] vs. –0.48 [IQR –0.56 to –0.31], $P = 0.070$), as well as between DD_{HMP}-KT and DD_{CS}-KT without retained sC5b-9 generation (0.15 [IQR –0.36 to 0.37] vs. 0.13 [IQR –0.09 to 0.51], $P = 1.00$). The same was true when excluding the only patient in DD_{HMP}-KT with retained sC5b-9 generation.

To evaluate the association between CIT and IIIS activation in response to IRI, a receiver operating characteristic analysis was performed showing a cutoff threshold for CIT of 522.4 minutes (73% specificity, 83% sensitivity) for prompt generation of sC5b-9 at 1-minute postreperfusion ([Figure 4](#)).

DISCUSSION

In this cohort study of uremic patients undergoing KT, we utilized for the first time an extensive panel of activation markers of IIIS to uncover a simultaneous and prompt activation of the complement, coagulation, and kallikrein-kinin cascade systems following reperfusion, indicating a thrombo-inflammatory response to IRI. This complex inflammatory response was predominantly observed in patients receiving DD_{CS}

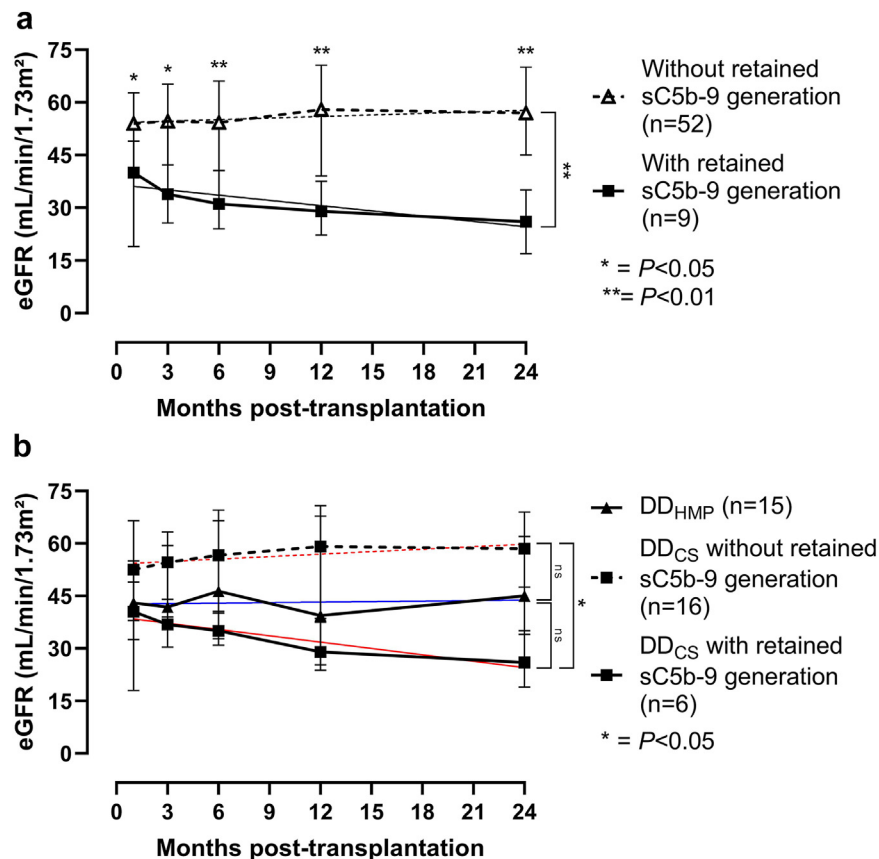


Figure 3. Progression of median eGFR (mL/min per 1.73 m²) with interquartile range from 1 to 24 months post-transplantation stratified (a) for retained sC5b-9 generation (defined as sC5b-9 generation at 30 minutes following reperfusion) within the study cohort and (b) for modalities DD_{HMP} and DD_{CS} with and without retained sC5b-9 generation. The eGFR slopes, calculated between 1 and 24 months, are represented by filled and dashed lines in the figures. The lines are color-coded in red for DD_{CS} and blue for DD_{HMP}. Two LD-KT patients with missing data on 30-minute sC5b-9 are excluded from the analyses. In addition, 2 patients (DD_{CS}-KT and LD-KT) are excluded from the slope analyses due to patient death on day 64 and loss to follow-up on day 69, respectively. DD_{CS}, deceased donor kidney preserved with static cold storage; DD_{HMP}, deceased donor kidney preserved with hypothermic machine perfusion; eGFR, estimated glomerular filtration rate.

