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CLINICAL RESEARCH

Urine and Plasma Complement Ba Levels During Disease Flares in Patients With Antineutrophil Cytoplasmic Autoantibody–Associated Vasculitis

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Introduction: Although the alternative complement pathway has been implicated in the pathogenesis of antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV), the specific nature of its involvement is unclear. This study measured levels of urine and plasma complement fragment Ba at multiple time points in a group of patients with AAV.

Methods: The complement fragment Ba was measured by enzyme-linked immunosorbent assay in serial urine and plasma samples from 21 patients with AAV who developed a renal flare, 19 who developed a nonrenal flare, and 20 in long-term remission. Urine Ba levels were corrected for urine creatinine concentration. Changes in Ba levels were modeled using mixed linear-effect models. A logistic regression model was fit to predict a renal flare using Ba levels at the time of flare versus the nonrenal flare and long-term remission groups.

Results: Data from 60 patients with AAV were used for this analysis; 53% were male, 93% were White, and 74% had antiproteinase3-ANCA. Urine Ba levels increased at renal flare ($P < 0.001$) but remained stable during a nonrenal flare or long-term remission. Plasma Ba levels were stable over time in all groups. Urine Ba levels predicted a renal flare with an area under the curve of 0.76 ($P < 0.001$), with a cutoff of 12.53 ng/mg urine creatinine yielding a sensitivity of 76.2% and a specificity of 68.4%.

Conclusion: Urine Ba levels, but not plasma Ba levels, are increased at the time of a renal flare in AAV, suggesting intrarenal complement activation and highlighting the potential use of this biomarker for surveillance of active renal vasculitis.

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AAV is a group of systemic autoimmune diseases characterized by necrotizing inflammation of small-to-medium–sized vessels of many organ systems, including the kidneys where they manifest with a necrotizing and pauci-immune crescentic glomerulonephritis [#197].[1,2] In AAV, loss of tolerance to antigens typically present in neutrophil granules leads to the

formation of autoantibodies. Neutrophil priming is followed by surface overexpression of autoantigens, that, when engaged by their respective ANCAs, cause neutrophil activation and degranulation culminating in tissue injury and inflammation.[1,2] The contribution of the complement system to the pathogenesis of AAV has been increasingly appreciated. Experiments in murine models of myeloperoxidase (MPO)-AAV revealed that complement depletion is protective against the development of crescentic glomerulonephritis, an effect that is mainly mediated by the alternative complement pathway, specifically the complement factor 5a (C5a).[3,4] This led to the clinical development of the C5a receptor antagonist avacopan that is currently approved for treatment of AAV.[5,6]

Given the involvement of the complement in the pathogenesis of AAV, complement activation products have been explored as biomarkers of disease activity. Plasma levels of C5a, Bb, and soluble C5b-9 (sC5b-9) were elevated at the time of active disease and decreased after treatment in 20 patients with AAV who had kidney involvement.[7] Urine levels of Bb, C3a, C5a, and sC5b-9) were higher in patients with active renal vasculitis than patients with inactive renal vasculitis,[8] supporting the potential utility of these biomarkers. However, longitudinal assessment of plasma and urine complement activation products levels leading to flare, the relationship between the plasma and urine levels of these markers, and their specificity for kidney involvement has not been explored. The current study evaluated the utility of plasma and urine levels of Ba as biomarkers of vasculitis activity in patients with AAV with and without kidney involvement.

METHODS

Patient Cohort

The analysis used data and biospecimens from 60 patients with AAV participating in the Vasculitis Clinical Research Consortium Longitudinal Studies. The Vasculitis Clinical Research Consortium is an integrated network of academic medical centers, patient support organizations, and clinical research resources dedicated to conducting clinical research in vasculitis. The eligibility criteria for patients enrolled in the Vasculitis Clinical Research Consortium Longitudinal Studies are depicted in Supplementary Table S1. Patients were selected for analysis if (i) they had stored urine and plasma specimens collected at the time of a renal flare (ReF) or a nonrenal flare (NReF) and (ii) had stored urine and plasma specimens from 2 to 3 consecutive visits at a time they were in remission preceding their flare visit. A third cohort of patients who were in long-term remission (LTR) on 3 to 4 consecutive visits with available urine and plasma specimens were included as controls. For the purposes of this analysis, a renal flare was defined as a score in the renal components of the Birmingham Vasculitis Activity Score (BVAS/WG)[9] of $\geq$3, with new hematuria or red blood cell casts, or an increase in serum creatinine deemed by the treating physician to be due to active AAV. A nonrenal flare was defined as an increase in BVAS/WG of $\geq$2 or a new increase in the physician global assessment score of $>$3 without any of the renal flare features described previously. Visits were numbered $-$3, $-$2, $-$1, and 0, with visit 0 representing the flare visit in the ReF and NReF groups and the last available remission visit in the LTR group. The analysis was performed using visit number because the periods between visits were not uniform.

