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Diagnostics of preeclampsia based on Congo red binding to urinary components: rationales and limitations

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Abstract:	Preeclampsia is a disease in pregnancy that is one of the main causes of death in pregnant women. This disease occurs after the 20th week of pregnancy and is characterized by arterial hypertension, proteinuria, fetoplacental, and multiple organ dysfunctions. Despite the long history of studying preeclampsia, its etiology and pathogenesis remain poorly understood, and therapy is symptomatic. One of the factors in the development of the disease is presumably misfolded proteins that are prone to form amyloid aggregates. Diagnostic tests based on the binding of the amyloid-specific dye Congo red to urine components (CRD tests) show high efficiency in the diagnosis of preeclampsia. However, it was revealed that the CRD test gives a positive result in other diseases characterized by proteinuria, which is supposedly associated with the development of concomitant amyloidosis. This study aimed to assess the congophilia of urine samples from patients with different etiologies of proteinuria to determine the limitations of CRD tests. Patients with amyloidosis and non-amyloid nephropathy were analyzed. Congophilia values were shown to have a high correlation with urinary protein levels in all experimental groups. The mean values in patients diagnosed with amyloidosis did not differ from those in patients with proteinuria not associated with amyloid accumulation. Separation of urine fractions with different molecular weights indicates that the congophilia of urine samples is due primarily to binding to monomeric forms of the protein, including serum albumin. Our results confirm the presence of limitations in the use of the CRD tests in relation to pregnant women with renal disorders not associated with preeclampsia and amyloidosis and indicate the need for further study of the mechanisms of binding of Congo red with urine components.
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Diagnostics of preeclampsia based on Congo red binding to urinary components: rationales and limitations

Short title

Congo red tests for diagnosis of preeclampsia

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Abstract

Preeclampsia is a disease in pregnancy that is one of the main causes of death in pregnant women. This disease occurs after the 20th week of pregnancy and is characterized by arterial hypertension, proteinuria, fetoplacental, and multiple organ dysfunctions. Despite the long history of studying preeclampsia, its etiology and pathogenesis remain poorly understood, and therapy is symptomatic. One of the factors in the development of the disease is presumably misfolded proteins that are prone to form amyloid aggregates. Diagnostic tests based on the binding of the amyloid-specific dye Congo red to urine components (CRD tests) show high efficiency in the diagnosis of preeclampsia. However, it was revealed that the CRD test gives a positive result in other diseases characterized by proteinuria, which is supposedly associated with the development of concomitant amyloidosis. This study aimed to assess the congophilia of urine samples from patients with different etiologies of proteinuria to determine the limitations of CRD tests. Patients with amyloidosis and non-amyloid nephropathy were analyzed. Congophilia values were shown to have a high correlation with urinary protein levels in all experimental groups. The mean values in patients diagnosed with amyloidosis did not differ from those in patients with proteinuria not associated with amyloid accumulation. Separation of urine fractions with different molecular weights indicates that the congophilia of urine samples is due primarily to binding to monomeric forms of the protein, including serum albumin. Our results confirm the presence of limitations in the use of the CRD tests in relation to pregnant women with renal disorders not associated with preeclampsia and amyloidosis and indicate the need for further study of the mechanisms of binding of Congo red with urine components.

Introduction

Preeclampsia is a severe complication of pregnancy that is characterized by elevated blood pressure, proteinuria, seizures, failure of multiple organs, and even death [1–7]. An accurate and early diagnosis of this condition is an unmet clinical need [8,9]. In 2014, Buhimschi et al. demonstrated that urine proteins in patients with preeclampsia exhibited properties similar to amyloid [10]. For diagnostic

purposes, they developed the Congo red dot (CRD) test, which assessed the retention of the amyloid-specific Congo red dye by urine proteins on a membrane (Congo red retention, CRR). Samples from preeclampsia patients retained the dye, while those from non-preeclampsia patients lost it when washed off (Fig. 1A). For use in clinical practice modified versions of the CRD test have been introduced known as CRD paper tests [11,12] where positive test results are determined by spot area (Fig. 1B). When tested in pregnant women, this modified test provided a sensitivity of 80.2% and specificity of 89.2%, making it more effective than well-known disease markers such as fms-like tyrosine kinase 1 and placental growth factor [11].

Fig 1. The representation of the CRD tests' results. The CRD membrane (A) and CRD paper (B) tests are shown. The representative images displayed are from five pregnant women with preeclampsia (1–5) and five control pregnant women (6–10).

