Podocyturia Predates Proteinuria and Clinical Features of Preeclampsia
Longitudinal Prospective Study


Abstract—Podocyturia, the shedding of live podocytes, is present at delivery in women with preeclampsia. The aim of this study was to test whether podocyturia is present earlier in pregnancy and predicts for preeclampsia. We also aimed to compare test characteristics of podocyturia with those of angiogenic factors previously implicated in the pathogenesis of this disorder. We prospectively enrolled 315 women who provided blood and urine samples at the end of the second trimesters of their pregnancies (median, 27 gestational weeks) and within 24 hours of their deliveries (median, 39.5 gestational weeks). Blood samples were analyzed for angiogenic markers, including placental growth factor, the soluble receptor fms-like tyrosine kinase receptor-1 for vascular endothelial growth factor, and endoglin. The urine sediments were analyzed for podocytes, identified by staining for podocin after culturing the urinary sediments for 24 hours. This analysis included all women who developed preeclampsia (n=15), gestational hypertension (n=15), and a subsample of women who remained normotensive throughout pregnancy (n=44), matched for maternal age and number of previous pregnancies to those who developed preeclampsia. At the second trimester collection, all women who developed preeclampsia had podocyturia, compared with none of those who remained normotensive or were diagnosed with gestational hypertension. Podocyturia in the second trimester had a significantly greater sensitivity and specificity for the subsequent diagnosis of preeclampsia than any single angiogenic marker or a combination thereof. Screening for podocyturia at the end of the second trimester may allow for accurate identification of pregnant women at risk for preeclampsia. (Hypertension. 2013;61:1289-1296.) • Online Data Supplement

Key Words: high-risk pregnancy • podocyturia • preeclampsia • pregnancy hypertension • proteinuria

Preeclampsia is a pregnancy-specific disorder affecting 3% to 5% of all pregnancies worldwide and remains the major cause of maternal and fetal morbidity and mortality.1 It occurs after 20 weeks of gestation and is marked by hypertension (>140/90 mm Hg) and proteinuria (>300 mg in a 24-hour urine).

Accumulating evidence suggests that endothelial dysfunction, caused by placental factors that enter the maternal circulation, may play a central role in the pathogenesis of preeclampsia.2 Endothelial dysfunction in preeclampsia has been associated with an imbalance of pro- and antiangiogenic factors. This imbalance is caused by a decrease in proangiogenic factors, such as vascular endothelial growth factor and placental growth factor (PIGF), and an increase in antiangiogenic factors, including the soluble vascular endothelial growth factor receptor fms-like tyrosine kinase receptor-1 (sFlt-1) and endoglin.3,4 However, it remains unclear how endothelial dysfunction, in general, and in preeclampsia in particular, affects glomerular epithelial cells (ie, podocytes) that form the final barrier to protein loss. Foot processes of the neighboring podocytes interdigitate and interconnect via specialized cell-to-cell junctions known as glomerular slit diaphragms,5 which provide the main size selective filtration barrier in the kidney. Several podocyte proteins, including podocin,5 nephrin,6 synaptopodin,8 and podocalyxin,9 maintain the structural and functional integrity of the glomerular slit diaphragm through complex interactions. Because mature podocytes lose their mitotic activity, podocyte loss, either attributable to apoptosis10-11 or through detachment from the glomerular basement membrane, followed by urinary podocyte loss (ie, podocyturia), may cause disruption of the filtration barrier.

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with resultant proteinuria. We have reported previously that podocyturia, as identified by the staining of urinary sediment for podocyte proteins, is present in preeclamptic women at the time of delivery. Because previous studies have suggested that podocyte shedding in the urine may occur earlier in the course of glomerular disease than proteinuria, we postulated that podocyturia can be detected in urine samples before overt proteinuria develops and, therefore, may serve as a harbinger of proteinuria and the subsequent diagnosis of preeclampsia.

The first aim of this study was to examine whether podocyturia can be detected earlier in pregnancy before overt proteinuria develops and whether it is predictive of the subsequent diagnosis of preeclampsia. To do so, we prospectively collected and analyzed serial urine samples for protein and podocytes in pregnant women at 2 points during their pregnancies: at the end of the second trimester and at delivery. In the second aim, we compared the diagnostic accuracy (ie, sensitivity and specificity) of podocyturia for the prediction and diagnosis of preeclampsia with the accuracies of the serum concentrations of sFlt-1, free PIGF, and endoglin, which were measured at the same time points (ie, at the end of second trimester and at delivery). Finally, in the third aim, we studied the correlation between the degree of podocyturia and the severity of proteinuria and the levels of the angiogenic markers in women who developed preeclampsia, both at the end of the second trimester and at delivery.

