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Valtanen, Rosa
Buhimschi, Catalin
Bahtiyar, Mert
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Conformation-dependent anti-Aβ monoclonal antibody signatures of disease status and severity in urine of women with preeclampsia

Rosa S. Valtanen, MD[1], **Catalin S. Buhimschi, MD, MBA**[2,3], **Mert O. Bahtiyar, MD**[3], **Guomao Zhao, BSc**[2,3], **Hongwu Jing, PhD**[2], **William E. Ackerman IV, MD**[2], **Charles G. Glabe, PhD**[1], **Irina A. Buhimschi, MD, MMS**[2,3]

[1]Department of Molecular Biology & Biochemistry, University of California, Irvine, California 92617

[2]Department of Obstetrics and Gynecology, University of Illinois at Chicago College of Medicine, Chicago, Illinois 60611

[3]Department of Obstetrics and Gynecology & Reproductive Sciences, Yale University School of Medicine, New Haven, CT 06520

Abstract

Prior research has shown that urine of women with preeclampsia (PE) contains amyloid-like aggregates that are congophilic (exhibit affinity for the amyloidophilic dye Congo red) and immunoreactive with A11, a polyclonal serum against prefibrillar β-amyloid oligomers, thereby supporting pathogenic similarity between PE and protein conformational disorders such as Alzheimer's and prion disease. The objective of this study was to interrogate PE urine using monoclonal antibodies with previously characterized A11-like epitopes. Over 100 conformation-dependent monoclonals were screened and three (mA11–09, mA11–89, and mA11–205) selected for further confirmation in 196 urine samples grouped as follows: severe features PE (sPE, n=114), PE without severe features (mPE, n=30), chronic hypertension (crHTN, n=14) and normotensive pregnant control (P-CRL, n=38). We showed that the selected conformation-specific monoclonals distinguished among patients with varying severities of PE from P-CRL and patients with crHTN. By use of latent class analysis (LCA) we identified three classes of subjects: Class 1 (n=94) comprised patients whose urine was both congophilic and reactive with the monoclonals. These women were more likely diagnosed with early-onset sPE and had severe hypertension and proteinuria; Class 2 patients (n=55) were negative for congophilia and against the antibodies. These were predominantly P-CRL and crHTN patients. Lastly, Class 3 patients (n=48) were positive for urine congophilia, albeit at lower intensity, but negative for

Address and correspondence to: Dr. Irina A. Buhimschi M.D., Department of Obstetrics and Gynecology, University of Illinois at Chicago College of Medicine, 820 South Wood Street CSN W204 (office) & COMRB 7100 (lab), Chicago, Illinois 60612, Phone: (312) 996-8786 | Fax: (312) 996-4238, irina@uic.edu.

Conflict of interest/disclosure

IAB, CSB and CG are named co-inventors on patents claiming conformational-dependent immunoreactivites and congophilia as diagnostic and therapeutic targets for preeclampsia.

monoclonal immunoreactivities. These women were diagnosed primarily as mPE or late-onset sPE. Collectively, our study validates conformation-dependent Aβ imunoreactivity of PE urine which in conjunction to urine congophilia may represent an additional indicator of disease severity.

Keywords

hypertension; Alzheimer's disease; amyloid; preeclampsia/pregnancy; congophilia

Introduction

Preeclampsia (PE) is a pregnancy specific disease that affects 5–10% of all pregnancies around the world [1]. Along with other hypertensive disorders of pregnancy, PE is responsible for 76,000 maternal and 500,000 fetal deaths annually, making PE a leading cause of maternal and fetal death globally.[1]. The disorder is characterized by high blood pressure associated with proteinuria or by other signs or symptoms, usually after 20 weeks of gestation [2]. The exact cause of PE is unknown but if left untreated, this hypertensive disorder may worsen (e.g. seizure, liver and renal failure, hemorrhage, stroke, death) and permanently damage multiple organ systems [2,3]. To date, other than early delivery of the baby and placenta there is no definitive cure for PE [4]. Overall, early diagnosis with proper gestational care and careful monitoring for signs and symptoms of end organ damage are key to allow survival without sequelae for both mother and baby [5].

As high blood pressure and proteinuria can be mimicked by other clinical conditions such as chronic hypertension and nephropathy, the diagnosis of PE on the basis of clinical characteristics such as hypertension, nonspecific proteinuria or headache has proven problematic [6,7,8]. Additionally, the varied presentation and progression of PE complicates the process of accurate and early diagnosis. As such, there is a need for better modalities to diagnose PE and to predict the women who are prone to develop severe complications such as HELLP (hemolysis, elevated liver enzymes, low platelet counts) syndrome or end-organ damage [9].

While the origin of PE is unknown, the placenta is thought to play an important role in the pathophysiology of the disorder because the symptoms generally fade away upon delivery. However, many PE cases debut or worsen in postpartum suggesting that the theory of placenta as the sole origin of PE may be overly reductionist. Abnormal development of placental vasculature, widespread endothelial dysfunction, oxidative stress, systemic inflammation, and disrupted balance of angiogenic and antiangiogenic factors are thought to be involved [7,10,11].

Our group previously demonstrated that urine of PE women is congophilic (affinity for the amyloidophilic dye Congo red) due to presence of amyloid aggregates, a characteristic of protein misfolding originally described for Alzheimer's disease (AD) and extended to other protein conformational disorders (proteopathies) [12]. Subsequent studies demonstrated that urine congophilia varied with PE severity and had potential to aid in prediction of PE and differential diagnosis [13]. This led us formulate the hypothesis that PE may share additional

features of disturbed proteostasis which may also participate in disease pathogenesis [14,15]. Among these, we demonstrated that urine of women with severe features of PE (sPE) was characterized by immunoreactivity with A11, a polyclonal serum specific marker for generic epitopes presented on prefibrillar oligomers [12,16]. Conversely, urine of women with PE without severe features reacted frequently with a polyclonal serum (Officer) antibody developed against annular protofibrils, a functionally distinct more mature type of amyloid prefibrillar oligomer [12,17].