Recently, McCarthy et al. assessed the effectiveness of CRD tests in pregnant and non-pregnant women with chronic kidney disease (CKD) with unspecified morphology, hypertension, and lupus nephritis [13]. In this study, the CRD test was positive in all patients with proteinuria, regardless of the diagnosis. Whether the renal amyloidoses and other proteinuric nephropathies yield CRD test positivity remains unknown. Another poorly investigated aspect [10] is whether urinary congophilia in patients with preeclampsia originates from urinary proteins themselves or amyloid-like aggregates. To address these gaps and identify potential limitations of the CRD test, we investigated congophilia and protein components mediating Congo red positivity in urine samples from patients with proteinuria of different etiology, including preeclampsia and histologically confirmed amyloid and non-amyloid nephropathies.

Materials and methods

Study design, patient selection, and data collection

Two study groups comprised patients with amyloid nephropathies (AN) and non-amyloid nephropathies (NA), confirmed by detailed clinical and histological evaluation. We also enrolled women with

preeclampsia (PE) as the positive controls. Women with normal pregnancy (NP) and patients with various non-proteinuric kidney diseases (Control) served as negative controls. Preeclampsia was diagnosed according to the clinical guidelines [7]. Inclusion criteria for Control and NP were 24-h albuminuria <30 mg and estimated glomerular filtration rate (eGFR) >60 ml/min/1,73m^2. At the time of enrollment, pregnancy was excluded in all women from the AN, NA, and Control groups. Serum creatinine was used to evaluate kidney function [14], while the eGFR was additionally calculated using the CKD-EPI 2009 formula [15] for the AN, NA, and Control groups. To assess proteinuria, the Quick Start Bradford Protein Assay kit (Bio-Rad, USA) was used.

All participants signed the informed consent as a part of Regular Medical documentation. The study was approved either by the Ethics Committees of Pavlov University (St. Petersburg, Russia) (No.: 21-250; date of approval 28 June 2021) or the Research Institute of Obstetrics (St. Petersburg, Russia) (No.: 97; date of approval 27 June 2019). Urine samples for this study were collected from 14.09.2019 to 30.12.2021 for PE and NP groups and from 12.09.2021 to 28.12.2021 for AN, NA, and Control groups.

Congophilia assays

The urine samples were stored at -80 °C, thawed on ice, and centrifuged at 4000 x*g*, 4 °C for 5 min before the experiments. In congophilia tests, 80 µl of each sample was mixed with 8 µl of a 0.2 % aqueous solution of Congo red dye, incubated for 10 min and applied on a nitrocellulose membrane (Amersham Protran 0.45 NC, GE Healthcare, USA) in two aliquots of 2 µl (membrane test) and FN 3 chromatography paper (thickness: 0.19 mm; square weight: 90 g/m^2) in two aliquots of 40 µl (paper test). The membrane was dried for 10 minutes, wetted with water, imaged using a camera in a lab-made box, then incubated for 1 min in 50 % and 70 % ethanol, and washed for 1 h in 90% ethanol with shaking. After rinsing successively in 70 % ethanol, 50 % ethanol, and water, the membrane was imaged again. The integrated intensity of each spot was determined using ImageJ (version 1.51j8) and the CRR was calculated as the ratio of the average intensities after washing and before washing. In the paper test, the stained samples were scanned 15 min after application on the paper, and the area of the spots was calculated in ImageJ as Congo red area (CRA, px).

For congophilia tests and polyacrylamide gel electrophoresis (PAGE), the following proteins were used: human serum albumin (HSA) (A3782, Sigma-Aldrich, USA), bovine serum albumin (BSA) (23209, Thermo Fisher Scientific, USA), and bovine gamma globulin (BGG) (500-0208, Bio-Rad, USA).

Separation of urine fractions

The separation of high molecular weight (MW) aggregates was carried out using an Optima MAX-XP ultracentrifuge (Beckman Coulter, USA) at 300,000 x*g* and 4 °C for 2 h. To isolate protein fractions within specific MW ranges, 200 µl of urine samples were applied onto Amicon Ultra-4 Centrifugal Filter Devices (Merck Millipore, Germany) with a cut-off of 30 kDa (UFC8030) and 100 kDa (UFC8100), followed by centrifugation at 7,000 x*g* for 10 min. Subsequently, 190 µl of filtrates and 10 µl of concentrates were collected (concentration factor 20). Concentrates were diluted by phosphate buffer, pH 7.4 (Merck, S-P4417) to 200 µl, concentrated once more (washing), and diluted to the volume of the original sample.

