OPEN

The Role of Complement Split-products as Biomarkers for Acute Antibody-mediated Rejection of Kidney Allografts

Bassam G. Abu Jawdeh, MD,[1] Begona Campos-Naciff, PhD,[2] Karthikeyan Meganathan, MS,[2] E. Steve Woodle, MD,[3] and Bradley P. Dixon, MD[4]

Background. Acute antibody-mediated rejection (AMR) is mediated by the activation of the classical complement system in addition to noncomplement-dependent inflammatory pathways. Complement fixation by donor-specific antibodies leads to cleavage of the complement proteins C4, C3, and C5 to produce multiple complement split-products (CSP) and the end-effector membrane attack complex, C5b-9. In this study, we investigate CSP as potential biomarkers for AMR. **Methods.** In an Institutional Review Board–approved, prospective, controlled study, CSP levels were measured in blood and urine samples from consecutive kidney transplant recipients with biopsy-proven AMR (n = 10), acute cellular rejection (ACR) (n = 5), or no rejection (n = 5). After obtaining informed consent, samples were collected at the time of biopsy (day 0) and days 15 (end of rejection treatment) and 30 postbiopsy for AMR and ACR patients. ELISA was used to measure C5a, C4d, and soluble C5b-9 concentrations in blood and urine, in addition to factor Bb (Bb) concentration in blood only. Kidney transplant histopathology was evaluated using the Banff 2013 classification. Rejection treatment and follow-up were performed per standard of care. **Results.** Blood and urine CSP levels adjusted to urine creatinine were not elevated in AMR compared to no rejection and ACR arms. There was significant variability in CSP concentration within each of the study groups. **Conclusion.** Our study does not support the utility of CSP as surrogate biomarkers of AMR; however, it is limited by the small sample size and larger studies may be warranted.

(*Transplantation Direct* 2022;8: e1366; doi: 10.1097/TXD.0000000000001366).

Received 28 May 2022. Revision received 24 June 2022.
Accepted 27 June 2022.

[1] *Division of Nephrology, Mayo Clinic, Phoenix, AZ.*
[2] *Division of Nephrology, University of Cincinnati, Cincinnati, OH.*
[3] *Division of Transplant Surgery, University of Cincinnati, Cincinnati, OH.*
[4] *Division of Pediatric Nephrology, Children's Hospital Colorado Anschutz Medical Campus, Aurora, CO.*
ORCID: 0000-0002-1530-6665

B.G.A.J. oversaw study conception, design and procedures, and wrote the article. B.C.-N. contributed to drafting the article. K.M. performed the statistical analysis. E.S.W. contributed to drafting and finalizing the article. B.P.D. contributed to study conception, sample analysis, and finalization of the article.
B.G.A.J. received funding from Dialysis Clinic, Inc (DCI). DCI had no role in study design, execution, or article preparation.
The authors declare no conflicts of interest.
Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantationdirect.com).

Correspondence: Bassam G. Abu Jawdeh, MD, Division of Nephrology, Mayo Clinic Arizona, 5777 E Mayo Blvd, Phoenix, AZ 85054. (abujawdeh.bassam@mayo.edu).

ISSN: 2373-8731
DOI: 10.1097/TXD.0000000000001366

Acute antibody-mediated rejection (AMR) triggered by donor-specific antibodies (DSAs) against HLA and non-HLA antigens is a major cause of poor allograft outcomes in kidney transplant recipients.[1] AMR can be triggered by preformed or post-transplant de novo DSAs often leading to allograft loss.[1] Kidney transplantation, however, offers a better quality of life and a clear survival advantage compared to chronic dialysis.[2,3] This holds true even in patients who are sensitized to HLA antigens and at risk for AMR; hence, more programs are offering transplants to the highly sensitized patient population.[4] As a result, the burden of AMR is not expected to decrease anytime soon.

