UNIVERSITY OF BELGRADE

MEDICAL FACULTY

Vesna D. Garović

PODOCYTURIA AS DIAGNOSTIC AND PROGNOSTIC MARKER OF PREECLAMPSIA

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Vesna D. Garović

DIJAGNOSTIČKI I PROGNOSTIČKI ZNAČAJ PODOCITURIJE KOD ŽENA SA PREEKLAMPSIJOM

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Mentor:

Dr Darko Plećaš, profesor, Medicinski fakultet Univerziteta u Beogradu

Članovi komisije:

Dr Amira Egić, profesor, Medicinski fakultet Univerziteta u Beogradu

Dr Tanja Simić, profesor, Medicinski fakultet Univerziteta u Beogradu

Dr Milan Radović, profesor, Medicinski fakultet Univerziteta u Beogradu

Dr Natasa Milić, docent, Medicinski fakultet Univerziteta u Beogradu

Dr Marko Babić, profesor, Medicinski fakultet Univerziteta u Beogradu

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PODOCYTURIA AS DIAGNOSTIC AND PROGNOSTIC MARKER OF PREECLAMPSIA

SUMMARY

Preeclampsia is a pregnancy-specific disorder clinically characterized by hypertension and proteinuria that occurs after 20 weeks of gestation. Affecting 5% of pregnancies, it remains one of the leading causes of both maternal and fetal morbidity and mortality worldwide. Preeclampsia covers a spectrum of conditions, with eclampsia (its convulsive form) and HELLP syndrome representing its most severe forms. Despite recent advances in the field of angiogenesis and anti-angiogenesis in preeclampsia, urine and serum measurements of circulating angiogenic proteins have not provided a reliable screening tool for preeclampsia with current techniques. Emerging evidence suggests that podocyte plays a critical role in the evolution of kidney injury in this disorder. Studies of human tissue show that the expressions of podocyte-specific proteins (including nephrin and synaptopodin) are severely affected by preeclampsia. In addition, the detection of podocyte products and live podocytes in the urine (podocyturia) may serve as clinically useful tools for prediction and diagnosis of preeclampsia. Identification of podocytes using the techique of culturing and staining of urinary sediments is time consuming and requires special expertise. To overcome these limitations, a new technique using Mass spectrometry was developed that allows to confirm the presence of urinary podocytes trough identification of the podocyte-specific proteins. In addition, it was shown that women with preeclamptic pregnancies demonstrate signs of small vessel disease and less favorable cardiovascular risk profile both at the time of delivery and years after their affected pregnancies. One possible mechanism for this relationship is that hypertensive pregnancy disorders (preeclampsia, in particular) and CVD share several common risk factors (obesity, diabetes mellitus, and renal disease) or, alternatively, hypertension in pregnancy may induce long-term metabolic and vascular abnormalities that may increase and overall risk for cardiovascular disease later in life. Therefore, improved screening, preventive and treatment strategies may both

optimize management of hypertensive pregnancy disorders, and may have long-term impact on women's cardiovascular events and outcomes years after the affected pregnancies.

Key words: preeclampsia, proteinuria, podocyturia, glomerular epithelial cells, cardiovascular disease, women's health

Scientific field: Medicine

Sub scientific field: Gynecology and obstetrics

DIJAGNOSTIČKI I PROGNOSTIČKI ZNAČAJ PODOCITURIJE KOD ŽENA SA PREEKLAMPSIJOM

REZIME

Preeklampsija je oboljenje koje se javlja u trudnoći i klinički se karakteriše pojavom hipertenzije i proteinurije nakon dvadesete nedelje trudnoće. Javlja se u 7 – 10% svih trudnoća i čini vodeći uzrok mortaliteta i morbiditeta trudnica i fetusa. Klinička slika ovog oboljenja varira od lakih do najtežih formi, koje mogu da se manifestuju pojavom konvulzija tj. razvojem eklampsije, kao i razvojem hemolize, poremećaja funkcije jetre i koagulacije u okviru tzv. HELP sindroma. Uprkos nedavnom napretku u razumevanju uloge proteina angiogeneze u patofiziologiji preeklampsije, postojeći dokazi iz kliničkih studija još uvek su nedovoljni za korišćenje u ranom otkrivanju trudnoća koje su pod rizikom za razvoj preeklampsije. Prvi objavljen rad iz serije radova o dijagnostičkom i prognostičkom značaju podociturije kod žena sa preeklampsijom pokazao je da je pojava proteinurije u preeklampsiji povezana sa podociturijom, odnosno pojavom i gubitkom glomerularnih epitelnih ćelija (podocita) u mokraći, kao i sa promenama u ekspresiji strukturnih proteina (nefrina i sinaptopodina) ovih ćelija. Ovi klinički podaci uputili su na zaključak da se podociturija javlja pre proteinurije i hipertenzije, te da može da posluži kao rani pokazatelj i dijagnostički marker ovog oboljenja. S obzirom da je tehnika identifikacije podocita koja se bazira na kulturi sedimenta urina komplikovana i da zahteva posebnu obučenost za interpretaciju rezultata, u nastavku studije razvijena je nova tehnika identifikacije podocita koja se bazira na masnoj spektrometriji. Ovom tehnikom, potvrđeno je da se podociturija moze dijagnostikovati identifikacijom peptida koji je specifičan za podocin. U nastavku istraživanja i sledećim publikovanim radovima, pokazano je, dodatno, da žene sa

preeklampsijom pokazuju znake oboljenja malih krvnih sudova i povišene faktore rizika za pojavu kardiovaskularnih i bubrežnih bolesti, kako u vreme trudnoće, tako i decenijama nakon porođaja. Hipertenzija u trudnoći prepoznata je kao novi faktor rizika za kardiovaskularnu bolest u žena, ukazujući na činjenicu da žene koje su imale trudnoću komplikovanu hipertenzijom treba redovno kontrolisati i nakon trudnoće, kako bi se hipertenzija i ostali tradicionalni faktori rizika za razvoj kardiovaskularnih i bubrežnih bolesti pravovremeno dijagnostikovali i lečili.

Ključne reči: preeklampsija, proteinurija, podociturija, glomerularne epitelijalne ćelije, kardiovaskularna bolest, zdravlje žena

Naučna oblast: Medicina

Uža naučna oblast: Ginekologija i akušerstvo

CONTENT

1. INTRODUCTION

Preeclampsia, a hypertensive disorder unique to pregnancy, remains a leading cause of fetal and maternal morbidity and mortality worldwide.[1, 2] Preeclampsia, unlike other hypertensive pregnancy disorders, is a systemic disease with multi-organ involvement, which is commonly, but not invariably, accompanied by either sudden onset or worsening of pre-existing proteinuria. It is estimated that 5% of otherwise uncomplicated pregnancies will be affected by preeclampsia and that as many as 25% of pregnant women with preexisting hypertension will develop superimposed preeclampsia. Preeclampsia commonly is viewed as one of the hypertensive pregnancy disorders, with these disorders covering a spectrum of clinical presentations from chronic hypertension (i.e. hypertension occurring prior to 20 weeks of gestation) and gestational hypertension (i.e. hypertension occurring after 20 weeks of gestation) to more severe forms, including preeclampsia, eclampsia (its convulsive form), and HELLP syndrome (**H**emolysis, **E**levated **L**iver enzymes, and **L**ow **P**latelets).[3] The rationale to treat these disorders as a continuum comes from clinical evidence demonstrating that either chronic or gestational hypertension may progress to preeclampsia (commonly evidenced by new-onset or worsening of proteinuria), while preeclampsia may progress to more severe forms, such as eclampsia or HELLP syndrome. An alternative theory is that preeclampsia is a separate disease entity. It is a heterogeneous disease, regardless of whether it is viewed as a distinct disease entity, or one within the spectrum of the hypertensive disorders of pregnancy. Different clinical subtypes may reflect distinct underlying pathological mechanisms.[4] It is common in clinical practice, for example, to subcategorize preeclampsia as early versus late (before and after 34 weeks of gestation, respectively),[5] and mild versus severe,[6] based on the absence/ presence of severe hypertension, defined as a blood pressure ≥160/110 mm Hg, neurological/renal/cardiac impairment, or signs of HELLP. Recent evidence suggests that women with early severe preeclampsia, who are at particularly high risk for adverse pregnancy outcomes, may have a more pronounced imbalance between pro- and anti-angiogenic factors than those with late preeclampsia and more favorable outcomes.[7] Active research in this field[8] may delineate the mechanisms of the subtypes of preeclampsia, commonly referred to as "placental" versus "maternal" preeclampsia, based on their etiologies and origins.[9] These causal pathways, regardless of mechanisms, are believed to converge at the point of systemic endothelial dysfunction, which leads to hypertension and proteinuria (**Figure 1**).