Urine protein concentrating

Control urine samples (20-80 ml) of NP were lyophilized in a freeze dryer (Labconco Corporation, USA), dissolved in water to a volume of 1.5-2 ml, and centrifuged for 10 min at 4,000 x*g*. The supernatant was analyzed by 10% PAGE followed by Coomassie brilliant blue staining. Spectra Multicolor Broad Range Protein Ladder (26634, Thermo Scientific, USA) was used to assess MW of proteins separated in PAGE.

Statistics

Data are presented as median with interquartile range or mean ± standard deviation for continuous variables and frequencies with % for categorical variables. Parameters among the groups were compared by analysis of variance. For continuous and categorical variables comparison, the Mann-Whitney U-test and the chi-square test were applied, respectively. The mean CRRs, CRAs, and proteinuria were compared between groups by a two-sided randomization test in the Drosophila Courtship Lite v. 1.3 [16,17]. The 95 % confidence intervals (C. I.) for means were calculated by bootstrapping (10,000 iterations) [18]. The 95 % C. I. and *p*-values for the Spearman rank-order

correlation coefficient were assessed by *t*-test. To determine the linear approximation (coefficient of determination, R^2) Microsoft Excel 2016 was used. Statistical significance was assumed at $p < 0.01$.

Results

Patient description

AN group presented with 5 cases of serum amyloid A amyloidosis and 22 cases of immunoglobulin light chain (AL)-amyloidosis. NA group included patients with focal segmental glomerulosclerosis (n = 5), non-amyloid type of monoclonal immunoglobulin-related kidney disease (n = 5), membranous nephropathy (n = 4), diabetic nephropathy (n = 4), immunoglobulin A nephropathy (n = 4), anti-neutrophil cytoplasmic antibodies associated glomerulonephritis (n = 2), lupus nephritis (n = 1), C3-glomerulopathy (n = 1), and idiopathic membranoproliferative glomerulonephritis (n = 1). The control group comprised patients with diabetes mellitus (n = 13), cardiovascular disease (n = 12), systemic autoimmune disorder (n = 2), aplastic anemia (n = 2), Cushing disease (n = 1), human immunodeficiency virus (n = 1), multiple myeloma (n = 1), and four healthy persons. Demographic and clinical findings of the studied groups are presented in Table 1.

Table 1. Demographic and clinical data in studied groups

Parameters[a]	**AN (n = 27)**	**NA (n = 27)**	**Control (n = 36)**	**PE (n = 13)**	**NP (n = 31)**
Woman/man, n/n	18/9	15/12	24/12	13/0	31/0
Age, years	59±11	49±15	48±17	32±4	32±4
BMI, kg/m²	24.2 (20.4; 25.9)	25.4 (22.1; 30.3)	27.2 (23.0; 31.0)	30 (26; 36)	25 (24; 28)
Serum creatinine, mg/dl[b]	1.13 (0.9; 2.77)	1.55 (0.9; 2.23)	0.81 (0.69; 0.94)	0.76 (0.7; 0.8)	0.73 (0.68; 0.81)
eGFR CKD-EPI, ml/min/1,73m² [b]	54 (23; 86)	43 (27; 95)	85 (76; 102)	n/a	n/a
24-h proteinuria, g/24-h	8 (5.6;12.4)	6.6 (3.6; 13.1)	n/a	5 (2.5; 5.5)	n/a

24-h albuminuria, mg/24-h	n/a	n/a	8 (6;14)	n/a	10 (6; 15)

AN, amyloid nephropathies; BMI, body mass index; Control, patients without proteinuria; eGFR, estimated glomerular filtration rate; NA, non-amyloid nephropathies; NP, normal pregnancy; PE, preeclampsia.

[a] No parameters differed significantly between AN and NA when compared in pairs, as well as between PE and NP.

[b] In the ANOVA analysis, the Control group significantly differed from AN and NA in terms of eGFR and serum creatinine (both with $p < 0.001$).

Congophilia of urine samples did not differ in groups of amyloid and non-amyloid nephropathies

The mean CRAs in the AN and NA groups were 2.5 and 2.2 times higher, respectively, compared with the Control group (Fig. 2A). Similarly, the mean CRA was two-fold higher in PE compared with the NP group. The mean CRAs did not differ between AN and NA groups. This trend was consistent when comparing mean CRR in the membrane test (Fig. 2B) and protein concentrations between groups (Fig. 2C).

