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On renal pathophysiology in preeclampsia

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Abstract

Preeclampsia is a significant cause of maternal and fetal morbidity and mortality worldwide. A clinically useful screening test that can predict development of preeclampsia at an early stage is urgently needed. The detection of podocyuria by immunohistochemistry after cell culture has been noted as a reliable marker for preeclampsia. However, this method is laborious and carries the risk of cell-culture contamination. The aim of this study was to investigate the diagnostic value of qPCR as a rapid method to detect preeclampsia.

Clean-catch urine samples were collected from preeclamptic(n=35), healthy pregnant(n=34), and healthy non-pregnant(n=12) women. Furthermore, a control group of women with gestational hypertension(n=5) was included. qPCR analysis was performed for podocyte-specific markers. ROC-curve analyses were performed.

Significantly elevated mRNA levels of nephrin, podocin and VEGF were detected in preeclamptic women compared to healthy pregnant and healthy non-pregnant controls. In addition, significantly elevated levels of nephrin mRNA were detected in urine of preeclamptic women compared to women with gestational hypertension. A positive correlation ($\rho=0.82$, $p<0.0001$) was observed between nephrin and VEGF mRNA levels in preeclamptic women. ROC-curve analyses demonstrated a strong ability of this method to discriminate between the different study groups.

qPCR analysis of podocyte-specific molecules in urine samples is a rapid and reliable method to quantify podocyuria. We demonstrate that this method distinguishes preeclamptic patients from healthy controls and women with gestational hypertension. This method may be a tool for the detection of preeclampsia at an earlier stage, thereby preventing maternal and fetal morbidity and mortality.

Introduction

Preeclampsia affects 2-8% of all pregnancies and is an important cause of maternal and fetal morbidity and mortality worldwide. Preeclampsia is a pregnancy-specific, multisystemic disorder, with the kidney as one of the major target organs. Proteinuria and new-onset hypertension after 20 weeks of gestation are the defining diagnostic criteria for preeclampsia.¹

Proteinuria arises when there is damage to the glomerular filtration barrier (GFB). The GFB consists of a basement membrane between a layer of fenestrated endothelium and a layer of glomerular visceral epithelial cells, known as podocytes. The histological hallmark of preeclampsia in the kidney is glomerular endotheliosis. Endotheliosis is characterized by the swelling of endothelial cells, enlarged glomerular volume with hypertrophy, and loss of glomerular endothelial fenestrae. Podocyte damage and foot process effacement are visible with electron microscopy.²

Although the exact pathophysiologic mechanisms leading to preeclampsia-related proteinuria remain unknown, it is evident that preeclampsia is associated with elevated serum levels of soluble Fms-like tyrosine kinase 1 (sFlt-1) and Endoglin (sEng) and reduced serum levels of Vascular Endothelial Growth Factor-A (VEGF-A).³⁻⁵ VEGF-A regulates angiogenesis in all endothelial beds and is required in the early stages of vascular development. In the kidney, VEGF-A is expressed in tubular epithelial cells and in differentiated podocytes in the glomerulus. It appears that a paracrine/juxtacrine VEGF-signaling pathway is responsible for the cross-talk between endothelial cells and podocytes. It is thought that this signaling is crucial for the formation and maintenance of the glomerular filtration barrier.⁶

There is increasing evidence that podocytes play an important role in the renal manifestations of preeclampsia. It was demonstrated that a podocyte-specific conditional knock-out of VEGF-A in mice results in a loss of podocytes, the induction of

proteinuria, and glomerular abnormalities, such as thrombotic microangiopathy (TMA).⁷ This mechanism also appears to be relevant in humans, as was clearly demonstrated by a study in which patients with malignancies were treated with anti-VEGF therapy and developed glomerular endotheliosis, proteinuria and TMA, similar to what is observed in women with preeclampsia.⁷