Since A11 and Officer are polyclonal antisera that contain multiple individual antibody specificities, it is still not clear if urine of women with PE contains a single or multiple conformational epitopes perhaps generated from the different amyloidogenic proteins found co-aggregated in congophilic PE urine [12,18]. Identifying a monoclonal antibody that reliably binds to select protein conformations in PE urine would provide additional insight into the types of prefibrillar oligomers characterizing urine congophilia. Here, we examined whether three distinct monoclonal antibodies that recognize epitopes within the A11 repertoire are reactive with PE urine. The relationships of the respective epitopes with urine congophilia, clinical features of PE, and whether these antibodies differentiate among clinical disease phenotypes were further explored.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Design, Patients and Biological Samples

Using a case control study design, we tested urine samples from 196 pregnant women of whom 158 had hypertensive pregnancy disorders. Women were grouped clinically as follows: **1)** chronic hypertension (crHTN, n=14, gestational age [GA] median [interquartile range]: 37 [36–38] weeks), **2)** PE without severe features (mPE, n=30, GA: 36 [33–37] weeks), **3)** PE with severe features (sPE, n=114, GA: 31 [28–34] weeks) and **4)** healthy pregnant women [P-CRL, n=38, 29 [25–30] weeks). Patients with sPE were further grouped as 72 were early-onset sPE (≤34 weeks GA) (Eo-sPE, n=72) and late-onset sPE (Lo-sPE, n=42).

Pregnant women were enrolled in the antepartum clinics and Labor and Delivery wards at Yale-New Haven Hospital (New Haven, CT) from March 2004 to January 2013. Exclusion criteria included congenital anomalies, abnormal fetal karyotype, and known presence of viral infections (hepatitis, human immunodeficiency virus). All women provided written informed consent under research protocols approved by the Institutional Review Board of Yale University and University of Illinois at Chicago where the data analyses were performed. P-CRL women attended the antenatal clinic for routine prenatal care and had a normal pregnancy outcome with delivery of a healthy term baby in the absence of a diagnosis of PE or other hypertensive disorder. Clinical management of patients with hypertensive disorders was left to the discretion of medical providers. For sPE women, delivery was recommended for worsening maternal or fetal status, which included persistent cerebral or visual symptoms, epigastric or right upper-quadrant pain, pulmonary

edema, oliguria, placental abruption, worsening laboratory parameters such as evidence of impaired liver function, increased maternal serum creatinine levels (>1.1 mg/dL), worsening manifestations of HELLP syndrome, and abnormal fetal heart rate testing [19,20]. Among women diagnosed with crHTN none were diagnosed with preeclampsia during the index pregnancy.

GA was determined based on the last menstrual period confirmed by an ultrasound examination prior to 20 weeks [21]. The ACOG Task Force definition of hypertensive disorders of pregnancy was applied [22]. sPE was defined as systolic blood pressure >160 mmHg or diastolic >110 mmHg on at least two occasions 4 hours apart, plus either of the following clinical severity features: neurologic or visual symptoms, renal insufficiency (serum creatinine >1.1 mg/dL), pulmonary edema, right upper quadrant pain, impaired liver function tests (elevated blood liver transaminases to twice the normal concentration) or thrombocytopenia (platelet count <100,000/μL). The following clinical features were considered manifestations of HELLP syndrome: hemolysis (total bilirubin >1.2 mg/dL), impaired liver function tests, and/or low platelet count irrespective of blood pressures or proteinuria. mPE was defined as blood pressure elevation of systolic ≥140 or diastolic ≥90 mmHg in addition to new onset proteinuria >300 mg in 24 hours or >0.3 urinary protein/creatinine ratio on two occasions 4–6 hours apart, in the absence of signs or symptoms consistent with a diagnosis of sPE [22]. crHTN was defined as a sustained elevated blood pressure prior to pregnancy or before 20 weeks gestation. Urine samples were collected as previously described [12,23]. Briefly, a random urine sample (5–10 mL) was collected by standard use of sterile containers using “straight catheterization” or “clean catch” sterile techniques. Every effort was made to enroll patients and collect the urine sample as close to the time of admission and diagnosis as possible. sPE women had a Foley catheter placed to allow for accurate monitoring of urinary output. Seventy percent of sPE women were enrolled and samples collected before seizure prophylaxis (magnesium sulfate) or steroid administration if preterm. Urine samples were spun at 3,000g at 4°C for 15 min. Supernatants were collected and stored in aliquots at −80°C.

Prefibrillar Oligomer and Annular Protofibril Monoclonal Antibodies

Monoclonal antibodies were developed in rabbit hybridoma cells under contract with Epitomics, Inc (Burlingame, CA). New Zealand white rabbits were immunized with homogenous populations of Aβ42 prefibrillar oligomers conformations (mA11 series monoclonals) or Aβ42 fibrils (mOC series monoclonals) prepared as described [17,17, 24,25]. Rabbits were vaccinated a total of 7 times at 3 week intervals. The serum was screened for specific titer and the rabbit with the highest titer chosen for monoclonal production. The supernatants from the resulting hybridomas were initially screened by ELISA with Aβ prefibrillar oligomers, Aβ annular protofibrils, Aβ fibrils and Aβ monomer. Wells that produced an optical density of greater than 1.0 and background reactivity on Aß fibrils and monomer were chosen for secondary screening. Approximately 100 wells were chosen for secondary screening against Aβ annular protofibrils, alpha hemolysin pores, Aß fibrils, Aβ prefibrillar oligomers, Aβ monomer and Aβ in 0.1 % SDS using dot blotting. The binding specificity of the resulting 28 monoclonal antibodies (22 mOC type and 6 mA11 type antibodies) was determined by epitomic analysis of random sequences that bind to

the antibodies [26]. Next, pilot experiments were conducted with a small number of urine samples of pregnant women with and without PE (n=15) to select the monoclonals yielding promising signals against the yet uncharacterized conformations in PE urine. The criteria for selection of the promising antibodies was negative reactivity for all P-CRL samples and positive reactivity in one or more of the sPE samples. For antibodies showing the same reactivity pattern in the pilot samples, the one yielding the more intense signal was selected. mA11–09, mA11–89, and mA11–205 monoclonals were selected based on this initial screening and additional experiments were conducted to optimize the dot blot procedure (e.g., amount of protein per spot, blocking solution, primary antibody concentration).