Fig 2. The results of the CRD tests and proteinuria levels in studied groups. The values of congophilia observed in the paper (A) and membrane (B) tests, as well as the levels of proteinuria (C), are shown. The box plots present the 25th and 75th percentiles (box), the maximum and minimum values, the median (line in the box), and the mean (cross). AN, amyloid nephropathies; Control, patients without proteinuria; CRA, Congo red area; CRR, Congo red retention; NA, non-amyloid nephropathies; NP, normal pregnancy; PE, preeclampsia.

The urine protein excretion and CRR values strongly correlated in AN, NA, and PE groups (Fig. 3).

Fig 3. The quantitative relationship between urine congophilia and proteinuria in three groups with nephropathies. The Spearman rank-order correlation coefficients (Spearman's correlation), 95 % confidence intervals (C. I.), *p* values (*t*-test), and scatter charts with linear trend lines are shown for each group. AN, amyloid nephropathies; CRR, Congo red retention; NA, non-amyloid nephropathies; PE, preeclampsia.

To examine Congo red binding to the main urine proteins, we conducted the CRD membrane tests on a series of dilutions of HSA and BGG in phosphate buffer, pH 7.4, and revealed a positive correlation

between CRR and the concentration of these proteins (Fig. 4A and 4B). The average ratio of CRRs to protein levels in HSA samples and the PE group did not differ (Fig. 4C).

Fig 4. The quantitative relationship between congophilia and concentrations of BGG and HSA in comparison with preeclampsia samples. The reliability of the linear approximation (coefficient of determination, R^2) of CRR dependence on concentrations of BSA (A) and HSA (B) is shown. Section B also shows urinary congophilia in the PE group (grey circles). (C) The ratios of the CRRs to the protein concentrations in HSA solutions and PE samples are shown. CRR, Congo red retention; HSA, human serum albumin; PE, preeclampsia.

Urinary congophilia was determined by binding to proteoforms of the 30-100 kDa range

The contribution of large urine protein aggregates to CRR value and the MW of the urine proteins binding to Congo red were estimated. After isolating the aggregates from urine samples by ultracentrifugation, the CRR values of the supernatants still were high. The mean CRRs did not differ between supernatants after centrifugation at 300,000 x*g* and 4,000 x*g* (p = 0.432, Fig. 5).

Fig. 5. Congophilia of urine samples from patients of different groups before and after ultracentrifugation. On the left, sample preparation conditions, mean CRRs (n = 11), and 95 % C. I. are indicated. AA, serum amyloid A amyloidosis; AL, Immunoglobulin light chain amyloidosis; ANCA, anti-neutrophil cytoplasmic antibody-associated glomerulonephritis; CF, centrifugation; CRR, Congo red retention; IgA, Immunoglobulin A nephropathy; MM, multiple myeloma; MN, membranous nephropathy; PE, preeclampsia.

Separation of protein fractions in HSA solution and urine samples using the centrifugal concentrators revealed congophilic components within the 30-100 kDa range (Fig. 6A). According to PAGE analysis, the main two protein bands in the urine samples were around 45-50 and 70 kDa (Fig. 6B).

Fig 6. Congophilia of urine samples and HSA solution after centrifugation on concentrators with cut-offs of 30 and 100 kDa. (A) Samples before centrifugation (original samples) and after centrifugation (concentrates and filtrates) are analyzed using a membrane test. At the left, urine protein fractions are listed. (B) 10 % polyacrylamide gel electrophoresis of urine samples with 15 μg of protein is shown. Proteins in the gel are stained by Coomassie

185 brilliant blue. AL, Immunoglobulin light chain amyloidosis; HSA, human serum albumin; MW, molecular weight; PE,
186 preeclampsia.

187 Concentrated urine was less congophilic than HSA solution

188 The effectiveness of the CRD tests was previously assessed by utilizing concentrated urine samples from
189 healthy pregnant women as control subjects [10]. We compared the CRR values between concentrated
190 urine samples and an HSA solution with the same protein concentration given that the ratio of
191 congophilia to protein is equal in non-concentrated samples and the HSA solutions (Fig. 3). Four control
192 urine samples of pregnant women with a protein level less than 0.3 mg/ml were concentrated by
193 lyophilization, dissolved in water, and centrifuged at 4,000 x*g*. The volume of the supernatant was
194 approximately equal to that of the precipitate, and its protein concentration was approximately 2.5
195 times lower than the concentration in the samples before lyophilization multiplied by the concentration
196 factor (Fig. 7A). Thus, the majority of the protein after dissolution remained in the sediment. The
197 membrane test showed a lower CRR in each concentrated sample (supernatant) compared to an equal
198 concentration of HSA (Fig. 7B). PAGE analysis of the original and concentrated samples revealed no
199 apparent qualitative changes in the protein composition. In nearly all cases, a distinct band, presumably
200 attributed to HSA, was observed (Fig. 7C).