It is, therefore, prudent to continue to investigate strategies to better characterize and treat AMR. With the availability of costly potential therapies, such as the terminal complement C5-blocking monoclonal antibody, the exact understanding of the pathogenesis of a specific AMR episode will allow better allocation of treatment based on the underlying pathobiology.[5]

AMR is mediated by the activation of the classical complement pathway, which is initiated by binding of the Fc portion of DSA to C1q, whereas the alternative pathway—involving complement protein Bb—is not believed to play a major role in AMR pathogenesis.[6] Complement fixation leads to a cascade of downstream interactions, which

include cleavage of complement proteins, C4, C3, and C5, to produce multiple complement split-products (CSP), including C3a, C3d, C4d, and C5a. The terminal complement component, C5, is cleaved by the C5 convertase to produce C5b, the precursor of the effector C5b-9 complex which induces endothelial cell injury and activation.[6] Other AMR mechanisms were proposed later. These include C3a-mediated, C5a-mediated, and Fc receptor–mediated recruitment of neutrophils, macrophages, and natural killer cells to the allograft and direct DSA-induced activation of endothelial cells.[6] Moreover, although immunoglobulin isotypes IgG1 and IgG3 are typically complement fixing, IgG2 and IgG4 are injurious to the allograft by inflammatory, noncomplement activating pathways.[7] Various AMR pathologic mechanisms are not mutually exclusive; therefore, understanding the relative contribution of each pathway may determine the appropriate treatment modality. In this study, we investigate blood and urine CSP as pathogenesis-based biomarkers of AMR with potential for allowing individualization of therapy.

PARTICIPANTS AND METHODS

This study was funded by Dialysis Clinic Inc and approved by the University of Cincinnati Institutional Review Board. Study procedures were in accordance with the International Conference on Harmonization Guidelines for Good Clinical Practice and with the ethical principles laid down in the Declaration of Helsinki.

The study design is noninterventional, observational, and prospective investigating CSP as potential biomarkers in 10 consecutive kidney transplant recipients with AMR (AMR group) and comparing them with 5 with acute cellular rejection (ACR) (control group 1) and 5 recipients with no rejection (NR) (control group 2). Patients with mixed rejection were included under the AMR group.

Study Protocol

After the study has been fully explained, written informed consent was obtained from the subjects. Blood and urine specimen collection was initiated after patients' consent. In the AMR and ACR groups, blood and urine specimens were collected just after diagnosis of rejection, at enrollment (day 0), and after treatment completion at 15 (±5) and 30 (±5) days (**Table S1, SDC**, http://links.lww.com/TXD/A440). C5a, C4d, soluble C5b-9 (sC5b-9), and factor Bb (Bb) levels were measured in blood samples. C5a, C4d, and sC5b-9 levels were measured in urine samples.

In the NR group, blood and urine specimens were collected at study entry after the kidney biopsy read (day 0) (**Table S1, SDC**, http://links.lww.com/TXD/A440). An additional 10–20 mL of blood and urine per patient (throughout the study) were collected with standard of care labs (**Table S1, SDC**, http://links.lww.com/TXD/A440).

Treatment of rejection followed the transplant center's standard of care protocols. AMR was treated with plasmapheresis, followed by bortezomib and low dose intravenous immunoglobulin with or without rituximab. ACR treatment included methylprednisolone for Banff 1A/1B ACR and antithymocyte globulin for Banff 2/3 and ACR refractory to corticosteroids.

Sample Handling and Analysis

At the point of collection, blood (EDTA plasma) and urine samples were labeled with the study subject and specimen identifier codes (deidentified from the electronic medical records) that were used for processing and analyzing the samples. The study samples were kept in a locked −80 °C freezer separate from clinical samples. Blood C5a, C4d, sC5b-9 and Bb, and urine C5a, C4d, and sC5b-9 levels were measured using enzyme-linked immunoassays at Cincinnati Children's Nephrology Lab. Urine CSP levels were adjusted to spot urine creatinine (UCr) and denoted as CSP/UCr ratio.

Statistical Analysis

Descriptive statistics are used to summarize results in tables and graphs. Categorical variables are summarized as count (n) and proportion (%). Continuous variables are summarized using median, first quartile (Q1) and third quartile (Q3), or minimum and maximum. Differences in medians of continuous variables across groups are evaluated using the nonparametric Kruskal-Wallis test. All statistical tests utilized $P < 0.05$ as the significance threshold. All statistical analyses were performed using SAS (9.4 SAS Institute, Cary, NC).

RESULTS

Baseline characteristics of the subjects including demographics, transplant type, induction, maintenance immunosuppression, DSA, serum creatinine, and spot urine for protein to creatinine ratio are shown in Table 1.

