CLINICAL KIDNEY JOURNAL

ORIGINAL ARTICLE

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

Qianqian Li*, Xiaohan Yuan*, Sufang Shi, Lijun Liu, Jicheng Lv, Li Zhu and Hong Zhang

Renal Division, Peking University First Hospital; Institute of Nephrology, Peking University; Key Laboratory of Renal Disease, Ministry of Health of China; Key Laboratory of CKD Prevention and Treatment, Ministry of Education of China; Research Units of Diagnosis and Treatment of lmmune-Mediated Kidney Diseases, Chinese Academy of Medical Sciences, Beijing, China

*These authors contributed equally to this work.
Correspondence to: Li Zhu; E-mail: funnyzhuli@bjmu.edu.cn

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.
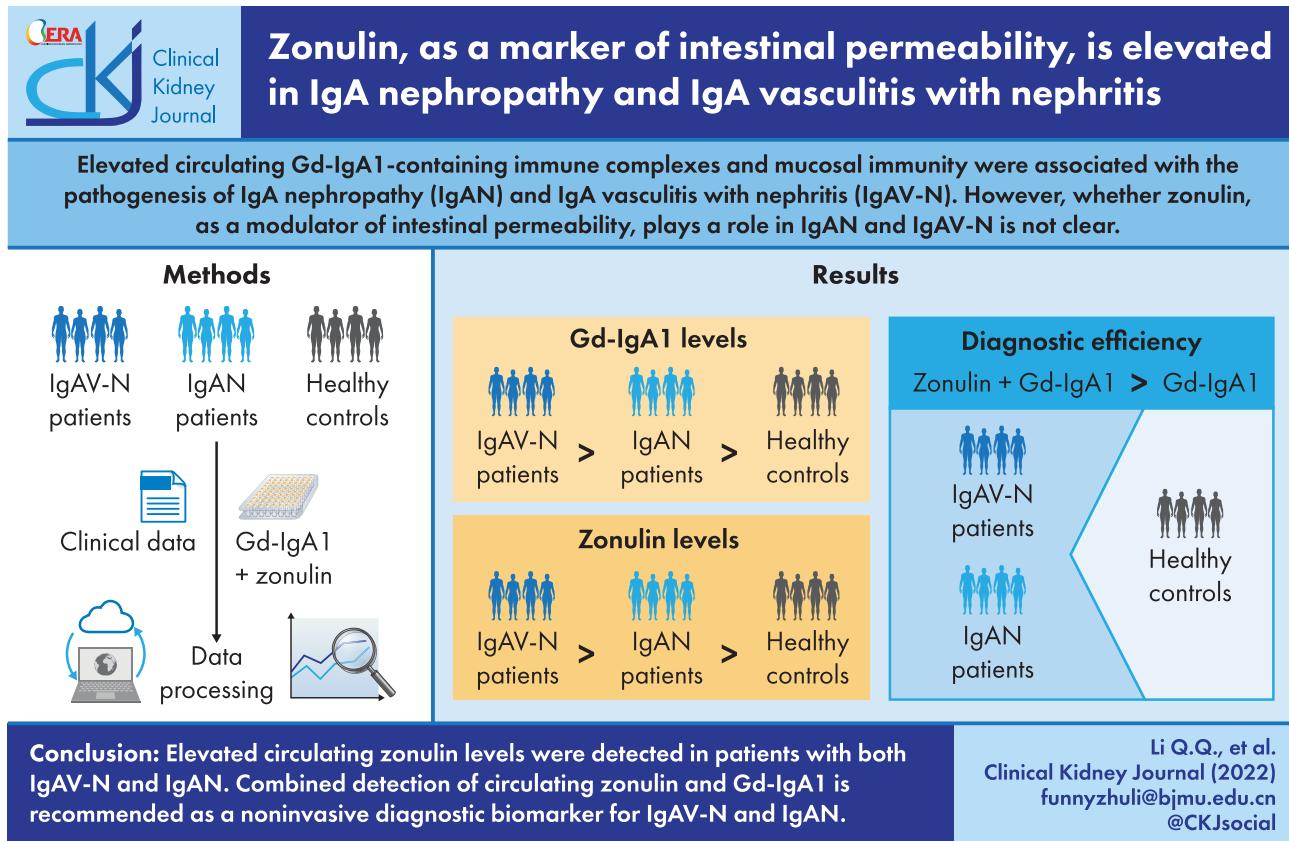**Results.** Compared with healthy controls, we found that both IgAV-N and IgAN patients had elevated zonulin and Gd-IgA1 levels ($P < .001$). Additionally, patients with IgAV-N presented with even higher circulating zonulin levels than patients with IgAN ($P = .020$). The addition of zonulin to Gd-IgA1 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.

