Nephrin in maternal urine applied to a point-of-care kit to predict preeclampsia: a prospective study.

Kyong-No Lee[1], Subeen Hong[2], Tae Eun Kim[3], Eun Ji Oh[1], Ki-seok Kim[4], Ju-Hyung Kang[4], Min-Young Yeo[4], Hyeon Ji Kim[1], and Jee Yoon Park[3]

[1]Seoul National University Bundang Hospital Department of Obstetrics and Gynecology
[2]Catholic University of Korea School of Medicine
[3]Seoul National University Bundang Hospital
[4]Aptamer Sciences Inc

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Abstract

Objective: Nephrin is a protein in the glomerular podocyte slit diaphragm; therefore, its presence in urine implies damage to podocytes. This study aimed to determine the efficacy of nephrin as a biomarker in maternal urine to predict preeclampsia (PE). Design and setting: This prospective study included pregnant women admitted for delivery at Seoul National University Bundang Hospital: March 2019 – May 2020. Population: Patients who had been diagnosed with PE were included and patients without a history of underlying diseases were recruited for the control group. Important clinical data has been collected. Methods: Urine samples were obtained, and nephrin signaling was detected through test strips using a lateral flow assay. Main Outcome Measures: The results of the point-of-care test were compared between the 2 groups: patients with PE, and without (control group) using the exact concentration of nephrin by enzyme-linked immunosorbent assay (ELISA). Results: Clinical characteristics – maternal age, rate of nulliparity, proportion of twin pregnancies, height, weight, cesarean section rate – were comparable between the PE and control groups. Nephrin signals were classified into four groups. In the PE group, signals 0, 1, 2, and 3 were found in 18.4% (9/49), 44.9% (22/49), 24.5% (12/49), and 12.2% (6/49) of participants, respectively. This was significantly different from the control group, in which 84.3% (43/51) were found to have signal 0 ($P<0.001$). Conclusions: Nephrin signaling in maternal urine could be a noninvasive and useful test for predetecting severity of PE.

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[a]Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul National University Bundang Hospital, Seongnam, Gyeonggi, Republic of Korea

[b]Aptamer Sciences Inc., Seongnam, Gyeonggi, Republic of Korea

[c]Department of Obstetrics and Gynecology, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea

Correspondence to:

Jee Yoon Park, MD

Department of Obstetrics and Gynecology Seoul National University Bundang Hospital

82 Gumi-ro, 173 beon-gil, Bundang-gu, Seongnam-si, Gyeonggi-do, 13620, Republic of Korea

Fax: +82-31-787-4054

Tel: +82-31-787-7266

E-mail: *jyparkmd08@snubh.org*

Running Title: Predicting severity of preeclampsia with nephrin

Abbreviations:

- Area under the curve (AUC)
- Enzyme-linked immunosorbent assay (ELISA)
- Placental growth factor (PlGF)
- Preeclampsia (PE)
- Soluble endoglin (sEng)
- Soluble fms-like tyrosine kinase 1 (sFlt-1)

Abstract

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Methods: Urine samples were obtained, and nephrin signaling was detected through test strips using a lateral flow assay.

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Results : Clinical characteristics – maternal age, rate of nulliparity, proportion of twin pregnancies, height, weight, cesarean section rate – were comparable between the PE and control groups. Nephrin signals were classified into four groups. In the PE group, signals 0, 1, 2, and 3 were found in 18.4% (9/49), 44.9% (22/49), 24.5% (12/49), and 12.2% (6/49) of participants, respectively. This was significantly different from the control group, in which 84.3% (43/51) were found to have signal 0 (P <0.001).

Conclusions : Nephrin signaling in maternal urine could be a noninvasive and useful test for predetecting severity of PE.

Funding: Seoul National University Bundang Hospital Research Fund [grant number 06-2019-198].

Keywords: preeclampsia, proteinuria, nephrin, biomarker, preterm birth

Tweetable abstract for social media:

This study aimed to determine the efficacy of nephrin as a biomarker in maternal urine to predict preeclampsia (PE). Patients who had been diagnosed with PE were included and patients without a history of underlying diseases were recruited for the control group. Urine samples were obtained, and nephrin signaling was detected. Nephrin signals were classified into four groups. In the PE group, signals 0, 1, 2, and 3 were found in 18.4%, 44.9%, 24.5%, and 12.2% of participants. In the control group, in which 84.3% were found to have signal 0 (P <0.001). The severity of PE was, however, implied according to the intensity of signals in this study.