Urine Dot Blot Immunoassay Profiling

Six identical nitrocellulose membranes were prepared for the final experimentation. A 12×18 grid was drawn onto the membranes and 2-μL of normalized urine samples (8 μg/spot) was carefully spotted into each cell. The protein concentration of the urine was normalized by dilution or concentration to 4 mg/mL as measured with the bicinchoninic acid/cupric sulfate reagent (BCA kit, Thermo Fisher). To prevent non-specific binding of the primary antibody, the membranes were blocked with 10% milk in low-Tween (0.05%) Tris-buffered saline (TBS-T) and incubated overnight in the cold room (4°C) on a shaker. The following day, the membranes were washed three times with TBS-T solution. mA11–09, mA11–89, and mA11–205 were diluted to concentrations of 1:500, 1:10, and 1:100 respectively, each in 1% BSA in TBS-T. Each membrane was incubated with one of the three primary antibodies, and each trial was repeated. All membranes were incubated on a shaker overnight in the cold room. The following day, membranes were washed three times with TBS-T. A 1:10,000 concentration of HRP-conjugated anti-rabbit secondary solution was created in 5% milk in TBS-T. Membranes were incubated in this secondary antibody solution for one hour. Then, membranes were again washed three times TBS-T. The final wash was poured out and replaced with chemiluminescence mixture (Amersham ECL, Cytiva, Marlborough, MA). The membranes were agitated for one minute. Blots were sandwiched in between a plastic sheet protector and placed under a Nikon D700 camera. The exposure time was set to two minutes and the lights in the dark room were turned off. Images were processed and labeled on Adobe Photoshop (Adobe Systems Inc., San Jose, CA). Images were uploaded onto ImageJ (https://imagej.nih.gov/ij/index.html) which was used to quantify the amount of protein binding on each membrane against a standard curve generated from Aβ42 prefibrillar oligomers.

Urine Congophilia

Urine congophilia was measured using a nitrocellulose-based array after samples were normalized for total protein concentration as previously published [12]. Total protein was measured with the bicinchoninic acid assay (BCA assay kit, Sigma). Congophilia results are expressed as % Congo Red Retention (%CRR), which is a measure of spot redness after removing unbound Congo Red. For the purpose of this study urine congophilia was measured for all cases from the same aliquot as the one for detection of conformational immunoreactivity. The calculation of %CRR was accomplished using the automated image analysis protocol implemented on a mobile phone device that was validated in a prior

study [27]. Samples measuring %CRR ≥15% in protein-normalized urine were classified as positive for congophilia as previously described [12].

Statistical Analysis

Statistical analyses were performed with SigmaPlot 14.0 (Systat Software, San Jose, CA). Normality testing was performed using the Shapiro-Wilk test. Groups were compared with Kruskal-Wallis ANOVA on ranks followed by Dunn's tests or Mann Whitney Rank Sum test if only two groups were compared. Correlations were estimated using the Person's product moment correlation. Comparisons between proportions were done with χ^2 tests. Agreement between dichotomous variables was measured by kappa coefficient and interpreted by the scale proposed by Altman [28,29]. Heatmaps were generated in R version 3.0.3. Multivariable stepwise linear regression was used to adjust for possible influences of GA and total proteinuria. Variables were entered into the model based on $P<0.05$ and removed if $P>0.1$.

Bayesian latent class analysis (LCA), which is a statistical method that uncovers hidden patterns in data, was performed using Latent Gold (v.5.1, Statistical Innovations, Arlington MA) [30,31]. LCA models were compared for relative fit based on reductions in the following indices: Bayesian information criterion (BIC), Akaike Information Criterion (AIC) and log likelihood (LL). For our final model we chose the most parsimonious (the lowest BIC, AIC and LL) [32]. The input of each indicator or co-variate to the final model was measured by Wald statistic. High Entropy R2 (range 0–1) indicates clear delineation of classes [33]. A posterior probability was calculated for each case and the final class assignment was determined based on the highest probability. A P value of <0.05 was considered significant throughout the analysis.

Results

Characteristics of the Clinical Groups

Demographic and outcome characteristics of the women who contributed urine samples are presented in Table 1. Women with crHTN were significantly older and had higher gravidity and parity. Women in the hypertensive groups had significantly elevated blood pressures and those with PE had higher proteinuria. Manifestations of HELLP syndrome were present in 25% (29/114) of women diagnosed with sPE while fetal growth restriction (FGR) was diagnosed in 14% of cases (16/114). Women with sPE were more often delivered preterm (84%) and/or by Cesarean section (68%) and their babies had lower birthweights compared to all other groups.

Screening and Selection of Conformation-dependent Monoclonals Reactive with Preeclampsia Urine

Like A11 serum, A11 monoclonal antibodies bind to generic conformation-dependent epitopes independent of protein sequence, but they also distinguish different types of oligomers within this class that vary in their size distribution as well as their immune reactivity [18]. Fig. 1A exemplifies two pilot experiments where monoclonals were tested either individually (Strip 2–3) or mixed in cocktail (Strip 4) and compared to reactivity

for A11 (Strip 1). When cocktails yielded positive immunoreactivity, additional strips and samples were tested with the cocktail's component antibodies on an individual basis (Fig. 1B). Ultimately, after our screening protocol described in the Methods three monoclonals were deemed promising and selected for further validation (noted thereafter as mA11–09, mA11–89, and mA11–205). All these conformational state antibodies were raised against Aβ42 prefibrillar oligomers but demonstrated to cross-react with oligomers from other amyloid forming proteins (e.g. prion protein, α-synuclein) [17,18]. In a recent epitomic analysis of the specificity of these antibodies identified A-E-[F/Y]-R as the highest fitness epitope recognized by mA11–09 [26]. The same study identified that mA11–89 binds to a mimotope consisting of H-L-[A/D]-C-A-[A/D/G/V]-C-R, and mA11–205 preferentially binds to A-E-F-R-H [26].

Conformational Immunoreactivity Patterns Associated with Preeclampsia

Next, we examined how well the three selected monoclonal antibodies performed in distinguishing between PE from normal pregnancy urine samples and whether they were capable of revealing differences among women with different types of hypertension. Fig. 2 presents three representative dot blot arrays with identical sample arrangement, each reacted with one of the three monoclonals. Inspection of the images revealed that there are large differences in the immunoreactivities of the samples and that generally similar immunoreactivity patterns are observed for all 3 monoclonals. Additionally, a number of the individual samples displayed significant differences in immunoreactivity between the three antibodies. For example, mA11–09 (Fig. 2A) stained samples U0208 (Eo-sPE), U0164 (Eo-sPE), and U0263 (mPE) (dot blot positions b6, h4 and f7, respectively) that were not recognized by mA11–89 (Fig. 2B) or mA11–205 (Fig. 2C), while mA11–89 stained samples U0001 (Eo-sPE), U0014 (Lo-sPE), and U0015 (mPE) (dot blot positions a1, g1 and h1, respectively) that were not stained by mA11–09 or mA11–205.

Scatterplots of dot blot reactivity grouped by enrollment groups are shown in Fig. 3 with the sPE patients further separated in Eo-sPE and Lo-sPE. The pattern of differences between groups was similar for mA11–09 (Fig 3A), mA11–89 and mA11–205 (Fig 3C). All PE groups had higher median reactivity values compared to P-CRL ($P<0.001$) and both sPE groups differed from crHTN ($P<0.001$) but not from mPE. Similar pattern of group differences were noted for urine congophilia (Fig. 3D).