Based on experimental models, it was suggested that the appearance of podocytes in urine could be a specific marker for glomerular disease. In different *in vivo* models of podocyte damage with transient or continuing injury, evidence was found that urinary excretion of viable podocytes is confined to the phase of active, ongoing glomerular damage, whereas the detection of proteinuria cannot distinguish between ongoing damage and persistent glomerular defects in the barrier's function.⁸ Garovic *et al.* showed for the first time that podocyturia as detected by immunohistochemistry on cultured podocytes is a highly sensitive and specific method for the detection of preeclampsia at disease onset.⁹ Subsequently, Aita *et al.* showed that podocyturia was still present in patients with preeclampsia one month following delivery, while proteinuria disappeared.¹⁰

Although prospective studies are still awaited, the publication by Garovic *et al.* holds the promise of a novel, possibly even predictive biomarker for preeclampsia. The method described by Garovic *et al.* has important drawbacks. Cell culture and immunohistochemistry are laborious protocols and carry the risk of cell-culture contamination. We therefore investigated the potential of quantitative polymerase chain reaction (qPCR) to detect the mRNA level of podocyte-specific molecules in urine-samples of women with preeclampsia. This method has been shown to be rapid and accurate in other clinical settings.^{11,12} We therefore hypothesized that this technique may be an alternative way to diagnose preeclampsia.

Methods

PATIENTS

A case-control study was conducted to investigate the potential of qPCR-based analysis of podocyturia as a biomarker for preeclampsia. Preeclamptic(n=35), healthy pregnant(n=34) and healthy non-pregnant(n=12) women were included. Furthermore, a control group of women with gestational hypertension(n=5) was included.

According to the ACOG-guideline¹³ the definition of preeclampsia is a new-onset hypertension (defined as an arterial systolic blood pressure of >140mm Hg or arterial diastolic blood pressure >90 mmHg) and proteinuria (>300 mg in a 24-hour urine sample) after at least 20 weeks of gestation. Gestational hypertension was defined as new-onset hypertension, but without concurrent proteinuria. Hypertension and/or proteinuria had to be resolved within 12 weeks following delivery. Patients with known hypertension, renal disease or proteinuria before pregnancy were excluded. At the time of urine collection, all patients with preeclampsia were admitted to the Department of Obstetrics of the LUMC. To prevent contamination of the maternal urine with amniotic fluid or fetal urine, urine was collected from patients whose membranes were intact.

Healthy pregnant women were matched with the patients for parity, gestational age (± 2 weeks) and age (± 5 years). These women had no medical history of preeclampsia, other hypertensive disorders or renal disease. Healthy pregnant women were monitored at the obstetrical outpatient clinic of the LUMC.

Healthy non-pregnant women were residents of the Department of Obstetrics and Gynecology of the LUMC. These women were matched with the healthy pregnant women for parity.

Urine samples were collected following the guidelines of the medical ethical review commission of the LUMC and in accordance with the recent guideline of the Dutch Federation of Scientific Societies. All samples were coded and processed anonymously.

URINE COLLECTION AND CELL ISOLATION

Clean-catch urine samples were obtained and processed within two hours of collection. Urine-samples were transferred to tubes and centrifuged at 1500 rpm for 5 minutes. Centrifuged urine samples were stored at -20°C. Albumin and creatinine levels were measured from these urine samples. Pellets of centrifuged urine samples were washed with phosphate buffered saline (PBS) and centrifuged again at 1500 rpm for 5 minutes. The pellets were suspended in RNA-later and stored at -20°C until RNA isolation.

