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Original Article

ORIGINAL ARTICLE

Measurement of urinary exosomal phospholipase A2 receptor is a sensitive method for diagnosis of PLA2R-associated membranous nephropathy

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ABSTRACT

Background. The discovery of phospholipase A2 receptor (PLA2R) and its antibody (aPLA2Rab) has paved the way for diagnosing PLA2R-associated membranous nephropathy (PLA2R-MN) with a high specificity of 98%. However, the sensitivity was only 40% to 83.9%, and there is ongoing discussion around determining the optimal threshold for diagnosis. Recent advancements in the use of exosomes, a novel form of "liquid biopsy," have shown great promise in identifying markers for various medical conditions.

Methods. Protein mass spectrometry and western blot were applied to verify the existence of PLA2R antigen in the urine exosome. We then evaluated the efficacy of urinary exosomal PLA2R antigen alone or combined with serum aPLA2Rab level to diagnose PLA2R-MN.

Results. The urinary exosomes contained a high abundance of PLA2R antigen as evidenced by protein mass spectrometry and western blot in 85 PLA2R-MN patients *vs* the disease controls (14 secondary MN patients, 22 non-MN patients and 4 PLA2R-negative MN patients) and 20 healthy controls. Of note, urinary exosomal PLA2R antigen abundance also had a good consistency with the PLA2R antigen level in the renal specimens of PLA2R-MN patients. The sensitivity of urinary exosomal PLA2R for diagnosing PLA2R-MN reached 95.4%, whereas the specificity was 63.3%. Combining detection of the urinary exosomal PLA2R and serum aPLA2Rab could develop a more sensitive diagnostic method for PLA2R-MN, especially for patients with serum aPLA2Rab ranging from 2 to 20 RU/mL.

Conclusions. Measurement of urinary exosomal PLA2R could be a sensitive method for the diagnosis of PLA2R-MN.

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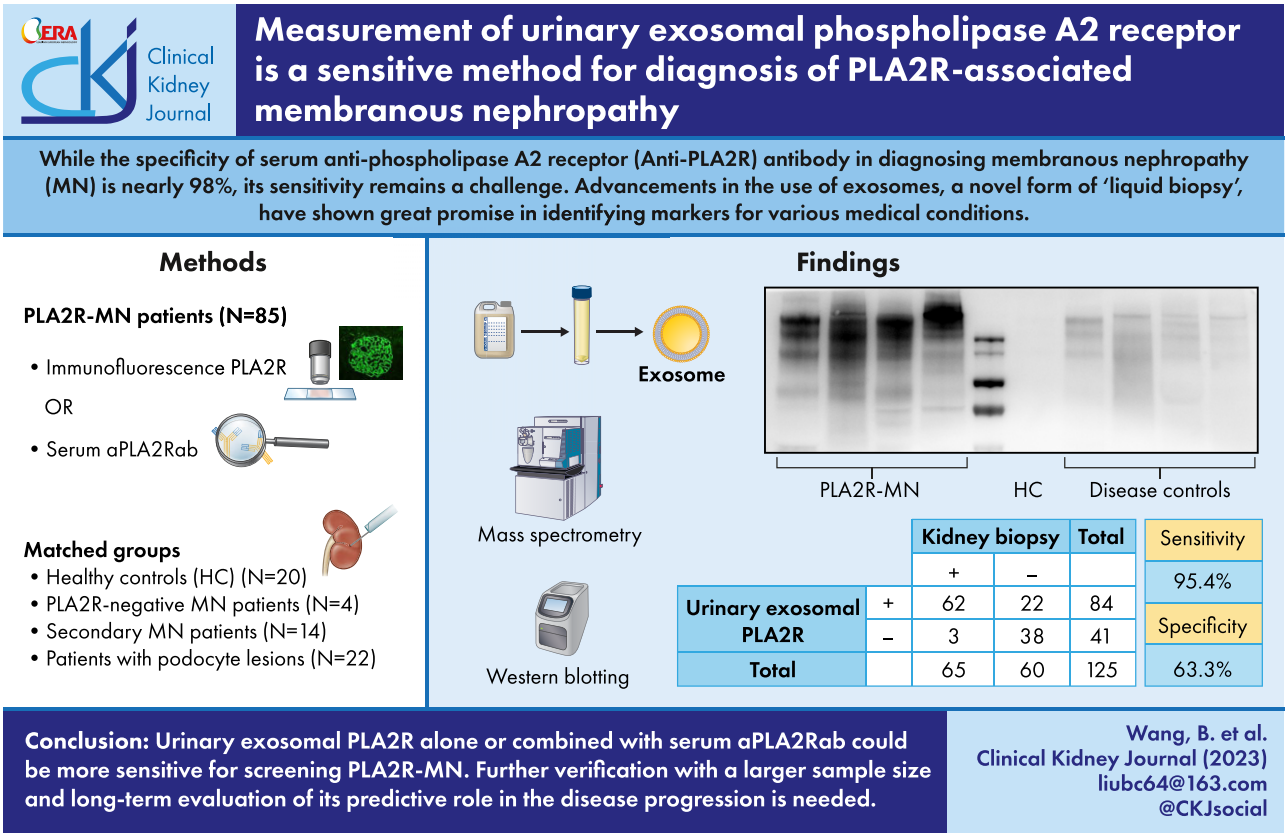
GRAPHICAL ABSTRACT



Keywords: biomarker, exosome, phospholipase A2 receptor, PLA2R-associated membranous nephropathy

INTRODUCTION

Membranous nephropathy (MN) is a common cause of nephrotic syndrome in adults, with primary membranous nephropathy (PMN) accounting for 80% of cases, and secondary membranous nephropathy (SMN) associated with other conditions (rheumatologic disorders, infection, malignancy, and use of some drugs or exposure to toxic agents) making up the remaining 20% [1–4].