Table 2 presents the Pearson correlation coefficients between immunoreactivity of the three different monoclonals along with the correlation of each of the monoclonals with urine congophilia. While all correlations were significant at $P<0.001$ there were also differences. The differences and similarities among immunoreactivity patterns can be visually appreciated in the heatmap presented in Fig 3A. We further dichotomized the dot blot immunoreactivities based on signal-to-noise ratio as positive (> 2 standard deviations of P-CRL samples reacted with the same antibody) or negative. The previously reported 15% cut-off in CRR was used for dichotomization of urine congophilia as negative (<15%CRR) or positive (≥15% CRR) [12]. As shown, while kappa agreement values were in the range of "good" or "very good" for the paired comparisons between the monoclonal reactivities and "moderate" for the agreement of the antibody reactivities with urine congophilia.

Among P-CRL group, one urine sample tested negative for all three monoclonal antibodies but displayed congophilia (CRR=38%). Upon further review, this woman was enrolled at 25 weeks in absence of any signs of symptoms of PE. As her pregnancy progressed, she reported occasional headaches and visual disturbances for which she had multiple PE work-ups which revealed progressive increases in proteinuria (up to 3 grams/24 hours at term) all in the context of normal blood pressures. She ultimately delivered at term a normal birthweight baby.

Among women enrolled as mPE or sPE all but four cases were positive for urine congophilia. The four PE cases that tested negative for urine congophilia (two mPE, one Eo-sPE and one Lo-sPE) were also negative for all three monoclonal antibodies. Notably, only one of these cases fulfilled the sPE diagnostic criteria (mild range hypertension + proteinuria + headache). The other 3 cases were classified clinically as PE based on mild hypertension and neurological symptoms (two for headache and one for isolated hyperreflexia).

To explore for possible determinants of monoclonal antibody signatures we conducted multivariable linear regression analyses and found that urine congophilia (%CRR) was the sole determinant of mA11–09 reactivity ($r=0.718$, $p<0.001$) whereas the combination of total proteinuria and urine congophilia were both significant determinants for mA11–89 (CRR $r=0.694$; total proteinuria $r=0.284$, $P=0.001$ for both) and mA11–205 (CRR $r=0.590$; total proteinuria $r=0.279$, $P=0.001$ for both) reactivities. The clinical diagnosis of preeclampsia, gestational age, maternal age and parity were eliminated from the model based on $P>0.1$

Relationships between Conformational Immunoreactivity, Urine Congophilia and Clinical Features Revealed by Latent Class Analysis (LCA)

Fig. 4A presents a clustered heatmap of CRR and conformational immunoreactivities for the three monoclonals along for each individual case color coded by clinical group. Among the PE cases that were positive for urine congophilia, a significant proportion did not stain with any of the three monoclonals. This proportion was higher among mPE cases (57%, 17/30) compared to Lo-sPE (26%, 11/42) or compared to Eo-sPE (14%, 10/72).This suggests that there are protein aggregates participating to urine congophilia still unrecognized by the 3 monoclonals selected in this study. To further understand what components of the PE syndrome associate with conformational immunoreactivity, we subjected the data to LCA using the dichotomized conformation-dependent immunoreactivity and the dichotomized CRR as indicators (variables that participate at class assignment) and the demographic and clinical variables from Table 1 as covariates (variables that provide additional information but are not used for class delineation). A 3-class model was identified as the most parsimonious solution compared to one-, two-, and four-class models (Supplemental Table S1). The probabilities for class assignment into Class 1, Class 2 and Class 3 were 0.989, 0.999 and 0.988 suggesting that each case can be assigned to a class with little ambiguity. The strength of association of indicator and covariate variables with the three resulting LCA classes is shown in Supplemental Table S2. The profile plot of the three LCA Classes is presented in Fig 4B. Class 1 cases (n=94) were comprised by those more likely to react positive with one or more conformational antibodies and to have positive urine congophilia.

These women were more likely to have severe range hypertension, proteinuria and to be part of the Eo-sPE enrollment group. Class 2 cases (n=54), were more likely to be negative for all four features, in particular for mA11–205, and to be part of the P-CRL or crHTN groups. Class 3 cases (n=48) were more likely to be congophilic in the context of negative monoclonal immunoreactivity. These cases presented more often with mild hypertension and proteinuria or with non-specific neurological symptoms such as headache (Supplemental Table S3). Frequency of HELLP manifestations or FGR were not significant covariates among the 3 classes. Together, the LCA analyses suggests that while the reactivity for the monoclonal antibodies does not appear to relate to HELLP or FGR, its co-occurrence with congophilia appears to be a feature more frequently associated with severe hypertension, proteinuria, and Eo-sPE.

Lastly, among women diagnosed with PE, we explored the relationship between LCA Class assignment and urine congophilia as a continuous variable (Fig. 4C). As expected, there was a significant difference in urine congophilia between Class 1 and Class 2 ($P<0.001$). Class 3 cases (which were more likely to be positive of congophilia in the context of negative conformational immunoreactivity) had intermediate congophilia levels that were significantly higher than those of Class 2 ($P<0.001$), but lower than those observed for Class 1 ($P<0.001$). Samples retrieved from patients positive for all three monoclonal antibodies were all positive for urine congophilia with median %CRR of 81.8 [66.1–96.7] whereas those positive for only one or two of the monoclonals had significantly lower %CRR levels: 54.4 [30.0–85], $P=0.013$.

Discussion

The current study shows that urine of some women with PE contains unique protein aggregates that are reactive with one or more conformation-dependent monoclonal antibodies. The evidence presented in this study supports once again the assertion that PE is a protein conformational disorder [12]. The low amounts of immunoreactivity to mA11–09, mA11–205, and mA11–89 in P-CRL and crHTN patients compared to the higher immunoreactivity in Eo-sPE and Lo-sPE patients, and intermediate levels in mPE, suggest severity of the PE syndrome may relate to the profile of conformational pre-fibrilar oligomers. Assuming these conformational changes are involved in the pathological progression or onset of the disease, targeted therapies could be designed to either prevent or reverse the PE symptoms. Such therapeutic avenues are currently explored in AD and age-related neurodegeneration [34]. For example, BAN2401, a humanized IG1 version of a mouse monoclonal analogous to the ones tested in this study is currently undergoing clinical trials in patients with mild AD against several clinical and biomarker endpoints [35].