kidneys, as the local generation of sC5b-9 was nearly absent in the LD-KT population (Figure 1). The simultaneous and consecutive measurements of key activation products of the blood cascade systems enabled us

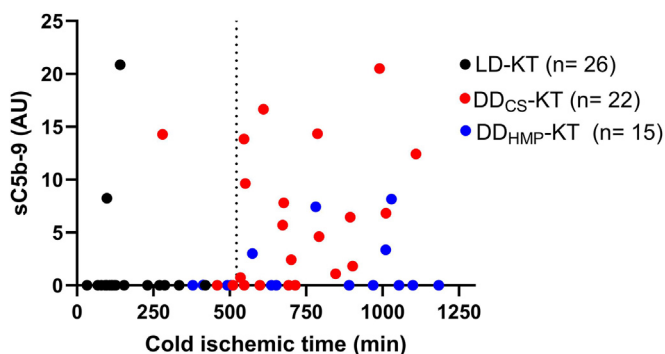


Figure 4. Scatterplot presenting levels of sC5b-9 by cold ischemic time. A dashed line denotes 522.4 minutes on the x-axis. DD_{CS}, deceased donor kidney preserved with static cold storage; DD_{HMP}, deceased donor kidney preserved with hypothermic machine perfusion; LD, living donor.

to further investigate the correlations within and between the proteolytic cascades. Apart from the strong interactions within each individual system, we found changes in cross-cascade correlations over time that occurred promptly postreperfusion. For instance, although complement activation product sC5b-9 showed no correlations with other plasma proteins at baseline in DD_{CS} kidneys, it became promptly associated with activation products of both coagulation (FXIa, thrombin) and kallikrein-kinin systems (FXIIa, KK) after reperfusion. These strong cross-cascade correlations endured throughout the sampling period but were completely absent in LD-KT (Figure 2).

Considering these findings, in conjunction with the inherently low exposure to graft ischemia within the LD-KT population, we argued that the prompt thrombo-inflammatory response in DD_{CS} kidneys is a direct consequence of increased graft immunogenicity evoked by early events during DD management, procurement, and organ preservation. Ischemia is

incumbent on these early events and a known risk factor for early graft dysfunction and premature graft loss in the DD-KT population.^{20–25}

To further elucidate the relationship between IRI-induced thromboinflammation and ischemia, the relationship between CIT and sC5b-9 generation was investigated. Soluble C5b-9 is widely recognized as a robust marker of complement activation. However, in the context of IRI, the concurrent generation of sC5b-9 alongside its strong association with other activation markers of IIIS prompted us to recognize sC5b-9 as a response marker of thromboinflammation. We found that a CIT cutoff threshold of 522.4 minutes (73% specificity, 83% sensitivity) was associated with generation of sC5b-9 postreperfusion (Figure 4). Notably, the median CIT in the DD-KT population was restricted to 11 hours and 32 minutes, which is below previously proposed risk threshold values for inferior transplant outcomes.^{26,27} Admittedly, the overall low exposure to CIT may explain the comparable overall graft-survival and patient survival between the LD-KT and DD-KT populations in our study. Nevertheless, we found that the generation of sC5b-9 was associated with DGF in DD-KT patients, even with a supposedly low burden of ischemia. Furthermore, we noticed that retained generation of sC5b-9 at 30 minutes postreperfusion was associated with mid-term (24 months) allograft dysfunction (Figure 3a). Indeed, patients with retained sC5b-9 generation belonged to the subpopulation experiencing a progressive decline in eGFR, whereas in patients with no retained sC5b-9 generation, mid-term kidney function was preserved. Given these findings, it is conceivable to assume a proportional relationship between the duration of CIT and the risk increment for an injurious thrombo-inflammatory response following reperfusion. The relationship between graft injury and CIT, even in cases of relatively shorter CIT durations, is not a new discovery. A recent meta-analysis focusing on LD-KT revealed that a CIT exceeding 4 hours increased the risk of DGF and has a detrimental effect on both 1-year and 5-year graft survival.²⁸

HMP has proven to be superior to static cold storage in preservation of kidneys from both expanded criteria and donation-after-circulatory-death donors.^{29–32} Preclinical evidence suggests that HMP might reduce the immunogenicity of kidney grafts by clearance of cell debris and toxic metabolites, optimization of renal vascularization, and active preservation of endothelial integrity.^{6,33–36} Interestingly, HMP exhibited an attenuation of the thrombo-inflammatory response to IRI, as evidenced by the disappearance of cross-cascade correlations at 10 and 30 minutes, in contrast to cold stored DD kidneys (Figure 2). Moreover, this attenuation was associated with

preserved eGFR over a 24-month follow-up period (Figure 3b). Notably, the homogeneity in kidney donor risk index and CIT among DD kidneys undergoing both preservation modalities adds further interest to these findings.