Since Beck *et al.* identified M-type phospholipase A2 receptor (PLA2R) as the pathogenetic antigen in PMN patients in 2009 [5], subsequent clinical studies have confirmed that serum antibodies against PLA2R (aPLA2Rab) are instrumental in PMN diagnosis [6–8], and in predicting the efficacy of immunosuppressive agents and prognosis [9, 10]. Most antibody-positive and even antibody-negative patients have positive PLA2R staining in glomeruli, suggesting that 85% of PMN are PLA2R-mediated [11]. THSD7A is responsible for 3%–5% of PMN cases [12], while the etiology of the remaining 10% of PMN cases is still unknown. Although some new antigens have been discovered recently [13], PLA2R-associated MN (PLA2R-MN) constitutes the majority of PMN, and detecting serum aPLA2Rab has become a common clinical practice for PMN screening. The test for PMN using serum aPLA2Rab is specific but not very sensitive. This is because the antibody needs to be saturated in the kidneys before it can be detected in the blood. This means that 30% of PMN patients cannot be diagnosed with this test and need a kidney biopsy. It is also unclear what the cut-off value for the test should be when the values are between 2 and 20 RU/mL. Despite serum aPLA2Rab demonstrating a specificity of nearly 98% for diagnosing PMN, its sensitivity ranges from 40% to 80% measured by enzyme-linked immunosorbent assay (ELISA) [14–16]. A standardized recombinant cell–based indirect immunofluorescence assay (RC-IFA) and chemiluminescence immunoassay (ChLIA) can provide higher sensitivity up to 83.9% and 83.2%, respectively, but the lack of a finely graduated quantification of antibody titers should also be considered [17]. Furthermore, approximately 24% of PLA2R-MN patients tested negative for serum aPLA2Rab despite the positive results of glomerular PLA2R staining. Van De Logt *et al.* have attributed this to a "reservoir effect," whereby the antibody is only detectable in the circulation after being locally saturated in the kidneys [18]. Hence, there is a "hysteresis" effect in diagnosing PMN by serum PLA2R antibody, and approximately 30% of PMN cases require invasive kidney biopsy [18]. Furthermore, there is the ongoing controversy surrounding the optimal diagnostic cut-off value for serum aPLA2Rab testing [19, 20], particularly in cases where the serum values range between 2 and 20 RU/mL. To solve this problem, a combination of two different antibody measurements (ELISA-based and immunofluorescence assay–based) could be considered [21].

Exosomes are a subclass of extracellular vesicles with an average diameter of about 100 nm that carry proteins, lipids, nucleic acids and other molecules, regulating intercellular communication, including transferring the information relevant to cell damage and inflammation [22]. Exosomes and their contents are stable in various body fluids, especially in serum and urine, which renders them an excellent source for the noninvasive detection of individual pathological status [23]. Several studies have demonstrated the potential of exosomal molecules in urine as innovative biomarkers to diagnose and monitor various kidney diseases [24, 25]. Chen *et al*. found that the concentrations of exosomal miR-194-5p and miR-23b-3p were positively correlated with the urine protein content and were markedly higher in the high urine protein group of a cohort of 129 children with nephrotic syndrome [26]. Of diabetic nephropathy conditions, the association of urinary exosomal levels of miR-424 and miR-218 with renal damage in T1-type diabetes patients was clarified [27]. Our previous studies have found that urinary exosomal *CCL2* mRNA, *CD2AP* mRNA and miRNA-29c could serve as the new biomarkers for indicating the histologic injury severity in patients with chronic kidney disease [28–30].

Recently, aPLA2Rab has been demonstrated to be present in the urine of PMN patients [31], and the presence of PLA2R in urine exosomes has also been reported [32]. Since urine is a complex mixture of secreted and filtered proteins, salts and metabolites, potential biomarkers in exosomes may not be detected due to their dilution in whole urine [33]. In the present study, we directly evaluated the levels of PLA2R antigen in harvested urinary exosomes from our cohort and found that quantification of urinary exosomal PLA2R may be a more promising and reliable noninvasive diagnostic method for PLA2R-MN.

MATERIALS AND METHODS

Recruitment of patients

In this study, 85 PLA2R-MN patients, 14 SMN patients, 22 non-MN patients and 4 PLA2R-negative MN patients (PLA2R staining negative and serum aPLA2Rab test <2 RU/mL) diagnosed between October 2017 and April 2021 were enrolled from the Zhongda Hospital of Southeast University, the Third Affiliated Hospital of Soochow University and Jiangsu Taizhou People's Hospital. For the biopsy-proven PLA2R-MN cohort, the images of light microscopy were reviewed, excluding those with a proliferation of endothelial and mesangial cells, subendothelial or mesangial location of immune and electron-dense deposits, "full house" immunostaining and C1q immunofluorescence staining positive. In our study, PMN patients with serum aPLA2Rab test results of more than 2 RU/mL or positive PLA2R immunofluorescence staining results were placed in the PLA2R-MN group. The severity staging was conducted by electron microscopy.

For immunofluorescence staining, the sections of kidney biopsies were stained with polyclonal anti-human PLA2R antibody (ATLAS ANTIBODIES, HPA012657, 1:500). The immunofluorescence intensity of PLA2R was ranked by an expert pathologist as level 1 (negative) and level 2–5 (+, ++, +++ and ++++, respectively) (Supplementary data, Fig. S1). Another pathologist would reexamine the ranking of PLA2R staining and if there were disagreements about the previous judgments, the final results would be determined by discussion with the third expert. All of these PLA2R-MN patients were clinically ruled out for SMN, including tumor-related nephropathy, lupus nephritis, hepatitis B virus (HBV)-associated glomerulonephritis and drug/heavy metal–induced kidney injury. The groups of SMN patients and patients with podocyte lesions were all diagnosed by pathological biopsies.

As a comparison, 20 healthy subjects were recruited from the Healthy Physical Examination Center of Zhongda Hospital. According to the World Medical Association's Declaration of Helsinki, the CIOMS International Ethical Guidelines for Biomedical Research Involving Human Subjects, and the Chinese Ministry of Health's Ethical Review Measures for Biomedical Research Involving Human Subjects (Trial Implementation, 2007), the collection, handling, informed consent, privacy protection and protection of the rights of sample donors for this study were in accordance with ethical standards, as approved by the ethics committee. The serial number of the ethics committee approval is 2017ZDSTLL107-T02.

Data collection

The medical records were retrospectively reviewed to acquire information on patient characteristics and laboratory data, including age, gender, clinical diagnosis, albumin, estimated glomerular filtration rate and the value of serum aPLA2Rab.