GRAPHICAL ABSTRACT

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

Elevated circulating Gd-IgA1-containing immune complexes and mucosal immunity were associated with the pathogenesis of IgA nephropathy (IgAN) and IgA vasculitis with nephritis (IgAV-N). However, whether zonulin, as a modulator of intestinal permeability, plays a role in IgAN and IgAV-N is not clear.

Methods

IgAV-N patients   IgAN patients   Healthy controls

Clinical data   Gd-IgA1 + zonulin

Data processing

Results

Gd-IgA1 levels

IgAV-N patients > IgAN patients > Healthy controls

Zonulin levels

IgAV-N patients > IgAN patients > Healthy controls

Diagnostic efficiency

Zonulin + Gd-IgA1 > Gd-IgA1

IgAV-N patients   IgAN patients   Healthy controls

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

Li Q.Q., et al.
Clinical Kidney Journal (2022)
funnyzhuli@bjmu.edu.cn
@CKJsocial

Keywords: Gd-IgA1, IgA nephropathy, IgA vasculitis with nephritis, intestinal permeability, zonulin

INTRODUCTION

Imunoglobulin 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 deposit diseases, 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 a 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) according to the manufacturer's instructions, as reported previously [28, 29]. 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 (IBL 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 (χ^2) 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 m^2, 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 (44.1%) 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[a]	P-value[b]
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.
[a]P-value among IgAV-N patients, IgAN patients and healthy controls.
[b]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[a]
Clinical manifestations			
Hypertension (yes, %)	24 (38.1)	30 (44.1)	.484
eGFR (mL/min/1.73 m^2)	80.24 (45.53–111.21)	63.28 (33.15–86.92)	.027
24 h-proteinuria (g)	1.46 (0.44–3.32)	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.
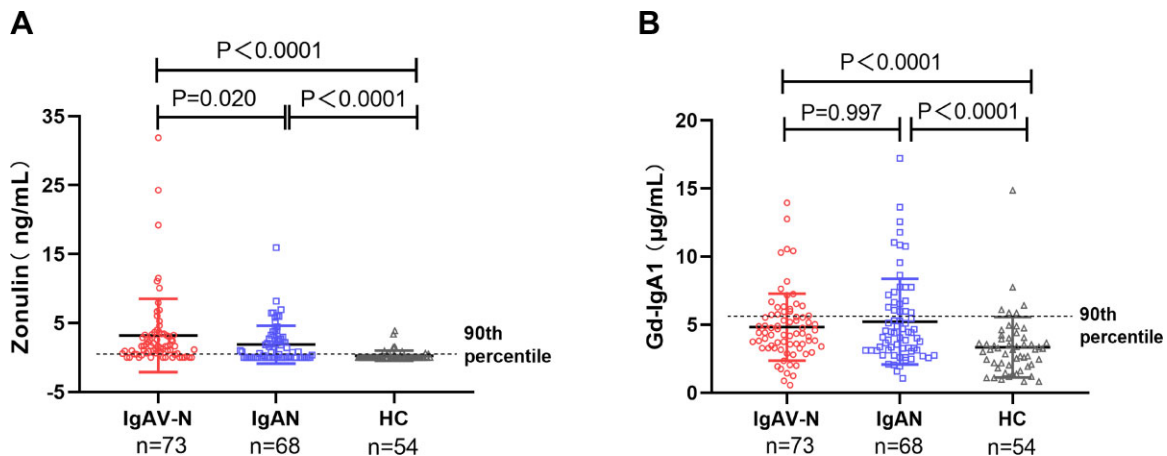[a]P-value between the IgAV-N patients and IgAN patients.