Limited clinical studies have investigated the initial engagement of IIIS in response to IRI, with a specific focus on individual cascade systems. For example, the complement system, assessed by sC5b-9 generation, has been shown to be promptly activated postreperfusion and found to be predictive for DGF and graft dysfunction at 1 year.^{11,37} Instant kallikrein-kinin system activation during liver transplant surgery has been linked to hemodynamic instability postreperfusion.³⁸ In addition, in liver recipients, prompt activation of the coagulation system has been reported postreperfusion.³⁹ Our study expands upon current knowledge by providing evidence from human transplant recipients with IRI of an intricate and comprehensive inflammatory response engaging all proteolytic cascades of the IIIS following IRI. This finding carries important clinical implications because it may explain the limited effectiveness observed in clinical trials that exclusively focus on targeting individual components of the IIIS, such as complement therapeutics, to mitigate IRI.^{12–14}

Interestingly, a clinical trial addressing IRI after DD-KT has shown promise in improving long-term transplant outcomes by using CIINH, a protease-inhibitor that effectively targets key components of the complement, kallikrein-kinin, and coagulation cascades.⁴⁰ Another recent study in a nonhuman primate model adopted a more comprehensive approach, combining CIINH with the anticoagulant heparin, resulting in a substantial reduction in DGF and systemic cytokine release following KT.⁴¹ Although the authors primarily attribute their success to complement system blockade, their findings challenge this assumption and align with our own, suggesting that therapeutic strategies targeting thromboinflammation may surpass limitations associated with interventions focused on individual cascade systems.

In our study, the lack of pathway-specific biomarkers, apart from MASP1 and MASP2, limits the precise identification of the underlying pathway responsible for IRI-induced thromboinflammation. Additional studies and pathway-specific biomarkers will be essential in uncovering the precise cascade activation sequence. Nevertheless, in DD_{CS} kidneys, the strong correlation between the generation of MASP2 and other markers of IIIS including sC5b-9, FXI, FXII and KK indicated a potential involvement of the lectin pathway. This notion finds support in preclinical evidence showing abnormal L-fucose expression on tubule cells in kidneys exposed to ischemia,

inducing autonomous binding to collectin-11, and activating the lectin pathway via MASP-2 interaction following reperfusion.⁴²

The study has points for critical discussion. Considering the single-center study design and relatively small patient population, we did not perform multiple testing for all study variables to prevent spurious associations. Instead, the generation of sC5b-9 was analyzed as a response biomarker of IRI-induced thromboinflammation. This consideration was conceivable due to the strong correlations of sC5b-9 to other key components of the proteolytic cascades postreperfusion. Next, the study population did not include donation-after-circulatory-death donors, a modality that was implemented at our center after the study period. Combined with a restricted overall CIT, the IIS response to more severe ischemic injury could not be investigated. Furthermore, kidney biopsies for parallel histological evaluations of tissue inflammation were not performed.

Nevertheless, we present a high-resolution investigation of the IIS suggesting that IRI can induce a prompt and interlinked activation of all proteolytic cascade systems involved in the IIS, leading to a harmful thrombo-inflammatory response. This indicates that individual components, such as the complement system, are part of a larger and more intricate response to IRI rather than being the sole primary effector system. Moreover, the generation of sC5b-9, in addition to serving as a complement activation marker, may also reflect the thrombo-inflammatory response triggered by IRI, because it strongly correlates with activation markers of other cascade systems. Lastly, the efficacy of HMP to mitigate IRI-induced thromboinflammation underscores the importance of preemptive interventions aimed at reducing graft immunogenicity before allograft reperfusion.

DISCLOSURE

All the authors declare no competing interests. No AI-associated technologies were used in the writing process.