Our study represents an example of interdisciplinary collaboration among physicians with interest in preeclampsia and scientists with interest in neurodegenerative disorders. Of the three conformational polyclonals initially tested in the study published in 2014 [12], only A11 was commercially available at that time and a few monoclonals were in the process of being characterized. To date, the Glabe lab has developed 28 conformational state monoclonals that have been thoroughly characterized in published studies [18,24,26,36]. The antibodies in the A11 series may recognize antiparallel β-sheet amyloids because they

react with hemolysin pores and β-cylindrins that are known to contain intermolecularly hydrogen bonded antiparallel ß strands.[37,38] This is in contrast to the OC series monoclonal antibodies that appear to recognize parallel stranded β-sheet amyloid fibrils [24]. Of the monoclonals tested in this study, mA11–205 has been previously shown to react with prefibrillar oligomers prepared from Aβ, prion protein (PrP 109–149) and α-synuclein but not with amyloid plaques in human AD brain (which contain Aβ in fibrillar conformation) mA11–09 and mA11–89 were originally raised against Aβ42 annular protofibrils [17,18]. Prior studies from the Glabe lab demonstrated that these antibodies bind to β-barrel structures shared by annular protofibrils and α-hemolysins [37]. Consistent with the reported properties of A11, none of these three antibodies stained amyloid plaques in human AD brain (which contain Aβ in fibrillar conformation) and mA11–09 was demonstrated to stain intraneuronal amyloid suggestive of distinct reactivities [16,42]. In a recent study, mA11–09 and mA11–205 were determined to display binding site patterns which could be computationally predicted against Aβ sequence. In contrast, the epitomic pattern of mA11–89 was unrelated to Aβ but predicted against other amyloid sequences which are currently under characterization [17].

The amount of urine immunoreactivity with mA11–09, mA11–205, and mA11–89 correlated significantly with clinical disease severity and with urine congophilia. Remarkably, a subgroup of patients with less severe clinical manifestations and moderate levels of urine congophilia lacked these specific conformations. As a result, there may be clinical applications to detection of conformational antigens in conjunction to urine congophilia. Specifically, detection of prefibrillar oligomer conformations in women positive for urine congophilia could serve monitoring biomarkers of PE severity to guide treatment decisions such as initiation of magnesium sulfate or mandated delivery. Additionally, positivity for these quaternary conformations in congophilic women could serve as gatekeepers to future treatments where the risk-benefit ratio of continued pregnancy or early delivery outweighs that of the drug itself. A probabilistic risk model combining reactivity for multiple types of conformations similar to the one derived from our LCA analysis could be envisioned as an aid to complex clinical decision making.

The Aβ oligomer hypothesis represented a sea change in our understanding of AD pathogenesis, shifting the villain protagonist from the amyloid plaque to the soluble ligand-like Aβ oligomer [39]. The discovery at the basis of the oligomer hypothesis was that fibril free synthetic preparations of Aβ exhibited potent neurotoxicity at nanomolar concentrations causing selective neuronal cell death [39]. Prior to this, the scientific community has tried for many years to establish correlations between amyloid plaque burden and dementia. Yet, many healthy subjects with prominent brain amyloid deposition will remain cognitively unimpaired throughout life [40,41]. Many different laboratories have embarked in the characterization of amyloid oligomers showing that they differ in size, morphology, toxicity, method of preparation or purification, raising the question of structural relationships among Aβ oligomer preparations that can be linked to neurotoxicity [42,43]. As the protein misfolding theory began to extend to other diseases, it became clear that the toxic oligomeric conformations are universal across many diseases characterized by proteotoxic aggregation. Along with Congo red and thioflavin T (ThT) which provided the first common features of misfolded proteins, conformation-dependent antibodies have been at the experimental

forefront of unifying seemingly unrelated diseases under a common pathogenic umbrella and the number is steadily growing [44,45,46].

Our group was the first to employ conformational-state antibodies to interrogate protein misfolding in pregnant women with PE [12,23]. In our first publication describing presence of misfolded SERPINA1 we tested weather placentas of women who delivered in the context of PE reacted with the ATZ11, an antibody raised against oligomeric misfolded SERPINA-1 [47]. We found an intense staining pattern in sPE placentas with predominant endothelial and perivascular localization, whereas the villi of the control placentas remained entirely free of staining. As we did not expect for all our sPE subjects to harbor the piZ mutation which is the primary trigger for SERPINA-1 oligomer deposition, we remained intrigued by this observation and searched for additional reagents that could help us test the generalized misfolding hypothesis in PE.

Although our group previously reported that urine congophilia and ThT-induced fluorescence are present in urine and/or serum of sPE women [12,15], the dye-binding property of misfolded proteins is also shared by metastable proteins [48]. Therefore, the correlation between immunoreactivity for conformational monoclonals and CRR described in the current study suggests that urine congophilia is an intrinsic property of PE urine which results from excess excretion of quaternary non-random structures present in native urine whose composition and functional significance relative to PE symptomatology remains to be further fully characterized.

While our study has many strengths including the novelty of seeking these types of structural biomarkers in PE, it also has several limitations. First is that we did not include in this report urine samples collected prior to disease manifestations. In our prior studies we showed that urine congophilia precedes manifest disease, has the potential to predict PE, and can guide physicians in their clinical judgement when faced with complex and overlapping co-morbidities [12,13]. Recently this was further confirmed by another group is a different clinical setting. At this time, we do not know if in the course of the disease the type of monoclonal immunoreactivity changes in addition to the level of immunoreactivity, but this is possible at least theoretically. We demonstrated that a proportion of women with mPE were negative for mA11–08, mA11–89 and mA11–205 despite positive medium level congophilia. This does not exclude presence of non-random aggregations that have not yet been characterized or for which monoclonal antibodies have not been yet developed. While we have knowledge of a handful of candidate protein culprits responsible for urine congophilia, several other hundred may participate to the *PE misfoldome* with different combinations associating with different disease phenotypes as suggested by our pilot data [49]. We further know that the origin of congophilic proteins is mixed maternal, fetal and placental which adds to the complexity of disentangling the PE misfoldome [50]. While fragments of the Alzheimer precursor protein (APP) including Aβ were previously shown to contribute to the PE misfoldome, [12] the universe of co-aggregated proteins and physico-chemical characteristics of the environment influence the formation of the quaternary conformations revealed in this study. The *in silico* modeling of conformational epitopes resulting from protein misfolding is highly complex and to our knowledge these methods have not been perfected for epitopes resulting from aggregation of different

proteins (hetero-aggregation) which we believe characterizes the pathological aggregation in PE.