For the values of serum aPLA2Rab, the serum samples were collected on the day of performing renal biopsies and subsequently measured by ELISA in Zhongda Hospital of Southeast University and the Third Affiliated Hospital of Soochow University (Euroimmun), while Jiangsu Taizhou People's Hospital assessed serum aPLA2Rab by commercially indirect immunofluorescence (IIF) test (Euroimmun). Laboratory professionals carried out both measurements according to the manufacturer's instructions. Sixty-one patients tested by ELISA were included in the subsequent statistical analysis, and we discussed the situation where 2 and 20 RU/mL were set as different cut-off values, respectively [20].

Urine samples

Urine samples of all patients obtained the day before the kidney biopsies were used to measure the values of 24-h proteinuria, calculated by urine protein content (g/L) multiplied by the total amount of urine in 24 h (L). In addition, 25 mL of urine from each patient was taken for exosome extraction. Urine samples from the healthy controls were collected during their physical examinations. Follow-up results were available for 17 patients with urine samples collected after 3-month individualized immunosuppressive therapies (prednisone plus tacrolimus according to advised prescription by physicians). Written informed consent from each included patient was obtained. All urine samples were centrifuged at 4°C, 2000*g* for 20 min to eliminate the urine-exfoliated cells, and stored at –80°C.

Isolation and purification of urine exosomes

The urine exosomes were isolated by gradient centrifugation [34]. In brief, 25 mL urine samples were centrifuged at 2000*g* for 20 min and 13 500*g* for 30 min to eliminate the debris and microvesicles.

Then, the supernatants were transferred to the hypervelocity centrifugal tube and centrifuged for 2 h at 4°C, 200 000*g* (Type 70 Ti rotor, Beckman Coulter Optima L-80 XP). The translucent pellets of urine exosomes were washed in sterile phosphate-buffered saline (PBS). Then, after centrifugation at 200 000*g* for 2 h again, the urine exosomes were re-suspended with 80 μL PBS and cryopreserved at –80°C for later use.

Transmission electron microscopy

Urine exosome suspension (5 μL) was mixed with 2.5% glutaraldehyde (5 μL) for fixation for 20 min. The mixture was dropped onto a copper grid. Then specimens were stained with 2% phosphotungstic acid (5 μL) for 5 min. After air drying, samples were photographed with transmission electron microscopy (FEI Tecnai G2 Spirit).

Nanoparticle tracking analysis

Urine exosomes were appropriately diluted with PBS, and nanoparticle tracking analysis measurements were recorded and analyzed by ZetaView PMX 110 (Particle Metrix) at 11 positions to measure the particle size.

Detection of urine exosomal PLA2R by liquid chromatography–tandem mass spectrometry

The urine exosomes were lysed on ice for 30 min with RIPA buffer, and centrifuged at 12 000*g* for 30 min. The supernatants were collected to measure the protein concentration using the BCA protein assay kit (Thermo Fisher). To load the protein as much as possible, the protein amount in each lane ranged from 100 to 600 μg [the same volume (50 μL) of loading samples with different protein concentrations]. Then the prominent protein bands of a fraction over 70 kDa (depicted in Supplementary data, Fig. S2) were excised from the native PAGE gel following staining Coomassie brilliant blue (Service). The protein bands were cut into 1-mm^3 gel particles, transferred into low protein binding tubes, and rinsed twice with ultrapure water. Destaining buffer was added for decolorization for 30 min to complete decolorization. The proteins were digested with trypsin; the resulting peptides were separated by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using ESI-QUAD-TOF (OE Biotech Co., Ltd, Shanghai, China). The peptides were using the UniProt protein database.

The label-free experimental principle was adopted for quantitative mass spectrometry and LC-MS was directly carried out to analyze the peptides after enzymatic hydrolysis. Quantitative information about PLA2R was obtained by calculating the signal response intensity of mass spectrometry or the number of spectrograms.

Detection of urine exosomal PLA2R by western blotting

The protein samples of urine exosomes were diluted with lysis buffer and configured into the solutions of the same volume (20 μL) and the same concentration (1 μg/μL) by adding the appropriate amount of loading buffer. The loading protein of each lane was 20 μg (Supplementary data, Fig. S3). Then, the samples were thoroughly mixed, boiled for 5 min, and cooled to room temperature, preparing for SDS-PAGE electrophoresis under nonreducing conditions. After the electrophoresis, these protein samples were transferred from the gel to the PVDF membrane and blocked with 5% bovine serum albumin for 1 h, then incubated with PLA2R antibody (ATLAS ANTIBODIES, HPA012657, 1:1000) at room temperature for 2 h. The membrane was washed three times with TBS-T and incubated with an anti-rabbit secondary antibody (CST, 7074, 1:3000) at room temperature for 1 h. The membrane was rewashed three times. The ECL chemiluminescence solution was used for blots, and the bands were obtained by 30 s of regular exposure (Tanon exposure machine) for gray value measurement. The gray value of each target strip was framed and measured with a fixed area (180 kDa) by ImageJ software. The maximum gray value of the 20 healthy controls was taken as the cut-off value (gray value 97 140): the value exceeding the cut-off value was defined as positive; the value less than or equal to the cut-off value was regarded as negative. The gray values measured are shown in the box selection and each subject's selected range was fixed (Aera 2040 measured by ImageJ software). For the PLA2R-MN group, the gray value of urinary exosomal PLA2R was further classified into levels II–V according to calculated quartiles as these measured values did not conform to the normal distribution.

Statistical analysis

The values of 24-h proteinuria and serum aPLA2Rab were expressed as median and interquartile ranges because they did not conform to a normal distribution (Shapiro–Wilk method was used to check the normal distribution). The intergroup comparisons of PLA2R gray values, which conformed to a normal distribution, were analyzed by one-way analysis if variance test and independent-samples T-test. The internal consistency coefficient of urinary exosomal PLA2R and renal biopsy was analyzed by Cohen's kappa test. The correlation between the gray values of urinary exosomal PLA2R and PLA2R immunofluorescence intensity in renal biopsy was analyzed by Spearman's rank coefficient of correlation. All analyses were conducted with SPSS 22.0. The differences were considered significant with a *P*-value $<.05$.

RESULTS

The composition and characteristics of the cohorts of PLA2R-MN patients vs the disease controls

Between October 2017 and April 2021, a total of 156 PMN patients identified by kidney biopsies were screened out of 241 MN patients initially, preclusive of 85 patients with SMN conditions including autoimmunity, malignancy, medication or heavy metal poisoning, and virus infection (referring especially to HBV, HCV and HIV).