Blood CSP Levels

Bb median (interquartile range [IQR]) μg/mL was 0.92 (0.91–1.22), 0.69 (0.66–0.79), and 0.9 (0.69–1.27) on day 0 in the NR, ACR, and AMR groups, respectively ($P = 0.05$); 0.67 (0.56–0.83) and 0.72 (0.58–0.84) on day 15 in the ACR and AMR groups, respectively ($P = 1.00$); and 0.71 (0.65–0.76) and 1.03 (0.65–1.44) on day 30 in the ACR and AMR groups, respectively ($P = 0.20$). C5a median (IQR) ng/mL was 12.70 (9.74–29.24), 6.62 (6.16–6.62), 9.36 (5.26–10.86) on day 0 in the NR, ACR, and AMR groups, respectively ($P = 0.04$); 7.11 (5.52–8.39) and 6.82 (2.22–11.16) on day 15 in the ACR and AMR groups respectively ($P = 1.00$); and 7.38 (5.52–9.83) and 7.87 (6.64–11.26) on day 30 in the ACR and AMR groups, respectively ($P = 0.67$). C4d median (IQR) μg/mL was 1.96 (1.12–2.38), 1.19 (0.49–2.66), and 1.58 (1.05–2.52) on day 0 in the NR, ACR, and AMR groups, respectively ($P = 0.90$); 1.16 (0.39–3.85) and 0.46 (0.21–0.56) on day 15 in the ACR and AMR groups, respectively ($P = 0.23$); and 1.58 (1.33–3.15) and 1.09 (0.63–2.03) on day 30 in the ACR and AMR groups, respectively ($P = 0.20$). sC5b-9 median (IQR) ng/mL was 347.46 (280.92–441.43), 142.29 (127.48–227.98), and 240.01 (197.87–251.44) on day 0 in the NR, ACR, and AMR groups, respectively ($P = 0.06$); 196.84 (133.73–266.20) and 190.57 (178.40–252.04) on day 15 in the ACR and AMR groups, respectively ($P = 0.89$); and 227.69 (164.93–243.82) and 271.62 (246.02–410.34) on day 30 in the ACR and AMR groups, respectively ($P = 0.07$) (Fig. 1).

The C4d level significantly decreased within the AMR group at day 15; however, rebounded at day 30 ($P < 0.01$). The rest of the P values from Kruskal-Wallis test comparing

TABLE 1.

Patient characteristics

	NR; n = 5	ACR; n = 5	AMR; n = 10
Demographics			
Age, y[a]	62.4 (43.3–71.1)	61.0 (35.4–67.7)	41.2 (26.8–68.8)
Sex, %			
Male	60.0	80.0	70.0
Race, %			
Black	60.0	0.0	30.0
Transplant type, %			
DDKT	80.0	60.0	50.0
LRKT	20.0	40.0	20.0
LUKT	0.0	0.0	30.0
cPRA[a] (at time of transplant)	1.0 (0.0–73.0)	0.0 (0.0–1.0)	n = 9 30.0 (0.0–86.0)
HLA mismatches[a]	4.0 (2.0–4.0)	4.0 (1.0–5.0)	n = 9 4.0 (2.0–6.0)
Induction			
Thymoglobulin, %	80.0	60.0	70.0
Campath, %	0.0	0.0	0.0
Simulect, %	20.0	40.0	0.0
Methylpredisone, %	0.0	0.0	10.0
Unknown, %	0.0	0.0	20.0
Maintenance immunosupression (at time of biopsy)			
Tacrolimus, %	100.0	100.0	100.0
Cyclosporin, %	0.0	0.0	0.0
Mycophenolate, %	100.0	100.0	90.0
Prednisone, %	0.0	40.0	30.0
Time from transplant to biopsy			
Time, y[a]	0.2 (0.0–0.2)	0.3 (0.0–5.2)	2.7 (0.1–11.0)
iDSA[b]			
Baseline[a] (MFI)		n = 1 3700.0	n = 3 12400.0 (6800.0–23000.0)
Day 0[a] (MFI)			n = 9 10600.0 (3100.0–25800.0)
Day 30[a] (MFI)		n = 1 8100.0	n = 8 5200.0 (1200.0–23000.0)
Creatinine, mg/dL			
Baseline[a]	1.1 (1.1–3.0)	1.3 (1.0–4.1)	1.4 (0.8–8.3)
Day 0[a]	1.8 (1.5–4.4)	2.3 (1.7–5.0)	1.7 (1.2–8.3)
Day 30[a]		n = 4 1.9 (1.8–2.4)	1.8 (1.2–9.0)
Protein/creatinine ratio			
Baseline[a]	0.1 (0.1–2.1)	n = 4 0.4 (0.2–1.6)	n = 9 1.2 (0.2–5.1)
Day 0[a]	0.3 (0.1–3.5)	n = 4 1.0 (0.3–1.6)	n = 6 1.0 (0.3–5.1)
Day 30[a]		n = 4 0.4 (0.2–1.9)	n = 3 0.2 (0.2–2.3)