Second, we have yet to demonstrate the direct functional relevance of immunogenic aggregates in PE beyond the association with clinical symptoms. Pathophysiologic consequences of amyloid-like aggregates in neurodegenerative disease include calcium dysregulation, oxidative stress, and inflammation - three processes also part of PE pathogenesis [10,51]. However, demonstrating directly that amyloid-like aggregation induces PE will be difficult unless there is knowledge on which clients, chaperones and associated proteins are participating so the process can mimicked in cell cultures or relevant animal models. Our group has demonstrated as proof of concept that at least one candidate pregnancy-specific chaperone, pregnancy zone protein (PZP) produced by extravillous trophoblasts modifies Aβ oligomeric toxicity and these aggregates are excessively deposited in PE placenta [52]. The number of reports studying amyloid hetero-aggregation is steadily increasing [53]. It is important to note that the conformational immunoreactivity in PE validated in this study could not have been predicted through linear genomics, transcriptomics, proteomics approaches to biomarker discovery as these non-random quaternary state conformations are not encoded in the genome or epigenome. As the field of supramolecular pathology evolves, it will be important to take advantage of new experimental and computational methods that along with the monoclonal antibodies tested in this study or developed in the future will help disentangle the complex nature of the PE syndrome and provide insight into targeted therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- This study adds to the evidence that preeclampsia is a protein conformational disorder characterized by proteostasis imbalance similar to Alzheimer's disease albeit characteristic to pregnancy.
- Urine of some women with preeclampsia contains supramolecular aggregates reactive with monoclonal conformational-state antibodies raised against different oligomeric aggregation states of β-amyloid.
- There was a high level of correlation between the conformational-state monoclonal signature and urine congophilia although we found preeclampsia cases which were congophilic but lacked the antibody immunoreactivity. These cases were more often diagnosed as preeclampsia without severe features.
- Conformational-state immunologic signatures vary with disease severity and pending further validation may complement urine congophilia to identify patients at risk for severe complications.

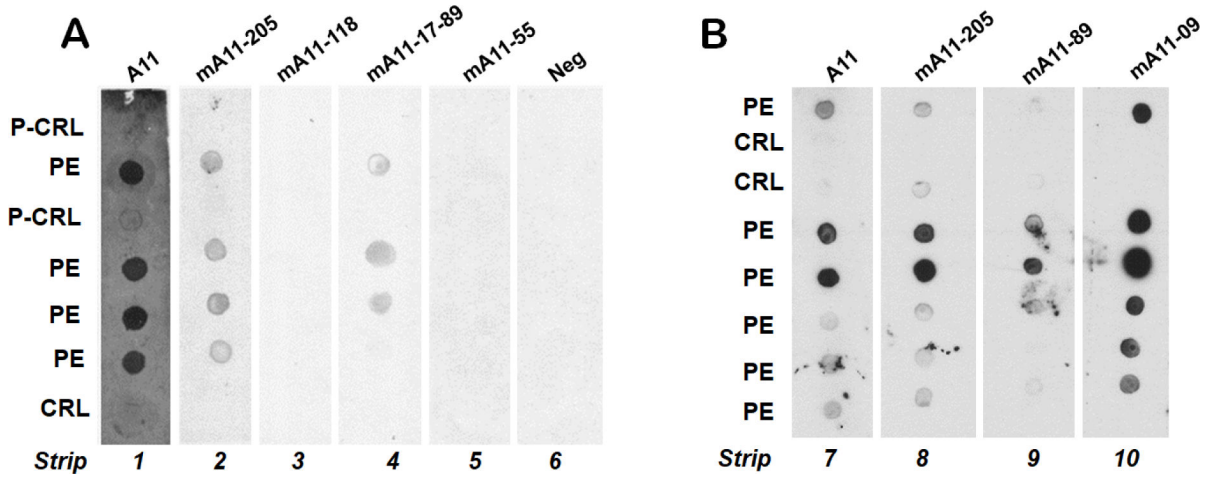

Figure 1. Screening of pregnancy urine for reactivity against b-amyloid conformational-state monoclonal antibodies.

(A) Immuno-dot blot strips with urine from representative pregnant control (P-CRL) and severe preeclampsia (sPE) women showing presence of prefibrillar oligomers reactive with polyclonal serum A11 (lane 1) and monoclonal antibodies applied individually (lanes 2, 3, 5) or as a cocktail (lanes 3 & 4). **(B)** Immuno-dot blot strips from the second round of screening with A11 (lane 7) showing presence of prefibrillar oligomers in sPE samples as revealed by the three monoclonals selected for validation.

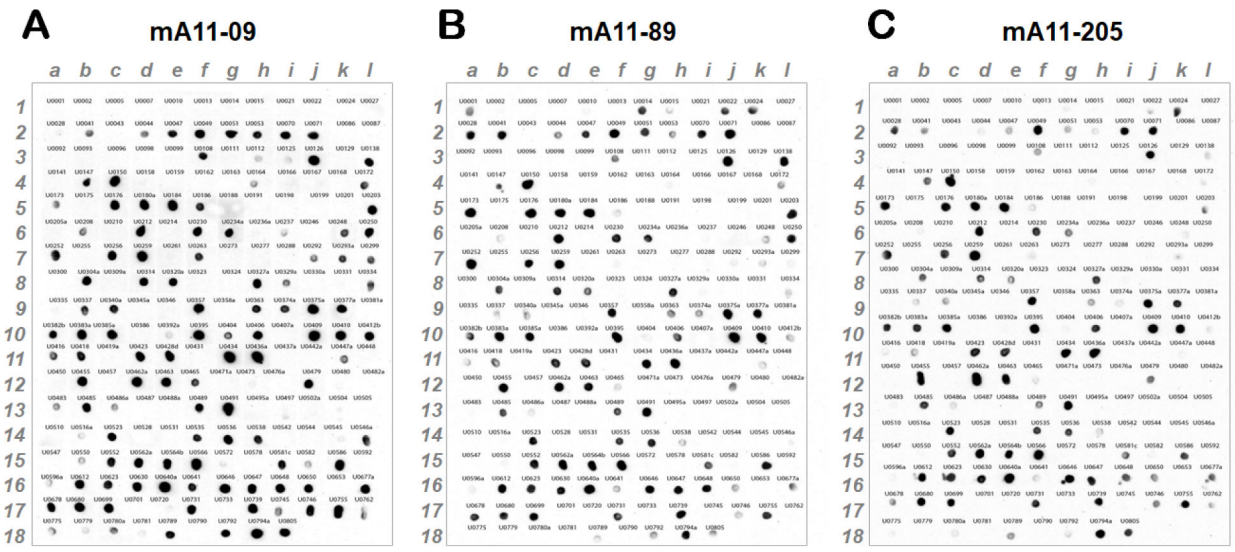

Figure 2. Representative dot blot arrays probed with the three prioritized A11 monoclonals.