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 = .002). 90th percentile: 90th percentile for healthy controls (0.880 ng/mL) as upper normal reference value for zonulin level. (**B**) Compared with healthy controls, both IgAN and IgAV-N patients showed significantly elevated Gd-IgA1 levels (P < .001), while there was no significant difference between IgAN and IgAV-N patients (P = .097). 90th percentile: 90th percentile for healthy controls (5.86 μg/mL) as upper normal reference value for Gd-IgA1 level. HC: healthy control.

median 11.24 (IQR 0.00–18.97), P < .001] and a lower percentage of endocapillary hypercellularity lesions (focal/diffuse) [6 (8.8%)/0 (0.0%) versus 31 (43.1%)/7 (9.7%), P < .001]. The percentage of segmental sclerosis was comparable between IgAN patients and IgAV-N patients [median 3.64 (IQR 0.00–9.78) versus median 2.67 (IQR 0.00–7.92), P = .230].

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-

Table 3: Multivariate logistic regression analyses for the risk of IgAN and IgAV-N.

	IgAN vs HC						IgAV-N vs HC					
Variables	B	SE	Wald	P-value	OR	95% CI	B	SE	Wald	P-value	OR	95% CI
Gd-IgA1	0.264	0.101	6.795	.009	1.964	1.289–2.991	0.218	0.123	3.130	.077	1.243	0.977–1.582
Zonulin	1.276	0.310	16.968	<.001	1.303	1.068–1.589	1.276	0.310	16.968	<.001	3.583	1.952–6.576

HC: healthy controls; OR: odds ratio.

Table 4: The clinical and pathological manifestations of patients with and without elevated zonulin levels.

	IgAN patients (*n* = 68)			IgAV-N patients (*n* = 73)		
Variables	Without elevated zonulin levels group (*n* = 37)	With elevated zonulin levels group (*n* = 31)	P-value[a]	Without elevated zonulin levels group (*n* = 21)	With elevated zonulin levels group (*n* = 52)	P-value[b]
Zonulin (ng/mL)	0.00 (0.00–0.80)	2.73 (2.10–5.82)	<.001	0.00 (0.00–0.501)	2.54 (1.48–3.94)	<.001
Gd-IgA1(μg/mL)	4.46 (2.94–6.69)	3.87 (3.11–6.28)	.922	4.76 (2.92–6.21)	4.38 (3.56–5.63)	.826
Clinical baseline						
Age (years)	35 (27–48)	31 (22–37)	.071	32 (27–48)	32 (23–45)	.239
Gender (male, %)	24 (64.9)	21 (67.7)	.803	9 (42.9)	36 (69.2)	.036
Hypertension (yes, %)	18 (48.6)	12 (38.7)	.411	9 (45.0)	15 (34.9)	.441
eGFR (mL/min/1.73 m^2)	68.64 (32.63–86.76)	62.87 (33.54–89.08)	.975	80.34 (51.26–112.54)	79.40 (44.89–111.20)	.757
24 h-proteinuria (g)	1.82 (0.58–4.23)	1.95 (0.63–3.04)	.872	1.15 (0.82–2.64)	1.62 (0.42–3.40)	.848
Pathological findings						
Glomerular sclerosis (%)	16.67 (3.51–46.22)	16.67 (6.67–46.88)	.592	14.81 (5.12–19.52)	8.82 (0.00–23.77)	.447
Segmental sclerosis (%)	3.57 (0.00–8.22)	3.70 (0.00–12.50)	.730	5.56 (0.00–9.84)	2.56 (0.00–6.90)	.741
Percentage of crescent (%)	3.57 (0.00–8.17)	0.00 (0.00–11.86)	.707	12.00 (3.2–21.53)	10.00 (0.00–19.05)	.449
Endocapillary hypercellularity (focal/diffuse, %)	3 (8.1)/0 (0.0)	3 (9.7)/0 (0.0)	.820	7 (33.3)/1(4.8)	24 (47.1)/6 (11.8)	.252

