Zonulin, as a marker of intestinal permeability, is elevated in IgA nephropathy and IgA vasculitis with nephritis

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ABSTRACT

Background. Immunoglobulin A nephropathy (IgAN) and IgA vasculitis with nephritis (IgAV-N) are considered related diseases and share some similar clinicopathologic phenotypes. Elevated circulating galactose-deficient IgA1 (Gd-IgA1)-containing immune complexes and mucosal immunity were associated with the pathogenesis of IgAN and IgAV-N. Recently, studies have identified that the zonulin level, as a modulator of intestinal permeability, is significantly elevated in several inflammatory and autoimmune-related diseases. However, whether zonulin also plays a role in IgAN and IgAV-N is not clear.

Methods. A total of 73 IgAV-N patients, 68 IgAN patients and 54 healthy controls were assessed for circulating zonulin and Gd-IgA1 levels by enzyme-linked immunosorbent assay. The diagnostic efficiency of the combination of zonulin with Gd-IgA1 was evaluated by the area under the receiver operating characteristic curve (AUC) and integrated discrimination improvement (IDI) analysis.

Results. Compared with healthy controls, we found that both IgAV-N and IgAN patients had elevated zonulin and Gd-IgA1 levels (P < .001). Additionally, patients with IgAV-N presented with even higher circulating zonulin levels than patients with IgAN (P = .020). The addition of zonulin to Gd-IgA1 showed better predictive performance than Gd-IgA1 alone in the diagnosis of both IgAN and IgAV-N, as illustrated by a significantly increased AUC (IgAN: 0.805 versus 0.708, P = .0021; IgAV-N: 0.886 versus 0.673, P < .001) and significant IDI (IgAN: IDI 0.136, P < .001; IgAV-N: IDI 0.281, P < .001).

Conclusion. Elevated circulating zonulin levels were detected in both patients with IgAV-N and those with IgAN. Combined detection of circulating zonulin and Gd-IgA1 is recommended as a noninvasive diagnostic biomarker for IgAV-N and IgAN.
INTRODUCTION

Immunoglobulin A nephropathy (IgAN) and IgA vasculitis with nephritis (IgAV-N) have indistinguishable kidney injury-related clinical and histological phenotypes, including hematuria and glomerular injury with IgA-dominant immune deposits [1, 2]. Patients with IgAN and IgAV-N typically present with macroscopic or gross hematuria within 24 h after upper respiratory and/or gastrointestinal infections [3]. Therefore, IgAN and IgAV-N are often considered closely related diseases. In contrast to IgAN, which is restricted to the kidneys, IgAV-N is a systemic disease with multiorgan involvement [4]. In addition to hematuria, patients with IgAV-N additionally present with cutaneous purpura, arthritis and gastrointestinal pain or bleeding, which are not reported in patients with IgAN.

Although the exact pathogenesis of IgAN and IgAV-N is still not well defined, increasing evidence suggests some shared pathogenesis of IgAN and IgAV-N [5]. Elevated levels of circulating galactose-deficient IgA1 (Gd-IgA1)-containing immune complexes were found in patients with IgAN and IgAV-N and are regarded as major pathogenic factors for both IgAN and IgAV-N [3].

Since the disease onset of IgAN and IgAV-N often coincides with mucosal infection, mucosal immunity has been investigated extensively in IgAN as well as IgAV-N in recent years [6, 7]. A genome-wide association study of IgAN revealed multiple susceptible genes involved in immunity against intestinal pathogens [8]. In addition, numerous studies found that the intestinal permeability in adult or pediatric IgAN patients as well as IgAV-N patients was significantly higher than that in healthy controls [9–12]. More recently, Rivas et al. [13] identified that intestinal barrier dysfunction was associated with secretory IgA leakage and IgA-C3 immune complex deposition in cardiovascular lesions, which indicated that targeting mucosal barrier dysfunction may also be applicable to IgA vasculitis and IgAN.