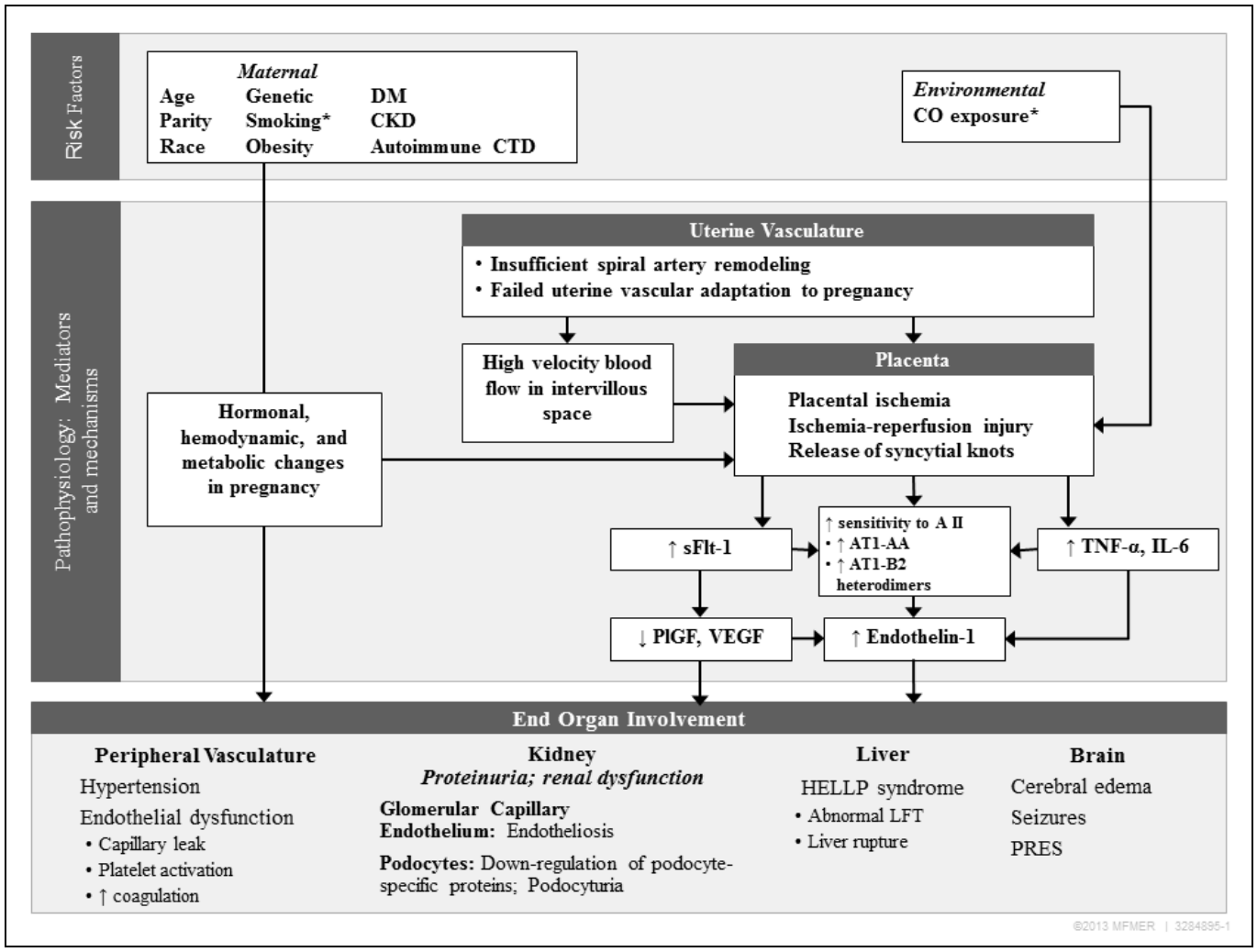


Figure 1. *Etiologies and pathophysiology of preeclampsia*. Several different signaling pathways may play a role, ultimately converging at the point of systemic endothelial dysfunction, hypertension, and proteinuria. Abbreviations: AT1-AA, autoantibodies to the angiotensin II type 1 receptor; AT1-AA-B2 heterodimers, angiotensin II type 1 receptor-bradykinin type 2 receptor heterodimers; carbon monoxide; CKD, chronic renal disease; CTD, connective tissue disease; DM, diabetes mellitus; HELLP, hemolysis, elevated liver enzymes, low platelet count; IL-6, interleukin 6; LFT, liver function tests; PlGF, placental growth factor; PRES, posterior reversible encephalopathy syndrome; sFlt-1, soluble fms-like tyrosine kinase 1; TNFα, tumor necrosis factor α; VEGF, vascular endothelial growth factor.* Reduced risk for preeclampsia

1.1. Endothelial Dysfunction in Preeclampsia

Increasing evidence suggests that endothelial dysfunction plays a central role in the pathophysiology of preeclampsia. The fact that hypertension rapidly resolves upon the removal of the fetus and placenta has led to several theories implicating placental hypoxia as an early event that may cause placental production of soluble factors leading to endothelial dysfunction. Preeclampsia has been associated with elevated levels of the soluble receptor for vascular

endothelial growth factor (VEGF) of placental origin.[10] This soluble receptor, commonly referred to as soluble fms-like tyrosine kinase receptor-1 (sFlt-1), may bind and neutralize VEGF, and thus limit the availability of free VEGF placental growth factor (PlGF) for fetal and placental angiogenesis. Several rodent models simulate preeclampsia after exogenous sFlt-1 administration. In the most direct model, intraperitoneal sFlt-1 injections produce short-term elevations of sFlt-1.[11] Animals develop hypertension, proteinuria, and altered podocyte protein expression in the hours after sFlt-1 injection, but do not develop glomerular endotheliosis, the classical renal lesion of preeclampsia. Administration of an adenoviral vector encoding sFlt-1[10] leads to longer-term sFlt-1 exposure in rats. This model reproduces the findings of hypertension, proteinuria, and glomerular endotheliosis. Elevated levels of another anti-angiogenic factor, soluble endoglin, have been subsequently implicated in neutralizing transforming factor-β, with resultant vascular damage in preeclampsia and HELLP syndrome.[12] These anti-angiogenic factors are commonly viewed as the missing link between abnormal placentation and the maternal syndrome. However, these factors are likely a consequence, rather than the cause, of placental ischemia in preeclampsia. Theylikely play an important role in "placental" preeclampsia, in which placental ischemia is present, but not in "maternal" preeclampsia, which occurs in the absence of placental ischemia,[8] or in postpartum preeclampsia[13] which occurs after delivery in the absence of the placenta. The underlying signaling mechanisms by which dysregulation of angiogenic factors leads to renal injury in preeclampsia are not well understood.

1.2. Renal injury in preeclampsia

Renal pathology in preeclampsia in the form of endotheliosis has long been recognized, and the kidney manifestations of preeclampsia form the basis for a "nephrocentric" view of the disease in research and clinical arenas.[14] The obstetrical literature, in contrast, questions the importance of kidney injury (as demonstrated by proteinuria) in the diagnosis of preeclampsia, suggesting that a subclass of "non-proteinuric preeclampsia" should be added,[15] or that detection of proteinuria should not be mandatory for a preeclampsia diagnosis.[16] However, similar to other renal diseases, proteinuria in preeclampsia may represent a late marker of renal injury. It also is unknown how endothelial dysfunction affects glomerular endothelial cells, podocytes, which form the final barrier to urinary protein loss.

1.3. Glomerular Epithelial Cells and Slit Diaphragm

The glomerulus is a highly specialized filtration apparatus with selective permeability that allows free passage of water and solutes, but not protein. It is comprised of three layers that are structurally and functionally distinct. The first layer consists of capillary endothelial cells that are highly fenestrated and allow free passage of albumin. The second is the glomerular basement membrane that is negatively charged and thus repels negatively charged proteins. The third layer is composed of visceral glomerular epithelium, with podocytes situated on the outer aspect of the glomerular basement membrane. These cells originate from mesenchyme, in contrast to most other non-renal epithelial cells that are derived from ectoderm.[17] Immature podocytes maintain a high proliferation index during glomerular development.[18] They lose their mitotic activity and develop a highly differentiated cytoarchitecture with acquisition of a mature phenotype. Mature podocytes consist of cell bodies, major processes, and foot processes. Foot processes are anchored to the glomerular basement membrane by $\alpha_3\beta_1$ integrin[19] and α-β–dystroglycans.[20] Neighboring foot processes interdigitate and connect by specialized cell-to-cell junctions, also known as glomerular slit diaphragms. The slit diaphragm appears to be a modified adherens junction[21] that provides the main size selective filtration barrier in the kidney. Several proteins that localize either to the slit diaphragm or foot process cytoskeleton have been identified, including nephrin,[22] podocin,[23] synaptopodin,[24] and podocalyxin.[25] These proteins maintain the structural and functional integrity of the slit diaphragm through complex interactions.

1.4. Slit Diaphragm in Congenital and Acquired Nephrotic Syndromes

The critical role of the slit diaphragm in the normal function of the glomerular filtration barrier has been supported by studies of inherited nephrotic syndromes. NPHS1, the gene mutated in the congenital nephrotic syndrome of the Finnish type, was cloned in 1998.[22] The gene product, nephrin, was localized to podocytes where it likely represents the major structural component of the slit diaphragm. Several new proteins that localize either to the slit diaphragm or foot process cytoskeleton subsequently have been identified over the past few years which maintain the structural and functional integrity of the slit diaphragm and podocytes through complex interactions. These include Neph-1, a homologue of nephrin,[26] and podocin,[23] mutations of which result in recessive familial forms of early-onset proteinuria resistant to steroid treatment.[27] Other relevant proteins include, CD_2AP, a protein that localizes to the cytoplasmic portion of the slit diaphragm that may play an important role in protecting the filtration barrier,[28] and synaptopodin, which is linked to the formation of foot processes, and thus considered to be a

marker of the differentiated podocyte phenotype.[29,30] The remainder of the proteins presented in **Figure 2** will not be the focus of this study; hence will not be discussed in detail.[31, 32]

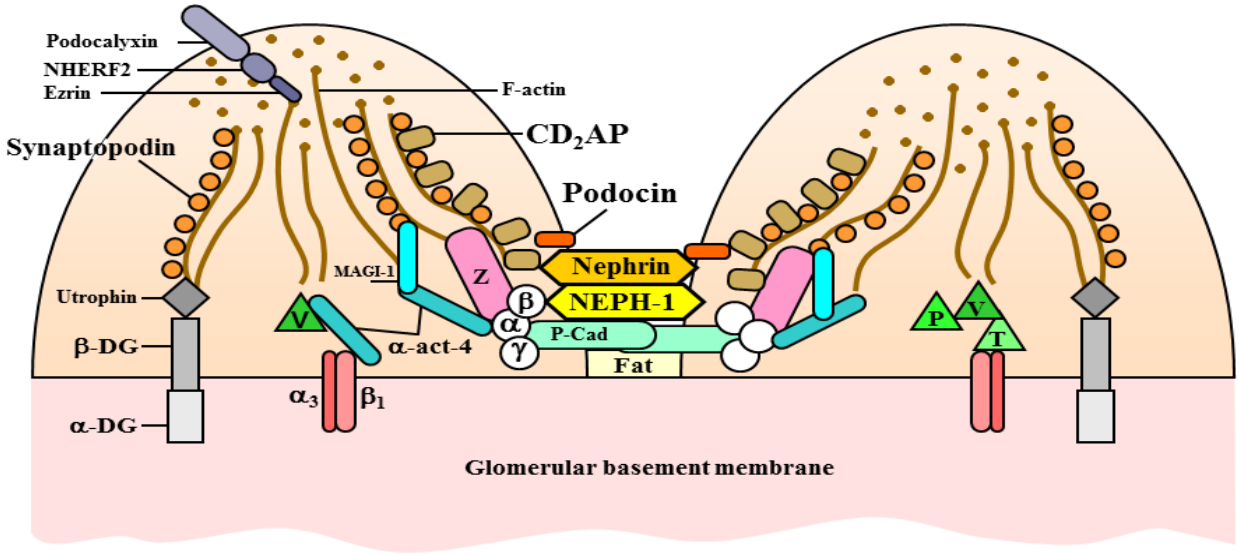


Figure 2. *Podocyte foot process cytoskeleton and slit diaphragm* α-act-4, α-actinin-4; α3β1, α3β1 integrin; α-DG, α-dystroglycan; β-DG, β-dystroglycan; P, paxillin; P-cad, P-cadherin; Z, ZO-1; V, vinculin; NHERF2, Na+/H+ exchanger regulatory factor. Modified from Mundel and Shankland.[101]

1.5. Preeclampsia and future cardiovascular disease

Studies in the 1970's and 1980's argued that hypertension which resolves after delivery, as in preeclampsia, did not herald hypertension later in life.[33] Subsequent studies,[34-36] however, reported a positive association of hypertension in pregnancy with hypertension later in life, as well as with increased risks for future cardiovascular disease (CVD),[37, 38] cerebrovascular events,[38, 39]and more recently, renal disease, both chronic kidney disease (CKD) and end-stage renal disease (ESRD).[40] The results of published studies were summarized in a systematic review[41] and a meta-analysis.[35] These studies, for the most part, did not control for traditional cardiovascular risk factors, were registry-based, reported a limited number of outcomes (such as cardiovascular deaths), and did not assess the impact of hypertension in pregnancy on the age of onset of the events, all of which may be clinically useful when individualizing risk profiles and intervention strategies.