Materials and Methods

Human Subjects

The study was approved by the Mayo Clinic institutional review board, and all participants gave written informed consent before inclusion in the study. A total of 315 pregnant women were enrolled prospectively at their first obstetric visit and followed until 4 to 8 weeks postdelivery. Blood and urine samples were collected at the end of the second trimester (median, 27 gestational weeks [GW]; interquartile range, 25–28) and within 24 hours of delivery (median, 39.5 GW; interquartile range, 39–40). The second trimester collection coincided with scheduled routine prenatal visits, that is, the customary screening of all pregnant women for gestational diabetes mellitus at 24 to 28 GW.

Pregnancy outcomes were ascertained at the time of hospitalization for delivery and included normotensive pregnancy, preeclampsia, gestational hypertension, and pregnancy complications other than preeclampsia and gestational hypertension (such as gestational diabetes mellitus, intrauterine growth retardation, and preterm delivery). The diagnoses of preeclampsia and hemolysis, elevated liver enzymes, and low platelet count syndrome were confirmed based on the accepted clinical criteria, including laboratory findings of proteinuria by either 24-hour urine collection or a dipstick method (Appendix in the online-only Data Supplement). Pregnant women who developed hypertension without proteinuria after 20 GW were diagnosed with gestational hypertension. Pregnancies were classified as high-risk in the presence of comorbidities that are known to be associated with a greater risk for preeclampsia (chronic kidney disease, diabetes mellitus, chronic hypertension, and systemic lupus erythematosus), or if previous pregnancies had been affected by gestational hypertension, gestational diabetes mellitus, preeclampsia, or eclampsia.

The sample for the present study included all women who developed preeclampsia or gestational hypertension and a subsample of the remaining women who remained normotensive and without other complications, matched for maternal age and number of previous pregnancies with the preeclamptic women.

Experimental Methods

Serum and Urine Chemistries and Podocyturia Assay

Serum samples obtained at the end of the second trimester and at delivery were analyzed for levels of angiogenic markers, and the corresponding urine samples were analyzed for protein/creatinine ratio and podocyturia (Appendix in the online-only Data Supplement). Aliquots of the midstream urine samples (25–50 mL, each) were collected in sterile containers and processed as previously described. Aliquots of the midstream urine samples (25–50 mL, each) were collected in sterile containers and processed as previously described. Aliquots of the midstream urine samples (25–50 mL, each) were collected in sterile containers and processed as previously described.

Statistical Methods

The statistical analysis was designed to assess the predictive value of angiogenic factor levels and podocyturia for the following diagnoses: (1) preeclampsia and (2) preeclampsia or gestational hypertension. Levels of angiogenic markers are influenced by gestational age. Because the angiogenic markers were measured at different times in the second trimester (between 25–28 GW), we derived gestational age–specific normalized Z scores for the angiogenic marker levels using 204 women with normal pregnancies as a reference group. Z scores were generated (individual value minus the gestational age–specific mean divided by its respective SD) for each angiogenic marker. Using the Z scores obtained at the second trimester collection, the predictive value of the level of each angiogenic marker was assessed by univariate logistic regression models. Receiver operating characteristic curves were derived for the level of each angiogenic marker and for podocyturia and were compared using the method of Delong and Delong.

The quantitative association between podocyturia and proteinuria was assessed by regressing podocyte count on urine protein concentration using a model that controlled for urine creatinine concentration and gestational time, at the end of second trimester and at delivery. In addition, a multiple variable model, which incorporates the levels of all 3 angiogenic markers simultaneously, was used to study the correlation between angiogenic marker levels and the podocyturia, both at the end of the second trimester and at delivery.