Those who lacked available urine samples at admission and the patients whose urinalysis suggested urinary system infection but which lacked both the results of PLA2R immunofluorescence staining and serum aPLA2Rab detection were subsequently excluded. The final cohort of 85 PLA2R-MN patients was formed after screening out 18 out of 103 PMN patients who lacked the results of PLA2R immunofluorescence staining and serum aPLA2Rab detection. During this process, four PMN patients with PLA2R-negative immunofluorescence staining and serum aPLA2Rab <2 RU/mL constituted the PLA2R-negative MN control group.

The disease controls included 4 PLA2R-negative MN patients, 14 SMN patients and 22 non-MN patients. The SMN group consisted of 12 patients diagnosed with lupus nephritis, 1 with HBV-associated nephritis and 1 with renal lesions caused by heavy metals. Those non-MN patients were diagnosed with focal segmental glomerulosclerosis, membranoproliferative glomerulonephritis, minimal change disease, C3 glomerulonephritis and diabetic nephropathy, respectively (Fig. 1).

The median value of 24-h proteinuria of 85 PLA2R-MN patients was 5.180 g/24 h (interquartile range 2.294–7.772 g/24 h), while that of 14 SMN patients, 22 non-MN patients and 4 PLA2R negative MN patients were 3.299 g/24 h (interquartile range 1.467–4.944), 3.712 g/24 h (interquartile range 2.157–6.429 g/24 h)

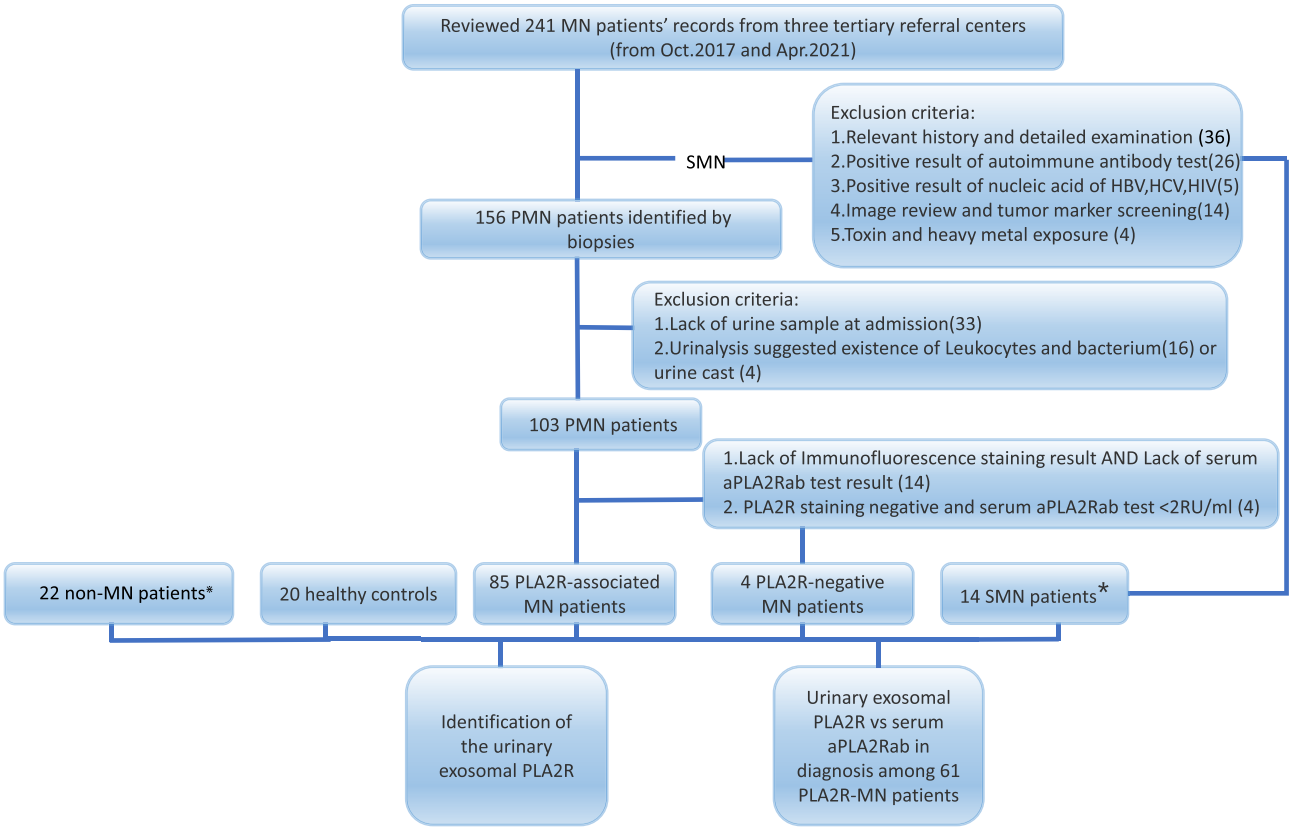


Figure 1: Flow chart of patients' inclusion. ⁎, includes three patients diagnosed with focal segmental glomerulosclerosis (FSGS), 5 with membranoproliferative glomerulonephritis (MPGN), 3 with minimal change disease (MCD), 3 with C3 glomerulonephritis and 8 with diabetic nephropathy. All of these patients were confirmed by pathological evidence. *: includes 12 patients diagnosed with lupus nephritis, one with HBV-associated nephritis and one with renal lesion caused by heavy metal.

and 1.184 g/24 h (interquartile range 0.314–6.571 g/24 h), respectively. Sixty-seven PLA2R-MN patients had serum aPLA2Rab tests during their hospitalization, 61 of whom were tested by ELISA, and the median value was 30.72 RU/mL (interquartile range 7.82–95.42 RU/mL). The results of the other six patients tested by IIF were shown in titer (Supplementary data, Table S1). All patients from each cohort were confirmed by pathological evidence, among which 71 patients in the PLA2R-MN group had PLA2R immunofluorescence results divided into five stages according to the intensity of PLA2R staining (Table 1, Supplementary data, Table S1).