2. AIMS

The overarching hypothesis of this project is that endothelial dysfunction in preeclampsia is associated with podocyte dysregulation and that endothelial dysfunction which persists after the affected pregnancies, may lead to an increased risk for future CVD, independent of traditional risk factors. Hence, we propose the following specific aims:

Aim 1. To test the hypothesis that proteinuria preeclampsia is associated with down-regulation of podocyte-specific proteins (nephrin, synaptopodin, and podocin) in the renal tissue of affected women

- Rationale for Aim 1: Studies of nephrotic syndrome in human renal tissues have indicated that these proteins play an important role in maintaining the structural and functional integrity of the slit diaphragm. In addition, studies using an animal model of sFlt-1 infusion that closely mimics preeclampsia have shown that a possible mechanism of proteinuria relates to down-regulation of nephrin.[11] However, no data are available regarding the expressions of nephrin and other podocyte-specific proteins in the kidney tissues of preeclamptic women.

Aim 2. To test the hypothesis that proteinuria in preeclampsia is associated with urinary loss of viable podocytes, i.e., podocyturia

- Rationale for Aim 2: Viable podocytes, as confirmed by culturing urinary sediments and staining for podocyte-specific proteins, are present in the urines from patients with a variety of glomerular diseases associated with proteinuria. Podocyturia seems to be confined to active disease only, in contrast to proteinuria, which is present during active and chronic phases of glomerular damage.[42, 43] Given the acuteness of kidney injury in preeclampsia, we postulate that urinary podocyte loss occurs concurrently with proteinuria during the acute kidney injury and correlates with its severity.

Aim 3. To develop a new method for identification of urinary podocytes; this would be operator-independent and a highly reproducible technique

- Rationale for Aim 3: Despite increasing evidence suggesting that podocyturia may serve both as a reliable diagnostic tool for preeclampsia,[44-46] and as a marker of the active

phases of other proteinuric diseases, reservations regarding the clinical utility of this test exist, mainly due to its technical complexity, length of time to obtain results, and the level of expertise and training required for interpretation of the results.[47] Thus, a new operator-independent and highly reproducible technique will facilitate future studies of renal injury in preeclampsia.

Aim 4. To test the hypothesis that hypertensive pregnancy disorders are an independent risk factor for future cardiovascular disease

- Rationale for Aim 4. Published studies of the association between hypertensive pregnancy disorders and future CVD did not control for traditional cardiovascular risk factors.[35] Understanding whether this association is dependent or independent of traditional risk factors is clinically useful when individualizing risk profiles and intervention strategies, and for developing guidelines for CVD prevention in women.

3. MATERIAL AND METHODS

3.1. General Methods

Participants: All studies which involved human subjects were approved by the Mayo Clinic – Rochester Institutional Review Board, Rochester, MN, and all women were consented prior to inclusion in the study. The diagnosis of preeclampsia was made in the presence of the following criteria: a) hypertension after 20 weeks' gestation, defined as a blood pressure of 140/90 mm Hg or higher, b) proteinuria, defined as 300 mg of protein or greater in a 24-hour urine specimen, and/or 1+ (30 mg/L) dipstick urinalysis in the absence of urinary tract infection, and/or a predicted 24-hour urine protein of more than 300 mg on a random urine collection c) resolution of hypertension and proteinuria by 12 weeks postpartum. We also included women with severe forms of preeclampsia such as eclampsia, the convulsive form of preeclampsia, and HELLP syndrome, the diagnosis of which was confirmed based on previously published criteria.[3] Healthy, normotensive pregnant women without hypertension and proteinuria served as controls.

3.1.1. Podocyturia by culturing and staining of urinary sediments

Random urine samples (25-50 mL each) were centrifuged for 8 minutes at 700 x g at room temperature. The pellets were rinsed twice with hemodiafiltration (HDF) solution. The pellets then were resuspended in Dulbecco's Modified Eagle Medium (DMEM F-12) with 10% fetal bovine saline (FBS) supplemented with antibiotics for prevention of bacterial contamination. 1-mL aliquots were plated in 4-chamber, collagen coated tissue culture slides, followed by overnight incubation at 37^0 C in 5% CO_2. The next day, media was removed, followed by two phosphate buffered saline (PBS) washes. Slides were fixed with 1 mL of ice -cold methanol for 10 minutes at -20 C^0. Each of the four slide chambers was incubated with one of four different antibodies to podocyte proteins: podocalyxin (dilution 1:40), podocin (1:200), nephrin (1:100), and synaptopodin (undiluted). After washing with PBS, a secondary FITC-labeled antibody was added at a dilution of 1:40 for 30 minutes. The sediment was counterstained with Hoechst nuclear stain to facilitate differentiation of whole cells from cell fragments. Coverslips were mounted with Vectashield (Vector Labs) and slides were viewed with a fluorescence microscope (Zeiss). Nucleated, positive staining cells were considered to be podocytes. A renal pathologist, Joseph P. Grande, blinded to the clinical diagnosis and laboratory findings, evaluated each

sample to determine the number of cells present and the percentage of cells that stained for podocyte markers.

Podocyturia was expressed as a ratio of the number of podocytes to the creatinine content of the respective urine sample; this was performed for each podocyte marker of interest.

3.2. Specific Methods I

3.2.1. Human renal tissue experiments

Renal tissue was obtained from the autopsy materials from seven women who developed severe preeclampsia during the second half of their pregnancies, up to 48 hours following delivery, and who subsequently died (**Table 1**). Autopsy materials from two women who died accidentally during the second half of their otherwise normal pregnancies were used as controls. Kidney sections for 6 cases (cases 1-6) were obtained through collaboration with the University of Cape Town, South Africa; one case (case 7) and both controls were obtained through the Department of Laboratory Medicine and Pathology at Mayo Clinic – Rochester. Light microscopy and immunohistochemical stains for nephrin, synaptopodin, and podocin were performed on representative sections prepared from paraffin embedded material.
Immunocytochemistry: All studied samples were obtained during autopsies: renal tissue was fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin, per standard techniques. Representative samples were stored at room temperature from 5 (case 2) to up to 50 years (case 3) (**Table 1**).

Histologic sections, 4-5 microns in thickness, were prepared. Representative sections were stained with nephrin (Alpha Diagnostics, International), synaptopodin (Biodesign), and podocin (Alpha Diagnostics, International) antibodies. Antigen retrieval was performed by steam heating in 0.5 mmol EDTA for 30 minutes (for nephrin and synaptopodin), followed by enzyme treatment with trypsin for 10 minutes at 37°C (nephrin, synaptopodin, and podocin). Commercially available kits (Vectastain ABC kit, Vector Laboratories, and Envision Plus HRP kit, DakoCytomation) were used for the blocking, secondary antibody, and amplification steps. Color development was performed using NovaRed (Vector Laboratories) followed by hematoxylin counterstain. Most of the stains were performed on the Dako Autostainer (Dako), an automated staining machine, to facilitate consistency between staining batches.

Table 1. Clinical presentations and outcomes of seven cases of preeclampsia and two controls

Case number / Year of death	Clinical features and pertinent findings	Protein expression*		
		nephrin	synaptopodin	podocin
Case 1 1995	32-year old, full-term pregnancy, complained of headache at home, died during the third eclamptic seizure in hospital from intracerebral hemorrhage.	1+	0-1+	3+
Case 2 2000	20-year old, presented at 39 weeks of gestation with epigastric pain, blurred vision and preeclampsia. Underwent emergent C-section, postoperatively progressed to HELLP syndrome and died of hemorrhagic stroke.	0	0	3+
Case 3 1954	30-year old who developed hypertension, proteinuria and edema towards the end of pregnancy. Had 3 seizures at home. Upon arrival to hospital, suffered from a ruptured hepatic hematoma, became hemodynamically unstable and died.	1-2+	0-1+	3+
Case 4 1964	40-year old, who delivered a stillborn at full-term pregnancy. Had post-partum eclamptic seizure and was found to be hypertensive, with 4+ proteinuria. 2 days after delivery developed profuse uterine bleeding and liver failure, became hemodynamically unstable and died.	1-2+	0-1+	3+
Case 5 2001	36-year old, who developed epigastric pain one day after normal vaginal delivery. Developed hemolysis, elevated liver enzymes and low platelet count (HELLP syndrome) and oliguria, died with multiorgan failure.	1-2+	1+	3+
Case 6 2001	31-year old, presented at 34 weeks of gestation of her fourth pregnancy with hypertension and proteinuria, and subsequently had a seizure. Died 6 hours after delivery from intracerebral hemorrhage.	1-2+	1+	3+
Case 7 1990	30-year old, developed hypertension and proteinuria at 31 weeks of gestation, progressed to HELLP syndrome, had C-section, but died on the 5th postoperative day.	0	0	3+
Control 1 1988	21-year old, first pregnancy, 28 weeks pregnant, no obstetric complications, killed in a MVA	3+	3+	3+
Control 2 1988	18-year old, first pregnancy, 36 weeks pregnant, no obstetric complications, killed in a MVA	3+	3+	3+

Abbreviations: HELLP, Hemolysis, Elevated Liver enzymes, and Low Platelets, MVA motor vehicle accident * 0-absent, 1-mild, 2-moderate, 3-strong expression

3.2.2. Animal experiments

Anti-VEGF antibody and sFlt-1 studies were performed using wild-type CD1 mice, as previously described.[11] Briefly, five mice in each group were injected with a single intravenous injection of either anti-VEGF antibody or a soluble sFlt-1/Fc chimera at a concentration of 32.5 pM (picomole per liter), which corresponds to ten times the molar concentration of normal

plasma VEGF (3.25 pM). One-hundred microliters of urine were collected at 0, 1, 3, 5, and 24 hours following the initial injection. Mice developed significant proteinuria 3 hours after the intravenous injections and maintained the same level of proteinuria for the subsequent 7 hours. The amount of proteinuria gradually abated to normal urine protein levels within 24 hours. Some mice were sacrificed 5 hours after the injection to collect kidneys for immunocytochemistry. Mice injected with IgG served as controls. In rescue experiments, 32.5 pM of human recombinant VEGF-165 was injected about 5 minutes after the injection of the VEGF antibody or sFlt-1/Fc. Immunofluorescence staining was performed for nephrin, podocin, and synaptopodin. Antibodies and staining for nephrin and podocin were reported in a previous publication.[11] The expression of synaptopodin was studied using primary antibodies to synaptopodin (a gift from Dr. P. Mundel), followed by incubation with fluorescein isothiocyanate (FITC)-labeled secondary antibodies, as previously described.[11]

3.3. Specific Methods II

A cross-sectional study was conducted and blood and urine samples were collected close to, and typically 24 hours or fewer prior to delivery. In total, 67 women were recruited. Preeclampsia was present in 33 of the patients and HELLP was diagnosed in 11 patients; 23 normotensive pregnancies served as controls (**Table 2**).