Results

Demographic and Clinical Variables

Of the 315 participants enrolled, there were 26 early pregnancy losses (<20-week gestation), 14 participants who were lost to follow-up or delivered elsewhere, and 2 women who had not delivered by the time of analysis. Because multiple gestations can have unpredictable effects on the levels of angiogenic factors, 6 women with twin pregnancies were excluded from the analysis. Of the remaining 267 participants, 33 women were excluded because of ≥1 of the following pregnancy complications other than preeclampsia and gestational hypertension: gestational diabetes mellitus (n=16), intrauterine growth restriction (n=5), and preterm delivery (n=17). Fifteen women developed preeclampsia, 2 of whom demonstrated the characteristic findings of hemolysis, elevated liver enzymes, and low platelet count syndrome; 15 developed gestational hypertension; and 204 had normotensive pregnancies. For the analysis, the subsample of the 44 of 204 women with normotensive pregnancies was constructed by selecting 3 normotensive controls, matched for maternal age and number of previous gestations with each preeclamptic woman. A random selection was applied for cases when >3 controls were available. One preeclamptic woman had only 2 controls attributable to lack of an
appropriately age- and gestation-matched third control with a normal pregnancy.

The women with normotensive pregnancies did not differ significantly from those with either gestational hypertension or preeclampsia in age (28.8±4.5, 30.0±3.4, and 29.2±6.4 years, respectively; \( P = 0.69 \)) or nulliparous status (70%, 67%, and 73%, respectively; \( P = 0.92 \); Table 1). Women who developed preeclampsia, compared with normotensive women, were more likely to be smokers (27% versus 5%; \( P = 0.014 \)), and to have a high-risk pregnancy (33% versus 2%; \( P = 0.001 \)). Table 2 summarizes the angiogenic markers (sFlt-1, PI GF, and endoglin), podocyturia, and urine protein/creatinine ratios in the 3 groups of pregnant women at the end of the second trimester and at delivery.

Podocyturia at the End of the Second Trimester of Pregnancy

Of the 15 women who developed preeclampsia, 100% had podocyturia at the end of the second trimester, (Figure; Appendix in the online-only Data Supplement) compared with none of either the 15 who developed gestational hypertension or the 44 controls who remained normotensive throughout pregnancy (Table 2). The presence of podocyturia at the end of the second trimester demonstrated 100% sensitivity and 100% specificity in distinguishing a subsequent diagnosis of preeclampsia from normotensive pregnancies (95% confidence interval for sensitivity 78–100, for specificity 92–100), from gestational hypertension (95% confidence interval for sensitivity 78–100, for specificity 75–100), or from both groups combined (95% confidence interval for sensitivity 78–100, for specificity 94–100).

Angiogenic Markers in the Prediction of Preeclampsia

For each angiogenic marker, the distribution was compared among normotensive, gestational hypertension, and preeclamptic pregnancies measured at the end of the second trimester (Figure). A significant overlap in angiogenic marker values was observed between women with preeclampsia and those with normotensive pregnancies, both at the end of the second trimester and at delivery.

Univariate associations of the angiogenic marker levels with subsequent pregnancy outcomes are shown in Table 3. In the univariate analysis, low levels of PI GF were associated with the outcomes of preeclampsia and of preeclampsia plus gestational hypertension, with the exception of the comparison between preeclampsia versus gestational hypertension, for which none of the markers reached statistical significance. Neither the level of sFlt-1 nor endoglin was predictive of the outcomes of preeclampsia, or preeclampsia plus gestational hypertension. When taking all 3 markers together, the improvement over the best single marker (ie, PI GF) was present in some, but not all comparisons (Table 3).

Test Characteristics of Markers in the Prediction and Diagnosis of Preeclampsia

The operating characteristics of both podocyturia and angiogenic marker levels for the prediction and diagnosis of preeclampsia (with the clinical diagnosis based on proteinuria and hypertension serving as the gold standard) were assessed through the generation of receiver operating characteristic curves, and by estimations of their sensitivities and specificities (Table 4), and comparisons of areas under the curve (Table 5). The presence of podocyturia in the second trimester and at...
delivery had significantly greater diagnostic accuracy (ie, sensitivity and specificity) for the prediction and diagnosis of preeclampsia than any of the measured angiogenic markers.

The podocyte count was independently associated with the urine protein concentration ($P=0.04$), after adjusting for the significant effects of urine creatinine concentration ($P<0.0001$) and gestational time ($P<0.0001$). We have studied the correlations between the angiogenic markers and podocyturia, both at the end of the second trimester and at delivery. In a multiple variable model, there was no association between podocyturia and any of the angiogenic markers (endoglin, $P=0.83$; PlGF, $P=0.16$; sFlt1, $P=0.31$). However, at delivery, there was a statistically significant association between podocyturia and PlGF ($P=0.03$), a trend toward an association with sFlt-1 ($P=0.06$), and no association with endoglin ($P=0.87$).