Complement Fragment Measurements

Sandwich enzyme-linked immunosorbent assays were used to measure plasma and urine Ba levels (Quidel Corporation, San Diego, CA) according to the manufacturer's recommendations. In brief, the samples were diluted at ratios of 1:15, 1:30, or 1:300 for urine and 1:1000 or 1:2000 for plasma. Furthermore, 100 μl of diluted patient and Ba control samples were pipetted into wells coated with antihuman Ba murine monoclonal antibody and incubated for 60 minutes, washed with wash buffer, then incubated for 60 minutes with horseradish peroxidase-conjugated murine antihuman Ba suspended in a stabilizing buffer. After another wash cycle, they were incubated with substrate solution (3,3′,5,5′-tetramethylbenzidene) for 15 minutes after which color development was stopped with 1N HCl. Enzyme-linked immunosorbent assay plates were read at an optical density of 450 nm. All samples were measured in duplicate. Samples with measurement pairs with an interassay coefficient of variation $>$20% had measurements repeated. Samples with measurements above the upper limit of the standard curve were also repeated using a higher dilution. Urine Ba levels were corrected for urine creatinine (Cr) measured using a colorimetric assay (Enzo Life Sciences Inc., Farmingdale, NY). Urine Ba/Cr ratios (ng/mg) were used for analysis. For this study, urine C5a, Bb, and sC5b-9 were also measured but only Ba is reported here as $>$50% of the samples had C5a, Bb, and sC5b-9 levels below the lower limit of assay detection. Urine C5a and Ba were well correlated in previous studies of glomerular diseases.[10]

Statistical Analysis

For descriptive statistics, mean ($\pm$SD) or median (interquartile range) was used where appropriate. Log transformation was applied to plasma and urine Ba measurements. Parametric and nonparametric tests

were used for between-group comparisons and association according to the underlying sample distribution. Plasma and urine Ba levels were correlated to the estimated glomerular filtration rate (estimated using the CKD-EPI equation[11]), C-reactive protein level, and BVAS/WG score. Changes in plasma and urine Ba levels were modeled using mixed linear effects models. The variables included in these models were the visit number (fixed effect) and subject (random effects). Other variables were not included due to the small sample size. Separate models for the 3 subgroups (LTR, NReF, ReF) were used due to variability (differing mean square errors). The term "predictor" is used throughout this manuscript when reporting the results of the model and not to signify that the variable proves utility in providing insight into a future outcome. Least-square means were used to create figures. Analysis of variance was used to compare Ba levels at each visit between the 3 groups. Pairwise comparisons were done using Tukey's HSD *post hoc* test. Five logistic regression models were fit to predict a renal flare using plasma or urine Ba levels at visit 0 or −1, or the difference between −1 and 0, of the ReF group versus the NReF and LTR groups.

RESULTS

Patient Cohort

Clinical data, urine, and plasma collected during 233 visits of 60 patients with AAV were used in this study. There were 20 patients in the LTR group, 19 patients in the NReF group, and 21 patients in the ReF group. Each patient in the LTR and NReF groups had 4 visits, whereas 14 of 21 patients in the ReF group had 4 visits, with the other 7 patients having had 3 visits. The mean age of the participants was 54 (±16) years at the time of visit 0. Of 60 patients, 32 (53%) were male, 53 of 60 (88%) had a history of a positive test result for ANCA by indirect immunofluorescence or enzyme-linked immunosorbent assay, and 56 of 60 (93%) were White (Table 1). At the time of a flare, most patients in the ReF and NReF had ear/nose/throat and pulmonary involvement (Table 1).