ACKNOWLEDGMENTS

The authors thank Mrs. Helena Pollard for her assistance. This work was supported by the National Kidney Foundation, Swedish governmental funding of clinical research [2020-0106], and Research and Development Council of Region Skåne, Sweden [2022-1200], the Swedish Research Council [2020-05762], the Swedish Heart-Lung Foundation [HLF 2020-0398] and by faculty grants from Linnaeus University as well as in part by the DFG-grant CRC1149 A01 [INST 40/479-2].

Data Availability Statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Table S1. Multiple comparisons analysis of variance of aligned rank-transformed data for analysis of relationships between IIS activation markers and the 2 independent variables time and modality. Data are shown as median (1st–3rd quartile).

Tables S2A–4D. Assessment of the relationship between activation markers of IIS within and across the proteolytic cascade systems at all sampling time points for LD-KT (S2a-d), DD_{CS}-KT (S3a-d), and DD_{HMP}-KT (S4a-d) populations.

STROBE Statement.

REFERENCES

1. Ekdahl KN, Fromell K, Mannes M, et al. Therapeutic regulation of complement activation in extracorporeal circuits and intravascular treatments with special reference to the alternative pathway amplification loop. *Immunol Rev.* 2023;313: 91–103. <https://doi.org/10.1111/imr.13148>
2. Nilsson B, Eriksson O, Fromell K, Persson B, Ekdahl KN. How COVID-19 and other pathological conditions and medical treatments activate our intravascular innate immune system. *Front Immunol.* 2023;13:1030627. <https://doi.org/10.3389/fimmu.2022.1030627>
3. Zhao H, Alam A, Soo AP, George AJT, Ma D. Ischemia-reperfusion injury reduces long term renal graft survival: mechanism and beyond. *EBiomedicine.* 2018;28:31–42. <https://doi.org/10.1016/j.ebiom.2018.01.025>
4. Biglarnia AR, Huber-Lang M, Mohlin C, Ekdahl KN, Nilsson B. The multifaceted role of complement in kidney transplantation. *Nat Rev Nephrol.* 2018;14:767–781. <https://doi.org/10.1038/s41581-018-0071-x>
5. Ramirez GA, Rovere-Querini P, Sabbadini MG, Manfredi AA. Parietal and intravascular innate mechanisms of vascular inflammation. *Arthritis Res Ther.* 2015;17:16. <https://doi.org/10.1186/s13075-015-0528-2>
6. Yang Z, Zhong Z, Li M, et al. Hypothermic machine perfusion increases A20 expression which protects renal cells against ischemia/reperfusion injury by suppressing inflammation, apoptosis and necroptosis. *Int J Mol Med.* 2016;38:161–171. <https://doi.org/10.3892/ijmm.2016.2586>
7. Cravedi P, Heeger PS. Complement as a multifaceted modulator of kidney transplant injury. *J Clin Invest.* 2014;124:2348–2354. <https://doi.org/10.1172/JCI72273>
8. Arumugam TV, Shiels IA, Strachan AJ, Abbenante G, Fairlie DP, Taylor SM. A small molecule C5a receptor antagonist protects kidneys from ischemia/reperfusion injury in rats. *Kidney Int.* 2003;63:134–142. <https://doi.org/10.1046/j.1523-1755.2003.00737.x>
9. de Vries B, Matthijsen RA, Wolfs TGAM, Van Bijnen AA, Heeringa P, Buurman WA. Inhibition of complement factor C5 protects against renal ischemia-reperfusion injury: inhibition