In 2000, Fasano et al. [14] first identified a novel protein, zonulin, as a modulator of intestinal permeability. Gluten (gliadin and glutenin) and bacteria are two powerful triggers for inducing the release of zonulin in intestinal lumina [15]. In addition to its function in regulating tight junctions, zonulin was recently shown to be associated with inflammation, autoimmunity and cancer. Upregulated expression of zonulin was reported in several autoimmune diseases, including celiac disease, type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus and Kawasaki disease [13, 16–21]. However, no such information about zonulin in IgAN or IgAV-N has been reported before.

Considering the important role of zonulin in the leakage of secretory IgA, in this study, we investigated circulating zonulin levels in patients and explored its role in IgAN and IgAV-N.
MATERIALS AND METHODS

Study population

From March 2018 to July 2021, a total of 73 patients were diagnosed with IgAV-N at Peking University First Hospital. Using propensity score matching analyses, we enrolled 68 age- and sex-matched IgAN patients from an independent cohort of IgAN patients diagnosed during the same period as the recruited IgAV-N patients. The diagnosis of IgAN was based on the predominant IgA deposits in glomerular mesangial areas under immunofluorescence staining, as well as mesangial electron-dense deposits under electron microscopy. For IgA vasculitis patients, the criteria of diagnosis were based on the clinical and histopathological criteria according to the 2010 European Alliance of Associations for Rheumatology (EULAR), Paediatric Rheumatology International Trials Organization (PRINTO) and Paediatric Rheumatology European Society (PRES) [22, 23]. IgAV-N was diagnosed as IgA vasculitis along with glomerular damage, including hematuria, proteinuria and/or renal failure manifestations, as well as predominant IgA immune deposition in the glomerular mesangial area [24, 25]. Patients with secondary mesangial IgA deposits, such as chronic liver diseases, systemic lupus erythematosus and inflammatory bowel disease, were excluded. In addition, 54 age- and sex-matched healthy volunteers were enrolled as healthy controls.

For enrolled participants, ethylene-diamine-tetraacetic-acid (EDTA) anticoagulated peripheral venous blood was collected at the time of renal biopsy for patients with IgAN and IgAV-N. Peripheral venous blood samples were centrifuged at 4°C and 3000 r.p.m. for 15 min, and then plasma was stored in aliquots at −80°C until use. This study was in accordance with the Declaration of Helsinki and approved by the Medical Ethics Committee of Peking University First Hospital (2021-570). Written informed consent was obtained from all participants.

Demographic, clinical and pathological information

For patients with IgAN and IgAV-N, information at the time of renal biopsy, including demographic features (age and sex), clinical characteristics (serum creatinine, 24-h urine protein, systolic and diastolic blood pressure) and pathological findings (sclerosis, crescent and endocapillary hypercellularity), were collected from the medical records. Hypertension was defined as systolic blood pressure of 140 mmHg or greater, and/or diastolic blood pressure of 90 mmHg or greater, or taking antihypertensive medications. The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation [26]. The percentages of cellular and fibro-cellular crescents were combined as percentages of crescents for analysis. Diffuse endocapillary proliferative was defined as >50% of all glomeruli involvement with endocapillary proliferation, and one glomerular lesion involved more than half of the glomerular tuft [25, 27].

Circulating zonulin and Gd-IgA1 detection

Circulating zonulin levels were detected using a commercial enzyme-linked immunosorbent assay (ELISA) kit (CSB-EQ027649HU, Cusabio, Hubei, China) following the manufacturer’s instructions. Briefly, the diluted standards and plasma samples (100 μL) were added to 96-well ELISA plates precoated with zonulin-specific antibody for 2 h at 37°C, followed by biotin-labeled zonulin antibody and horseradish peroxidase (HRP)-avidin for 1 h at 37°C. Finally, the plate was detected at an optical density of 450 nm/570 nm by an ELISA reader (Bio-Rad 550, USA).