Data are presented as median (IQR) unless otherwise stated.
[a]P-value between the group without elevated zonulin levels and the group with elevated zonulin levels in IgAN patients.
[b]P-value between the group without elevated zonulin levels and the group with elevated zonulin levels in IgAV-N patients.

centage of patients with elevated zonulin levels was significantly higher in IgAV-N than in IgAN (*P* = .004, Fig. 1A). However, in our IgAN and IgAV-N patients, we failed to find any significant difference in clinical and pathological phenotypes between patients with and without elevated zonulin levels (Table 4).

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) compared with 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% confidence interval (95% CI) 0.616–0.799] for IgAN and 0.673 (95% CI 0.579–0.768) for IgAV-N (Table 5, Fig. 2). Additionally, we found that zonulin also performed well for distinguishing IgAN or IgAV-N patients from healthy controls, with an AUC of 0.730 (95% CI 0.641–0.819) for IgAN and 0.859 (95% CI 0.791–0.926) 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* < 0.001] (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
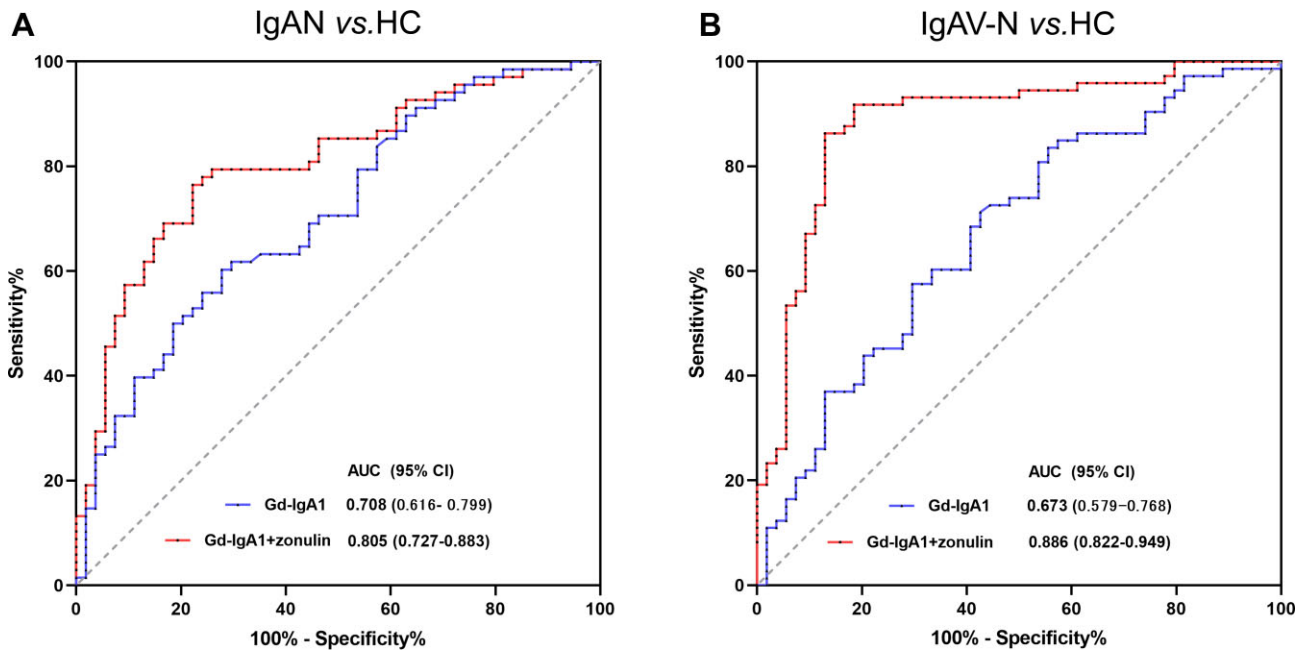
Figure 2: ROC curve analysis for discriminating patients with IgAV-N or IgAN from healthy controls. (**A**) ROC curves for distinguishing IgAN patients from healthy controls. (**B**) ROC curves for distinguishing IgAV-N patients from healthy controls. Blue curve: ROC curve of Model 1 (Gd-IgA1); red curve: 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	
Biomarkers	Model 1 Gd-IgA1	Model 2 Gd-IgA1 + zonulin	Model 1 Gd-IgA1	Model 2 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[a]		.0021		<.001
IDI	1 [reference]	0.136 (0.081–0.191)	1 [reference]	0.281 (0.213–0.350)
P-value[b]		<.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.
[a]P-value for the AUC difference between Model 1 and Model 2.
[b]P-value for the IDI difference between Model 1 and Model 2.