- of late apoptosis and inflammation. *Transplantation*. 2003;75:375–382. <https://doi.org/10.1097/01.TP.0000044455.05584.2A>
10. Zhou W, Farrar CA, Abe K, et al. Predominant role for C5b-9 in renal ischemia/reperfusion injury. *J Clin Invest*. 2000;105:1363–1371. <https://doi.org/10.1172/JCI8621>
 11. Błogowski W, Dołęgowska B, Salata D, Budkowska M, Domański L, Starzyńska T. Clinical analysis of perioperative complement activity during ischemia/reperfusion injury following renal transplantation. *Clin J Am Soc Nephrol*. 2012;7:1843–1851. <https://doi.org/10.2215/CJN.02200312>
 12. Kaabak M, Babenko N, Shapiro R, Zokoyev A, Dymova O, Kim E. A prospective randomized, controlled trial of eculizumab to prevent ischemia-reperfusion injury in pediatric kidney transplantation. *Clin J Am Soc Nephrol*. 2018;22:e13129. <https://doi.org/10.1111/ajtpetr.13129>
 13. Schroppel B, Akalin E, Baweja M, et al. Peritransplant eculizumab does not prevent delayed graft function in deceased donor kidney transplant recipients: results of two randomized controlled pilot trials. *Am J Transplant*. 2020;20:564–572. <https://doi.org/10.1111/ajt.15580>
 14. Kassimatis T, Greenlaw R, Hunter JP, et al. Ex vivo delivery of microcept: a dose-finding study in pig kidney after showing a low dose is insufficient to reduce delayed graft function in human kidney. *Am J Transplant*. 2021;21:1012–1026. <https://doi.org/10.1111/ajt.16265>
 15. Ekberg H, Tedesco-Silva H, Demirbas A, et al. Reduced exposure to calcineurin inhibitors in renal transplantation. *N Engl J Med*. 2007;357:2562–2575. <https://doi.org/10.1056/NEJMoa067411>
 16. Nyman U, Grubb A, Larsson A, et al. The revised lund-malmö GFR estimating equation outperforms MDRD and CKD-EPI across GFR, age and BMI intervals in a large Swedish population. *Clin Chem Lab Med*. 2014;52:815–824. <https://doi.org/10.1515/ccm-2013-0741>
 17. Kozarcanin H, Lood C, Munthe-Fog L, et al. The lectin complement pathway serine proteases (MASPs) represent a possible crossroad between the coagulation and complement systems in thromboinflammation. *J Thromb Haemost*. 2016;14:531–545. <https://doi.org/10.1111/jth.13208>
 18. Back J, Lood C, Bengtsson AA, Ekdahl KN, Nilsson B. Contact activation products are new potential biomarkers to evaluate the risk of thrombotic events in systemic lupus erythematosus. *Arthritis Res Ther*. 2013;15:R206. <https://doi.org/10.1186/ar4399>
 19. Nilsson EK, Nilsson B, Pekna M, Nilsson UR. Generation of iC3 at the interface between blood and gas. *Scand J Immunol*. 1992;35:85–91. <https://doi.org/10.1111/j.1365-3083.1992.tb02837.x>
 20. Peters-Sengers H, Houtzager JHE, Idu MM, et al. Impact of cold ischemia time on outcomes of deceased donor kidney transplantation: an analysis of a national registry. *Transpl Direct*. 2019;5:e448. <https://doi.org/10.1097/TXD.0000000000000088>
 21. Helanterä I, Ibrahim HN, Lempinen M, Finne P. Donor age, cold ischemia time, and delayed graft function. *Clin J Am Soc Nephrol*. 2020;15:813–821. <https://doi.org/10.2215/CJN.13711119>
 22. Siedlecki A, Irish W, Brennan DC. Delayed graft function in the kidney transplant. *Am J Transplant*. 2011;11:2279–2296. <https://doi.org/10.1111/j.1600-6143.2011.03754.x>
 23. Perico N, Cattaneo D, Sayegh MH, Remuzzi G. Delayed graft function in kidney transplantation. *Lancet*. 2004;364:1814–1827. [https://doi.org/10.1016/S0140-6736\(04\)17406-0](https://doi.org/10.1016/S0140-6736(04)17406-0)
 24. Yarlagadda SG, Coca SG, Formica RN, Poggio ED, Parikh CR. Association between delayed graft function and allograft and patient survival: a systematic review and meta-analysis. *Nephrol Dial Transplant*. 2009;24:1039–1047. <https://doi.org/10.1093/ndt/gfn667>
 25. Damman J, Bloks VW, Daha MR, et al. Hypoxia and complement-and-coagulation pathways in the deceased organ donor as the major target for intervention to improve renal allograft outcome. *Transplantation*. 2015;99:1293–1300. <https://doi.org/10.1097/TP.0000000000000500>
 26. Barba J, Zudaire JJ, Robles JE, et al. Is there a safe cold ischemia time interval for the renal graft? *Actas Urol Esp*. 2011;35:475–480. <https://doi.org/10.1016/j.acuro.2011.03.005>
 27. Opelz G, Döhler B. Multicenter analysis of kidney preservation. *Transplantation*. 2007;83:247–253. <https://doi.org/10.1097/01.tp.0000251781.36117.27>
 28. van de Laar SC, Lafranca JA, Minnee RC, Papalois V, Dor FJMF. The impact of cold ischaemia time on outcomes of living donor kidney transplantation: a systematic review and meta-analysis. *J Clin Med*. 2022;11:1620. <https://doi.org/10.3390/jcm11061620>
 29. Savoye E, Macher MA, Videcoq M, et al. Evaluation of outcomes in renal transplantation with hypothermic machine perfusion for the preservation of kidneys from expanded criteria donors. *Clin Transpl*. 2019;33:e13536. <https://doi.org/10.1111/ctr.13536>
 30. Jiao B, Liu S, Liu H, Cheng D, Cheng Y, Liu Y. Hypothermic machine perfusion reduces delayed graft function and improves one-year graft survival of kidneys from expanded criteria donors: a meta-analysis. *PLoS One*. 2013;8:e81826. <https://doi.org/10.1371/journal.pone.0081826>
 31. Kox J, Moers C, Monbaliu D, et al. The benefits of hypothermic machine preservation and short cold ischemia times in deceased donor kidneys. *Transplantation*. 2018;102:1344–1350. <https://doi.org/10.1097/TP.0000000000002188>
 32. Tingle SJ, Figueiredo RS, Moir JA, Goodfellow M, Talbot D, Wilson CH. Machine perfusion preservation versus static cold storage for deceased donor kidney transplantation. *Cochrane Database Syst Rev*. 2019;3:CD011671. <https://doi.org/10.1002/14651858.CD011671.pub2>
 33. Vaziri N, Thuillier R, Favreau FD, et al. Analysis of machine perfusion benefits in kidney grafts: a preclinical study. *J Transl Med*. 2011;9:15. <https://doi.org/10.1186/1479-5876-9-15>
 34. Gallinat A, Efferz P, Paul A, Minor T. One or 4 h of “in-house” reconditioning by machine perfusion after cold storage improve reperfusion parameters in porcine kidneys. *Transpl Int*. 2014;27:1214–1219. <https://doi.org/10.1111/tri.12393>
 35. Chatauret N, Coudroy R, Delpech PO, et al. Mechanistic analysis of nonoxygenated hypothermic machine perfusion’s protection on warm ischemic kidney uncovers greater eNOS phosphorylation and vasodilation. *Am J Transplant*. 2014;14:2500–2514. <https://doi.org/10.1111/ajt.12904>
 36. Taylor MJ, Baicu SC. Current state of hypothermic machine perfusion preservation of organs: the clinical perspective. *Cryobiology*. 2010;60(suppl):S20–S35. <https://doi.org/10.1016/j.cryobiol.2009.10.006>