[a]Numerical values are reported as median (minimum, maximum).
[b]iDSA represents DSA with the highest MFI value.
ACR, acute cellular rejection; AMR, antibody-mediated rejection; cPRA, calculated panel reactive antibody; DDKT, deceased donor kidney transplant; iDSA, immunodominant donor-specific antibody; LRKT, living-related kidney transplant; LUKT, living-unrelated kidney transplant; MFI, mean fluorescence intensity; NR, no rejection.

blood CSP levels across time points within ACR and AMR groups were not significant (Fig. 1).

Urine CSP Levels Expressed as CSP/Ucr

C5a/UCr median (IQR) ng/mL/mg/dL was 0.10 (0.05–0.27), 0.23 (0.22–0.38), 0.23 (0.17–0.58) on day 0 in the NR, ACR and AMR groups, respectively (P = 0.70); 0.26 (0.05–0.66) and 0.17 (0.06–0.42) on day 15 in the ACR and AMR groups, respectively (P = 0.78); and 0.13 (0.03–0.26) and 0.15 (0.06–0.22) on day 30 in the ACR and AMR groups, respectively (P = 0.89). C4d/UCr median (IQR) µg/mL/mg/dL was 0.02 (0.01–0.04), 0.05 (0.02–0.07), and 0.01 (0.01–0.06) on day 0 in the NR, ACR, and AMR groups, respectively (P = 0.48); 0.04 (0.00–0.10) and 0.04 (0.01–0.04) on day 15 in the ACR and AMR groups, respectively (P = 0.67); and 0.01 (0.00–0.03) and 0.02 (0.01–0.05) on day 30 in the ACR and AMR groups, respectively (P = 0.16). sC5b-9/UCr median (IQR) ng/mL/mg/dL was 3.10 (2.89–8.94), 6.59 (0.85–12.77),