Urine samples were applied in numeric sequence to nitrocellulose sheets which were blotted with three monoclonals: mA11–09 **(A)**, mA11–89 **(B)** and mA11–205 **(C)**. Approximately half of the samples displayed no detectable immunoreactivity. A number of the individual samples stained differentially with the three antibodies.

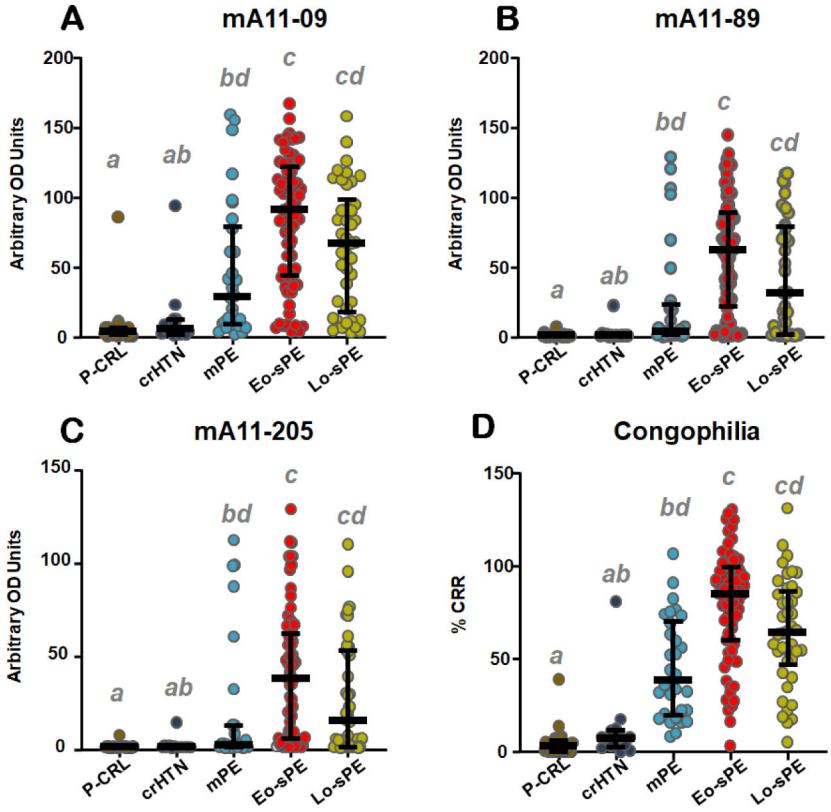

Figure 3. Conformation-dependent immunoreactivity in healthy pregnant controls and in w omen with hypertensive conditions of pregnancy.

Optical density (OD) of urine prefibrillar oligomer immune-reactivity detected with mA11–09 **(A)**, mA11–89 **(B)** mA11–205 **(C)** along with urine congophilia expressed at % Congo Red Retention (CRR) **(D)** in pregnant controls (P-CRL, n=38), chronic hypertension (crHTN, n=14), preeclampsia without severe clinical feature (mPE, n=30), early-onset preeclampsia with severe clinical features (Eo-sPE, n=72) and late-onset severe features preeclampsia (Lo-sPE, n=42). Scatterplots are presented with group median (horizontal line) and interquartile range (vertical bars). Kruskal-Wallis analysis of variance followed by multiple comparisons with Dunn's test. Groups sharing a common letter are not significant at $P>0.05$.

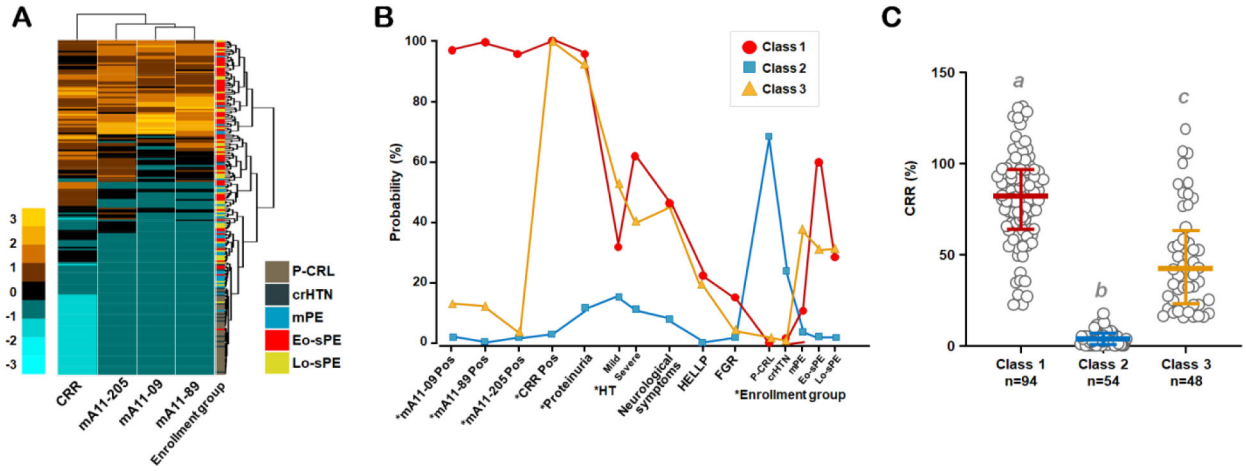

Figure 4. Relationships of conformation-dependent immunoreactivities with urine congophilia.

(A) Heat map with unsupervised hierarchical clustering showing the relationships among the immunoreactivities (measured by optical density of chemiluminescence signals) of conformation-dependent monoclonal antibodies (mA11–09, mA11–89, and mA11–205) in relation to urine congophilia, expressed as % Congo Red Retention (CRR), in standardized urine spots (8 μg/spot) from specimens (n=196) stratified according to classification at time of enrollment: pregnant controls (P-CRL, n=38), chronic hypertension (crHTN, n=14), preeclampsia without severe clinical feature (mPE, n=30), early-onset preeclampsia with severe clinical features (Eo-sPE, n=72) and late-onset severe features preeclampsia (Lo-sPE, n=42). **(B)** Profile plot of the 3-class solution derived from Latent Cluster Analysis (LCA) applied to the subjects in the study. The x-axis lists the discriminative indicators with their modal characteristic for which the probability level expected to manifest in each of the latent clusters is displayed on the y-axis. The variables marked with asterisk are statistically significant and included the monoclonal imunoreactivities, CRR, hypertension (HT), proteinuria and the clinical enrollment grouping. **(C)** Scatter plot of urine congophilia displayed as continuous variable CRR for the patients groups by latent class assignment. Different letters denote statistical significance.