noninvasive diagnostic biomarker for IgAN [31]. In this study, we have shown that IgAN patients as well as IgAV-N patients had significantly increased circulating zonulin levels. More-over, 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 IgAN-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 multiethnic 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.

AUTHORS' CONTRIBUTIONS

Research idea and study design were by L.Z.; experiments were performed by Q.-q.L. and X.-h.Y.; data acquisition was carried out by Q.-q.L. and X.-h.Y.; data and statistical analysis were performed by Q.-q.L. and X.-h.Y.; S.-f.S., L.-j.L. and J.-c.L. recruited patients and collected the clinical data; and L.Z. and H.Z. provided supervision or mentorship. Each author contributed important intellectual content during manuscript drafting or revision and accepts accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved.

DATA AVAILABILITY STATEMENT

Data are available upon reasonable request.

CONFLICT OF INTEREST STATEMENT

All the authors declare no conflicts of interest.

REFERENCES

1. Heineke MH, Ballering AV, Jamin A *et al*. New insights in the pathogenesis of immunoglobulin A vasculitis (Henoch-Schönlein purpura). *Autoimmun Rev* 2017;**16**:1246–53. http://dx.doi.org/10.1016/j.autrev.2017.10.009.
2. Suzuki H, Yasutake J, Makita Y *et al*. IgA nephropathy and IgA vasculitis with nephritis have a shared feature involving galactose-deficient IgA1-oriented pathogenesis. *Kidney Int* 2018;**93**:700–5. http://dx.doi.org/10.1016/j.kint.2017.10.019.
3. Gesualdo L, Di Leo V, Coppo R. The mucosal immune system and IgA nephropathy. *Semin Immunopathol* 2021;**43**:657–68. http://dx.doi.org/10.1007/s00281-021-00871-y.
4. Pillebout E, Sunderkötter C. IgA vasculitis. *Semin Immunopathol* 2021;**43**:729–38. http://dx.doi.org/10.1007/s00281-021-00874-9.
5. Pillebout E. IgA vasculitis and IgA nephropathy: same disease? *J Clin Med* 2021;**10**:2310. http://dx.doi.org/10.3390/jcm10112310.
6. Floege J, Feehally J. The mucosa-kidney axis in IgA nephropathy. *Nat Rev Nephrol* 2016;**12**:147–56. http://dx.doi.org/10.1038/nrneph.2015.208.
7. He JW, Zhou XJ, Lv JC *et al*. Perspectives on how mucosal immune responses, infections and gut microbiome shape IgA nephropathy and future therapies. *Theranostics* 2020;**10**:11462–78. http://dx.doi.org/10.7150/thno.49778.
8. Kiryluk K, Li Y, Scolari F *et al*. Discovery of new risk loci for IgA nephropathy implicates genes involved in immunity against intestinal pathogens. *Nat Genet* 2014;**46**:1187–96. http://dx.doi.org/10.1038/ng.3118.
9. Davin JC, Forget P, Mahieu PR. Increased intestinal permeability to (51 Cr) EDTA is correlated with IgA immune complex-plasma levels in children with IgA-associated nephropathies. *Acta Paediatr Scand* 1988;**77**:118–24. http://dx.doi.org/10.1111/j.1651-2227.1988.tb10609.x.
10. Rostoker G, Wirquin V, Terzidis H *et al*. Mucosal immunity in primary glomerulonephritis. III. Study of intestinal permeability. *Nephron* 1993;**63**:286–90. http://dx.doi.org/10.1159/000187211.
11. Kovács T, Kun L, Schmelczer M *et al*. Do intestinal hyperpermeability and the related food antigens play a role in the progression of IgA nephropathy? I. Study of intestinal permeability. *Am J Nephrol* 1996;**16**:500–5. https://www.ncbi.nlm.nih.gov/pubmed/8955761.
12. Kovács T, Mette H, Per B *et al*. [Relationship between intestinal permeability and antibodies against food antigens in IgA nephropathy]. *Orv Hetil* 1996;**137**:65–9. https://www.ncbi.nlm.nih.gov/pubmed/8721870.
13. Noval Rivas M, Wakita D, Franklin MK *et al*. Intestinal permeability and IgA provoke immune vasculitis linked to cardiovascular inflammation. *Immunity* 2019;**51**:508–21.e6. http://dx.doi.org/10.1016/j.immuni.2019.05.021.
14. Fasano A, Not T, Wang W *et al*. Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. *Lancet* 2000;**355**:1518–9. http://dx.doi.org/10.1016/S0140-6736(00)02169-3.