Circulating Gd-IgA1 levels were detected using a commercial ELISA kit (JBL 27600, Immuno-Biological Laboratories Co., Ltd, Japan) following the manufacturer’s instructions. Briefly, 100 μL standards and serum samples (1:200) were added to 96-well ELISA plates precoated with anti-Gd-IgA1 (KM55) rat IgG antibody for 1 h at room temperature, followed by HRP-conjugated anti-human IgA (38B1) rat IgG antibody for 0.5 h at room temperature. Finally, tetramethyl benzidine was added to each well, and the optical density was measured at 450 nm/570 nm by an ELISA reader (Bio-Rad 550, USA).

Statistical analyses

Statistical data analysis was performed using SPSS 22.0 software (Chicago, IL, USA), and figures were drawn with GraphPad Prism 8.0 (San Diego, CA, USA). Quantitative variables with a normal distribution are described as the mean ± standard deviation. For variables with normal distribution, comparison between groups was performed by independent-samples t-test or one-way analysis of variance. For nonnormally distributed variables, the Mann–Whitney U test and Kruskal–Wallis test were used. Categorical variables were reported as percentages and compared by using the chi-square (χ²) test. A logistic regression model was used to assess the predictive effect of zonulin and Gd-IgA1 on the development of IgAV-N and IgAN. The diagnostic efficiency of variables was evaluated by receiver operating characteristic (ROC) curve analysis. To compare the discrimination capacity of different prediction models, integrated discrimination improvement (IDI) was used. Two-sided P < .05 was considered statistically significant.

RESULTS

Baseline demographic and clinicopathological characteristics of patients with IgAN and IgAV-N

In this study, we included 73 IgAV-N patients with a median age of 32 [interquartile range (IQR) 24–47] years. Among them, 45 (61.6%) were males (Table 1). At the time of renal biopsy, the median eGFR was 80.24 (IQR 45.53–111.21) mL/min/1.73 m², the median 24-h proteinuria was 1.46 (IQR 0.44–3.32) g and 24 (38.1%) patients presented with hypertension. Among them, the percentage of glomerular sclerosis was 9.74 (IQR 2.71–20.0) %, the percentage of segmental sclerosis was 2.67 (IQR 0.00–7.92) % and the percentage of crescents was 11.24 (IQR 0.00–18.97) %. Focal or diffuse endocapillary hypercellularity was found in 31 (43.1%) and 6 (8.8%) patients, respectively (Table 2).

In addition, 68 IgAN patients were enrolled in this study, with age and sex comparable to those of patients with IgAV-N (Table 1). For clinical characteristics (Table 2), IgAN patients presented with lower eGFR levels [median 63.28 (IQR 33.15–86.92) versus median 80.24 (IQR 45.53–111.21), P = .027] than IgAV-N patients, while the 24-h proteinuria levels [median 1.82 (IQR 0.58–3.49) versus median 1.46 (IQR 0.44–3.32), P = .353] and the percentage of hypertension were comparable [30 (41.4%) versus 24 (38.1%), P = .484]. Regarding pathological findings (Table 2), compared with IgAV-N patients, IgAN patients presented with a higher percentage of glomerular sclerosis [median 16.67 (IQR 4.86–46.27) versus median 9.74 (IQR 2.71–20.00), P = .044], a lower percentage of crescents [median 3.51 (IQR 0.00–8.33) versus
Table 1: The zonulin levels in different groups of participants.

|                      | IgAV-N patients (n = 73) | IgAN patients (n = 68) | Healthy controls (n = 54) | P-value<sup>a</sup> | P-value<sup>b</sup> |
|----------------------|--------------------------|------------------------|---------------------------|----------------------|--------------------|
| Age (years)          | 32 (24–47)               | 33 (24–46)             | 29 (25–44)                | .859                 | .967               |
| Gender (male, %)     | 45 (61.6)                | 45 (66.2)              | 29 (53.7)                 | .370                 | .576               |
| Zonulin (ng/mL)      | 1.64 (0.57–3.31)         | 0.74 (0.00–2.72)       | 0.00 (0.00–0.00)          | <.001                | .020               |
| Gd-IgA1 (μg/mL)      | 4.406 (3.33–5.74)        | 4.40 (3.11–6.45)       | 3.18 (2.00–4.01)          | <.001                | 997                |

Data are presented as median (IQR) unless otherwise stated.
<sup>a</sup>P-value among IgAV-N patients, IgAN patients and healthy controls.
<sup>b</sup>P-value between the IgAV-N patients and IgAN patients.