Identification of the PLA2R in urine exosomes

The morphological characteristics and particle size distribution of the exosomes extracted were determined by the transmission electron microscopy and nanoparticle tracking analyzer, respectively (Fig. 2). Then LC-MS/MS was performed to provide evidence for the identification of PLA2R because of the detection of specific peptides of PLA2R (Supplementary data, Fig. S2). Moreover, an increased level of urinary exosomal PLA2R of PLA2R-MN patients was detected by western blotting, which was significantly higher than that of the SMN, non-MN and PLA2R-negative MN patients (Figs 3 and 4). The results of quantitative LC-MS also indicated that the average intensity of the PLA2R-MN group was about to be at 9 orders of magnitude, which was 100–1000 times higher than that of the healthy group, the PLA2R-negative MN, the SMN group or the podocyte injury group (Supplementary data, Figs S4 and S5).

Prediction of urinary exosomal PLA2R in diagnosing PLA2R-MN

Fourteen SMN patients, 4 PLA2R-negative MN patients and 22 non-MN patients were regarded as the disease controls. The measurement results of urinary exosomal PLA2R for 85 PLA2R-MN patients and 34 disease controls were compared according to their pathologic biopsy judgments which included PLA2R staining results for PLA2R-MN diagnosis as the gold standard. Sixty-five of 85 PLA2R-MN patients had positive PLA2R immunofluorescence staining results, and 62 of them were detected positive for urinary exosomal PLA2R. The other 20 PLA2R-MN patients and 34 disease controls did not conform to the gold standard, and 22 of them tested positive for urine exosome PLA2R, including 3 patients in the SMN group, 5 in the non-MN group and 14 in the PLA2R-MN group (of which 12 PLA2R-MN patients lacked the PLA2R immunofluorescence staining results) (Table 2). Identified with the gold standard for kidney biopsy, the sensitivity and specificity for measurement of urinary exosomal PLA2R were 95.4% and 63.3%, respectively.

To further explore the consistency of the gray values of urinary exosomal PLA2R and immunofluorescence staining grades, 71 out of 85 PLA2R-MN patients were analyzed. The linear weighted kappa coefficient was calculated at 0.510 [$P < .001$, 95% confidence interval (CI) 0.373–0.648, Table 3], indicating that

Table 1: Characteristics and main laboratory and pathology findings of 85 PLA2R-MN patients, 14 SMN patients, 22 non-MN patients and 4 PLA2R-negative patients.

Characteristics	PLA2R-MN patients	SMN patients	Non-MN patients	PLA2R-negative patients
No. of patients	85	14	22	4
Median age at initial diagnosis, years	56 (45–65)	55 (31–66)	61 (46–69)	58 (43–59
Male (%)	54 (63.5)	5 (35.7)	16 (72.7)	2 (50.0)
Median 24-h proteinuria, g/24 h	5.180 (2.294–7.772)	3.299 (1.467–4.944)	3.712 (2.157–6.429)	1.184 (0.314–6.571)
Measurement of serum aPLA2Rab (%)	67 (78.8)	14 (100.0)	12 (54.5)	4 (100)
Median aPLA2Rab value, RU/mL	30.72[c] (7.82–95.42)	2.00 (2.00–2.00)	2.00 (2.00–2.18)	<2[d]
Kidney biopsies	85	9	21	4
PLA2R staining performed (%)	71/85 (83.5)			4/4 (100)
–	6 (7.1)			4 (100)
+	18 (21.4)			
++	14 (16.7)			
+++	21 (25.0)			
++++	12 (14.3)			
EM staging (%)	66/84 (78.6)			3/4
I	19 (22.6)			1 (25.0)
II	33 (39.3)			1 (25.0)
III	10 (11.9)			1 (25.0)
IV	4 (4.7)			0

Non-MN patients included focal segmental glomerulosclerosis, membranoproliferative glomerulonephritis, minimal change disease, C3 glomerulonephritis and diabetic nephropathy.
[a]Median 24-h proteinuria at admission.
[b]Median 24-h proteinuria after 3 months' treatment.
[c]The serum aPLA2Rab values of six patients were displayed in titer, so the calculated median value only included the 61 patients whose antibody values were numerical.
[d]The serum aPLA2Rab values of all PLA2R-negative MN patients tested <2 RU/mL as defined above.

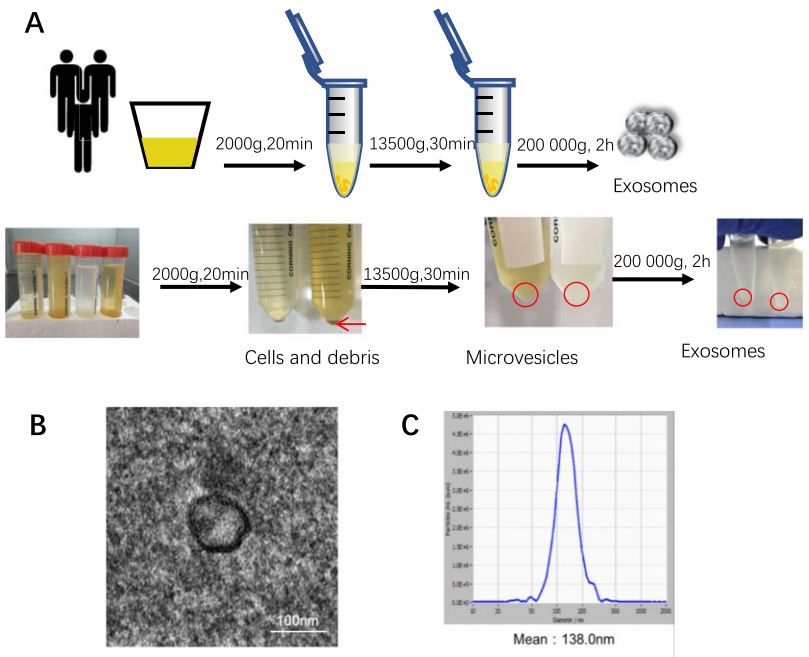


Figure 2: Extraction and identification of urine exosome. (**A**) Schematic (top) and physical (bottom) diagram of extraction of urine exosome. The typical volume of urine used to prepare exosomes was 25 mL. (**B**) Morphology of exosome observed by transmission electron microscopy. The characteristic morphology of a circular double-layered membrane structure with a diameter of about 100 nm of urine exosomes was observed by particle size analyzer. (**C**) Diameter distribution of urine exosomes. The average diameter of exosomes was identified as 138.0 nm.

there was a moderately strong consistency between the two. Additionally, the Spearman's bivariate correlation coefficient of 24-h proteinuria and exosomal PLA2R among 85 PLA2R-MN patients was 0.458($P < .001$), suggesting a positive correlation between 24-h proteinuria and the exosomal PLA2R gray value of these patients.