An additional control group consisted of women with hypertension and proteinuria (n=11). Blood samples were obtained from all 67 women and urine samples for podocyturia were collected in a subset of 31 pregnant women (15 cases and 16 controls).

Table 2. Participant characteristics[a]

Variable	Normal (n = 23)	Preeclampsia (n = 33)	HELLP (n = 11)	Preeclampsia + HELLP (n = 44)
Maternal age (years)	28.7 ± 5.4	26.2 ± 5.1	33.0 ± 6.0	27.9 ± 6.1
Gestational age (weeks)	39.2 ± 2.2	34.3 ± 3.8*	33.5 ± 5.6*	34.1 ± 4.2*
Primiparous (%)	47.8	81.8	9.1	63.6
Systolic blood pressure (mm Hg)	110.5 ± 9.5	159 ± 19.8*	162.6 ± 23*	159.9 ± 20.3*
Diastolic blood pressure (mm Hg)	66.9 ± 9.8	97.8 ± 9.4*	98.3 ± 10.6*	97.9 ± 9.6*
Proteinuria (g/24 hr)	247 ± 294	2693 ± 3164	4373 ± 5962*	3113 ± 4032*
Platelet count	242,000 ± 35,519	232,333 ± 66,787	100,273 ± 42,245	199,318 ± 84,146

Abbreviations: HELLP, Hemolysis, Elevated Liver enzymes, and Low Platelets
a Data are presented as mean ±SD
* P<.05 compared to normotensive group

3.3.1. Serum studies

Blood samples for the determination of sFlt-1, free PlGF, and soluble endoglin levels were drawn 24 hours prior to delivery. Serum creatinine, liver function tests, and platelet counts were performed according to standardized laboratory procedures. Serum levels of sFlt-1, soluble endoglin, and free PlGF were measured using Quantikine ELISA kits (R&D Systems).

3.3.2.Urine chemistry

Clean-catch urine specimens (50-100 mL) were obtained concurrent with serum collections. Urine albumin, total protein, and creatinine concentrations were measured by standard methods on a Hitachi 911 Chemistry Analyzer (Roche Diagnostics). Urinary PlGF determinations were performed using the PlGF ELISA kit (R&D Systems).

3.3.3. Podocyturia

Podocyturia testing was performed as explained under General Methods. A ratio of the number of podocytes to the creatinine content of the respective urine sample was calculated for each of the four podocyte markers.

3.3.4. Statistical Methods

Descriptive statistics are reported for quantitative traits as means and standard deviations or as median and interquartile ranges, and for categorical traits as percentages. The operating characteristics of podocyte and angiogenic markers of preeclampsia were assessed by considering the trait either as a categorical measure (i.e., absence/presence) and estimating its sensitivity and specificity, or by considering it as a quantitative measure and generating the receiver operating characteristic (ROC) curve. The areas under the curve (AUC) were estimated with confidence intervals, and contrasted among markers using the method of Delong and Delong.[48] The effect of variation in gestational age on each marker was depicted graphically by plotting each variable as a function of gestational age and showing the regression line and individual data points for the normal subjects, along with box plots (i.e., median and interquartile ranges) for the preeclamptic patients within strata defined by intervals of gestational age. All statistical tests were carried out at the 2-sided 0.05 significance level.

3.4. Specific Methods III

Random urine samples were obtained from 13 preeclamptic and 6 preeclamptic/HELLP syndrome consecutive patients who agreed to participate in the study (**Table 3**). Urine samples were used both for the podocyturia assay (performed as described under General Methods) and LC-MS/MS (Liquid Chromatography coupled with tandem Mass Spectrometry) technology (n=15), or for LC-MS/MS technology only (n=4). Urines were obtained from 4 normotensive consecutive pregnant women at the time of delivery and used as controls.

Table 3. Demographic and clinical data of preeclamptic (n=19) and normotensive pregnant patients (n=4) who underwent podocyturia studies

Characteristics	**Preeclamptic pregnancies* Mean ± SD**	**Normotensive pregnancies Mean ± SD**	***P* value**
Maternal age (years)	30.5 ± 7.1	29.7 ± 0.6	1
Days of gestation	235.6 ± 24.3	280.3 ± 8.7	.0086
Gravidity	2.12 ± 1.5	1.33 ± 0.57	.3996
Parity	0.81 ± 1.12	0.33 ± 0.58	.5379
Systolic blood pressure (mm Hg)	154.4 ± 11.35	118.3 ± 8.02	.0085
Diastolic blood pressure (mm Hg)	93.3 ± 13.4	68 ± 3.46	.0139
Protein/creatinine ratio (grams/24 hour urine)	3.896 ± 3.22	0.109 ± 0.034	.0086
Podocyturia (number of podocytes/mg creatinine)	3.1 ± 2.2	absent	N/A

In addition to diagnostic criteria for preeclampsia, 6 patients had laboratory abnormalities characteristic of HELLP syndrome: **h**emolysis, with a mean LDH of 350 ± 32 (normal, 122-222 U/L); **e**levated **l**iver enzymes, with a mean AST of 188 ± 102 (normal 8-43 U/L); and a **l**ow **p**latelet count, with a mean platelet count of 67 ± 34 (normal 150-450x10^9/L).

3.4.1. Podocyturia assay

The podocyturia assay was performed as explained under General Methods. Podocyturia was expressed as a ratio of the number of podocytes to the creatinine content of the respective urine sample, and was confirmed in the presence of ≥0.85 podocin-positive cells/mg creatinine (**Table 3**). This threshold value was previously determined to provide 100% sensitivity and specificity of the method in the diagnosis of preeclampsia.[49]

3.4.2. LC-MS/MS technology: Materials and Methods

Recombinant human podocin was obtained from Novus Biologicals. The synthetic stable isotope labeled peptide, with the same sequence as the podocin tryptic peptide, was synthesized

by the peptide synthesis facility at the Mayo Clinic – Rochester, Rochester, MN. Random urine samples (~50 mL each) were collected. Each was centrifuged for 8 minutes at 700 x g at room temperature. The supernatant was discarded and the pellet was re-suspended in methanol fixative and stored at 4°C. Prior to digestion, the methanol fixed pellets were centrifuged at 600 x g for 10 minutes, the supernatant was removed, and the pellet was re-suspended in 50 µL of RapiGest™ SF at a concentration of 0.1% in 50 mM ammonium bicarbonate, pH 8.0. The sample was sonicated for five minutes, then 100 µg of trypsin was added and the sample was sonicated again for 5 minutes. The sample was then digested in a shaking incubator set at 37°C for 4 hours. After digestion, the sample was acidified with 2 µL of formic acid and centrifuged for 10 minutes at 14,000 x g. A volume of 18 µL of patient digest was put into each well of a 96-well sample tray. A stable isotope labeled internal standard peptide was added to each sample and then analyzed by LC-MS/MS.

3.4.3. Liquid Chromatography coupled with Tandem Mass Spectrometry

All samples were analyzed using a Thermo TLX-2 HPLC system, (Thermo Scientific) coupled to an ABSciex API 5000 triple quadrupole mass spectrometer. (Sciex) A 20 µL injection was made from each sample, and separations were carried out on a 100 x 3.0 mm Atlantis T3 column, with a 3 µm particle size and 120 Å pore size, run at a flow rate of 250 µL/minute. A gradient consisting of mobile phase A (100% water, 0.1% formic acid) and mobile phase B (100% acetonitrile, 0.1% formic acid) was used to resolve the peptides with a 15 minute gradient. All MS/MS conditions were optimized by infusing the synthetic stable isotope labeled podocin tryptic peptide,[39] QEAGPEPSGSGR.[50] The transition that gave the best signal-to-noise was the doubly charged precursor ion to the singly charged y_6 ion i.e., $[M+2H^+]^{+2} \rightarrow y_6^{+1}$. The Analyst™ software version 1.4.2 (Applied Biosystems) was used to control the instrument, and to acquire and process the data.

3.4.4. Statistical methods

Statistical analyses were performed using JMP version 7.0.0 (SAS Institute Inc.). All data are expressed as mean values +/- standard deviation (SD) and compared using the Wilcoxon rank sum test. A two-tailed probability value of a Pearson correlation coefficient was computed for correlation studies. A *P*-value of $< .05$ was pre-specified as being statistically significant.

3.5. Specific Methods IV

3.5.1. Participants

This study included 4,782 women from 2,443 sibships participating in the Family Blood Pressure Program (FBPP) study. The FBPP was established in 1995 to investigate the genetics of hypertension in non-Hispanic Blacks, Hispanic whites, Asians, and non-Hispanic whites.[50] The FBPP consists of four different research networks, all ascertaining families having individuals with elevated blood pressures or genetic predispositions to hypertension: GenNet, Genetic Epidemiology Network of Arteriopathy (GENOA), Hypertension Genetic Epidemiology Network (HyperGEN), and Stanford Asian Pacific Program in Hypertension and Insulin Resistance (SAPPHIRe). The specific recruitment strategy for each network has been described previously.[50]

For the Phase 1 (1996-2000) study examination, a questionnaire was developed to obtain subjects' personal and family medical histories, including their use of prescription medications, histories of menopause, and hormone replacement. Questions regarding pregnancy and hypertension in pregnancy (as described below) were only added to the questionnaires administered during the Phase 2 study visits (2000-2004). Therefore, only the Phase 2 questionnaire data were used in the analyses.