Table 2: The clinical and pathological manifestations of IgAN and IgAV-N patients.

|                      | IgAV-N patients (n = 73) | IgAN patients (n = 68) | P-value<sup>a</sup> |
|----------------------|--------------------------|------------------------|----------------------|
| Clinical manifestations |                          |                        |                      |
| Hypertension (yes, %) | 24 (38.1)                | 30 (44.1)              | .484                 |
| eGFR (mL/min/1.73 m<sup>2</sup>) | 80.24 (45.53–111.21)     | 63.28 (33.15–86.92)    | .027                 |
| 24 h-proteinuria (g)  | 1.46 (0.94–3.32)         | 1.82 (0.58–3.49)       | .353                 |
| Pathological findings |                          |                        |                      |
| Glomerular sclerosis (%) | 9.74 (2.71–20.00)         | 16.67 (4.86–46.27)     | .044                 |
| Segmental sclerosis (%) | 2.67 (0.00–7.92)          | 3.64 (0.00–9.78)       | .230                 |
| Percentage of crescent (%) | 11.24 (0.00–18.97)       | 3.51 (0.00–8.33)       | <.001                |
| Endocapillary hypercellularity (focal/diffuse, %) | 31 (43.1)/7 (9.7) | 6 (8.8)/0(0.0) | <.001 |

Data are presented as median (IQR) unless otherwise stated.
<sup>a</sup>P-value between the IgAV-N patients and IgAN patients.

Figure 1: Circulating zonulin and Gd-IgA1 levels in patients with IgAN and IgAV-N, as well as healthy controls. (A) Compared with healthy controls, both IgAV-N and IgAN patients showed significantly elevated zonulin levels (IgAV-N: P < .001; IgAN: P < .001). IgAV-N patients had even higher zonulin levels than IgAN patients (P = .020). Using the 90th percentile for healthy controls (zonulin level = 0.880 ng/mL) as the upper normal reference value, we found that 51 out of 73 (69.9%) IgAV-N patients and 31 out of 68 (45.6%) IgAN patients had elevated zonulin levels. The percentiles were 90th percentile for healthy controls (zonulin level = 0.880 ng/mL) as upper normal reference value for zonulin level.

Elevated zonulin levels in both IgAN and IgAV-N

Here, we found that circulating zonulin levels were significantly different among the three groups (P < .001, Table 1 and Fig. 1A). Plasma zonulin levels in both IgAV [median 1.64 (IQR 0.57–3.31) ng/mL] and IgAN [median 0.74 (IQR 0.00–2.72) ng/mL] were significantly higher than those in the control group [0.00 (IQR 0.00–0.00) ng/mL]. In addition, patients with IgAV-N presented with even higher circulating zonulin levels than patients with IgAN [median 1.64 (IQR 0.57–3.31) ng/mL versus median 0.74 (IQR 0.00–2.72) ng/mL, P = .020].

Using the 90th percentile for healthy controls (zonulin level = 0.880 ng/mL) as the upper normal reference value, we found that 51 out of 73 (69.9%) IgAV-N patients and 31 out of 68 (45.6%) IgAN patients had elevated zonulin levels. The per-
The diagnostic accuracy of zonulin for IgAV-N and IgAN

As previously reported, we found increased Gd-IgA1 levels in both IgAN and IgAV-N patients (P < .001) in both healthy controls, while no significant difference between IgAN and IgAV-N patients was found (Table 1, Fig. 1B). Additionally, using the 90th percentile for healthy controls (Gd-IgA1 = 5.86 μg/mL) as the upper normal reference value, we found that 17 out of 73 (23.3%) IgAV-N patients and 22 out of 68 (32.4%) IgAN patients had elevated Gd-IgA1 levels (P = .229). The percentage of patients with elevated Gd-IgA1 levels was comparable between IgAV-N and IgAN (P = .249, Fig. 1B).