Introduction

Preeclampsia (PE) is identified in approximately 4–5% of pregnancies and is the second most common cause of pregnancy-related maternal death. More than 90% of maternal deaths occur in low-and lower-middle-income countries[1,2]. PE leads not only to maternal death and short-term maternal morbidities but also to long-term cardiovascular sequelae[3]. By terminating pregnancy through early diagnosis, more than half of all hypertension-related mortality can be prevented[4]. The diagnosis of PE is established when women have high blood pressure and evidence of multi-organ involvement, such as proteinuria, thrombocytopenia, liver involvement, or cerebral symptoms[5,6]. However, this method requires considerable time and specialized facilities to obtain laboratory findings and evidence of abnormal protein excretion in urine. In countries where this method cannot be easily performed, the diagnosis and treatment of PE may be delayed. To date, efforts have been made to develop easy and simple methods for the diagnosis of PE. However, they are still in the developmental stage and have not been fully validated.

Nephrin is a transmembrane protein of the slit diaphragm in podocytes and consists of a renal filtration barrier[7]. In kidney injuries, damaged podocytes lead to alterations of the slit diaphragm and foot process structure, resulting in nephrinuria. Thus, urine nephrin is well known as an early biomarker for glomerular injury in glomerulonephritis and diabetic nephropathy[8-11]. In addition, some studies have revealed that women with PE have nephrinuria, which is considered a marker for possible renal damage and a predictive indicator of severe PE[12-15]. Using aptamer-based technology, we attempted to develop a point-of-care test quantifying nephrin excretion in urine, which could be used to rapidly diagnose PE. The purpose of this study was to develop an assay to verify the accuracy and performance of this test in the diagnosis of PE.

Methods

Development of the point-of-care test quantifying nephrin excretion

Modified systematic evolution of ligands by exponential enrichment (SELEX)

The advanced SELEX was used as described by Gold et al. Briefly, we prepared a DNA library containing 40-nucleotide randomized region in which dT is substituted with 5-(N-benzyl carboxyamide)-2'-deoxyuridine (Bz-dU) or 5-(N-naphthylcarboxyamide)-2'-deoxyuridine (Nap-dU). The randomized central region was flanked by two conserved regions of 17 nucleotides (5'-GAG TGACCGTCTGCCTG-40N-CAGCCACACCACCAGCC-3'). Twenty-five thermal cycles (93 for 30 s, 52 for 20 s, and 72 for 60 s) were conducted to amplify the library. The library was applied for the SELEX process and the process was performed at 37 with following steps. A mixture of 1 mmol of aptamer library dissolved in the buffer (40 mM HEPES/pH 7.5, 120 mM NaCl, 5 mM KCl, 5 mM MgCl2, 0.002% Tween 20) was heated at 95 for 3 min, and then slowly cooled to 37 at 0.1/s for re-folding. To eliminate the non-specific binder aptamers, the aptamer library solution was pre-incubated with His-tagged magnetic bead (Invitrogen, Grand Island, NY), and supernatant was collected. The aptamers in supernatant were incubated with 10 pmol of nephrin for 30 min and then the nephrin was captured through the His.

Binding affinity

The aptamer-protein equilibrium dissociation constants (Kd) were determined by the nitrocellulose-filter binding method. Before the binding assay, aptamers were dephosphorylated using alkaline phosphatase and their 5'-ends were radiolabeled using T4 polynucleotide kinase (New England Biolabs) and [^{32}P]-ATP (Amersham Pharmacia Biotech, Piscataway, NJ). The direct binding assays were conducted by incubation of 10pM of ^{32}P-labeled aptamers with recombinant nephrin at a concentration ranging from 1 mM to 10 fM in the selection buffer at 37. The fraction of aptamers bound to nephrin was quantified with a PhosPhorImager (Fuji FLA-5100 Image Analyzer, Tokyo, Japan). The data was corrected by subtraction of nonspecific binding signal generated by binding of radiolabeled aptamers to the nitrocellulose filter from the obtained signal.