The RNA was isolated using the Trizol method. Briefly, the cell suspension in RNA-later was centrifuged at 13000 rpm for 2 minutes. Pellets were then dissolved with Trizol and RNA was isolated as previously described.¹⁴

QPCR

cDNA was generated with AMV reverse transcriptase (20 U/μl) (Roche), according to manufacturer's instructions. For the qPCR reaction iQ™ SYBR® Green supermix was used. Expression of the different podocyte-specific markers was measured using gene-specific primers. The primer sequences used are shown in Table S1. We measured expression of genes that code for proteins that localize to the slit diaphragm of podocyte foot processes (nephrin and podocin). These markers are known to be podocyte-specific. GAPDH was used as a positive control. The levels of VEGF mRNA were also measured. In the kidney, VEGF is expressed in podocytes and in the proximal tubular epithelium. To exclude the possible presence of proximal tubular epithelial cells, mRNA levels of megalin were also measured. mRNA levels detected by qPCR were corrected for creatinine concentration and calculated per ml urine. To investigate whether the podocyte-specific mRNA levels correlated with the number of viable podocytes, mRNA was isolated from six serially diluted samples of immortalized podocytes (kindly provided by M. Saleem).¹⁵

CELL CULTURE

From a subgroup of patients 5 ml of each urine sample was used to culture the cells on 8-chamber culture slides as described previously.^{9,16} The following day the medium was removed, and the slides were fixed with methanol. All eight chambers were incubated with an anti-podocin antibody (kindly provided by Dr. C. Antignac of Hopital Necker, Paris, France) at a dilution of 1:500.¹⁷ The slides were scored blindly for the presence of podocytes. Nucleated, positive-stained cells were considered to be podocytes. Podocyturia is expressed as the ratio of the number of podocytes to the creatinine content.

STATISTICAL METHODS

Descriptive statistics are reported as the means ± SD. Patient characteristics were compared using an unpaired T-test. The mRNA levels of VEGF and GAPDH in the different groups were compared using nonparametric tests (Kruskal-Wallis and Mann-Whitney U-test). All measurements were carried out in duplicate. The mean measurement was used for the statistical analysis. Because there were non-detects for nephrin and podocin (Table S2) censored boxplots were drawn and p-values were calculated using R-package NADA (Nondetects And Data Analysis for environmental data) software, as described previously.¹⁸ Correlations between the expression of the different markers, cultured podocytes and clinical characteristics were calculated using a Spearman's coefficient. Using the receiver operating characteristic (ROC) curve, the area under the curve (AUC), sensitivity and specificity of three markers (VEGF, nephrin and podocin) treated separately and in combination were calculated. All significance tests were two-tailed and conducted at the 0.05 significance level using Graphpad Prism version 5.

Results

PATIENT CHARACTERISTICS

Patient characteristics of the different patient groups are shown in Table 1. As expected, women with preeclampsia had a significantly higher body mass index, elevated systolic and diastolic blood pressures, elevated levels of proteinuria, earlier deliveries and infants with lower birth weights compared to healthy pregnant controls. Women with gestational hypertension had significantly elevated systolic and diastolic blood pressures compared to healthy pregnant controls.

mRNA LEVELS

qPCR was performed for nephrin, VEGF, podocin, GAPDH and megalin. mRNA levels for these proteins were examined in urine-samples of preeclamptic, non-preeclamptic pregnant and non-pregnant control women. In all samples, expression of GAPDH was detectable and there were no differences in the mRNA levels of this gene between the groups.

Significantly elevated levels of podocin ($p<0.001$), nephrin ($p=0.01$) and VEGF ($p<0.001$) were detected in the urine samples of preeclamptic women compared to healthy pregnant controls (see Figure 1). The mRNA levels of podocin ($p<0.00001$), nephrin ($p<0.001$) and VEGF ($p=0.01$) were also found to be significantly elevated in samples from preeclamptic women compared to healthy non-pregnant controls. Furthermore, the mRNA levels of podocin ($p<0.01$) and VEGF ($p=0.03$) were significantly higher in the group of healthy pregnant women than in the healthy non-pregnant controls. For nephrin, the difference between these two groups was not significant. Non-detects (*i.e.* mRNA levels were under the detection limit of the qPCR) were observed for podocin and nephrin (Table S2). No megalin mRNA was detectable in any of the analyzed urine samples (data not shown). Furthermore, an additional control

group containing women with gestational hypertension was analyzed using qPCR. Significantly higher levels of nephrin mRNA were detected in the urine from the preeclamptic women than from the women with gestational hypertension ($p<0.05$). In addition, the levels of VEGF and podocin mRNA were increased 9.5-fold and 4.0-fold respectively, in the urine from preeclamptic patients, although these differences did not reach significance (data not shown).