### Discussion

Our study demonstrates that podocyturia was present in pregnant women who developed preeclampsia, both toward the end of the second trimester of gestation and at the time of delivery. Because the presence of podocytes was demonstrated at a time when hypertension and proteinuria were absent, our data suggest that podocyturia identifies subclinical disease among pregnant women who will subsequently develop the clinical characteristics diagnostic of preeclampsia. As such, it may serve as an early marker of preeclampsia. Finally, we found a positive correlation between the number of podocytes and the degree of proteinuria, suggesting that ongoing podocyte loss may be mechanistically related to the onset and severity of proteinuria.

Our previous study of preeclamptic women and normotensive pregnant participants at the time of delivery demonstrated 100% sensitivity and specificity of podocyturia (as...
determined by the presence of podocin-positive cells) for the diagnosis of preeclampsia, and this has been confirmed independently by other cross-sectional studies, by the presence of cells that stain positive for podocalyxin or nephrin. Our technique is based on culturing urinary sediments overnight and assumes that these cells retain the ability to attach to tissue culture plates, which indicates cellular viability. In addition, only nucleated cells that stained for podocin were considered to be podocytes, which allowed for the differentiation of intact cells from cell particles. In contrast, in a study that questioned the use of podocyturia as a diagnostic marker for preeclampsia, podocytes were identified based on positive synaptopodin staining of urinary cytopsin. Of note, synaptopodin is a podocyte-specific protein, which is classically considered to be a marker of intact, well-differentiated podocytes, with clinical studies demonstrating that it is downregulated/absent in disease entities that are associated with dysregulated podocyte phenotypes. Similarly, the glomerular expression of synaptopodin is decreased in kidney sections from women with preeclampsia. Therefore, identification of podocytes based on positive synaptopodin staining may be a suboptimal approach in preeclampsia because the podocytes that are shed in the urine may express synaptopodin at lower levels, thus leading to false-negative results. With respect to the cytospin technique, urine sediment can be filled with red blood cells or casts, which become fixed and stained during the normal cytospin procedure. The presence of these red blood cells or casts can lead to a strong background signal, and false-positive results are not unusual, particularly after delivery with ongoing vaginal bleeding. The results of the current prospective study indicate that the presence of viable urinary podocytes in the second trimester, as demonstrated by urine sediment culture and staining with podocin, may identify women who later develop preeclampsia, even when simultaneous blood pressure readings and proteinuria assessments are normal.

On the clinical side, our data suggest that podocyturia may serve as an early marker and as a diagnostic test of preeclampsia. With respect to the mechanism of renal injury in preeclampsia, these data suggest that podocyte loss, which predates proteinuria in preeclampsia, may contribute to the occurrence and degree of severity of proteinuria, which is further supported by our findings of a positive correlation between the degree of proteinuria and the number of podocytes identified in the cultured urinary sediments. Podocyturia does not seem to be merely a result of hypertensive kidney damage, as supported by the following: (1) its presence at the time when blood pressure readings were not elevated, and (2) its absence in women with gestational hypertension. Podocyturia, which predates the clinical syndrome of preeclampsia, may lead to a disruption of the glomerular filtration barrier and, ultimately, proteinuria. In addition, the correlation between podocyturia and PlGF levels at the time of delivery suggests that these may be mechanistically related.

Similar to previously published data, our results indicate a significant overlap of PlGF and sFlt-1 values in predicting preeclamptic versus normotensive pregnancies, particularly in full-term pregnancies, potentially leading to both false-positive and false-negative screening test results. In contrast, podocyturia was able to distinguish between normotensive and preeclamptic pregnancies, as well as between pregnancies with preeclampsia and those with gestational hypertension.