201 **Fig 7. Congophilia and protein composition in concentrated urine samples from women with uncomplicated**
202 **pregnancy.** (A) From left to right: initial protein concentrations and protein amounts in the four control urine
203 samples (Ctrl1-4); procedure scheme; protein concentrations and total amounts of protein (weights and
204 percentages of the initial amount) in the supernatant obtained by concentrating. (B) Mean CRRs of original
205 samples, concentrated samples, and HSA solutions at concentrations equal to concentrated samples are shown.
206 (C) 10 % polyacrylamide gel electrophoresis results for original and concentrated samples (1 µg of protein) are
207 shown. BSA (2 µg) was applied to localize the putative HSA in the analyzed samples. Proteins in the gel are stained
208 by Coomassie brilliant blue. BSA, bovine serum albumin; CF, centrifugation; CRR, Congo red retention; Ctrl, control
209 sample from a woman with uncomplicated pregnancy; RT, room temperature.

210 Discussion

Our study was the first to evaluate urine congophilia using the CRD test in morphologically verified proteinuric nephropathies, including non-amyloid diseases and amyloidosis. The latter is assumed to be more prone to urine congophilia [11]. The study's findings demonstrated that the CRD test yielded positive results in all proteinuric patients, including male patients and those with non-amyloid kidney diseases. Urinary congophilia was strongly correlated with the urine protein concentration, regardless of the etiology of proteinuria and sex (Fig. 2 and 3). The positivity of the CRD test in cases with proteinuria limits its clinical utility for diagnosing preeclampsia. Thus, pregnant women with pre-existing renal disease unrelated to pregnancy can yield positive results in the CRD tests. Conversely, when proteinuria is absent in cases of preeclampsia, the CRD test may also have reduced effectiveness, as evidenced by a recent meta-analysis [19].

Similar to our findings, the Buhimschi group demonstrated a correlation between proteinuria and urinary congophilia and suggested a formation of amyloid-like protein aggregates as substrates for Congo red binding [10]. In our experiments, urinary congophilia was associated with urine proteins with a MW less than 100 kDa (Fig. 6), and the CRD test was still positive after removing possible aggregates of urine proteins (Fig. 5). These findings make unlikely the CRD test positivity to be attributed to large protein aggregates. As serum albumin and immunoglobulins demonstrate congophilia (Fig. 4) and are typical in urines in various nephropathies [20,21], these proteins might be responsible for the urinary congophilia in any proteinuric conditions. The binding of Congo red to HSA monomers [22] and the presence of urinary congophilia only in the 30-100 kDa fraction (Fig. 6A) confirm our assumption. However, we cannot entirely rule out the presence of low-molecular protein aggregation either under specific urine microenvironments or induced by conditions of the CRD test conducting.

To demonstrate the efficacy of the CRD test previous studies compared urinary congophilia in samples from preeclampsia patients with samples from normally pregnant women which were preliminary concentrated to achieve the same concentration of proteins in both groups [10,13]. We showed that the lower congophilia observed in the healthy pregnant might be attributed to a distinct qualitative protein composition before and after concentrating since the proteins could potentially precipitate out (Fig. 7).

Our results shed light on the possible reasons for the inaccurate estimation of the CRD test effectiveness.

Conclusion

Tests for congophilia in diagnosing preeclampsia are simple, cheap, and fast for primary screening of pregnant women [23,24]. At the same time, the effectiveness of these methods can vary greatly in hospitals in different countries [23]. Our study showed that urinary congophilia is largely determined by protein concentration and is not associated with the presence of large protein aggregates in the sample. Positive test results are obtained for patients with renal disorders of various etiologies, some of which are not associated with the detection of amyloid deposits in the body. Further research focusing on molecular mechanisms of Congo red binding to specific urine proteins could propose the possible clinical applications of the congophilia phenomenon in preeclampsia and other nephropathies.