To investigate whether the mRNA levels of podocyte-specific markers correlated with the number of viable podocytes, mRNA was isolated from a serial dilution of a known quantity of immortalized podocytes (kindly provided by M. Saleem). The Spearman's correlation coefficient revealed that the number of podocytes was significantly correlated with nephrin mRNA levels ($R=0.98$, $p<0.01$) as shown in Figure 2.

To determine whether relative expression levels were different between the various podocyte markers, mRNA levels were correlated with one another. Using Spearman's correlation coefficient, a correlation of 0.82 ($p<0.0001$) between nephrin and VEGF was observed in the preeclamptic women and in the pregnant controls (see Figure 3). Likewise, a Spearman's correlation coefficient was measured to test whether the severity of proteinuria correlated with the mRNA levels of podocyte-specific markers. The albumin/creatinine ratio did not correlate with the mRNA levels of either the combined or separate podocyte-specific markers in the patients with preeclampsia or in the controls.

CELL CULTURE

To determine whether the mRNA levels of podocyte-specific markers were correlated with the number of viable podocytes in patient samples, cells were cultured from urine collected from a subgroup of patients, and each patient's nephrin mRNA level was plotted against the patient's corresponding podocyte count. A Spearman's correlation coefficient revealed that the number of podocytes correlated significantly ($R=0.72$, $p<0.05$) with the levels of nephrin mRNA.

TEST CHARACTERISTICS: ROC CURVE ANALYSIS

A Receiver Operating Characteristic (ROC) curve analysis was performed. The area under the curve (AUC), sensitivity and specificity for the three markers (VEGF, nephrin and podocin) separately (see Table 2) and combined (see Table 2 and Figure 4) were calculated to discriminate between the different groups. The three markers combined had a higher overall value for the different calculated characteristics than when the markers were treated separately. For the group of preeclamptic women compared to healthy pregnant controls, the AUC for the three markers combined was 0.82, with sensitivity and specificity values of 68.6 and 88.2, respectively. The test characteristics for the group of women with preeclampsia compared to healthy non-pregnant women, again combining the three markers, were as follows: the AUC was 0.99, the sensitivity 94.3 and the specificity 91.7. All of these results are summarized in Table 2.

Discussion

To our knowledge, this is the first study to describe the use of qPCR to quantify podocyturia in the context of preeclampsia. In the present study, significantly elevated levels of nephrin, podocin and VEGF mRNA were detected in the urine of preeclamptic women as compared to levels in healthy pregnant controls and healthy non-pregnant controls. Significantly higher levels of nephrin mRNA were detected in urine from preeclamptic women than in urine from women with gestational hypertension.

In addition, we observed significantly elevated mRNA levels of VEGF and podocin in the urine of healthy pregnant controls as compared to healthy non-pregnant controls. Furthermore, the AUC, sensitivity and specificity for the three markers (nephrin, podocin and VEGF) treated separately and in combination indicate that this method is capable of discriminating between preeclamptic and non-preeclamptic women. Taken together, qPCR-based analysis

of podocyturia is a highly promising, non-invasive method for detecting preeclampsia.