Binding competition assay

To select the aptamer pairs binding different regions of nephrin, binding competition assays were performed using [^{32}P]-labeled aptamer (hot aptamers) and unlabeled aptamers (cold aptamers). We tested all aptamer pair candidates to identify the best pair that did not compete with each other for binding nephrin. Hot aptamers (2,000 cpm) and 25 pmole cold aptamers were dissolved in S buffer (30 μL) and the solution was heated at 95 for 3 min and then slowly cooled to 25 at a rate of 0.1/s. After transferring to a 96 well plate containing 30 μL of 10 nM nephrin, the aptamer solution was incubated at 25 for 15 min. Then, 5.5 μL of Zorbax resin solution was added to the reaction mixture followed agitation in a Thermomixer for 1 min at 1,300 rpm. The mixture was applied to a PVDF filter plate, and the hot aptamers bound to nephrin were quantified using a phosphor-imager (Fuji FLA-5100 Imamge Analyzer).

Preparation of capture and detection aptamers for LFA

The aptamers were modified to be used as capture or detection aptamers. For capture aptamers, 5' biotin-labelled aptamers were conjugated with neutravidin to efficiently spot them on the nitrocellulose membrane. The biotin-labelled aptamers were dissolved in 5 μL of 1xPBS buffer at the concentration of 80 μM. The solution was heated at 95 for 5 min and cooled at 37 for 15 min, then mixed with 80 pmol of neutravidin (5 μL) and incubated at 37 for 1 hour. The detection aptamers were prepared by conjugating with streptavidin-gold nano particle (SA-GNP). The biotin-labelled aptamers were dissolved in 100 μL of PBS buffer at a concentration of 2 μM and the solution underwent heating and cooling as mentioned above. The solution was mixed with 100 μL of SA-GNP (10 O.D units/mL) and then 250 μL of 1xPBS was added. The mixture was incubated for 1 hour at room temperature with shaking, them the mixture was centrifuge at 4, 8,500 rpm. After removing the supernatant, the pellet was resuspended with 100 uL of 1xPBS buffer. We applied 6 uL of the detection aptamer solution for each strip.

Preparation of test strips

The test strip consisted of a sample pad, a nitrocellulose membrane, and an absorbent pad. A 0.8 mm sample pad was immersed in a sample pad solution (10 mM sodium phosphate (pH 7.0), 10% Tween 20) and dried at room temperature in a desiccator. To assemble the test strip, a nitrocellulose membrane was attached to the middle of the backing card, and then a sample pad and an absorbent pad were attached to the bottom and the top of the backing card, respectively. We spotted 0.5 μL of capture aptamer solution and the control aptamer solution onto a nitrocellulose membrane strip and dried for 2 days in room temperature. The schematic illustration of the LFA system using a pair of nephrin specific aptamers is described in figure 1.

Lateral flow assay

We prepared 100 μL of each sample in test tubes and mixed it with 6 μL of detection aptamer solution and 1 μL of $DxSO_4$. Then the samples were soaked via the sample pad on a test strip. We read the signal after 15 mins of incubation and imaged it with a scanner. To determine the limit of detection of LFA for nephrin, we tested with commercially available nephrin. Various concentrations of nephrin (0, 0.5, 1, 2, 5 μg/mL) were prepared by spiking in the LF running buffer or urine. Figure 2 demonstrates the competition of aptamers for binding to nephrin protein. Positive signals were observed from nephrin protein diluted from 5 μg/mL to 0.5 μg/mL. The limit of detection (LOD) of the aptamer-based LFA was 0.5 μg/mL or lower.

Study design and population

This study was conducted with women admitted to the Seoul National University Bundang Hospital between March 2019 and March 2020. Pregnant female volunteers were recruited for this study. The case group included women diagnosed with PE; and the control group consisted of women who did not present with PE or kidney disease up until delivery. After obtaining informed consent, we collected urine samples (15 ml) at admission from women diagnosed with PE and at the time of hospitalization for delivery from the normal control group. Spot urine samples were stored at 4 °C immediately after collection and tested within 48 h. The calculation of the number of target participants was based on a specificity of 92%, according to the literature on the number of samples required for the performance evaluation of the diagnostic kit. The

formula for the calculation is as follows:

$$N = P \ \times (1 - P) \times \left(\frac{\mathcal{Z}_{\frac{\alpha}{2}}}{d} \right)^2$$

When the margin of error (d) is calculated as 0.08, at the significance level α=0.05, the minimum number of participants required to show a specificity of 92% or higher according to the above formula is 44. Considering a 15% dropout rate, 51 patients were required per group. The results of the point-of-care test were compared (between the 2 groups) using the exact concentration of nephrin by enzyme-linked immunosorbent assay (ELISA). The ELISA assay was performed twice to measure the concentration of nephrin protein using the Human Nephrin DuoSet® ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. This study was approved by the Institutional Review Board of the Seoul National University Bundang Hospital (IRB No. B-1904/537-304).