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Figure 1

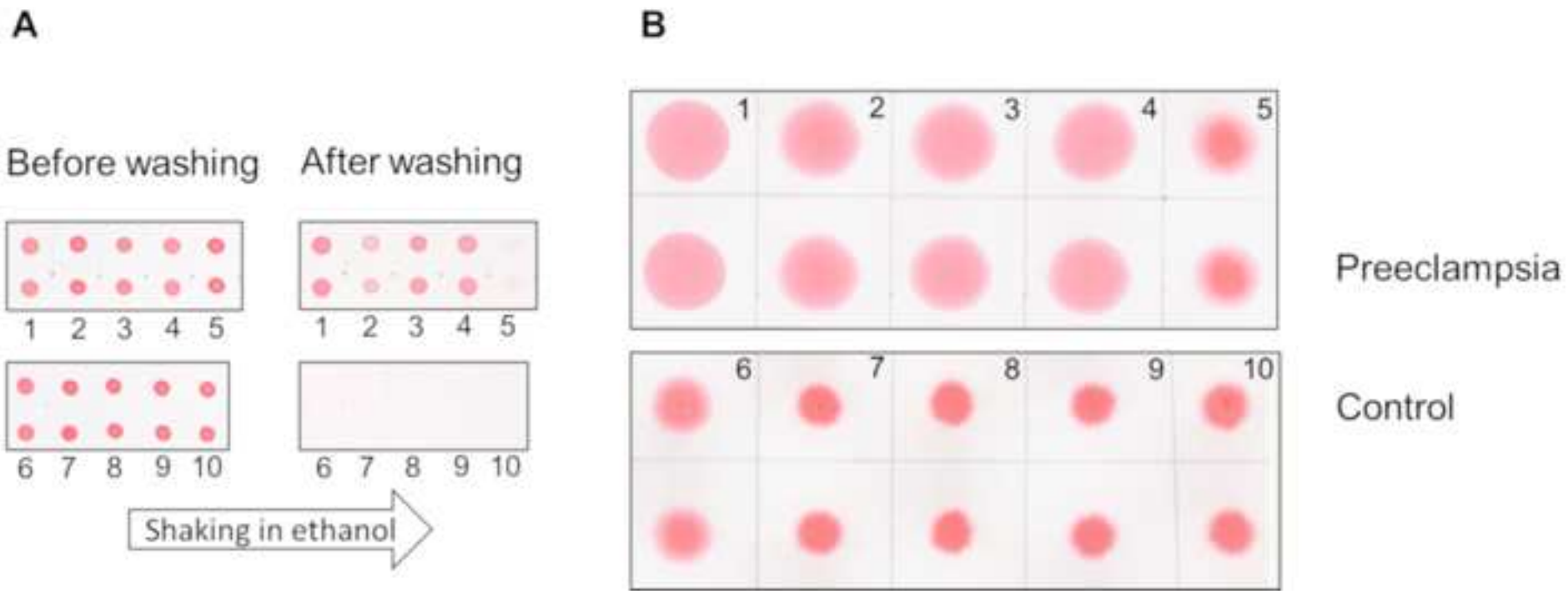

Figure 2

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