Despite decades of research into preeclampsia, predicting which women are at increased risk of developing this condition remains problematic. Many studies have attempted to identify a predictive biomarker. Numerous molecules in serum of patients with preeclampsia, such as sFlt-1, VEGF, sEng, Placental Growth Factor, P-Selectin, and Placental Protein 13, have been proposed as candidates for early detection of preeclampsia. However, no single molecule has proven to be reliable as a predictive tool.¹⁹

Our observation that mRNA levels of podocin, nephrin and VEGF are significantly elevated in the urine of preeclamptic women compared to those in non-preeclamptic patients is in agreement with the results of Garovic *et al.*⁹ In this case-control study, the authors report an impressive correlation between podocyturia detected by immunologic staining of podocyte-specific markers and the presence of preeclampsia. Podocyturia was a highly sensitive and specific (both 100%) predictor and the correlation with the condition was stronger than for any of the known angiogenic factors. Although our sensitivity and specificity values are not 100%, our confirmation of the correlation between podocyturia and preeclampsia as described by Garovic *et al.* is an important step for the use of podocyturia as a potential early biomarker for preeclampsia.

There are also several interesting differences between our data and the results of Garovic *et al.* that bear mentioning. In normotensive women, and in women with either hypertension or proteinuria but without preeclampsia Garovic *et al.* did not observe podocyturia as measured using a urinary podocin-staining protocol. In contrast with these results, our qPCR-based analysis revealed the expression of podocyte-specific molecules in the urine of both pregnant and non-pregnant healthy women. This finding indicates that in normal pregnancy - and even in non-pregnant states - there is loss of podocytes in the urine that can be detected using qPCR.

Interestingly, these results are in agreement with the most recent study by Garovic *et al.*, which illustrated that podocyuria, shown by the identification of a podocyte-specific peptide using mass spectrometry, was measurable in women with a normal pregnancy and was further increased in preeclampsia.¹⁶

Furthermore, we found that the mRNA levels of the podocyte-specific markers were lower in women with gestational hypertension than in preeclamptic patients, with nephrin being the most prominently reduced ($p < 0.05$). These results suggest that measuring the expression of podocyte-specific markers can distinguish between gestational hypertension and preeclampsia.

The ultimate goal of qPCR analysis of podocyuria is the detection of pre-clinical stages of preeclampsia prior to the occurrence of proteinuria, thereby identifying women at risk of developing the disease. In this stage, it is likely that the number of podocytes in the urine is relatively low. Therefore, a sensitive test is needed to detect small differences in the quantity of podocytes. In this context, qPCR analysis is a promising method.

One of the challenges in the current study was to ensure both the quality and quantity of the RNA isolated from urinary cells. To minimize the possibility that differences in mRNA levels were caused by variations in the processing methods, every urine sample was handled by following to a strict protocol. Because the RIN (RNA Integrity number) showed that the RNA in most samples was partially degraded, we used random primers to synthesize the cDNA. In addition, specific PCR primers were chosen to generate small amplification fragments. Although some samples were negative (non-detects) for nephrin and/or podocin (Table S2), the housekeeping gene GAPDH was detected in every sample, and its expression levels were similar among all study groups. This result suggests that the quality of RNA isolated from urinary cells was sufficient for amplification. Therefore, the possibility of non-detects being attributable to RNA degradation seems negligible.

The method used by Garovic *et al.* has several disadvantages

compared to the method described in the present study. Cell culturing followed by immunostaining of urinary podocytes carries the risk of contamination during cell culture. Moreover, this method is relatively time-consuming (a test result can be produced in two days at the earliest), whereas the detection of podocyuria using qPCR can be performed within four hours.

An important issue is the choice of the markers used in the experiments. Podocin and nephrin are proteins that are expressed in the slit-diaphragm of the podocyte foot process, and can therefore be used as podocyte-specific markers.^{20,21} VEGF was chosen as an additional marker because its expression is known to be high in podocytes.²² As VEGF is also expressed in the proximal tubular epithelium, we examined the expression of megalin, which is expressed on tubular epithelial cells only. Because we could not detect megalin mRNA in our samples, the observed VEGF mRNA was likely derived from podocytes.