37. de Vries DK, van der Pol P, van Anken GE, et al. Acute but transient release of terminal complement complex after reperfusion in clinical kidney transplantation. *Transplantation*. 2013;95:816–820. <https://doi.org/10.1097/TP.0b013e31827e31c9>
38. Scholz T, Backman L, Mathisen O, et al. Activation of the plasma contact system and hemodynamic changes after graft revascularization in liver transplantation. *Transplantation*. 1995;60:36–40. <https://doi.org/10.1097/00007890-199507150-00007>
39. von Meijenfeldt FA, Burlage LC, Bos S, Adelmeijer J, Porte RJ, Lisman T. Elevated plasma levels of cell-free DNA during liver transplantation are associated with activation of coagulation. *Liver Transpl*. 2018;24:1716–1725. <https://doi.org/10.1002/lt.25329>
40. Huang E, Vo A, Choi J, et al. Three-year outcomes of a randomized, double-blind, placebocontrolled study assessing safety and efficacy of C1 esterase inhibitor for prevention of delayed graft function in deceased donor kidney transplant recipients. *Clin J Am Soc Nephrol*. 2020;15:109–116. <https://doi.org/10.2215/CJN.04840419>
41. Eerhart MJ, Reyes JA, Blanton CL, et al. Complement blockade in recipients prevents delayed graft function and delays antibody-mediated rejection in a nonhuman primate model of kidney transplantation. *Transplantation*. 2022;106:60–71. <https://doi.org/10.1097/TP.0000000000003754>
42. Farrar CA, Tran D, Li K, et al. Collectin-11 detects stress-induced L-fucose pattern to trigger renal epithelial injury. *J Clin Invest*. 2016;126:1911–1925. <https://doi.org/10.1172/JCI83000>