Table 1.

Demographic clinical and outcome characteristics of the patient groups

Variable	P-CRL n = 38	crHTN n = 14	mPE n = 30	sPE n = 114	*P* value
Age, *years* *	27 [23 – 31]	36 [33 – 38]	27 [22 – 32]	27 [20 – 33]	**<0.001**
Gravidity *	2 [1 – 3]	4 [3 – 6]	2 [1 – 3]	1 [1 – 3]	**0.002**
Parity *	0 [0 – 1]	2 [0 – 2]	0 [0 – 1]	0 [0 – 1]	**0.043**
Nulliparity †	20 (53)	4 (29)	19 (63)	70 (61)	0.098
Race/ethnicity †					0.196
Non-Hispanic White	17 (45)	3 (22)	16 (53)	47 (41)	
Non-Hispanic Black	8 (21)	9 (64)	10 (33)	37 (32)	
Hispanic	11 (29)	2 (14)	3 (10)	26 (23)	
Other	2 (5)	0 (0)	1 (3)	4 (4)	
GA at collection, *weeks* *	29 [25 – 30]	37 [36 – 38]	36 [33 – 37]	31 [28 – 34]	**<0.001**
GA at collection †					**<0.001**
<34 weeks	22 (58)	7 (50)	4 (13)	72 (63)	
$34^{0/7}$–$36^{6/7}$ weeks	4 (11)	2 (14)	12 (40)	24 (21)	
≥$37^{0/7}$ weeks	12 (31)	5 (36)	14 (47)	18 (16)	
Twin pregnancy †	0 (0)	0 (0)	3 (10)	6 (5)	0.202
Peak systolic BP *	114 [110 – 124]	151 [127 – 161]	148 [140 – 159]	160 [150 – 171]	**<0.001**
Peak diastolic BP *	66 [60 – 70]	94 [76 – 101]	90 [87 – 99]	100 [90 – 107]	**<0.001**
Proteinuria, mg/24h *	NA	250 [120 – 395]	885 [422 – 1,868]	3,099 [1,487 – 5,005]	**<0.001**
HELLP manifestations †	0 (0)	0 (0)	0 (0)	29 (25)	**<0.001**
Fetal growth restriction †	0 (0)	1 (7)	0 (0)	16 (14)	**0.014**
Neurological symptoms including headache †	1 (3)	2 (14)	8 (27)	58 (51)	**<0.001**
Eclampsia †	0 (0)	0 (0)	0 (0)	6 (5)	0.217
GA at delivery, *weeks* *	39 [38 – 40]	38 [37 – 39]	37 [36 – 38]	32 [29 – 36]	**<0.001**
GA at delivery †					**<0.001**
<$34^{0/7}$ weeks	0 (0)	0 (0)	4 (13)	70 (61)	
$34^{0/7}$–$36^{6/7}$ weeks	0 (0)	2 (14)	12 (40)	26 (23)	
≥$37^{0/7}$ weeks	38 (100)	12 (86)	14 (47)	18 (16)	

Variable	P-CRL n = 38	crHTN n = 14	mPE n = 30	sPE n = 114	*P* value
Birth weight, grams *	3,400 [3,220 – 3,550]	2,670 [2,278 – 4,180]	2,803 [1,903 – 3,080]	1,450 [945 – 2,278]	**<0.001**
Cesarean delivery †	16 (42)	4 (3)	12 (40)	78 (68)	**<0.001**

*Data presented as median [interquartile range] and analyzed by Kruskal-Wallis ANOVA on Ranks.

†Data presented as n (%) and analyzed by χ^2 tests.

Significant *P* values are shown in bold.

Abbreviations: P-CRL, pregnant control; crHTN, chronic hypertension; mPE, preeclampsia without severe clinical features; sPE, preeclampsia with severe clinical features; HELLP, hemolysis, elevated liver enzymes, low platelet count; GA, gestational age; BP, blood pressure

Table 2.

Correlation and agreement coefficients between immunoreactivity of oligomeric monoclonals mA11–09, mA11–89 and mA11–205 and urine congophilia

Variable	mA11–09	mA11–89	mA11–205	CRR
All patient groups (n=196)				
M09	1	r = 0.854 $P = 5.1 \times 10^{-57}$ K = 0.847 [0.773–0.9211	r = 0.864 $P = 1.2 \times 10^{-59}$ K = 0.857 [0.785–0.9291	r = 0.718 $P = 2.2 \times 10^{-32}$ K = 0.513 [0.407–0.6181
M89		1	r = 0.905 $P = 8.7 \times 10^{-74}$ K = 0.867 [0.798–0.9371	r = 0.708 $P = 3.6 \times 10^{-31}$ K = 0.540 [0.436–0.6451
M205			1	r = 0.632 $P = 3.3 \times 10^{-23}$ K = 0.467 [0.363–0.5701
CRR				1
Patients enrolled in mPE and sPE groups (n=144)				
M09	1	r = 0.817 $P = 1.1 \times 10^{-35}$ K = 0.781 [0.672–0.8901	r = 0.849 $P = 3.8 \times 10^{-41}$ K = 0.790 [0.686–0.8941	r = 0.552 $P = 7.2 \times 10^{-13}$ K = 0.105 [0.008–0.2021
M89		1	r = 0.833 $P = 1.3 \times 10^{-48}$ K = 0.818 [0.721–0.9161	r = 0.603 $P = 1.4 \times 10^{-15}$ K = 0.111 [0.009–0.2131
M205			1	r = 0.532 $P = 7.2 \times 10^{-12}$ K = 0.088 [0.006–0.1711
CRR				1

r represents the Pearson coefficient of correlation followed by the associated *P* value. K represents the kappa coefficient of agreement between dichotomized variables followed by 95% confidence interval. Kappa agreement coefficients are interpreted as follows: < 0.20 Poor; 0.21 – 0.40 Fair; 0.41 – 0.60 Moderate; 0.61 – 0.80 Good; and 0.81 – 1.00 Very good.

Abbreviations: CRR, Congo Red Retention, a quantitative measure of urine congophilia; mPE, preeclampsia without severe clinical features; sPE, preeclampsia with severe clinical features.