We found a significant correlation ($p < 0.0001$) between the mRNA levels of VEGF and nephrin, indicating that the mRNA levels are a representative marker for the number of podocytes in the urine. Although preeclampsia has been associated with reduced VEGF signaling, the increased VEGF mRNA levels measured in our study seem to reflect the high abundance of podocytes in the urine of women with preeclampsia. Previous work performed by Baelde *et al.* demonstrated a strong correlation between the mRNA levels of nephrin and podocin and the number of podocytes as measured by WT-1 staining (a podocyte-specific stain) in kidney biopsies.²³

Importantly, it is difficult to discriminate between viable and apoptotic podocytes using qPCR analysis of urinary sediments. However, our finding that the number of cultured, viable podocytes correlates strongly with the expression levels of podocyte-specific markers suggests that measuring the expression of these markers provides a reliable estimate of the number of viable podocytes. However, based on this experiment, although we cannot exclude the possibility that qPCR may also detect mRNA from apoptotic cells,

our results nevertheless show that mRNA levels increase with an increase in the number of viable podocytes.

An interesting observation in the present study was the significant difference in the mRNA levels of VEGF and podocin in healthy pregnant controls compared to levels in healthy non-pregnant women. This finding suggests a physiologic shedding of podocytes during normal pregnancy. Previous studies show that preeclampsia is associated with a maternal systemic inflammatory response (MSIR).^{24,25} Interestingly, a milder MSIR is also seen during normal pregnancy. It is proposed that this MSIR only leads to the clinical symptoms of preeclampsia when one or more maternal systems decompensate. According to this model, the increased podocyturia seen in healthy pregnant controls may be the result of endothelial dysfunction due to the physiologic MSIR, whereas the further increase of podocyturia seen in preeclamptic women resembles the dysregulation and decompensation of one or more maternal systems.

The present study has two primary limitations. First, we measured mRNA levels in the urine of women with preeclampsia, gestational hypertension and healthy pregnant controls. Previous studies have reported significant higher levels of podocyte-specific mRNA detected using qPCR in patients with Diarrhea+ Hemolytic Uremic Syndrome (D+ HUS)¹¹, IgA nephropathy²⁶ and diabetic nephropathy^{26,27} relative to healthy controls. Therefore, it is difficult to rely solely on podocyturia to differentiate between preeclampsia and other underlying renal diseases.

Secondly, because we used a case-control study design, it is impossible to calculate the predictive value of our qPCR analyses. A previous study suggested that podocyturia might be more sensitive than proteinuria as a marker of glomerular disease severity.⁸ Interestingly, in the present study, the albumin/creatinine ratio was not correlated with the mRNA levels of podocyte-specific markers in patients with preeclampsia. Aita *et al.* reported that podocyturia can be present in patients with preeclampsia even after delivery, whereas

proteinuria has disappeared by this stage.¹⁰ In addition, several other studies have suggested the potential use of podocyturia for detecting ongoing glomerular damage.^{28,29}

In conclusion, a prospective study is needed to assess whether podocyturia at an early stage is a more reliable marker for preeclampsia than conventional biomarkers, such as proteinuria.

PERSPECTIVES

We have shown for the first time that qPCR-based analysis of podocyte-specific molecules in urine samples is a rapid and sensitive method for quantifying podocyturia in patients with preeclampsia. Our current results are promising and qPCR-detected podocyturia may be a useful diagnostic tool to detect preeclampsia during the early stages of pregnancy. A prospective study should assess whether this test can predict which women are predisposed to preeclampsia. If so, this would be an important, much-needed step toward improving the diagnosis of preeclampsia and possibly toward therapies for the prevention of maternal and fetal morbidity and mortality worldwide.

NOVELTY AND SIGNIFICANCE

What is New?

qPCR-based analysis of podocyte-specific molecules in urine samples for quantifying podocyturia in patients with preeclampsia.

What is Relevant?

- Preeclampsia is a common pregnancy-specific hypertensive disorder with serious maternal and fetal morbidity and mortality.
- A clinically useful screening test that can predict the development of preeclampsia at an early stage is urgently needed to prevent fetal and maternal morbidity and mortality worldwide.