3.5.2. Study visits

All individuals who participated in the FBPP gave informed consent; and the Institutional Review Board at each clinic site approved all protocols. Questionnaires were administered via in - person interviews by trained examiners. While each network used slightly different definitions for recruitment, the pooled data set used the standardized definitions of hypertension. The diagnosis of hypertension was confirmed at the Phase 2 study visit, if a prior diagnosis of hypertension and use of prescription anti-hypertensive medication had been reported, or if the average systolic or diastolic blood pressure (BP) was $\geq$ 140 mm Hg or $\geq$ 90 mm Hg, respectively. The diagnosis of coronary heart disease (CHD) was established by self-reports of a previous history of myocardial infarction, coronary bypass surgery, coronary angioplasty, balloon dilatation or stent placement, while the diagnosis of cerebrovascular disease was based on self-reports of stroke and/or cerebral hemorrhage. Diabetes mellitus was self-reported, while "ever" smoking was defined as having smoked more than 100 cigarettes in the past. The use of prescription medications in the previous month was recorded.

All participants underwent standardized physical examinations and blood tests. The diagnosis of dyslipidemia was confirmed if one or more of the following criteria were met: use of lipid lowering drugs or laboratory measurements at the Phase 2 examination (methods described below) revealing, a total cholesterol ≥ 200 mg/dL, triglycerides ≥ 150 mg/dL, or HDL ≤ 40 mg/dL.

Systolic and diastolic BPs were measured using an automated oscillometric BP-measurement device with a consistent protocol across networks. Height was measured while participants were standing without shoes, with heels together, against a vertically mounted ruler, and weight was measured on a balance. Body mass index (BMI) was calculated as weight (kg)/height (m).[2]

3.5.3. Pregnancy questionnaire

The standard questionnaire, which was previously validated,[51] was used for all networks. Female participants were asked, "Have you had at least one pregnancy that lasted more than 6 months?" Women who responded affirmatively were asked to report the number of pregnancies and to answer whether or not they had developed hypertension during any of the pregnancies that had lasted more than 6 months. If they confirmed a history of hypertension in pregnancy, they were asked whether it occurred only in the first pregnancy; in the first pregnancy *and* at least one subsequent pregnancy; or only in subsequent pregnancies. Preeclampsia was defined either by self-report of this condition, or by self-report of protein in the urine during the pregnancy with associated hypertension.

3.5.4. Laboratory Methods

Blood was drawn by venipuncture after an overnight fast of at least 8 hours. Serum glucose, total cholesterol, high-density lipoprotein (HDL)-cholesterol, and triglyceride concentrations were measured by standard methods on a Hitachi 911 Chemistry Analyzer (Roche Diagnostics). Low-density lipoprotein (LDL)-cholesterol was calculated using the Friedewald equation when triglycerides were < 400 mg/dL.[52]

3.5.5. Statistical Analysis

As participants were selected for being either hypertensive or members of hypertensive sibships, logistic regression for the presence or absence of hypertension later in life was deemed inappropriate. Survival analysis methods using age at onset of each outcome as the time variable were used alternatively. Kaplan-Meier curves were constructed to compare the unadjusted

probabilities for being free of hypertension after age 40 years, CHD, or stroke among: 1) nulliparous women, i.e., those who had no pregnancy lasting more than 6 months; 2) women with no history of hypertension during any pregnancy; 3) women with a history of hypertension in pregnancy; and 4) women with a history of preeclampsia. The age of 40 years for hypertension was chosen for the following reason. We aimed to correlate hypertension in pregnancy with the risk for hypertension later in life. Therefore, we wanted to exclude women with chronic hypertension that had occurred before pregnancy and then persisted after delivery. To accomplish this, the hazard ratio (HR) for the diagnosis of hypertension was assessed only for hypertension that was diagnosed after the age of 40, as most pregnancies are likely to occur by that age. The subject's reported age at diagnosis, or current age (in the case of hypertension diagnosed at the study visit), was used as the event time. Subjects who did not meet the diagnostic criteria for hypertension were considered censored free of hypertension as of their ages at the study visit. Similarly, subjects reporting CHD and stroke were considered as experiencing these events at the age reported, or were censored, i.e., considered to be free of these events, as of their current ages.

Cox proportional hazard models were used to estimate the adjusted risks and hazard ratios (HR) with respective 95 % confidence intervals (CI) for age at diagnosis of hypertension, CHD, and stroke. The models were fit using custom software in order to account for the potential correlation of outcomes among women within sibships, i.e., sisters.[53] We introduced 2 indicator variables calculated for all subjects: one for the presence of at least one pregnancy lasting 6 months, and a second for the presence of hypertension in at least one such pregnancy. When both indicator variables are included, the resulting coefficients can be interpreted as the contrast between "no pregnancy" and "normotensive pregnancy," and between "normotensive pregnancy" and "hypertensive pregnancy," respectively. Factors likely to be present throughout adulthood (e.g., race, education) were considered as adjustment variables in all of the following models. Model A, used to model the diagnosis of hypertension after 40 years, included race, network, a family history of cardiovascular disease, education, diabetes (time-dependent), smoking status, and BMI as adjustment variables. Model B, used to model the diagnosis of CHD and stroke, included the Model A variables plus the diagnosis of hypertension after age 40 years (time-dependent). Neither Model A nor B, which were fit in the pooled FBPP sample, included dyslipidemia as an adjustment variable, because the use of lipid lowering drugs was not available

for analyses in the pooled FBPP data set. Hence, two additional models (Model C and Model D) were fit only in a subset of GENOA participants for whom use of lipid lowering drugs was available for analyses. They comprised 37% of the pooled FBPP sample, i.e., 1,754 of 4,782 subjects. Model C, used to model the diagnosis of hypertension after age 40 years, adjusted for dyslipidemia, in addition to race, family history, smoking, and diabetes mellitus (time-dependent). Model D, used to model the diagnosis of CHD and stroke, adjusted for Model C variables plus the diagnosis of hypertension after age 40 years (time-dependent), thus controlling for all traditional risk factors when comparing cardiovascular event rates between pregnancy groups.

4. RESULTS

4.1. Human renal tissue experiments

We studied the expressions of proteins that localize either to the slit diaphragm (nephrin and podocin) or the cytoskeleton (synaptopodin) in the podocyte foot processes of the renal tissue from seven women who died during the course of preeclampsia. Renal autopsy materials of women who died accidentally during otherwise normal pregnancies were used as controls. Kidney tissue samples were obtained that were embedded in paraffin and had been stored from 5 to 50 years. The tissue preservation was variable, but in all cases and controls, the renal sections stained strongly for podocin, indicating that the immunoreactivity of the tissue samples was preserved. All kidney sections were stained initially with hematoxylin and eosin (H & E) and examined under light microscopy, looking for the presence of the glomerular lesion of endotheliosis, a classic pathologic renal lesion of preeclampsia. Once the presence of this lesion (capillary loop occlusion by swelling and hypertrophy of endocapillary cells) was confirmed, the remaining sections were stained for nephrin, podocin, and synaptopodin. Light microscopy indicated normal histology in both controls (controls 1-2, **Table 1**). In all cases (cases 1-7, **Table 1**), florid endotheliosis was present (**Figure 3A**); this frequently was associated with thrombotic microangiopathy (**Figure 3B**). Strong, diffuse capillary wall staining for both

3A **3B**

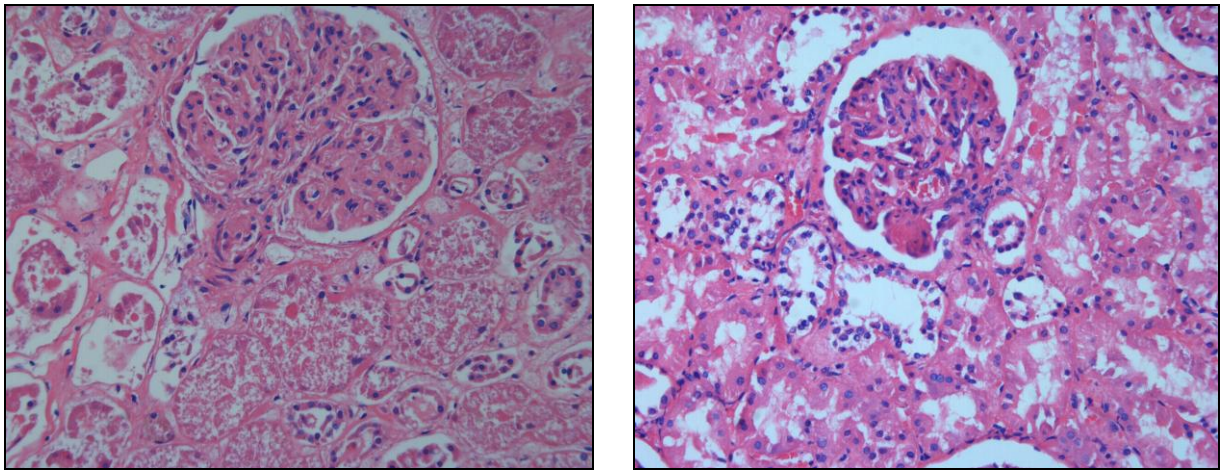
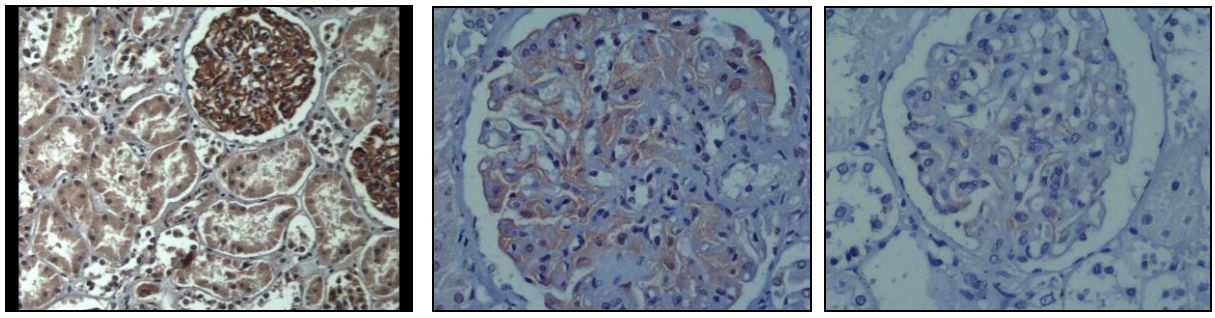