Plasma and urine Ba levels were measured for all patient visits, except for 1 urine measurement due to

Table 1. Patient and disease demographics

Patient and disease characteristics	Total (n = 60)	LTR (n = 20)	NReF (n = 19)	ReF (n = 21)	P value
Male	32 (53)	8 (40)	8 (42)	16 (76)	0.034
White	56 (93)	18 (90)	19 (100)	19 (90)	1
Non-Hispanic	54 (90)	18 (90)	19 (100)	17 (81)	0.173
Age (yr)	54 ± 16	52 ± 15	54 ± 20	55 ± 13	0.784
Disease duration at visit 0 (yr)	7.7 ± 6.7	11.1 ± 8.6	7.3 ± 5.9	4.9 ± 3.8	0.019
Follow-up time (mo)	21 (11–36)	37 (32–40)	12 (10–22)	14 (8–21)	<0.001
Clinical phenotype					
Granulomatosis with polyangiitis	44	14	14	16	
Microscopic polyangiitis	8	4	1	3	
Eosinophilic granulomatosis with polyangiitis	8	2	4	2	
ANCA status[a] positive/negative	53/7	17/3	15/4	21/0	
IIF: cANCA/pANCA	31/10	10/2	9/4	12/4	
ELISA: PR3/MPO-ANCA	34/12	12/3	8/5	14/4	
BVAS/WG (at visit 0)		0	3 (2.5–4.5)	4.5 (3.75–7)	
C-reactive protein (at visit 0)			9.2 (1.0–15.7)	6.8 (2.5–18.5)	
eGFR at visit 0 (ml/min per 1.73 m^2)	70 (50–94)	74 (61–101)	83 (74–104)	50 (38–59)	
Extrarenal manifestations					
Pulmonary			14 (74)	14 (67)	
Arthralgias			14 (74)	12 (57)	
Ear, nose, and throat			17 (89)	18 (86)	
Fatigue			14 (74)	19 (90)	
Nervous system			7 (37)	6 (29)	
Ophthalmic			9 (47)	6 (29)	
Skin			7 (37)	4 (19)	
Medications at visit 0					
Azathioprine			6 (32)	4 (19)	
Cyclophosphamide			1 (5)	2 (10)	
Methotrexate			3 (16)	1 (5)	
Rituximab			1 (5)	5 (24)	

ANCA, antineutrophil cytoplasmic antibody; BVAS/WG, Birmingham vasculitis assessment score/Wegener Granulomatosis; cANCA, cytoplasmic ANCA; eGFR, estimated glomerular filtration rate; ELISA, enzyme-linked immunosorbent assay; IIF, indirect immunofluorescence; LTE, long-term remission; NReF, nonrenal flare; P-ANCA, perinuclear ANCA; ReF, renal flare.
[a]By IIF or ELISA at any visit.
All values are depicted as number (%), average ± SD, or median (interquartile range).

sample inadequacy. Estimated glomerular filtration rate measurements were available for all visits. BVAS/WG was available for all but 2 visits. Overall, complete data were available for >99% of the visits. Visits with unavailable data were excluded from the analysis.

Plasma and Urine Ba Levels and Clinical Correlations

Correlation between plasma and urine Ba levels was observed across all groups (Supplementary Figure S1). However, the strength of correlations varied. Correlation was fair for the LTR group (Pearson's r = 0.384, $P < 0.001$, Supplementary Figure S1A), modest for the NReF (r = 0.419, $P < 0.001$, Supplementary Figure S1B), and strong for the ReF group (r = 0.727, $P < 0.001$, Supplementary Figure S1C).

At the time of a nonrenal flare, plasma but not urine Ba correlated with the BVAS/WG (Spearman's ρ = 0.577, $P = 0.010$; ρ = 0.236, $P = 0.347$, respectively) (Supplementary Table S2). In contrast, neither plasma nor urine Ba correlated with BVAS/WG at the time of a renal flare (Spearman's ρ = 0.213, $P = 0.366$; ρ = 0.421, $P = 0.064$, respectively). Plasma Ba also correlated with serum CRP (Spearman's ρ = 0.672, $P = 0.003$).

Ba Levels as Predictors of a Renal Flare

To assess whether plasma and urine Ba levels predict a renal flare, 5 logistic regression models were fit with a renal flare (flare vs. no flare) as the dependent outcome and different applications of the biomarkers as independent variables: (i) plasma Ba level at visit 0, (ii) the change of plasma Ba level from visit −1 to 0, (iii) urine Ba level at visit 0, (iv) the change of urine Ba level from visit −1 to 0, and (v) urine Ba at visit −1. The 5 models were all useful in predicting a kidney flare (Table 2). However, visit 0 urine Ba levels had the best predictive power with an area under the receiver operator curve of 0.76 ($P < 0.001$). At a urine Ba level cutoff of 12.53 ng/mg urine creatinine, the sensitivity and specificity for detecting a renal flare were 76.2% and 68.4%, respectively.