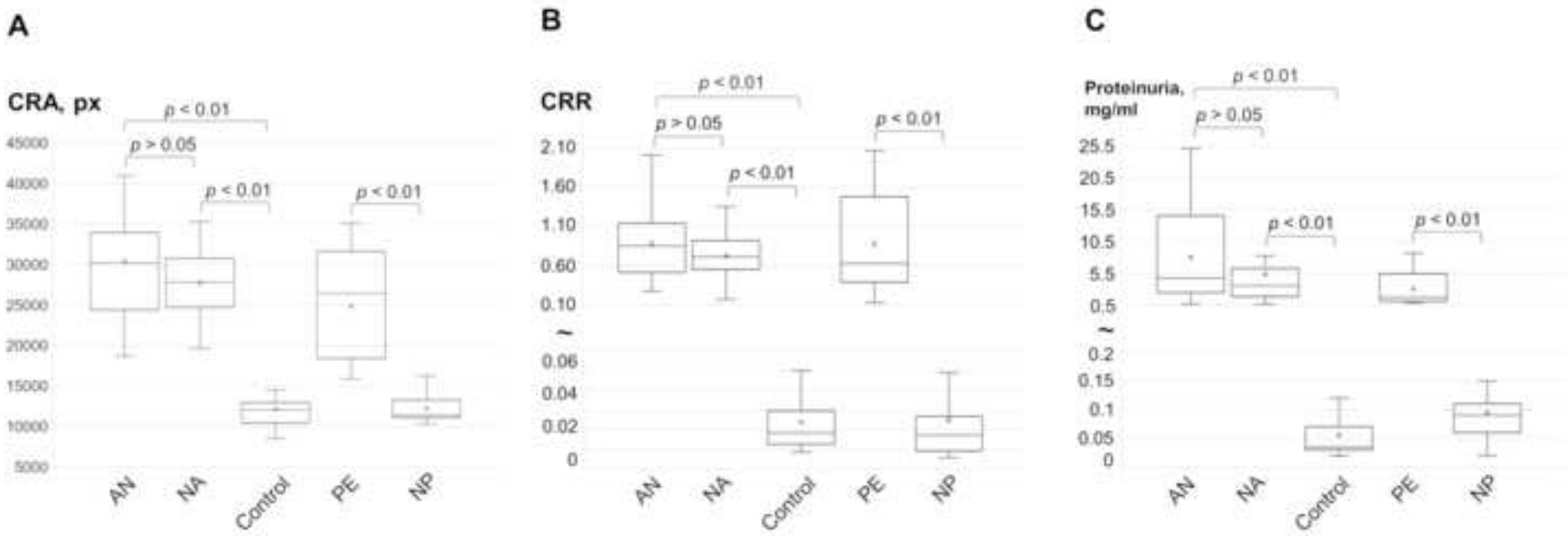

Figure 3

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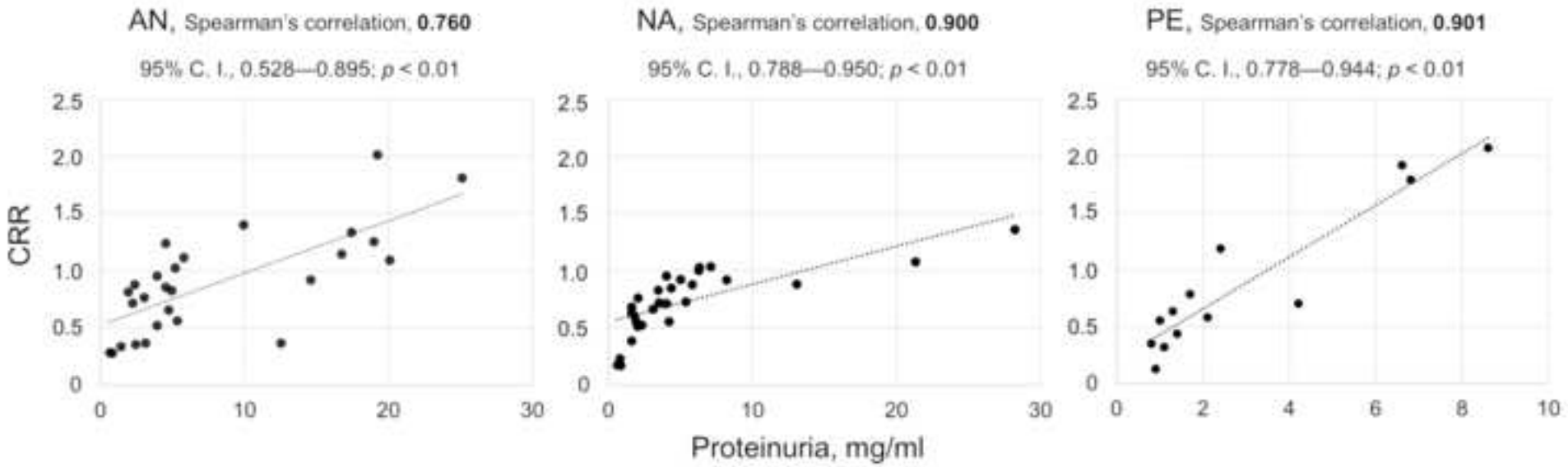