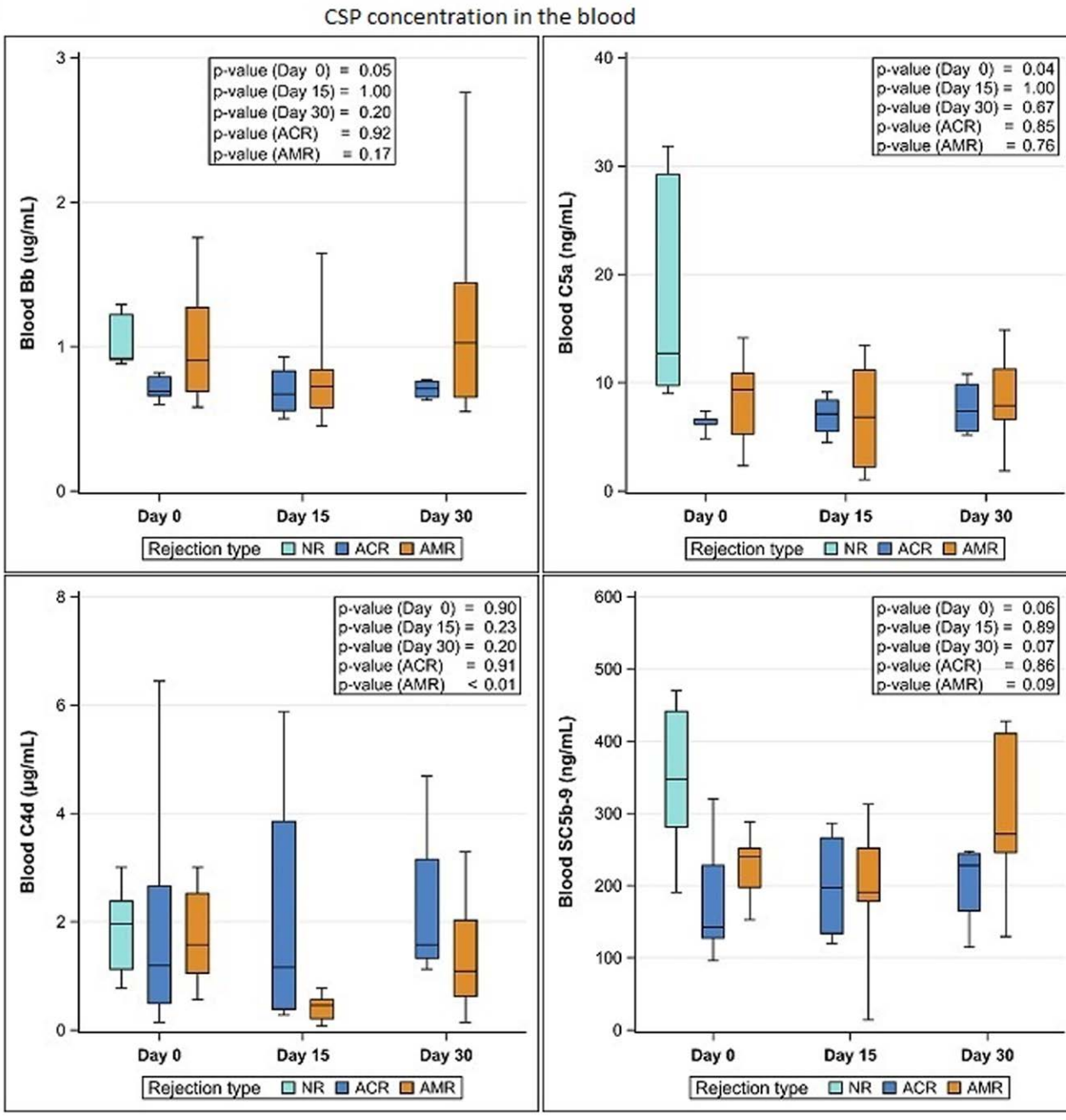

FIGURE 1. CSP concentration in the blood. ACR, acute cellular rejection; AMR, antibody-mediated rejection; Bb, factor Bb; CSP, complement split-products; NR, no rejection; SC5b-9, soluble C5b-9.

and 3.91 (1.75–19.20) on day 0 in the NR, ACR, and AMR groups respectively (P = 1.00); 1.07 (0.00–30.02) and 2.33 (0.73–7.82) on day 15 in the ACR and AMR groups, respectively (P = 0.48); and 4.93 (2.14–6.03) and 4.00 (0.34–9.43) on day 30 in the ACR and AMR groups, respectively (P = 0.78) (Fig. 2).

P values from Kruskal-Wallis test comparing Urine CSP/UCr levels across time points within ACR and AMR groups were not significant (Fig. 2).

DISCUSSION

Major strides toward diagnosis of AMR by both invasive and noninvasive molecular techniques have been achieved. Perhaps the breakthrough that allowed better diagnosis of AMR was the discovery of peritubular capillary C4d (a C4b split product) deposition by immunoperoxidase staining.[8] Subsequently, however, it has been shown that C4d staining is not highly sensitive, with about a third of AMR episodes being C4d negative.[9] This can be explained by either one of the following: (1) complement fixation and activation of the classical pathway is not the primary mechanism of AMR in these patients or (2) complement regulatory proteins (such as factor I), responsible for cleaving active CSP (C3b and C4b) to inactive forms (C3d and C4d) are functionally deficient. The latter, that is, lacking the mechanism to turn off the complement cascade, might explain the more severe phenotype often encountered in C4d-negative AMR.