15. Fasano A. Zonulin and its regulation of intestinal barrier function: the biological door to inflammation, autoimmunity, and cancer. *Physiol Rev* 2011;**91**:151–75. http://dx.doi.org/10.1152/physrev.00003.2008.

16. Clemente MG, De Virgiliis S, Kang JS et al. Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function. *Gut* 2003;**52**:218–23. http://dx.doi.org/10.1136/gut.52.2.218.

17. Watts T, Berti I, Sapone A et al. Role of the intestinal tight junction modulator zonulin in the pathogenesis of type i diabetes in BB diabetic-prone rats. *Proc Natl Acad Sci USA* 2005;**102**:2916–21. http://dx.doi.org/10.1073/pnas.0500178102.

18. Sapone A, de Magistris L, Pietzak M et al. Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. *Diabetes* 2006;**55**:1443–9. http://dx.doi.org/10.2337/db05-1593.

19. Caviglia GP, Dughera F, Ribaldone DG et al. Serum zonulin in patients with inflammatory bowel disease: a pilot study. *Minerva Med* 2019;**110**:95–100. http://dx.doi.org/10.23736/S0026-4806.18.05787-7.

20. Tajik N, Frech M, Schulz O et al. Targeting zonulin and intestinal epithelial barrier function to prevent onset of arthritis. *Nat Commun* 2020;**11**:1995. http://dx.doi.org/10.1038/s41467-020-15831-7.

21. Lai WT, Lee HC, Huang YH et al. Tight junction protein ZO-1 in Kawasaki disease. *BMC Pediatr* 2021;**21**:157. http://dx.doi.org/10.1186/s12887-021-02622-2.

22. Ozen S, Pistorio A, Iusan SM et al. EULAR/PRINTO/PRES criteria for Henoch-Schönlein purpura, childhood polyarteritis nodosa, childhood Wegener granulomatosis and childhood Takayasu arteritis: Ankara 2008. Part II: final classification criteria. *Ann Rheum Dis* 2010;**69**:798–806. http://dx.doi.org/10.1136/ard.2009.116657.

23. Hočevar A, Rotar Z, Jurčić V et al. IgA vasculitis in adults: the performance of the EULAR/PRINTO/PRES classification criteria in adults. *Arthritis Res Ther* 2016;**18**:58. http://dx.doi.org/10.1186/s13075-016-0959-4.

24. Pillebout E, Thervet E, Hill G et al. Henoch-Schönlein purpura in adults: outcome and prognostic factors. *J Am Soc Nephrol* 2002;**13**:1271–8. http://dx.doi.org/10.1097/01.ASN.0000013883.99976.22.