Combination of urinal exosomal PLA2R and serum aPLA2Rab values in diagnosing PLA2R-MN

Serum aPLA2Rab of 61 PLA2R-MN patients were displayed as numerical values and compared with their urinary exosomal PLA2R measurement results. Thirty-seven out of 61 (60.7%) and

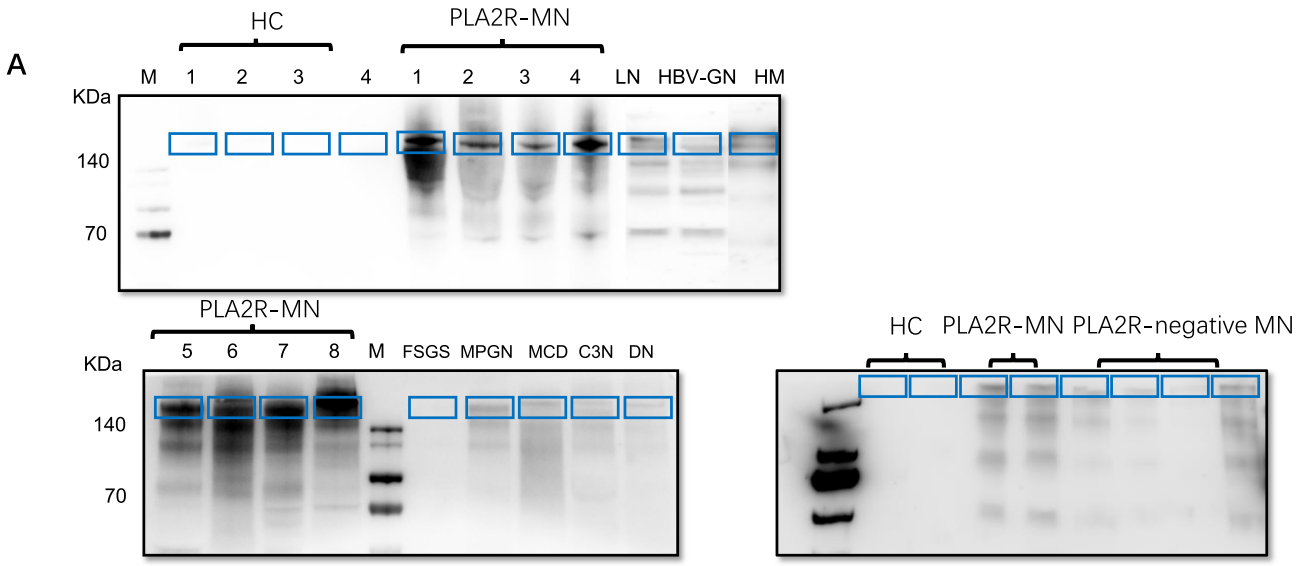


Figure 3: Examples of western blot determination of PLA2R in urine exosomes from PLA2R-MN patients and disease controls. The gray values measured are shown in the box selection and each subject's selected range was fixed (Aera 2040 measured by ImageJ software). For, healthy controls, 1–4 represent different subjects; for PLA2R-MN, 1–8 represent different subjects. HC, healthy controls; M, protein marker; LN, lupus nephritis; HBV-GN, HBV-associated nephritis; HM, renal lesion caused by heavy metal; FSGS, focal segmental glomerulosclerosis; MPGN, membranoproliferative glomerulonephritis; MCD, minimal change disease; C3N, C3 glomerulonephritis; DN, diabetic nephropathy.

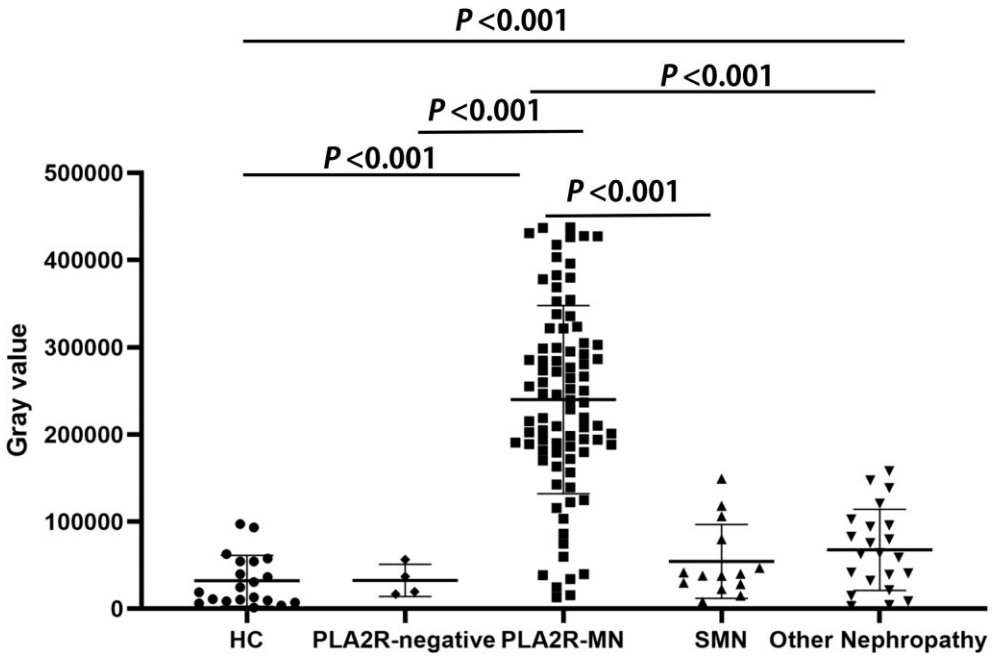


Figure 4: Measurement of urinary exosomal PLA2R by western blot experiment. The results of urine exosomal PLA2R of 20 healthy controls, 85 PLA2R-MN patients, 14 SMN patients, 22 non-MN patients and 4 PLA2R-negative MN patients, displayed as gray values, were measured by western blotting. The abundance of urinary exosomal PLA2R in PLA2R-MN patients was significantly higher than that in the healthy controls, the SMN group and the non-MN patients. The differences between each of the five groups are statistically different. HC, healthy controls.

53/61 (86.9%) of PLA2R-MN patients tested positive when the serum aPLA2Rab values were set at 20 and 2 RU/mL, respectively (Table 4A). However, of these 61 PLA2R-MN patients, the sensitivity of urinary exosomal PLA2R (ePLA2R+) reached 91.8% (56/61) (Table 4B).