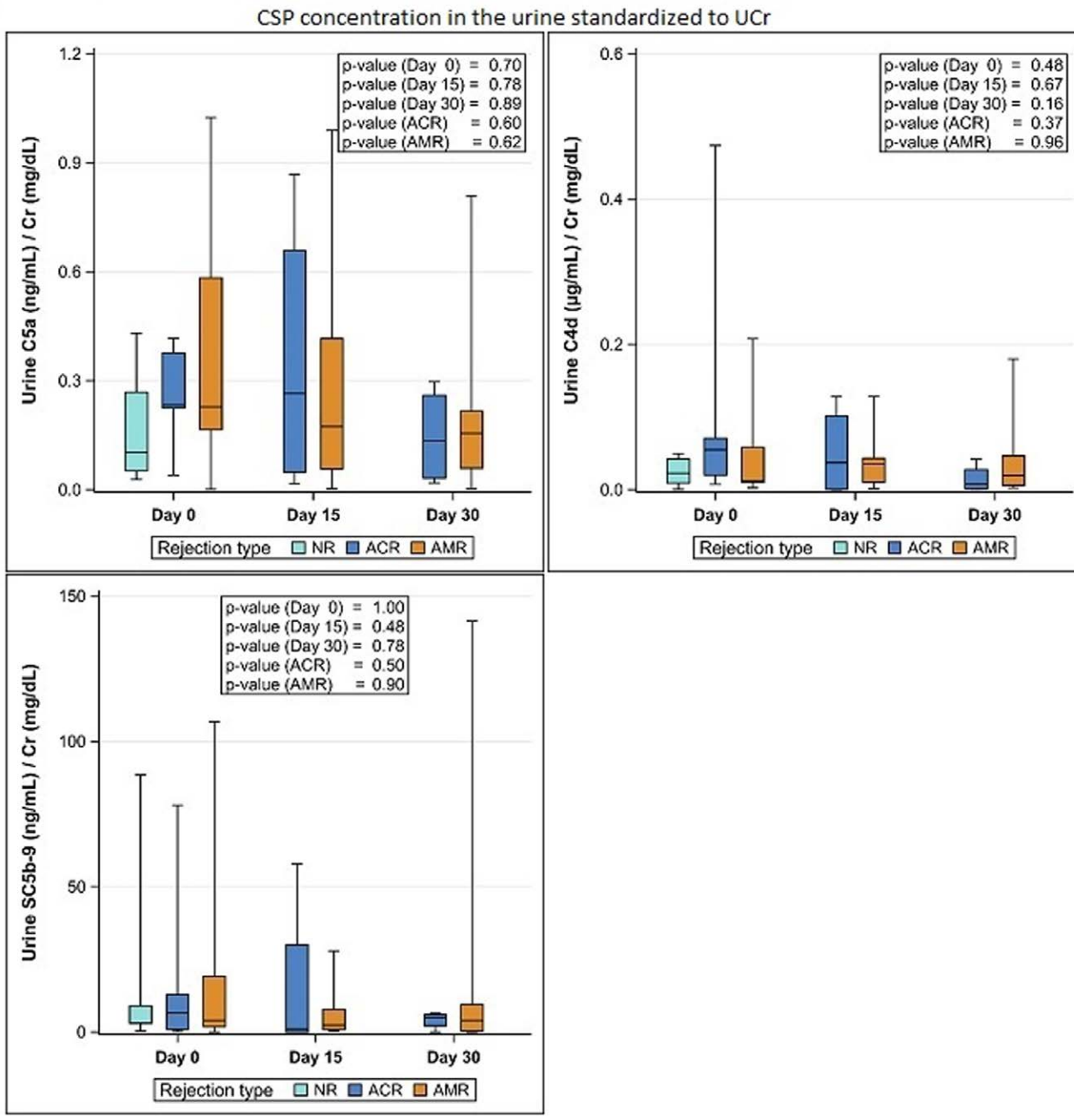

FIGURE 2. CSP concentration in the urine standardized to urine creatinine (UCr). ACR, acute cellular rejection; AMR, antibody-mediated rejection; CSP, complement split-products; NR, no rejection; SC5b-9, soluble C5b-9.