Table 2. Predictors of a renal flare

Predictor	Model *P* value	Area under ROC curve	95% Confidence interval[a]
Plasma Ba at visit 0	0.0024	0.72	(0.57, 0.85)
Change of Plasma Ba from visit −1 to visit 0	0.0716	0.60	(0.48, 0.76)
Urine Ba at visit 0	0.0002	0.76	(0.61, 0.88)
Change of urine Ba from visit −1 to visit 0	0.0014	0.72	(0.58, 0.85)
Urine Ba at visit −1	0.0682	0.63	(0.50, 0.77)

ROC, receiver operating characteristic.
[a]Based on 2500 bootstrap samples.

Change in Ba Levels Over Time

To assess the change of Ba levels over time, linear mixed models were fit at all time points for each group. In this longitudinal analysis, plasma Ba levels did not change during the visits leading up to and at flare in any of the 3 groups (Figure 1a). In contrast, urine Ba levels increased at the time of a renal flare, but not at the time of a nonrenal flare or during the 4 time periods in patients in LTR (Figure 1b). There was no difference between plasma or urine Ba levels among the 3 groups at visits −3, −2, or −1. However, plasma and urine Ba levels were higher at visit 0 in ReF compared with the NReF and LTR groups (analysis of variance $P = 0.015$ and $P = 0.001$, respectively; Tukey's *post hoc* test $P < 0.050$ for ReF vs. NReF and ReF vs. LTR, and >0.500 for NReF vs. LTR for plasma and urine). To assess whether the change of urine Ba levels in the ReF group was due to changes in glomerular filtration, the association of urine Ba levels and estimated glomerular filtration rate was tested at each visit. There was no association between urine Ba levels and estimated

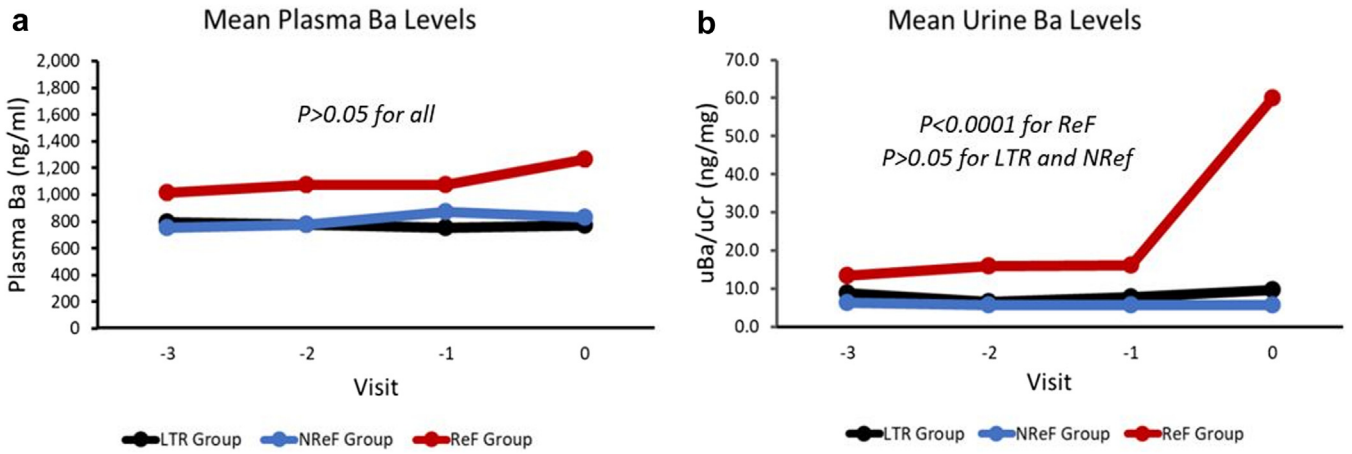

Figure 1. (a) Plasma and (b) urine Ba levels over time in patients with ANCA-associated vasculitis. Visit 0 indicates a flare visit in the long-term remission, nonrenal flare, and renal flare groups. ANCA, antineutrophil cytoplasmic autoantibody.

glomerular filtration rate at visits −3 or −2 (Pearson's r = −0.466, $P = 0.093$; and r = −0.363, $P = 0.106$, respectively). A significant association was observed at visit −1 (r = −0.690, $P = 0.001$). No association was observed at the time of a renal flare (r = −0.352, $P = 0.118$).