In the case of the 56 patients with positive urinary exosomal PLA2R, ePLA2R+ was further counted as 59.0% (36/61), 19.7% (12/61) and 13.1% (8/61) when the serum aPLA2Rab value is >20, 2–20 and <2 RU/mL, respectively. Only 5 of 61 PLA2R-MN patients were urinary exosomal PLA2R negative (8.2%), of whom 1 had a

Table 2: Fourfold table—the diagnosis efficacy of PLA2R-MN of urine exosomal PLA2R compared with kidney biopsy.

		Kidney biopsy		
		+	−	Sum
Urinary exosomal PLA2R	+	62	22	84
	−	3	38	41
Sum		65	60	125
Sensitivity			95.4%	
Specificity			63.3%	

The "positive" column of "kidney biopsy" of the fourfold table represents the combination of pathological characteristics of PMN and PLA2R immunofluorescence staining positive results as the gold standard. According to this standard, the "negative" column of "kidney biopsy" includes both the disease controls and the PLA2R-MN patients who lacked PLA2R staining results. Twenty-two patients in the "negative" column of "kidney biopsy" had positive results of urinary exosomal PLA2R, of which 3 patients were from the SMN group, 5 from the non-MN group and 14 from PLA2R-MN group (including 12 PLA2R-MN patients who lacked PLA2R immunofluorescence staining results).

serum value of aPLA2Rab >20 RU/mL (1.6%) and the other 4 had a value between 2 and 20 RU/mL (6.6%). None of the PLA2R-MN patients had both a negative urinary exosomal PLA2R result and a serological aPLA2Rab value <2 RU/mL (Table 4B).

DISCUSSION

In this study, we found for the first time that urinary exosomes from PLA2R-MN patients contained a high level of PLA2R antigen, which is well consistent with the levels observed in their renal specimens. We demonstrated that measuring urinary exosomal PLA2R was a new sensitive biomarker for diagnosing PLA2R-MN, and its combination with serum aPLA2Rab testing significantly improved the sensitivity of screening for PMN.

The discovery of serum aPLA2Rab has revolutionized the diagnosis of PLA2R-MN. However, the sensitivity of serum aPLA2Rab for detecting PLA2R-MN is relatively low compared with its high specificity (close to 100%). This may be explained by the hypothesis that circulating aPLA2Rab can only be detected after the local deposits of PLA2R antigen and aPLA2Rab *in situ* are saturated [18]. In fact, approximately 24% of PLA2R-MN patients tested negative for serum aPLA2Rab despite the positive results of glomerular PLA2R staining [35]. A meta-analysis showed that the pooled sensitivity of serum aPLA2Rab and glomerular PLA2R staining for differentiating PMN was 65% and 79%, respectively, suggesting that the detection of glomerular PLA2R antigen is more sensitive for diagnosing PMN in the early stage, as well as that the combination of serum aPLA2Rab and glomerular PLA2R immunofluorescence staining is preferable [16]. Since *in situ* glomerular PLA2R staining is an invasive procedure requiring biopsy, a noninvasive marker with better consistency with glomerular PLA2R antigen is needed.

It was demonstrated that the podocyte is the source of the autoantigen in PMN, of which PLA2R accounts for 85% [4]. Urinary exosomes were proven to be ideal biomarkers for kidney diseases because their increasing release could be a pathological sign of podocyte injury [36]. As shown in focal segmental glomerulosclerosis, a glomerulopathy characterized by podocyte injury, the overexpression of the Wilm's Tumor 1 protein in urine exosomes could be the damage signal [37], and also ceruloplasmin in urine exosome may act as a biomarker in immunoglobulin A nephropathy [38]. PLA2R is expressed on the cell body of human podocytes and their foot processes [5]. Hence, it is assumed that the presence of PLA2R antigens in urine exosomes could occur earlier than blood PLA2R antibodies. In this study, we found a high abundance of PLA2R antigen in the urine exosomes in PLA2R-MN patients by mass spectrometry and western blot experiments.

Interestingly, the quantity of PLA2R in urine exosomes is closely correlated with its expression extent in glomerular staining, and the level of 24-h proteinuria. Wang *et al*. measured the aPLA2Rab in urine among 28 PMN patients and 12 SMN patients. They found that 67.9% of PMN patients were antibody-positive [31], whereas our study indicated a sensitivity of 95.4% for urinary exosomal PLA2R in diagnosing PMN. This finding implies that urinary exosomal PLA2R could potentially serve as an indicator of podocyte damage in PLA2R-MN. Furthermore, the fact that eight patients with serological antibody levels <2 RU/mL were all positive for urinary exosomal PLA2R suggests that urinary exosomal PLA2R might represent an early manifestation of PLA2R-MN (Table 4B).

Table 3: Crosstab of measuring the urinary exosomal PLA2R positivity by western blot and PLA2R staining intensity by immunofluorescence.

			The intensity grades Crosstab of WB*IF					
			IF (number of PLA2R-MN patients)					
			I	II	III	IV	V	Total
A	WB (number of PLA2R-MN patients)	I	4	1	1	1	0	7
		II	1	11	3	3	0	18
		III	1	5	6	6	2	20
		IV	0	1	3	7	4	15
		V	0	0	1	4	6	11
	Total		6	18	14	21	12	71

	Weighting	Kappa	Asymptotic standard error	Z	P-value	Lower 95% asymptotic CI bound	Upper 95% asymptotic CI bound
B	Linear	.510	.070	6.494	<.0001	.373	.648

Seventy-one out of 85 PLA2R-associated MN patients had PLA2R immunofluorescence examination performed. (A) The linear weighted kappa coefficient was calculated at 0.510 ($P < .001$, 95% CI 0.251–0.599, B). (A) Negative results of PLA2R measurement in urine exosomes and its staining of biopsies were denoted as level I. The gray value of urinary exosomal PLA2R was further classified into levels II–V according to calculated quartiles, and the intensity grades of PLA2R staining results were one-to-one corresponded to levels II–V (+, ++, +++ and ++++ were recorded as level II–V). The grade performances were completely consistent in 34 PLA2R-MN patients (gray color) and inconsistent in the other 37 patients. (B) The linear weighted kappa coefficient was calculated at 0.510 ($P < .0001$, 95% CI 0.373–0.648).
WB, western blot; IF, immunofluorescence.