AMR is associated with poor graft outcomes and is notorious for lack of optimal response to treatment. This lack of response could be because of inadequate phenotyping of AMR cases. Most centers have standardized protocols for AMR treatment that include a combination of plasmapheresis, intravenous immunoglobulin, ± anti–B-cell antibody ± anti-plasma cell therapies ± anti-C5 monoclonal antibody. These protocols are often implemented irrespective of whether a patient has C4d staining or not, extent of glomerulitis and peritubular capillaritis, and type and titer of DSA. It is plausible that identifying biomarkers separating complement fixing from nonfixing pathways, and upstream versus downstream complement pathways would allow better dissection of various AMR episodes. This may lead to administration of tailored therapy, as opposed to a "one-size fits all" strategy, which is often costly and associated with suboptimal outcomes.

Investigating novel molecular techniques including microarray analysis, such as the molecular microscope, and donor-derived cell-free DNA have shown promise in enhancing rejection diagnostics.[10,11] To our knowledge, however, since Müller et al demonstrated that urinary C5a correlated with overall rejection in 1997, no other published paper attempted to reproduce similar findings by measuring blood and urine CSP in AMR.[12] This, nonetheless, could be the result of publication bias. Our goal from this study was to examine CSP as potential biomarkers with utility in pathogenesis-based stratification of AMR.

Except for a statistically significant decrease in blood C4d level within the AMR group at day 15, we did not observe any significant difference in other blood and urine CSP levels within or across different groups over time. The low blood C4d level at day 15 could be attributed to response to AMR treatment; however, this is negated by the fact that C4d level was not elevated in the AMR group compared with NR and ACR groups at day 0. Our study was not powered to examine associations between severity of AMR and CSP levels; however, we did not observe any signal of a positive correlation. Moreover, blood and urine CSP level is a result of complement activation in addition to tissue deposition and urinary excretion rate, which are hard to adjust for. In summary, this article does not support the utility of CSPs as biomarkers of AMR. Our sample size is small, however, and only suitable to detect a signal of a positive correlation between CSP and AMR if any. Therefore, similar but larger studies are warranted for more definitive conclusions.

REFERENCES

1. Djamali A, Kaufman DB, Ellis TM, et al. Diagnosis and management of antibody-mediated rejection: current status and novel approaches. *Am J Transplant*. 2014;14:255–271.
2. Evans RW, Manninen DL, Garrison LP Jr, et al. The quality of life of patients with end-stage renal disease. *N Engl J Med*. 1985;312:553–559.
3. Port FK, Wolfe RA, Mauger EA, et al. Comparison of survival probabilities for dialysis patients vs cadaveric renal transplant recipients. *JAMA*. 1993;270:1339–1343.
4. Montgomery RA, Lonze BE, King KE, et al. Desensitization in HLA-incompatible kidney recipients and survival. *N Engl J Med*. 2011;365:318–326.
5. Stegall MD, Diwan T, Raghavaiah S, et al. Terminal complement inhibition decreases antibody-mediated rejection in sensitized renal transplant recipients. *Am J Transplant*. 2011;11:2405–2413.
6. Stegall MD, Chedid MF, Cornell LD. The role of complement in antibody-mediated rejection in kidney transplantation. *Nat Rev Nephrol*. 2012;8:670–678.
7. Valenzuela NM, McNamara JT, Reed EF. Antibody-mediated graft injury: complement-dependent and complement-independent mechanisms. *Curr Opin Organ Transplant*. 2014;19:33–40.
8. Feucht HE, Schneeberger H, Hillebrand G, et al. Capillary deposition of C4d complement fragment and early renal graft loss. *Kidney Int*. 1993;43:1333–1338.
9. Sellarés J, Reeve J, Loupy A, et al. Molecular diagnosis of antibody-mediated rejection in human kidney transplants. *Am J Transplant*. 2013;13:971–983.
10. Halloran PF, Reeve J, Akalin E, et al. Real time central assessment of kidney transplant indication biopsies by microarrays: the INTERCOMEX study. *Am J Transplant*. 2017;17:2851–2862.
11. Bloom RD, Bromberg JS, Poggio ED, et al; Circulating Donor-Derived Cell-Free DNA in Blood for Diagnosing Active Rejection in Kidney Transplant Recipients (DART) Study Investigators. Cell-free DNA and active rejection in kidney allografts. *J Am Soc Nephrol*. 2017;28:2221–2232.
12. Müller TF, Kraus M, Neumann C, et al. Detection of renal allograft rejection by complement components C5A and TCC in plasma and urine. *J Lab Clin Med*. 1997;129:62–71.