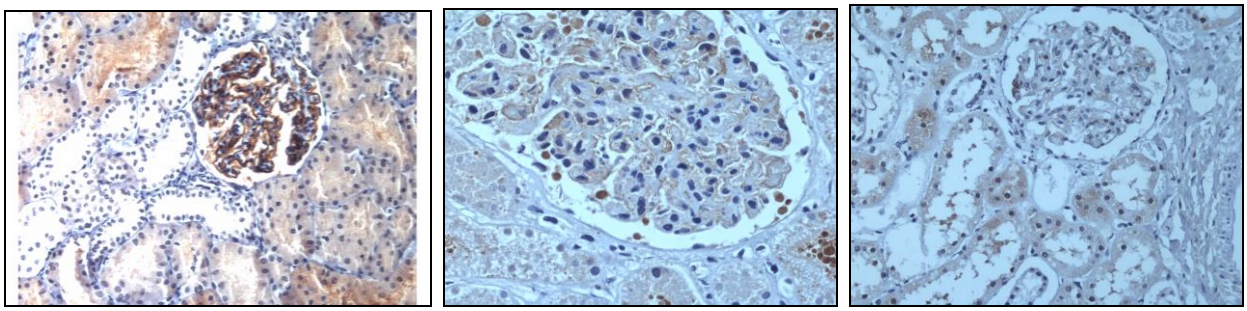
Figure 3A, 3B. Light microscopy of renal sections from autopsy material of patients with preeclampsia. Florid glomerular endotheliosis (Figure 3A, case 1 from Table 1) was present and frequently associated with thrombotic microangiopathy (Figure 3B, case 3 from Table 1). Hematoxylin and eosin, original magnification x 100

4A **4B** **4C**



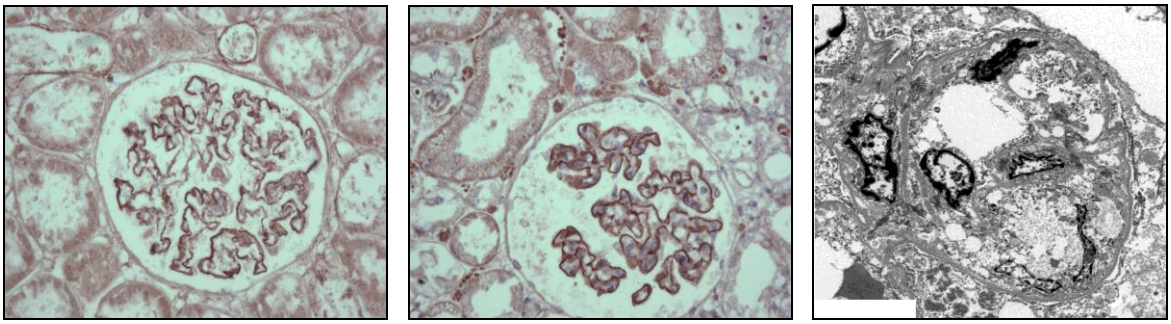
Figures 4A, 4B, 4C. Immunocytochemistry for nephrin. Uniform, strong staining in controls (Figure 4A, control 1 from Table 1); in cases, nephrin expression was either markedly reduced (Figure 4B, case 3 from Table 1), or absent (Figure 4C, case 2 from Table 1). Magnification x100, 400, and 200, respectively

5A **5B** **5C**



Figures 5A, 5B, 5C. Immunocytochemistry for synaptopodin. Normal expression in controls (Figure 5A, control 1 from Table 1), 100x with significant down-regulation (Figure 5B, case 5 from Table 1), 200x or complete absence of protein expression (Figure 5C, case 2 from Table 1), 200x in cases. Magnification x100, 200, and 200, respectively

6A **6B** **6C**

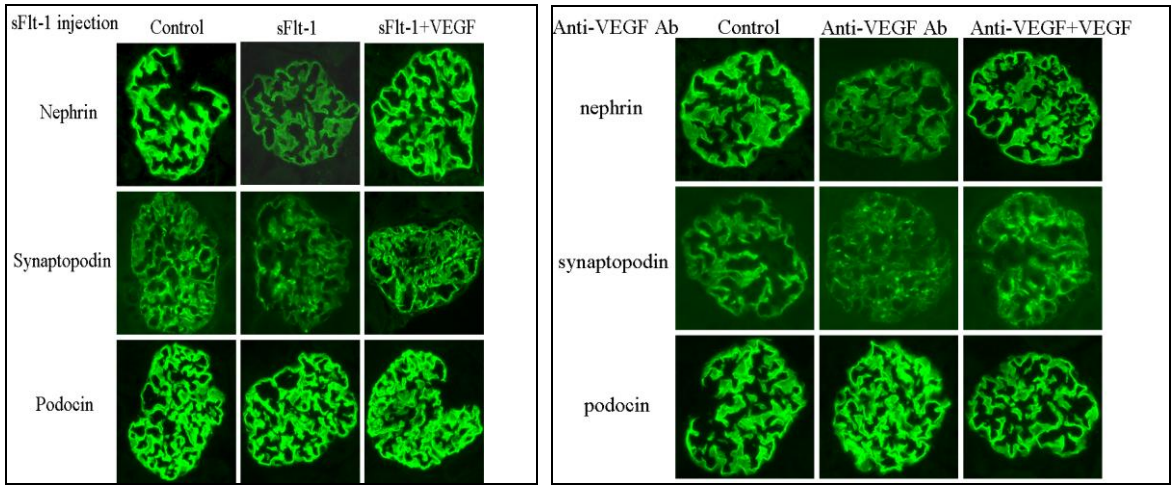


Figures 6A, 6B, 6C. Immunocytochemistry for podocin. Both controls (Figure 6A, control 1 from Table 1) and cases (Figure 6B, case 7 from Table 1) demonstrated strong, continuous podocin staining. Magnification x 200. Electron microscopy of kidney sections from a patient who died from severe preeclampsia/HELLP (Figure 6C, case 7 from Table 1), showing marked endothelial cell swelling and vacuolization, with compromise of capillary loop lumens and extensive epithelial foot process effacement. Magnification x 7400

nephrin and synaptopodin was present in the control kidneys (**Figures 4A** and **5B**, respectively). Expressions of both nephrin (**Figures 4B-4C**) and synaptopodin (**Figures 5B-5C**) were decreased in the cases compared to the controls; the degree of down-regulation varied from a marked decrease to almost complete absence of protein expression. Podocin did not appear to be affected, as both cases and controls demonstrated strong staining for podocin (**Figures 6A** and **6B**, respectively).

The expressions of nephrin, podocin, and synaptopodin were examined in the kidney sections from mice infused with either sFlt-1 (**Figure 7A**) or anti-VEGF antibodies (**Figure 7B**) at concentrations corresponding to ten times the molar concentration of normal plasma VEGF. The expression of nephrin was significantly reduced by both the anti-VEGF antibodies and sFlt-1 treatments, and that of podocin was unchanged. Both anti-VEGF antibodies and sFlt-1 significantly reduced the expression of synaptopodin that, similar to nephrin, was restored in rescue experiments when 32.5 pM of VEGF was delivered 5 minutes after infusion of either anti-VEGF antibodies or sFlt-1.

7A **7B**

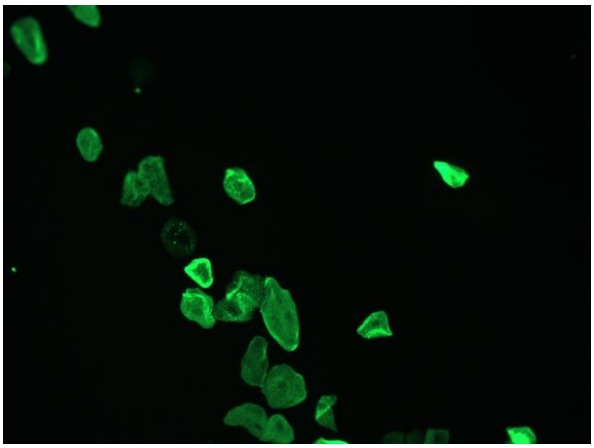


Figures 7A, 7B. Blocking of circulating VEGF reduces the expressions of nephrin and synaptopodin, but does not affect podocin. Immunofluorescence staining of kidney sections for nephrin, synaptopodin and podocin: control mice (injected with IgG1) versus those receiving 32.5 pM of either s-Flt-1 (Figure 8A) or anti-VEGF antibody (Figure 8B). Rescue experiments with equimolar VEGF treatments restored nephrin and synaptopodin expressions in both sFlt-1 and anti-VEGF pre-treated mice.

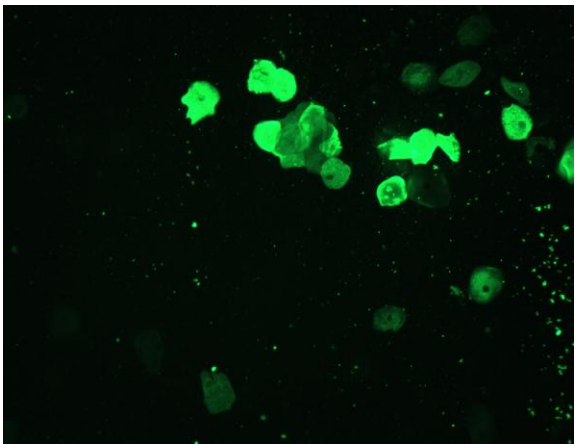
4.2. Podocyturia

In the women with urinary measures of podocyturia (i.e. 15 cases and 16 controls), those with preeclampsia or HELLP all had podocin-positive cells in the urine (Figure 8A), whereas none of the normotensive controls had any podocin-positive cells. Thus, the sensitivity and specificity of podocyturia for the diagnosis of preeclampsia, as determined by the podocin- positive cells, were both 100%. A positive correlation between the degree of albuminuria and podocyturia, as determined by podocin staining, was present (*P*=.04). Compared to podocin, measurements of podocyturia based on podocalyxin, nephrin, and synaptopodin stains (**Figures 8B, 8C, and 8D**, respectively), had both lower sensitivities and specificities (**Table 4**).