Statistical analysis

We used the Student's t-test for the analysis of continuous variables, and the chi-square test or Fisher's exact test for the analysis of categorical variables. To determine the signal intensity distribution of the aptamer-based assay according to the severity of PE, a linear-by-linear association was used. The Wilson interval method was used to evaluate the following: diagnostic performance, sensitivity and specificity, positive and negative predictive values, and positive and negative likelihood ratios with 95% confidence intervals. A P -value of <.05 was considered statistically significant. IBM® SPSS® Statistics version 25.0 software (IBM® Inc., Armonk, NY) was used for the analyses.

This study was supported by the Seoul National University Bundang Hospital Research Fund [grant number 06-2019-198].

Results

Table 1 presents the baseline characteristics of the study population. There was no difference between the PE group and the control group in age, parity, the proportion of twin pregnancies, preexisting hypertension or diabetes mellitus, or the proportion of gestational hypertension or diabetes mellitus. The gestational age at hospitalization was lower in the PE group than in the control group (32.8 weeks versus 37.0 weeks of gestation, respectively). The most common sign in the PE group was high blood pressure, followed by proteinuria, presence of PE-related symptoms, and elevation of liver enzymes. Increased levels of creatinine, thrombocytopenia, and pulmonary edema were observed in less than 10% of the patients. In the control group, no signs or symptoms except for high blood pressure and proteinuria were observed.

Table 2 shows the signal intensity distribution of the aptamer-based assay according to the severity of PE. Of the 50 women with PE, 37 (74%) showed severe symptoms after admission, and 13 (26%) were diagnosed with PE without severe symptoms. Of the 52 women with signal 0 in the assay, 43 (83%) were in the control group, and 9 (17%) were diagnosed with PE (regardless of the signal 0 in the assay). On the other hand, 80% of women with signal 2 and 71% of those with signal 3, respectively, showed PE with severe features. Only 14% of women with signals 2 or 3 were in the control group. The linear-by-linear association between the signal intensity and severity of PE was shown to be significant. The quantified concentration of nephrin measured from clinical samples by ELISA and the receiver operating characteristic curve are shown in Figure 3. The area under the curve (AUC) regarding the diagnosis of PE was 0.688, and the P -value was <0.005.

Table 3 shows the diagnostic performance of the assay in PE and PE with severe features when the intensity of the assay was [?]1 and [?]2, respectively. When signal 1 (or higher) was positive, the detection rate for PE was 82% and the detection rate for PE with severe features was 89%, which was significantly higher than that of signal 2 (or higher) when positive. When signal 1 (or higher) was positive, the negative predictive value for PE with severe features was 92.3%. However, when signal 2 (or higher) was positive, the false-positive rate (1-specificity) was 6%, which was significantly lower than that of signal 1 (or higher).

Discussion

Since PE is a very serious complication during pregnancy, researchers have been searching for various biological markers to enable early detection. To date, clinical signs such as high blood pressure and cerebral symptoms are considered to be the most important symptoms of PE; many studies have reported the predictive value of soluble fms-like tyrosine kinase 1 (sFlt-1) and placental growth factor (PlGF)[16-19]. However, few guidelines have selected the sFlt-1/PlGF ratio as a diagnostic tool for PE, and a major disadvantage of the sFlt-1/PlGF ratio is that it is measured in maternal blood, which means the test is available only in medical centers. Here, we identified the possibility of detecting a marker for PE in maternal urine. This has significant implications for the early detection of PE because urine tests are easier to perform and typically less costly than other tests using samples–such as serum.

Several studies have suggested podocyturia as a novel marker for the diagnosis of PE since significant excretion of podocytes in urine was found among women diagnosed with PE; these studies also demonstrated that acute but transient podocyturia in PE tends to be closely related to continued heavy proteinuria[20,21]. Nevertheless, diagnosing podocyturia has only been done in laboratory settings involving the incubation and staining of urinary cells with podocyte-related proteins such as podocin and nephrin[12,22-25]. To solve this time-consuming problem, many studies were conducted to discover the urinary podocyte-specific proteins associated with PE, and several of them showed that the urinary concentration of nephrin was significantly higher in patients with PE than in those with normal pregnancies[13,14,26].