Moreover, we found that Gd-IgA1 performed well for distinguishing IgAN or IgAV-N patients from healthy controls, with an area under the ROC curve (AUC) of 0.708 [95% CI 0.561–0.855] for IgAN and 0.639 [95% CI 0.557–0.711] for IgAV-N.

Considering the diagnostic value of both Gd-IgA1 and zonulin levels in IgAN and IgAV-N, we combined them in the logistic regression model, we identified that both Gd-IgA1 and zonulin were associated with the occurrence of IgAN or IgAV-N (Table 3). Using the predicted probability of the logistic model as weight coefficients of Gd-IgA1 and zonulin, we analysed their combined diagnostic value in IgAN and IgAV-N, respectively. Interestingly, according to the ROC curve analysis, compared with the traditional noninvasive biomarker Gd-IgA1, the addition of zonulin to Gd-IgA1 showed better predictive performance than Gd-IgA1 alone in the diagnosis of both IgAN and IgAV-N, as illustrated by a significantly increased AUC [IgAN: 0.805 (95% CI 0.727–0.883) versus 0.708 (95% CI 0.616–0.799), P = .0021; IgAV-N: 0.886 (95% CI 0.822–0.949) versus 0.673 (95% CI 0.579–0.768), P < .001] (Fig. 2) and significant integrated discrimination improvement (IDI) [IgAN: IDI 0.136 (0.081–0.191), P < .001; IgAV-N: IDI 0.281 (0.213–0.350), P < .001] (Table 5). These results suggested that zonulin combined with Gd-IgA1 may serve as a useful diagnostic biomarker for both IgAV-N and IgAN.

**DISCUSSION**

Zonulin, as a marker of intestinal permeability, has been reported to be elevated in many diseases [20, 21, 28, 30]. In addition, increasing evidence indicates the important role of Gd-IgA1 in the pathogenesis of IgAN and IgAV-N [2, 31, 32], and circulating Gd-IgA1 levels have been proposed as a useful
Zonulin in IgAN and IgAV-N

Figure 2: ROC curve analysis for discriminating patients with IgAV-N or IgAN from healthy controls. (A) ROC curves for distinguishing IgAN patients from healthy controls. (B) ROC curves for distinguishing IgAV-N patients from healthy controls. Blue curve: ROC curve of Model 1 (Gd-IgA1); red curve: ROC curve of Model 2 (combination of zonulin and Gd-IgA1). HC: healthy control.

Table 5: Comparison of different models for the diagnosis of IgAN and IgAV-N.

|                      | IgAN patients vs HC | IgAV-N patients vs HC |
|----------------------|---------------------|-----------------------|
|                      | Model 1             | Model 2               | Model 1               | Model 2               |
|                      | Gd-IgA1             | Gd-IgA1 + zonulin     | Gd-IgA1               | Gd-IgA1 + zonulin     |
| AUC (95% CI)         | 0.708 (0.616–0.799) | 0.805 (0.727–0.883)   | 0.673 (0.579–0.768)   | 0.886 (0.822–0.949)   |
| P-value               | .0021               | .001                  | <.001                 | <.001                 |
| IDI                   | 1 [reference]       | 0.136 (0.081–0.191)   | 1 [reference]         | 0.281 (0.213–0.350)   |
| P-value               | <.001               | <.001                 | <.001                 | <.001                 |

HC: healthy controls.

Model 1: the diagnostic value of Gd-IgA1 for IgAV-N or IgAN; Model 2: the diagnostic value of the combination of zonulin with Gd-IgA1 for IgAV-N or IgAN.