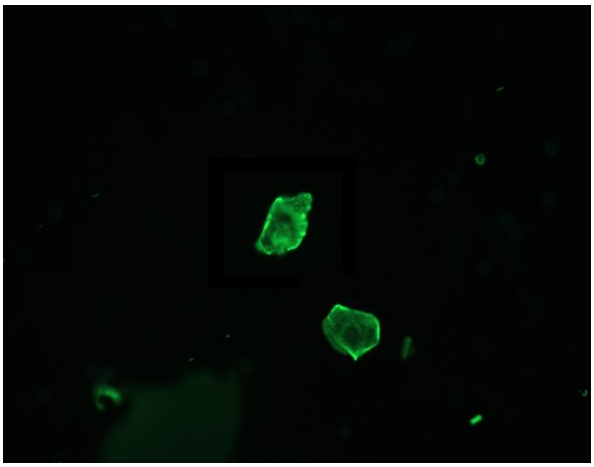
8A



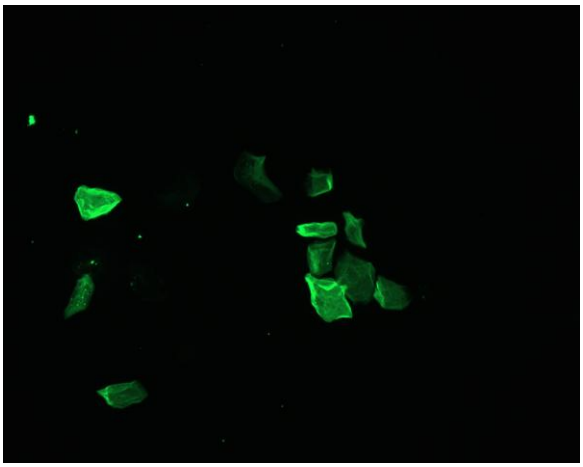
8B



8C



8D



Figures 8 A-D. Immunofluorescence of urinary cells plated on a collagen-coated slide cultured for 24 hours, as described in Methods; 8A, podocin, 8B, podocalyxin, 8C, nephrin, 8D, synaptopodin stains

We generated the receiver operator characteristics (ROC) curves for all four podocyte markers (**Figure 9**), and their respective areas under the curve (AUC) were compared as a measure of diagnostic accuracy. The analysis indicated that podocin had a greater diagnostic accuracy than podocalyxin (*P*=.04) or nephrin (*P*=.05), and possibly better than synaptopodin (p=0.08). The diagnostic accuracies of the other 3 markers (podocalyxin, nephrin and synaptopodin) did not differ. The rates of podocyte excretion (expressed as median cell number per mg of creatinine) for these cases were 3.7 for podocin and synaptopodin, 5.0 for podocalyxin, and 3.3 for nephrin.

The rates of podocyte excretion in the controls for synaptopodin were 0.6, and 0 for podocalyxin, podocin, and nephrin. Additional controls consisted of women with gestational hypertension (n=6), essential hypertension (n=2), and preexisting proteinuria (n=3) who did not develop the clinical signs and symptoms of superimposed preeclampsia. None of these 11 women demonstrated podocyturia, as determined by podocin staining.

Table 4. Test characteristics for markers of preeclampsia*

				Pretest probability for preeclampsia			
				5%		25%	
Test	**Cutoff**	**Sensitivity (%)**	**Specificity (%)**	Positive predictive value (%)	Negative predictive value (%)	Positive predictive value (%)	Negative predictive value (%)
sFlt-1*†	7,463 pg/mL	83	58	9.4	98.5	39.7	91.1
	9,795 pg/mL	71	68	10.5	97.8	42.5	87.6
Endoglin*†	21.3 ng/mL	94	58	58	10.5	99.5	42.7
	24.6ng/mL	86	63	10.9	98.8	43.7	93.1
Serum PIGF*‡	84.92 ng/mL	74	58	8.5	97.7	37.0	87.0
	102.7pg/mL	47	47	7.9	98.5	35.1	91.0
Urine PIGF*‡	1.22pg/mL§	79	50	7.7	97.8	34.5	87.7
	2.18pg/mL§	86	38	7.5	98.4	33.9	90.4
Podocin†	0.85 cells§	100.0	100.0	100.0	100.0	100.0	100.0
Nephrin†	0.75 cells§	93	75	16.4	99.5	55.4	97.0
Podocalyxin†	0.83 cells§	93	75	16.4	99.5	55.4	97.0
Synaptopodin†	1.11 cells§	93	81	20.5	99.5	62.0	97.2

* For sFlt-1, endoglin, and PlGF the sensitivities and specificities were calculated twice, with 2 different cutoffs to define a positive test. The cutoffs for podocyturia (podocin, nephrin, podocalyxin, and synaptopodin) are expressed as cells per milligram of creatinine.
† A positive test is defined as having a value higher than the cutoff
‡ A positive test has a lower value than the cutoff
§ Expressed per milligram of creatinine in the respective urine samples

4.3. Angiogenic markers of preeclampsia

Serum sFlt-1 levels were significantly higher in women with preeclampsia or HELLP syndrome than in the normotensive pregnant controls (17,326 ± 12,124 pg/mL vs 8,160 ± 5,186 pg/mL, *P*<.001) (**Table 5)**.

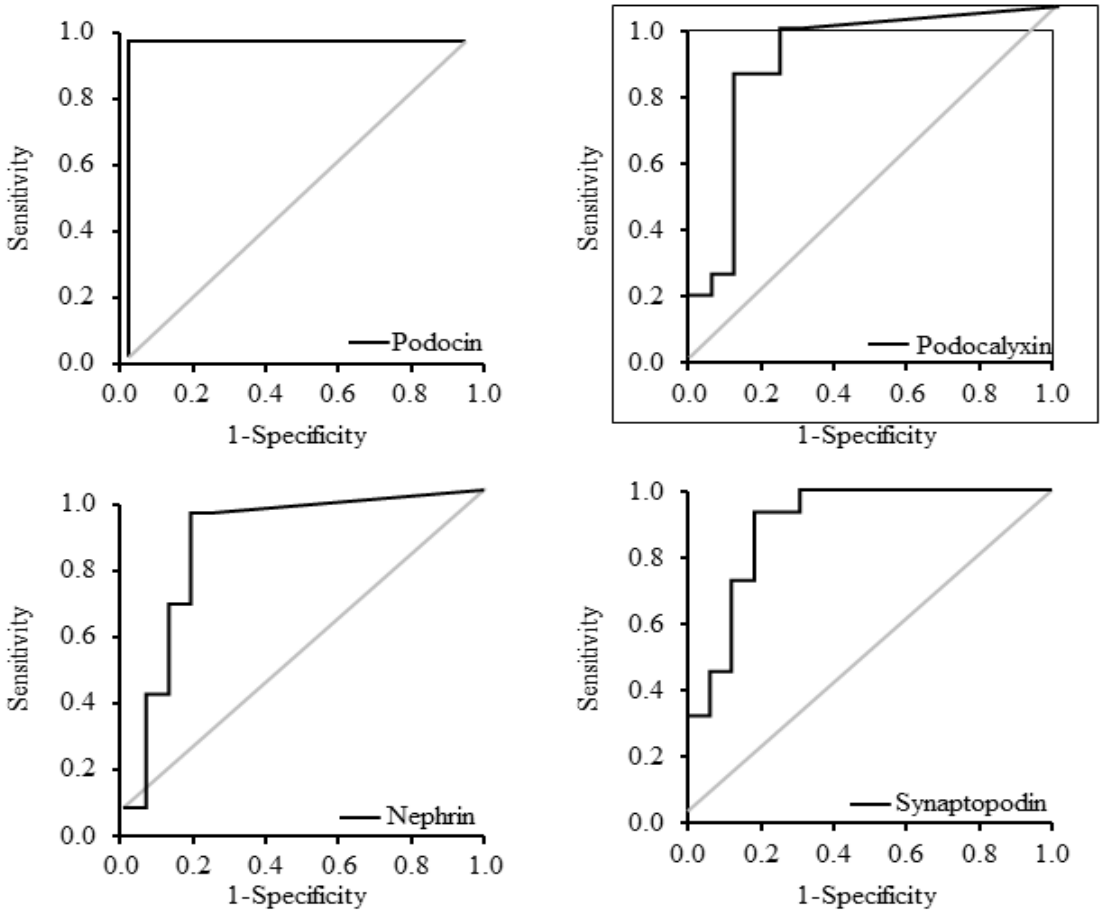
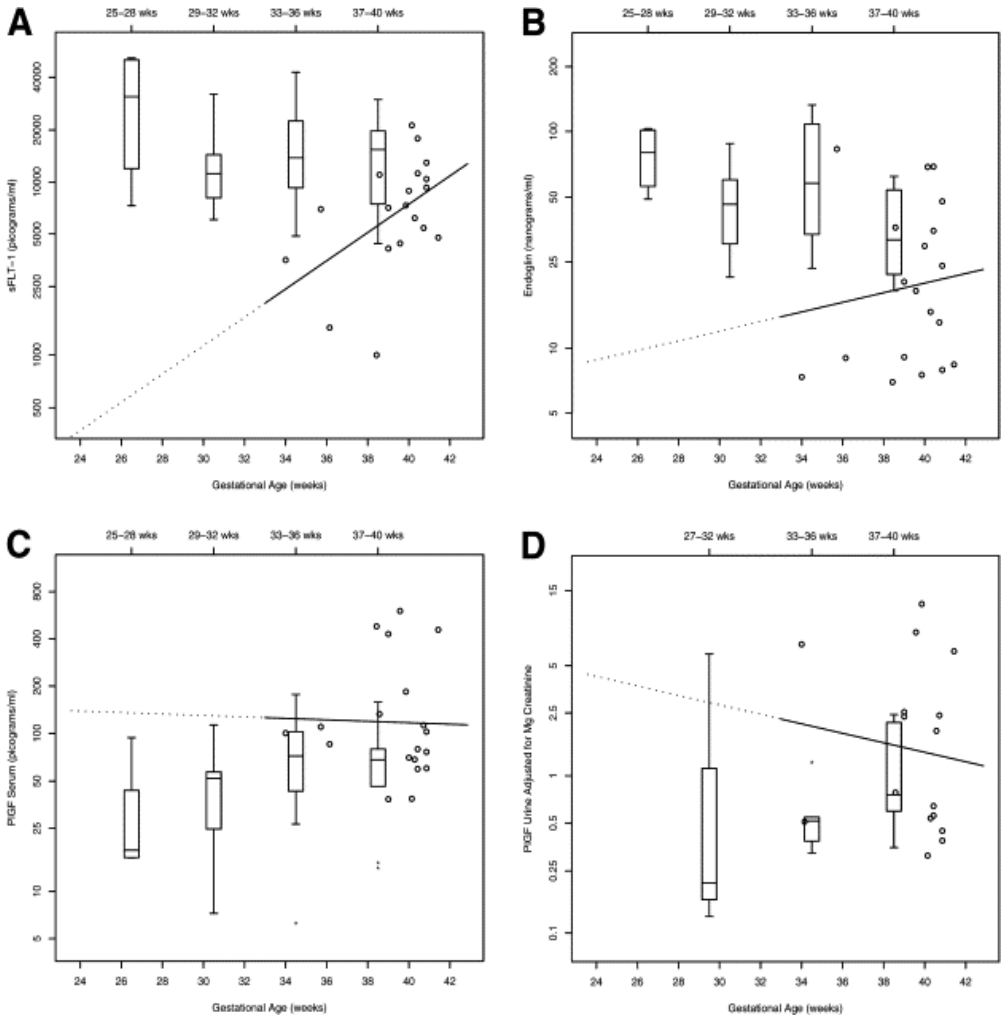


Figure 9. *ROC Curves for Podocyturia by staining for podocyte-specific proteins.* Receiver operator characteristics (ROC) curves for podocyturia, as determined by staining for podocyte proteins; podocin, podocalyxin, nephrin, synaptopodin

Table 5. Normal and preeclamptic levels of sFlt-1, endoglin, and PlGF

Variable	Normal (n = 23)	Preeclampsia (n = 33)	HELLP (n = 11)	Preeclampsia + HELLP
sFlt-1 (pg/mL)	8,160 ± 5186	18,231 ± 11,216*	14,711 ± 14,876*	17,326 ± 12,124*
Endoglin (ng/mL)	27.2 ± 23.9	56.5 ± 31.7*	52.1 ± 32.7*	55.4 ± 31.6*
Serum PlGF (pg/mL)	173 ± 175	66.2 ± 44.2*	59.8 ± 48.5*	64.6 ± 44.7*
Urine PlGF (pg/mL per mg creatinine)		2.94 ± 3.56	1.17 ± 1.54	† †

Serum sFlt-1 levels did not differ significantly between preeclamptic and HELLP patients (*P*=.11). **Figure10A** illustrates that patients with preeclampsia and HELLP displayed higher sFlt-1 levels than normal patients, if they delivered early in pregnancy. This difference became less apparent closer to full term delivery.