The main limitation of the present study is the small sample of women who went on to develop preeclampsia or gestational hypertension and the exclusion of women with other complications of pregnancy. However, this study was designed as a proof of principle, that is, to establish the presence of podocytes before the occurrence of proteinuria in preeclampsia. These data form the basis for future studies.
that will include women with other proteinuric diseases, either predating or occurring concomitantly with pregnancy. Of note, urinary podocytes may be present in both preeclampsia and active glomerular diseases. Current practice calls for close follow-up of pregnant women with either de novo or preexisting renal disease attributable to their increased risk for superimposed preeclampsia. In these patients, the differential diagnosis between preeclampsia and a renal disease flare relies on their clinical presentation and laboratory findings (such as urinary sediment findings and serologies). Of note, one of the largest studies of renal pathology in hypertensive pregnant patients indicated that only 96 of 176 (55%) displayed the renal lesion of preeclampsia, that is, glomerular endotheliosis only.23 A renal biopsy may provide a definitive answer and guide the treatment: delivery for preeclampsia and disease-specific therapies for glomerular diseases.24 For these patients, a screening test that will confirm or rule out preeclampsia with certainty, and change our current clinical practice, has yet to be developed. In addition, given the prospective character of our study, we were unable to focus on early preeclampsia (<34 GW), when alterations in angiogenic marker levels are most prominent.3 It is possible that, in this patient population, a head-to-head comparison between podocyturia and angiogenic marker levels may reveal different results; this important question needs to be addressed in future studies, which should test for the presence of podocyturia earlier in pregnancy, that is, before 27 GW.

Finally, the method that was used to detect podocyturia is complex, labor intensive, and not amenable to high throughput.25 We have reported recently that liquid chromatography, coupled with tandem mass spectrometry, is a reliable technology for the identification of urinary podocytes, based on the presence of podocyte-specific proteins in the urine.26 In addition, quantitative polymerase chain reaction recently has been reported as a rapid method to detect podocyturia in preeclampsia.27 These new techniques are operator-independent and highly reproducible, thus overcoming the limitations of the current podocyturia assay and may facilitate

### Table 3. Role of Angiogenic Markers (Z Scores) in the Prediction of Hypertensive Disorders of Pregnancy (PE or gHTN) vs Normotensive Pregnancies

<table>
<thead>
<tr>
<th>Variable</th>
<th>PE, gHTN vs NTP</th>
<th>PE vs gHTN, NTP</th>
<th>PE vs gHTN</th>
<th>PE vs NTP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate logistic model of angiogenic markers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endoglin</td>
<td>1.44 (0.92–2.23)</td>
<td>0.11</td>
<td>1.40 (0.94–2.08)</td>
</tr>
<tr>
<td></td>
<td>PlGF</td>
<td>0.66 (0.44–1.00)</td>
<td>0.052</td>
<td>0.52 (0.30–0.92)</td>
</tr>
<tr>
<td></td>
<td>sFlt-1</td>
<td>1.42 (0.90–2.24)</td>
<td>0.14</td>
<td>1.61 (0.94–2.74)</td>
</tr>
<tr>
<td></td>
<td>Multiple variable logistic model of angiogenic markers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endoglin</td>
<td>1.28 (0.75–2.19)</td>
<td>0.11</td>
<td>0.71 (0.1–1.74)</td>
</tr>
<tr>
<td></td>
<td>PlGF</td>
<td>0.60 (0.37–0.95)</td>
<td>0.038*</td>
<td>0.45 (0.23–0.85)</td>
</tr>
<tr>
<td></td>
<td>sFlt-1</td>
<td>1.50 (0.86–2.60)</td>
<td>0.90 (0.99–3.64)</td>
<td>1.84 (0.77–4.42)</td>
</tr>
</tbody>
</table>

CI indicates confidence interval; gHTN, gestational hypertension; NTP, normotensive pregnancy; OR, odds ratio; PE, preeclampsia; PlGF, placental growth factor; and sFlt-1, soluble fms-like tyrosine kinase receptor-1.

*This P value represents the improvement in predicting the outcome when taking all 3 markers together vs the best single marker (ie, PlGF) in the univariate model.