Figure 4

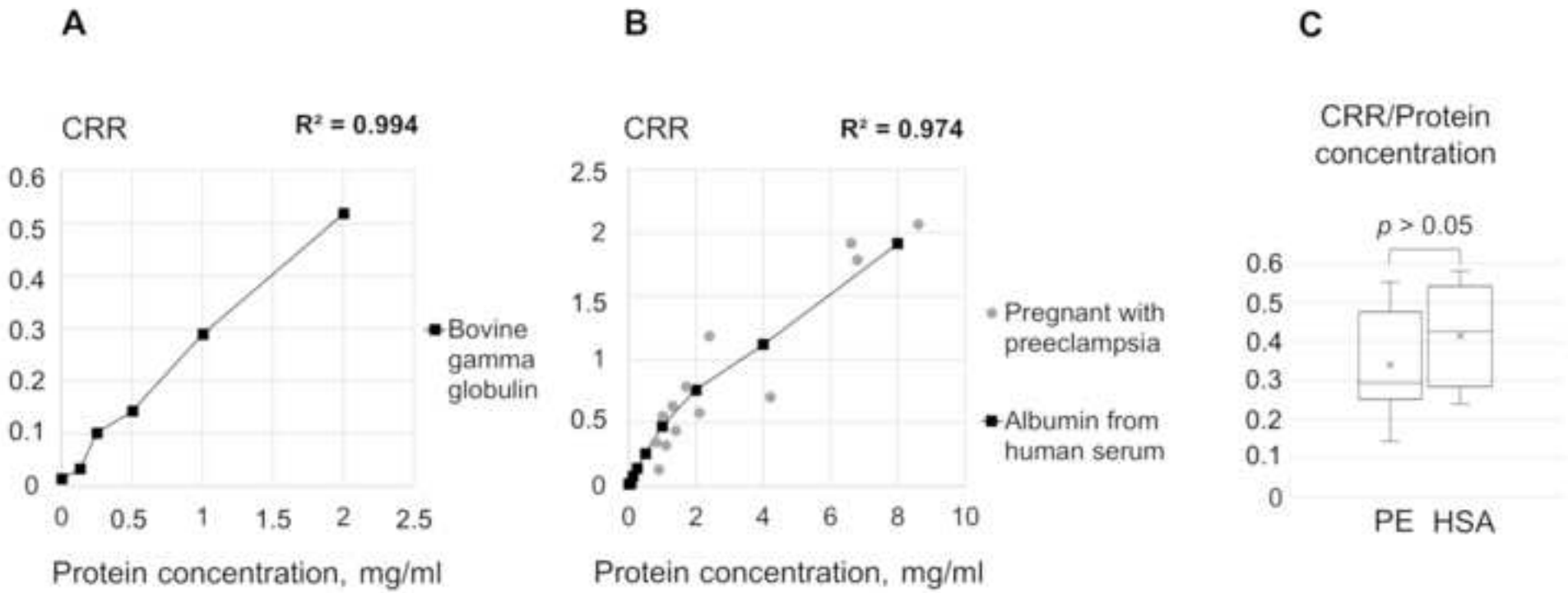

Figure 5

Diagnosis	PE 1	PE 2	PE 3	AL 1	AA 1	MM	IgA	MN	ANCA	AL 2	AA 2
Protein concentration, mg/ml	1.5	5.0	4.8	0.8	5.3	2.4	2.8	4.7	2.1	4.7	4.9

CF, 4 000 x*g*, 10 min
CRR, **0.90**, C.I., 0.759–1.029

CF, 300 000 x*g*, 120 min
CRR, **0.98**, C.I., 0.853–1.109

Figure 6

A

Diagnosis	PE	AL	HSA
Protein concentration, mg/ml	5.0	0.8	2.0
Original sample			
<30 kDa MW (filtrate)			
>30 kDa MW (concentrate)			
<100 kDa MW (filtrate)			
>100 kDa MW (concentrate)			

B

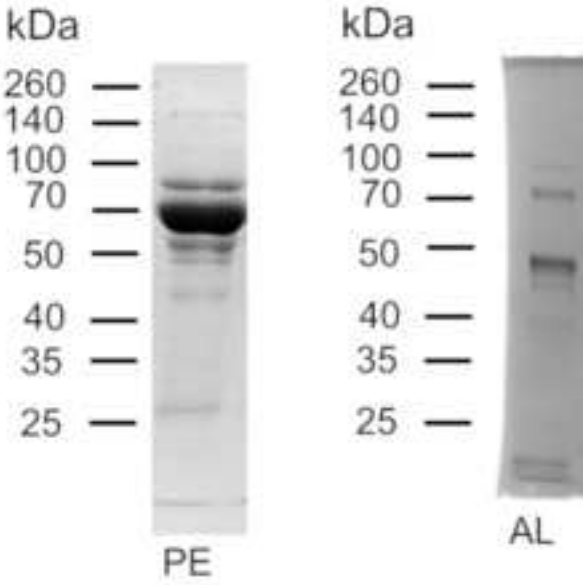

Figure 7