Main findings: In this study, we analyzed the diagnostic value of nephrin for PE. In addition to measuring the urine concentration of nephrin, we developed a point-of-care kit using nephrin-specific aptamers. The signals expressed by the kit were related to the presence and severity of PE. In the PE group: signals 1, 2, and 3 were found in 45%, 25%, and 12% of participants, respectively. In the control group: 84% was found to have signal 0, and the difference between the PE group and the control group was significant because signal 0 was found in only 18% of the PE group (P <0.001). The sensitivity and specificity of positive signals (1, 2, and 3) to predict PE were 82% and 84%, respectively. This study suggests the possibility of nephrin as a noninvasive and useful test to diagnose PE. Although the predictive values still need to be further studied and refined, the urine test kit using nephrin can be used to predict the development of PE in the general population.

Interpretation: The critical interval (of increasing or decreasing various biomarkers studied previously for the detection of PE) is relatively large. For example, the time interval in which soluble endoglin (sEng) increases is known to be from 5 weeks to 3 months after the onset of PE. For the sFlt-1/PlGF ratio, the positive predictive value reaches its highest point at 4 weeks of PE development. Compared to those markers, the level of nephrin in urine abruptly increases within 9 days of the onset of a clinical manifestation of PE[27,28]. This short interval of nephrin detection in urine related to the development of PE could be useful to predict the disease process early in pregnancy and to apply effective interventions to manage the disease[29-32].

For the last few decades, nephrin as a structural component of the podocyte slit diaphragm has been studied; however, the potential roles in extra-renal tissues and in acquired kidney diseases are not yet well known. Several groups studied the function of nephrin in various kidney diseases either by analyzing the production of nephrin mRNA and/or protein or by sequencing the nephrin gene for mutations in the samples of affected patients[10,33-36]. In a previous study, Jim et al. investigated whether the detection of nephrin in urine could be used as an early biomarker of diabetic nephropathy[10]. Another study investigated the association between nephrinuria and various traits causing renal dysfunctions in type 2 diabetes[37].

Strengths and limitations: A limitation of the present study is the relatively small sample size; however, the data collected prospectively were sufficient in showing one of the possibilities of nephrin: as a valuable predictive tool for PE, and to suggest the use of nephrin for an easy point-of-care test. Since urine is non-invasive and an easily obtainable sample, which can be acquired readily and repeatedly, using urinary markers to detect PE warrants the affirmative market feasibility in medical practice. Research on urine markers is especially promising because proteinuria and kidney malfunction are the main features of PE.

Since the samples were collected at the time of diagnosis for PE, the association between nephrin and the development of PE might not represent the predictive value of nephrin.

Conclusion:

The severity of PE was, however, implied according to the intensity of signals in this study. Future studies with large sample sizes are essential to compensate for the inconsistency of urine markers and to purify the specific markers, preventing any contamination caused by common urinary tract infections.

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Contribution to authorship :

Kyong-No Lee: Drafting of the manuscript

Subeen Hong: Drafting and editing of the draft

Tae Eun Kim: Editing of the draft

Eun Ji Oh: Editing of the draft

Ki-seok Kim, Ju-Hyung Kang, Min-Young Yeo: Planning, Analyzing data

Hyeon Ji Kim: Drafting and editing of the draft

Jee Yoon Park: Planning, analyzing data, drafting, and editing the draft.

Details of Ethics approval: This study was approved by the Institutional Review Board of the Seoul National University Bundang Hospital (IRB No. B-1904/537-304).

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Table 1. The baseline characteristics of study population

	Control (n=53)	Preeclampsia (n=50)	p value
Age (years)	34.8±4.4	35.2±4.0	0.624
Nulliparity	39 (73.6%)	36 (72.0%)	0.857
Twin pregnancy	16 (30.2%)	11 (22.0%)	0.345
Preexsiting hypertension	1 (1.9%)	3 (6.0%)	0.353
Gestational hypertension	3 (5.7%)	3 (6.0%)	1.000
Preexsiting diabetes	0 (0.0%)	1 (2.0%)	0.485
Gesetatioanl diabetes	7 (13.2%)	10 (20.0%)	0.353
Gestational age at admission	37.0±2.9	32.8±3.3	<0.001
Presence of severe symptom	0 (0.0%)	20 (40.0%)	<0.001
High blood pressure (>160/90mmHg)	7 (13.2%)	50 (100%)	<0.001
Proteinuria			
... Dipstick > 1+	7 (13.2%)	47 (94.0%)	<0.001