### Table 4. Test Characteristics for Angiogenic Markers and Podocyturia in the Second Trimester and at Delivery

<table>
<thead>
<tr>
<th>Variable</th>
<th>PE, gHTN vs NTP</th>
<th>PE vs gHTN, NTP</th>
<th>PE vs gHTN</th>
<th>PE vs NTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prediction of preeclampsia at median 27 gestational weeks (IQR, 25–28)</td>
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<tr>
<td>Endoglin, ng/mL</td>
<td>5.33</td>
<td>73</td>
<td>39</td>
<td>6.14</td>
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<tr>
<td>PlGF, pg/mL</td>
<td>165.44</td>
<td>87</td>
<td>19</td>
<td>624.10</td>
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<tr>
<td>sFlt-1, pg/mL</td>
<td>1326.70</td>
<td>87</td>
<td>34</td>
<td>1451.59</td>
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<tr>
<td>Podocyturia, no. of cells/mg creatinine</td>
<td>0.30</td>
<td>54</td>
<td>100</td>
<td>0.30</td>
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<tr>
<td>Diagnosis of preeclampsia at median 39–5 gestational weeks (IQR, 39–40)</td>
<td></td>
<td></td>
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<tr>
<td>Endoglin, ng/mL</td>
<td>10.66</td>
<td>89</td>
<td>39</td>
<td>11.57</td>
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<tr>
<td>PlGF, pg/mL</td>
<td>364.93</td>
<td>80</td>
<td>20</td>
<td>323.66</td>
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<tr>
<td>sFlt-1, pg/mL</td>
<td>4524.00</td>
<td>89</td>
<td>43</td>
<td>4524.00</td>
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<tr>
<td>Podocyturia, number of cells/mg creatinine</td>
<td>4.00</td>
<td>83</td>
<td>100</td>
<td>8.00</td>
</tr>
</tbody>
</table>

gHTN indicates gestational hypertension; IQR, interquartile range; N/A, not applicable (the ROC curve did not rise enough above the 45-degree line to warrant choosing among potential cutoff points); NTP, normotensive pregnancy; PE, preeclampsia; PlGF, placental growth factor; and sFlt-1, soluble fms-like tyrosine kinase receptor-1.
both cross-sectional and longitudinal studies of podocyturia in larger samples more broadly representative of pregnant women.

**Perspectives**

Urinary loss of viable podocytes may lead to a disruption of the glomerular filtration barrier and, ultimately, proteinuria in preeclampsia. As such, podocyturia may serve as an early marker and as a diagnostic test of preeclampsia, including women who develop symptoms and signs postpartum. Our results set the stage for studies of the mechanisms that regulate podocyte attachment in animal models of preeclampsia; these studies may not only provide information regarding the signaling pathways that underlie podocyte detachment and urinary loss, but may also provide novel therapeutic targets. On the clinical side, future clinical studies should include those of early renal injury in patients with nonproteinuric preeclampsia and renal involvement in the group of conditions that can imitate preeclampsia. These studies may improve our understanding of the different underlying pathological mechanisms that are related to specific clinical syndromes and may provide a tool for differential diagnosis.

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**Disclosures**

Dr Garovic is the inventor of technology referenced in this article. That technology has been patented by Mayo Clinic, but it is currently not licensed. The other authors have no conflicts to report.

**References**


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What Is New?

- We report that the urinary podocyte loss, that is, podocyturia, occurs by the end of the second trimester of pregnancy and predates clinical signs of preeclampsia, namely hypertension and proteinuria.

What Is Relevant?

- Screening for podocyturia at the end of the second trimester of pregnancy may allow for accurate identification of pregnant women at risk for preeclampsia.

Novelty and Significance

Summary

We have demonstrated that podocyturia identifies subclinical disease among pregnant women who will subsequently develop preeclampsia. As such, it may serve as an early marker of pre-eclampsia. Our data suggest that ongoing podocyte loss may be mechanistically related to the onset and severity of proteinuria in the affected women.
Podocyturia Predates Proteinuria and Clinical Features of Preeclampsia: Longitudinal Prospective Study

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PODOCYTURIA PREDATES PROTEINURIA AND CLINICAL FEATURES OF PREECLAMPSIA: A LONGITUDINAL PROSPECTIVE STUDY

Supplement


From the Divisions of Nephrology and Hypertension, Department of Internal Medicine, (I.M.C., S.J.W., S.T.T., V.D.G); and Division of Biomedical Statistics and Informatics (K.R.B., P.D.F.G., C.M.W.W.); and Division of Hematology, Department of Internal Medicine, (S.R.H); and Department of Obstetrics and Gynecology, (W.M.W., B.C.B., C.H.R.); and Department of Laboratory Medicine and Pathology, (J.P.G.) Mayo Clinic, Rochester, MN USA