In this study, we have shown that IgAN patients as well as IgAV-N patients had significantly increased circulating zonulin levels. Moreover, we found that the combination of circulating zonulin and Gd-IgA1 showed better diagnostic performance than Gd-IgA1 alone for both IgAN and IgAV-N, which suggested the involvement of zonulin in the pathogenesis of IgAN and IgAV-N.

In recent years, several studies have implied that intestinal mucosal immunity is involved in the pathogenesis of IgAN and IgAV-N [3, 7, 33, 34]. Zonulin, known as a regulator of epithelial and endothelial barrier functions, can reversibly increase intestinal permeability, activate the complement pathway (C3a and C5a) and promote the inflammatory response [35, 36]. Recently, several studies have shown that serum zonulin levels were elevated in patients with gastrointestinal and non-gastrointestinal diseases, including type 1 diabetes mellitus, rheumatoid arthritis, non-coeliac gluten sensitivity, ulcerative colitis, Kawasaki disease, irritable bowel syndrome, acne rosacea and autism [14, 17, 20, 21, 28, 30, 37]. In this study, compared with healthy controls, elevated circulating zonulin levels were detected in both IgAV-N patients and IgAN patients, probably suggesting impaired intestinal permeability in IgAV-N and IgAN, which was also supportive of the previously proposed theory that intestinal mucosal immunity might play an important role in the pathogenesis of both IgAV-N and IgAN. Additionally, we speculated that the increased zonulin in patients would induce intestinal barrier dysfunction and intestinal inflammation, which lead to immune dysregulation along with “leakage of IgA,” and finally accelerated the IgA deposition in kidney and vascular and contributed to IgAN and IgAV-N [13]. Furthermore, in our study, we found that the circulating zonulin levels in IgAV-N patients were significantly higher than those in IgAN patients. Currently, we still do not know whether the higher zonulin levels in IgAV-N were associated with abdominal symptoms, and whether it implies a more crucial role of intestinal permeabil-
ity and mucosal immunity in IgAV-N than IgAN awaits further investigation in future studies.

A recent study reported that zonulin combined with demographic and clinical characteristics can effectively discriminate non-coeliac gluten sensitivity and diarrhoea-predominant irritable bowel syndrome patients [28]. In addition, the concentration of serum zonulin performed well for the diagnosis of acne rosacea [38]. For IgAN and IgAV-N, their diagnosis is still based on renal biopsy, which is an invasive procedure. Researchers continue exploring some useful noninvasive biomarkers to evaluate disease development and progression more accurately. Currently, circulating Gd-IgA1 is the most popular biomarker for IgAN and IgAV-N, although renal biopsy is still the gold standard for the diagnosis of IgAN and IgAV-N [31]. In our study, we found that the combination of zonulin with Gd-IgA1 significantly increased the diagnostic performance for both IgAN and IgAV-N patients compared with Gd-IgA1 alone. Although the circulating zonulin levels in IgAV-N patients were significantly higher than those in IgAN patients, it was impossible to distinguish the IgAV-N and IgAN patients by circulating zonulin levels. Our findings encouraged the detection of circulating zonulin together with Gd-IgA1 for the supportive diagnosis of IgAN and IgAV-N.

Due to the limited sample size in our study, we failed to find any association of zonulin with clinical or pathological phenotypes. Unfortunately, we did not have an opportunity to perform an intestinal permeability test to confirm the role of zonulin. Validation studies in large multicentric and multietnic independent disease cohorts and in-depth functional studies of zonulin in IgAN and IgAV-N are needed in the future.

Taken together, we found elevated circulating zonulin levels in both patients with IgAV-N and those with IgAN, probably suggesting increased intestinal permeability, which might contribute to the pathogenesis of IgAV-N and IgAN and provided evidence of an "intestinal–renal axis" in IgAN and IgAV-N. Combined detection of circulating zonulin and Gd-IgA1 is recommended as a noninvasive diagnostic biomarker for IgAV-N and IgAN.

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