Summary

We have shown that qPCR-based analysis of podocyte-specific molecules in urine samples is a rapid and sensitive method for quantifying podocyturia in patients with preeclampsia.

Acknowledgments

We like to thank Clara Kolster-Bijdevaate and Marjolein Verhart for all their help with patient inclusion and collection of urine samples.

Characteristics	PE (n=35)	PC (n=34)	GH (n=5)	NPC (n=12)
Maternal age (years)	31.80 ± 5.49	31.49 ± 5.83	30.00 ± 2.34	32.17 ± 5.98
Parity	0.8 ± 0.93	0.77 ± 0.84	0.40 ± 0.55	0.75 ± 0.87
Body Mass Index	29.15 ± 9.62*	23.31 ± 3.21	26.47 ± 2.78	-
Blood pressure				
Systolic (mmHg)	145.3 ± 24.9*	114.86 ± 9.66	142.00 ± 9.75 *	-
Diastolic (mmHg)	105.6 ± 19*	69.0 ± 6.59	95.00 ± 6.12 *	-
Albumin/creatininee ratio	123.6*	0.6	Negative	-
Gestational age (weeks)				
at time of urine collection	31.46 ± 3.35	31.22 ± 3.73	36.17 ± 2.45	-
at time of delivery	32.7 ± 3.3*	39.8 ± 1.34	37.54 ± 0.80	-
Birth weight infants (g)	1716.5 ± 839.5*	3530.8 ± 518.0	2631.20 ± 673.93	-

Table 1: Patient characteristics

Data are given as the mean ± SD. * Denotes a statistically significant difference ($p < 0,01$) compared to the healthy controls. PE: preeclampsia, PC: pregnant control, GH: gestational hypertension, NPC: non-pregnant control.

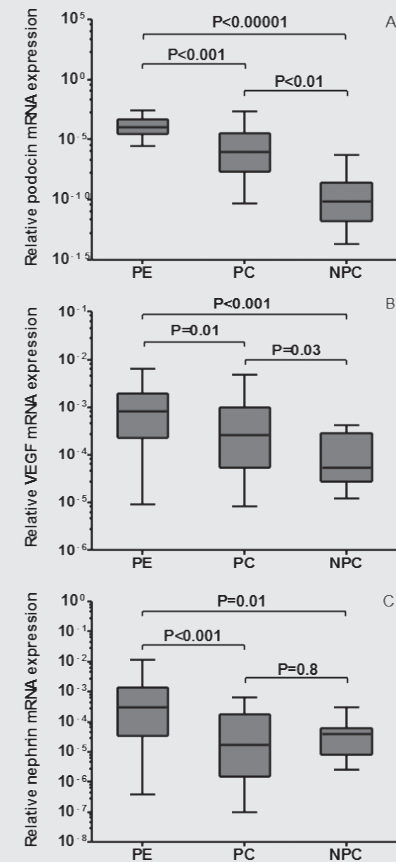
Table 2

Markers	AUC	Sensitivity	Specificity
1. PE vs. PC			
Podocin	0.69	37.1	88.2
VEGF	0.68	48.6	73.5
Nephrin	0.76	51.4	79.4
Combined	0.82	68.6	88.2
2. PE vs. NPC			
Podocin	0.85	74.3	100
VEGF	0.87	82.9	66.7
Nephrin	0.77	100	0
Combined	0.99	94.3	91.7
3. PC vs. NPC			
Podocin	0.76	100	0.0
VEGF	0.71	100	0.0
Nephrin	0.50	100	0.0
Combined	0.85	85.3	58.3

Test characteristics

In this table, the Area Under the Curve (AUC), sensitivity and specificity for the three markers (VEGF, nephrin and podocin) treated separately and in combination are shown. The test characteristics are calculated for the different groups. PE: preeclampsia, PC: pregnant control, NPC: non-pregnant control.