Short title: Podocyturia and preeclampsia

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Methods

Study participant diagnostic criteria
The diagnosis of preeclampsia was defined by the presence of: (1) hypertension after 20 weeks gestation (blood pressure ≥140/90 mm Hg); (2) proteinuria ≥300 mg of protein in a 24-hour urine specimen, and/or 1+ (30 mg/L) protein by dipstick urinalysis, in the absence of urinary tract infection; and (3) resolution of hypertension and proteinuria by 12 weeks post-partum. The diagnosis of Hemolysis, Elevated Liver enzymes, and Low Platelet count (HELLP) syndrome, believed to be a severe form of preeclampsia, was confirmed by the presence of microangiopathic hemolytic anemia, elevated liver enzymes, and thrombocytopenia. The diagnosis of chronic hypertension was defined as a history of hypertension predating pregnancy, or by hypertension occurring before 20 weeks of gestation in the absence of proteinuria. All study participants were included for only one pregnancy and were not re-enrolled for subsequent pregnancies.

Serum studies
Serum samples obtained in the second trimester (median 27 GW, IQR 25-28 GW) and at delivery (median 39.5 GW, IQR 39-40 GW) were collected in SST tubes, centrifuged at 1500 x g, and stored at -80º C until the time of assay. After thawing, samples were not re-frozen. Serum concentrations of angiogenic factors (total sFlt-1, free PlGF and soluble endoglin) were measured using Quantikine ELISA (enzyme-linked immunosorbent assay) kits (R&D Systems, Minneapolis, MN).

Urine chemistries
Concurrent with the serum samples at the end of the second trimester and at delivery, clean-catch urine samples (50-100 mL) also were collected. The concentrations of albumin, total urinary protein, and creatinine were measured using standard methods on a Hitachi 911 Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN).

Aliquots of the urine samples (25-50 mL each) were centrifuged for 8 minutes at 700 x g at room temperature. The pellets were rinsed twice with human diploid fibroblast (HDF) solution and re-suspended in Dulbecco’s modified eagle’s medium with 10% fetal bovine serum that was supplemented with penicillin, streptomycin, and fungisome for the prevention of bacterial and fungal contamination. One-milliliter aliquots were placed on collagen-coated tissue culture grade cover slips. Following overnight incubation at 37ºC in 5% CO2, the medium was removed, after which two gentle washes in phosphate-buffered saline (PBS) solution were performed. Cover slips were thoroughly dried and frozen at -80º C until the time of analysis.

Immunofluorescence
Cover slips were placed immediately in -20º C methanol for 10 minutes, followed by two rinses in PBS for 5 minutes each, without agitation. Permeabilization was achieved by incubating for 10 minutes in 0.25% Triton-X at room temperature, followed by two rinses in PBS for 5 minutes each, without agitation.

Each cover slip was incubated with antibodies to podocin (Sigma Rabbit anti-Podocin #P0372-200 ul) at a dilution of 1:50 overnight at 4º C. After two washes at room temperature with PBS for 5 minutes each, a fluorescein isothiocyanate (FITC) labeled goat anti-rabbit secondary antibody was added at a dilution of 1:50 for 1 hour at room temperature. This was followed by
two PBS washes of 5 minutes each at room temperature, without agitation. Coverslips were mounted using VectaShield mounting media with 4',6-diamidino-2-phenylindole (DAPI) nuclear stain to facilitate the differentiation of nucleated whole cells from cell fragments (Vector Labs, Burlingame, CA). The stained slides were viewed with a fluorescence microscope (Leica, Germany). Nucleated cells staining for podocin were considered to be podocytes. A renal pathologist (J.P.G.), who was blinded to the clinical diagnosis and laboratory findings, evaluated each sample to determine the number of cells that were present and the percentage of cells that stained for podocin. Podocyturia was expressed as a ratio of the number of podocytes to the creatinine content of the respective urine sample.
References


Figure S1. Immunofluorescence of urinary podocytes plated on a collagen-coated slide cultured for 24 hours and stained with podocin in a patient who developed preeclampsia at 35 weeks of gestation; (A) at 25 weeks of gestation (blood pressure 128/72 mm Hg, predicted 24 hour proteinuria of 128 mg); (B) at the time of delivery (blood pressure 160/100 mm Hg, predicted 24 hour proteinuria of 720 mg)