25. Yu B, Shi S, Hou W et al. Evaluation of the Oxford classification in immunoglobulin A vasculitis with nephritis: a cohort study and meta-analysis. *Clin Kidney J* 2021;**14**:516–25. http://dx.doi.org/10.1093/ckj/sfaa129.

26. Stevens LA, Claybon MA, Schmid CH et al. Evaluation of the Chronic Kidney Disease Epidemiology Collaboration equation for estimating the glomerular filtration rate in multiple ethnicities. *Kidney Int* 2011;**79**:555–62. http://dx.doi.org/10.1038/ki.2010.462.

27. Liu LJ, Li GT, Zhou Y et al. Clinicopathologic features and outcomes in endocapillary proliferative IgA nephropathy. *Nephron Clin Pract* 2010;**115**:c161–7. http://dx.doi.org/10.1159/000312880.

28. Barbaro MR, Cremon C, Morselli-Labate AM et al. Serum zonulin and its diagnostic performance in non-coeliac gluten sensitivity. *Gut* 2020;**69**:1966–74. http://dx.doi.org/10.1136/gutjnl-2019-319281.

29. Yin H, Pu N, Chen Q et al. Gut-derived lipopolysaccharide remodels tumoral microenvironment and synergizes with PD-L1 checkpoint blockade via TLR4/MyD88/AKT/NF-κB pathway in pancreatic cancer. *Cell Death Dis* 2021;**12**:1033. http://dx.doi.org/10.1038/s41419-021-04293-4.

30. Karagözlü S, Dalgıç B, İşeri E. The relationship of severity of autism with gastrointestinal symptoms and serum zonulin levels in autistic children. *J Autism Dev Disord* 2022;**52**:623–9. http://dx.doi.org/10.1007/s10803-021-04966-1.

31. Moldoveanu Z, Wyatt RJ, Lee JY et al. Patients with IgA nephropathy have increased serum galactose-deficient IgA1 levels. *Kidney Int* 2007;**71**:1148–54. http://dx.doi.org/10.1038/sj.ki.5002185.

32. Zhang X, Xie X, Shi S et al. Plasma galactose-deficient immunoglobulin A1 and loss of kidney function in patients with immunoglobulin A vasculitis nephritis. *Nephrol Dial Transplant* 2020;**35**:2117–23. http://dx.doi.org/10.1093/ndt/gfz151.

33. Fellström BC, Barratt J, Cook H et al. Targeted-release budesonide versus placebo in patients with IgA nephropathy (NEFIGAN): a double-blind, randomised, placebo-controlled phase 2b trial. *Lancet* 2017;**389**:2117–27. http://dx.doi.org/10.1016/S0140-6736(17)30550-0.

34. Wan F, Wang H, Wang M et al. Sustained release of Lactobacillus casei cell wall extract can induce a continuous and stable IgA deposition model. *J Pathol* 2022;**257**:262–73. http://dx.doi.org/10.1002/path.5884.

35. Rittirsch D, Flierl MA, Nadeau BA et al. Zonulin as pre-haptoglobin2 regulates lung permeability and activates the complement system. *Am J Physiol Lung Cell Mol Physiol* 2013;**304**:L863–72. http://dx.doi.org/10.1152/ajplung.00196.2012.

36. Sturgeon C, Fasano A. Zonulin, a regulator of epithelial and endothelial barrier functions, and its involvement in chronic inflammatory diseases. *Tissue Barriers* 2016;**4**:e1251384. http://dx.doi.org/10.1080/21688370.2016.1251384.

37. Singh P, Silvester J, Chen X et al. Serum zonulin is elevated in IBS and correlates with stool frequency in IBS-D. *United European Gastroenterol J* 2019;**7**:709–15. http://dx.doi.org/10.1177/2050640619826419.

38. Yüksel M, Ülfer G. Measurement of the serum zonulin levels in patients with acne rosacea. *J Dermatolog Treat* 2022;**33**:389–92. http://dx.doi.org/10.1080/09546634.2020.1757015.