Figure 1

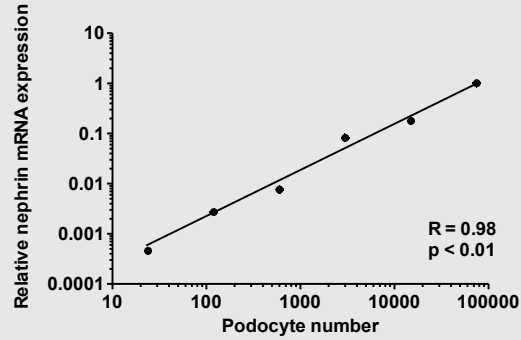


Boxplots for VEGF, nephrin and podocin

This figure shows the boxplots for podocin (A), VEGF (B) and nephrin (C) mRNA levels. For podocin and nephrin, censored boxplots are shown. A regular boxplot was drawn for VEGF. The detection limits for podocin and nephrin were 4.95×10^{-8} and 3.83×10^{-7} , respectively.

For podocin and VEGF, the calculated differences between the different groups were found to be statistically significant. Differences in nephrin mRNA levels were found to be statistically significant between the examined groups, except for levels between the healthy pregnant controls and the healthy non-pregnant controls. A logarithmic scale was used for all panels. PE: preeclamptic patients, PC: pregnant controls, NPC: non-pregnant controls.

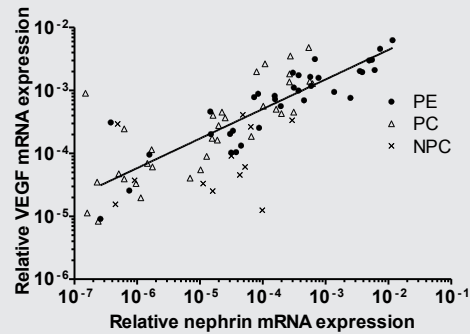
Figure 2



Correlation between number of immortalized podocytes and nephrin mRNA level

In this figure, the statistically significant correlation between the number of immortalized podocytes in six samples and nephrin mRNA level is shown. An R-value of 0.98 ($p < 0.01$) was calculated using a Spearman's coefficient.

Figure 3



Correlation between nephrin and VEGF mRNA expression levels

In this figure, the statistically significant correlation between nephrin and VEGF mRNA levels is shown. An R-value of 0.82 ($p < 0.0001$) was calculated using a Spearman's coefficient. Logarithmic scales are used for both axes. PE: preeclamptic patients, PC: pregnant controls, NPC: non-pregnant controls.

Figure 4

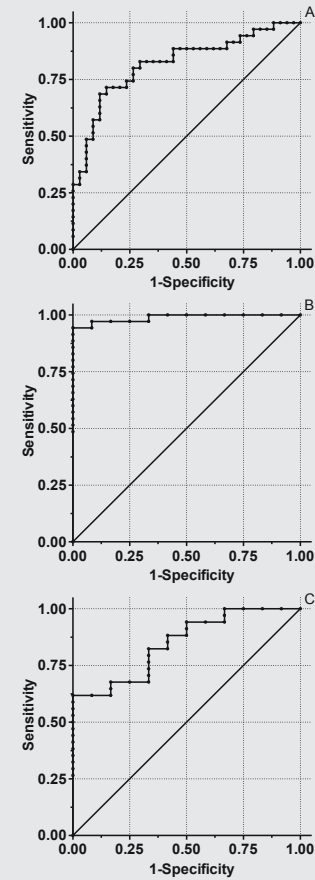


Figure 4: ROC curves

In this figure, the ROC curves for the three combined markers (VEGF, nephrin and podocin) are shown. In panel A, the ROC curve is shown for the preeclamptic group compared to the healthy pregnant controls. The calculated AUC was 0.82. Panel B shows the ROC curve for the preeclamptic group compared to the healthy non-pregnant women. Here, the AUC was 0.99. Panel C shows the ROC curve for the healthy pregnant women compared to the healthy non-pregnant women. The AUC for this analysis was 0.85.

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