DISCUSSION

This study explored the utility of plasma and complement levels of Ba as biomarkers of disease activity in patients with AAV. Correlations between plasma and urine complement Ba levels were found to depend on kidney involvement. The correlation was strong at the time of a renal flare, modest at the time of a nonrenal flare, and poor when vasculitis was in remission. The increased correlation at the time of a renal flare suggests systemic and intrarenal complement activation. The limited correlation (and limited amount of detectable urine Ba) at the time of a nonrenal flare suggests that intrarenal complement activation is the major contributor to urine complement activation products, an argument that is supported by an earlier study that revealed deposition of other complement activation products (Bb, C5b-9, and C3d) in glomeruli and renal vessels of patients with active renal AAV, and also found a correlation between glomerular Bb deposition and the proportion of crescentic glomeruli.[8] Alternatively, because the size of the Ba fragment is approximately 30 kD,[12] making it partially filtered,[13] it is possible that the increase in urine Ba is due to leakage through a damaged filtration barrier. However, earlier studies revealed an increase in levels of the much larger complement activation products Bb and sC5b-9 in urine of patients with active renal vasculitis, which cannot be solely explained by increased filtration.[8]

The findings from this study do not support the utility of plasma or urine Ba as biomarkers of nonrenal flares because plasma and urine Ba levels did not reveal a change at the time of a nonrenal flare compared with remission. In contrast, urine Ba levels increased at the time of a renal flare, identifying it as a promising biomarker for renal vasculitis activity. The implications of those observations are intriguing and may suggest that patients with renal involvement may have a more pronounced degree of complement activation than those with nonrenal vasculitis and that systemic complement activity may be more dependent on the organs involved rather than the disease severity.

The findings from this study highlight an important caveat of using cross-sectional data for biomarker discovery. As found in this study, a biomarker that has utility in a cross-sectional analysis may not be useful for longitudinal surveillance. In the current analysis, when measured at visit 0, both plasma and urine Ba were higher in patients with a renal flare, and the logistic regression model revealed that plasma and urine Ba levels predicted a renal flare. However, when followed longitudinally, only urine Ba levels change at the time of a flare. This observation is significant when seeking to use biomarkers for longitudinal disease surveillance. On the basis of these data, urine but not plasma Ba levels are good candidates for further study as a biomarker of renal vasculitis.

There was a significant variation in plasma and urine Ba levels between patients at the time of a renal flare, suggesting that the intensity of complement activation may be different between patients. The reason for this variation is not clear, although it has been previously reported that complement-related variables can influence the degree of complement activation during renal flare in lupus nephritis. For example, C3 activation at the time of a flare of lupus nephritis is influenced by a common variant in the complement regulator factor H that reduces its effectiveness.[14] In addition, an autoantibody to C3b is highly associated with lupus nephritis and influences the role of other complement measurements in monitoring lupus nephritis flare onset.[15] Whether the role of complement in AAV during a renal flare is also influenced by similar variations in the complement system is unclear. Owing to the low frequency of these genetic variants, studying all the factors that affect the degree of complement activation will require a larger sample than what was used in the current analysis. Nevertheless, these studies can help identify patients with more pronounced complement activation who can be a prime target for complement-targeting therapies.

This study has several strengths. It was conducted in a well-characterized cohort that was studied prospectively in centers that excel in the care of patients with AAV. In addition, the use of serial measurements eliminates many of the biases that arise when different patient cohorts are compared. However, this study is limited by the lack of a validation cohort and the small samples size, both of which are common challenges when studying a rare disease. In addition, given that most patients were White, these findings need to be confirmed in other ethnicities.

In conclusion, urine Ba levels are potential biomarkers for acute changes in renal activity in patients with AAV and should be validated in a separate cohort of patients with AAV before further development for clinical use.

DISCLOSURE

SA reports receiving consulting fees from Amgen, ChemoCentryx, Kezar, Aurinia, and Otsuka and research

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.

Figure S1. Correlation between (a) urine and plasma Ba levels in the long-term remission, (b) nonrenal flare, and (c) renal flare groups.

Table S1. Eligibility criteria for the Vasculitis Clinical Research Consortium longitudinal protocols for granulomatosis with polyangiitis, microscopic polyangiitis, and eosinophilic granulomatosis with polyangiitis.

Table S2. Correlation between clinical variables and plasma or urine Ba levels.

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