	Control (n=53)	Preeclampsia (n=50)	p value
... Proteinuria in 24 hour > 300mg or Protein/Creatinine ratio > 0.3	0 (0.0%)	38 (76.0%)	<0.001
Elevation of liver enzyme	0 (0.0%)	12 (24.0%)	<0.001
Elevation of Creatinine	0 (0.0%)	5 (10.0%)	0.024
Low platelet count < 10,000	0 (0.0%)	2 (4.0%)	0.233
Pulmonary edema	0 (0.0%)	2 (4.0%)	0.233

Table 2. Distribution of signal according to the severity of preeclampsia

	Control (n=53)	Preeclampsia without severe feature (n=13)	Preeclampsia with severe feature (n=37)	p-value
0 (n=52)	43 (82.7%)	5 (9.6%)	4 (7.7%)	<0.001
1 (n=29)	7 (24.1%)	6 (20.7%)	16 (55.2%)	
2 (n=15)	2 (13.3%)	1 (6.7%)	12 (80.0%)	
3 (n=7)	1 (14.3%)	1 (14.3%)	5 (71.4%)	

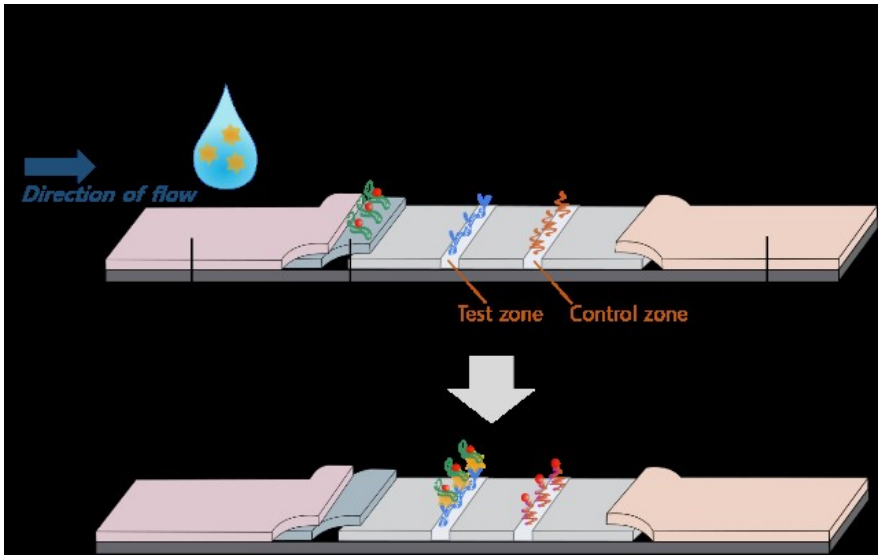
Table 3. Diagnostic performances of the point-of-care test using nephrin

	Sensitivity	Specificity	PPV	NPV	pLR	nLR	nLR
Signaling [?]1							
Preeclampsia with severe feature	89.2* (74.6-97.0)	72.7* (60.4-83.0)	64.7 (50.1-77.6)	92.3 (81.5-97.9)	3.27	3.27	0.15
Preeclampsia (total)	82.0* (68.6-91.4)	81.1** (68.0-90.6)	80.4 (66.9-90.2)	82.7 (69.7-91.8)	4.35	4.35	0.22
Signaling [?]2							
Preeclampsia with severe feature	46.0* (29.5-63.1)	92.4* (83.2-97.5)	77.3 (54.6-92.2)	75.3 (64.5-84.2)	6.06	6.06	0.58
Preeclampsia (total)	38.0* (24.7-52.8)	94.3** (84.3-98.8)	86.4 (65.1-97.1)	61.7 (50.3-72.3)	6.71	6.71	0.66

PPV, positive predictive value; NPV, negative predictive value; pLR, positive likelihood ratio; nLR, negative likelihood ratio

* P values <0.001 for the comparison between signaling [?]1 and signaling [?]2

** P values <0.05 for the comparison between signaling [?]1 and signaling [?]2



A

Nephrin		Hot aptamer	
		#01	#02
Cold aptamer	#01		
	#02		
Positive control		Without cold aptamer	
Negative control		Without protein	

B