Table 4: The diagnosis efficacy of measurement of urine exosomal PLA2R compared with that of aPLA2Rab for 61 PLA2R-MN patients.

	Measurement of PLA2R		No. of PLA2R-MN patients with positive results of urinary exosomal PLA2R or serum aPLA2Rab/No. of PLA2R-MN patients with available exosomal PLA2R or serum aPLA2Rab results (%)	
A	Urinary exosomal PLA2R+		62/65 (95.4)	
	Serum aPLA2Rab ≥20		37/61 (60.7)	
	Serum aPLA2Rab ≥2		53/61 (86.9)	
	Urinary exosomal PLA2R result	Serum aPLA2Rab result	No. of PLA2R-MN patients in the cut-off range of serum PLA2Rab value/No. of PLA2R-MN patients with numerical serum aPLA2Rab results (%)	
B	Exosomal PLA2R+	aPLA2Rab >20	36/61 (59.0)	No. of PLA2R-MN patients with urinary exosomal PLA2R positive results/
		2 ≤ aPLA2Rab < 20	12/61 (19.7)	No. of PLA2R-MN patients with numerical serum aPLA2Rab results (%)
		aPLA2Rab <2	8/61 (13.1)	56/61 (91.8)
	Exosomal PLA2R–	aPLA2Rab >20	1/61 (1.6)	No. of PLA2R-MN patients with urinary exosomal PLA2R negative results/
		2 ≤ aPLA2Rab < 20	4/61 (6.6)	No. of PLA2R-MN patients with numerical serum aPLA2Rab results (%)
		aPLA2Rab <2	None	5/61 (8.2)

(
A) The first row 1 represents 62 out of 65 PLA2R-MN patients who tested positive for urinary exosomal PLA2R. Rows 2–3 represent patients with positive aPLA2Rab serology with a cut-off value of 2 or 20 RU/mL, respectively. Sixty-one patients were included with available serum aPLA2Rab data (these 61 people were also among the 67 PLA2R-MN patients confirmed by biopsy). (B) The 61 patients with serological antibody data available were first grouped according to urinary exosomal PLA2R test results and each group was further classified according to different cut-off values of 2 and 20 RU/mL.

Previous studies have suggested the cut-off value was set at a minimum of 2 and a maximum of 20 RU/mL by classical ELISA [20]. In our study, the diagnostic sensitivity was only 60.7% when serum aPLA2Rab was >20 RU/mL by ELISA. In addition, even if the cut-off value was set to 2 RU/mL, the diagnostic sensitivity increased to 86.9% (Table 4A), which was still lower than 91.8%, the result calculated by the combination of urinary exosomal PLA2R and serum aPLA2Rab test regardless of the cut-off value setting (Table 4B). This indicated that a combination of the two might be a more sensitive way to diagnose PLA2R-MN. Bobart *et al*. reported that for patients with preserved renal function, IFA could serve as a complementary method for detecting PLA2R antibodies by ELISA within the range of 2–20 RU/mL [21]. In this study, we offered another measurement way to consider the level of urinary exosomal PLA2R in conjunction with serum aPLA2Rab, as this comprehensive approach can increase confidence in the diagnosis of PLA2R-MN without relying solely on a cut-off value [39, 40]. (The serum aPLA2Rab values of 12 out of 16 patients ranged from 2 to 20 RU/mL tested positive for urinary exosomal PLA2R, Table 4B.) Ultimately, using both assays can improve diagnostic sensitivity beyond the serum minimum cut-off value of 2 RU/mL, while also potentially reducing false positives for serum aPLA2Rab levels between 2 and 20 RU/mL.

Although urinary exosomal PLA2R has a high sensitivity to detect PMN, it should be noted that some SMN patients might also test positive when there is podocyte injury. Since PLA2R is expressed in normal podocytes [33], an increase in the secretion of urinary exosomal PLA2R is expected when podocyte damage occurs. In such cases, a combination of the detection of urinary exosomal PLA2R and serum aPLA2Rab is proposed to be more effective for diagnosing PLA2R-MN. For patients with serum aPLA2Rab values <2 RU/mL, we recommend performing a urinary exosomal PLA2R test subsequently. Exosomal PLA2R may be used as a supportive method with low suspicion PLA2R-MN cases particularly for those patients with contraindications to renal puncture.

The limitations of this study are as follows. (i) There are different sub-cohorts within the cohort of PLA2R-MN with some diagnosed by serum antibody positivity and some with biopsy immunofluorescence staining. Comparing the performance of urinary exosomal PLA2R separately in patients with serum antibody testing and biopsy immunofluorescence testing and then a combined PLA2R-MN would potentially signify the benefit of exosomal testing on top of the other two tests that are currently undertaken in clinical practice. (ii) The inclusion of an external validation cohort would provide an important validation step and strengthen the reliability of our results. Additionally, further verification with a larger sample size and long-term evaluation of its predictive role in the disease progression are needed. (iii) We applied western blot and quantitative MS techniques to semi-quantitatively and quantitatively analyze urine exosomal PLA2R, respectively. While efforts were made to ensure consistency within our laboratory, the standardization of PLA2R gray value measurements across different laboratories remains a challenge; the development of a urine exosome PLA2R assay kit will further promote the clinical application of this biomarker.

In conclusion, here we first demonstrated that urinary exosomal PLA2R alone or combined with serum aPLA2Rab could be more sensitive for screening PLA2R-MN.

SUPPLEMENTARY DATA

Supplementary data are available at *ckj* online.

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AUTHORS' CONTRIBUTIONS

B.W. and B.-C.L.: conceptualization; Y.-q.F. and J.-Y.C.: writing—original draft; B.W., Y.-q.F., B.-C.L: writing—review and editing; L.-j.X.: methodology; M.Y., M.L., T.-L.C.: resources; Y.-q.F. and L.-j.X.: data curation; X.-L.Z., L.-L.L., B.-C.L.: supervision; Q.L., Y.-q.F. and L.-j.X.: formal analysis; Y.-q.F. and L.-j.X.: visualization; B.-C.L.: project administration; B.W. and B.-C.L.: funding acquisition.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this article and its Supplementary data files. Further inquiries can be directed to the corresponding author.

CONFLICT OF INTEREST STATEMENT

All authors have nothing to disclose.

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