Figures 10A-D. *Angiogenic markers in normotensive and preeclamptic pregnancies as a function of gestational age.* Open circles represent individual patients values, with a solid line representing trend regression and a dashed line representing extrapolation. The box plots correspond to medians and interquartile ranges; A. sFlt-1 B. soluble endoglin C. serum PlGF D. urine PlGF

Serum soluble endoglin levels were significantly higher in women with preeclampsia or HELLP than in normotensive pregnant controls (55.4 ± 31.6 ng/mL vs 27.2 ± 23.9 ng/mL *P*<.001). Serum soluble endoglin levels did not differ significantly between preeclamptic

and HELLP patients (*P*=.69). The difference between normal and preeclamptic pregnancies was greater with an earlier delivery, and became less apparent in those patients delivering full term, as shown in **Figure10B**.

Serum free PlGF levels were lower in women with preeclampsia or HELLP than in normotensive pregnant controls (64.6 ± 44.7 pg/mL vs 173 ± 174.8 pg/mL *P*=.0005). Serum free PlGF levels did not differ significantly between preeclamptic and HELLP patients (*P*=.36). In those patients delivering at an earlier gestational age, free PlGF levels were lower in preeclamptic and HELLP patients vs controls, but this difference became less apparent as pregnancies were carried towards full term (**Figure10C**).

As shown in **Table 5**, there was a statistically insignificant trend towards lower urine PlGF levels in women with preeclampsia or HELLP compared to normotensive pregnant controls (1.17 ± 1.54 pg/mL/mg creatinine vs 2.94 ± 3.56 pg/mL/mg creatinine (*P*=.11). Urine PlGF levels in preeclamptic women were not different than normal women, regardless of gestational age at delivery **(Figure10D**).

4.4. Angiogenic factors as diagnostic tests for preeclampsia: comparison to podocyturia

ROC curves were generated for sFlt-1, soluble endoglin and both serum and urine PIGF (**Figure11)**. We calculated the positive and negative predictive values for podocyturia, as determined by the four podocyte-specific markers and the angiogenic factors that were evaluated (**Table 4**). As the value of a diagnostic test depends on the pretest probability of disease, the diagnostic accuracy of each test was estimated for 2 different pretest probabilities: 5%, which reflects the pretest probability for preeclampsia in the general population, and 25%, a commonly cited percentage risk in women with preexisting hypertension. The negative predictive value did not differ between the podocyturia and angiogenic factor tests in patients with a low, 5%, pretest probability.

However, in patients with a pretest probability of 25%, the negative predictive value was higher with podocyturia. The positive predictive value was higher with podocyturia when compared to the angiogenic factors tests, both in the low and high pretest probability groups.

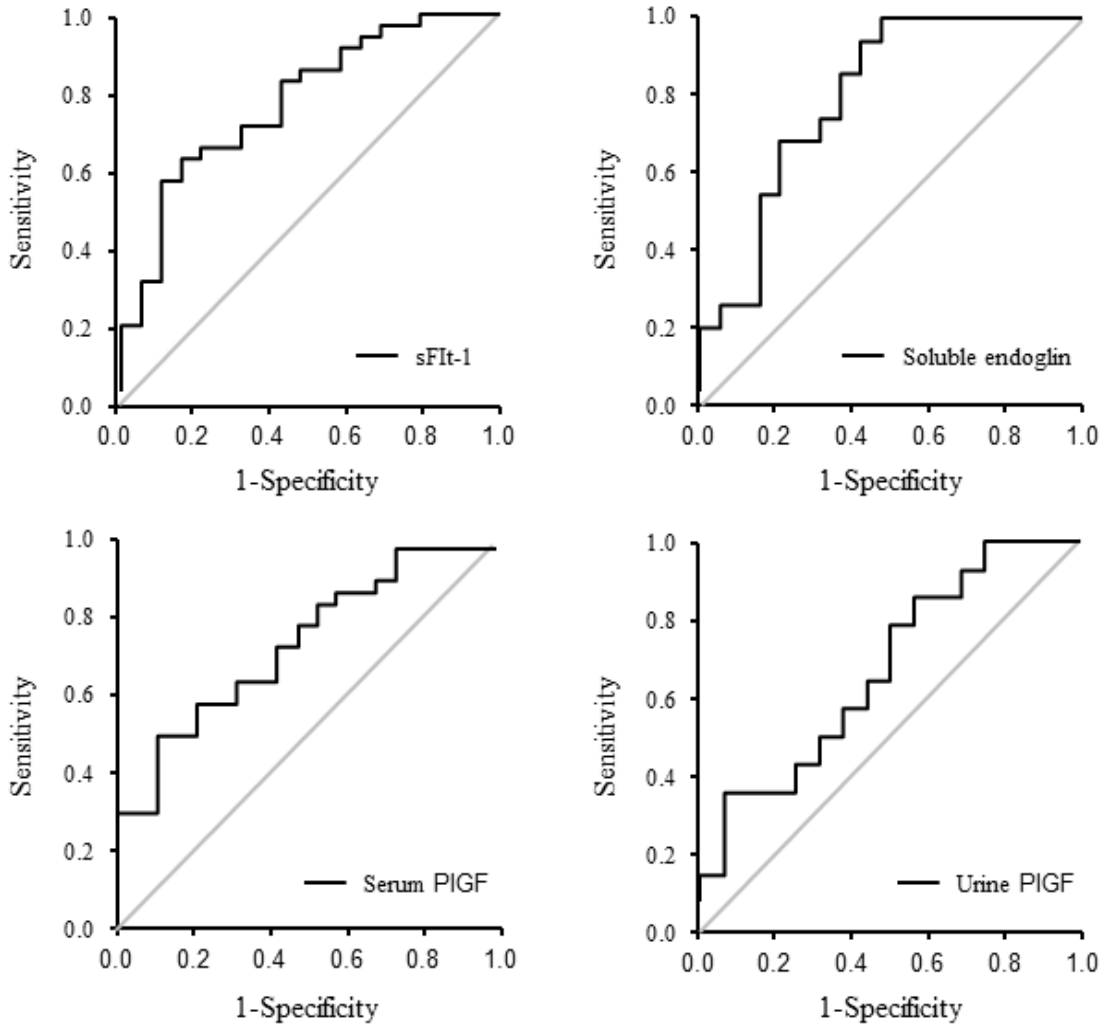
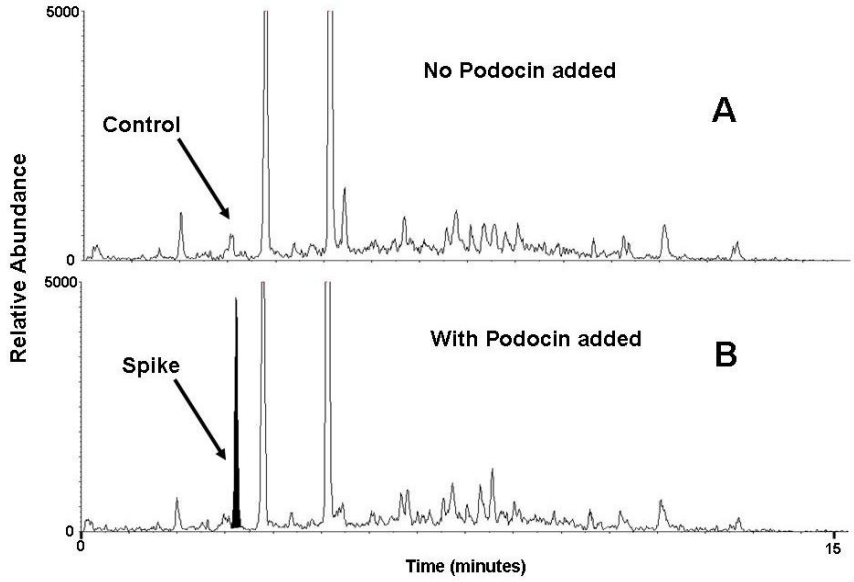


Figure 11. ROC Curves for Angiogenic Markers. Receiver operator characteristics (ROC) curves for angiogenic markers; sFlt-1, soluble endoglin, serum PlGF, urine PlGF

4.5. Tryptic Peptide from Podocin

LC-MS/MS methodology applied to our study assumes a 1:1 stoichiometric ratio between the tryptic fragment isolated from podocin and the concentration of intact podocin in the sample. Adding a known amount of stable isotope labeled internal standard (IS) peptide with the same amino acid sequence as the tryptic peptide, but with a different mass, allows the mass spectrometer to distinguish between the two forms, and thus provide relative quantification. The technique has recently grown in popularity, as there is a need for absolute quantification of proteins using the sensitivity and specificity of LC-MS/MS.[54,55,56]

Our choice of podocin was based on the fact that it is highly podocyte-specific,[47] and that podocin exhibited 100% sensitivity and specificity in the diagnosis of preeclampsia.[49] Its respective recombinant protein is also commercially available. The tryptic peptide, QEAGPEPSGSGR, was chosen as the best peptide for quantifying podocin from urine for the following reasons: this sequence is unique to human podocin, and gave the best response of all of the podocin tryptic peptides in a digest of recombinant podocin, as measured by LC-MS/MS. The identity of the peptide was confirmed by full scan MS/MS, and by standard addition of recombinant podocin digests to the frozen cell digests. The reproducibility of the digestion protocol was examined using frozen mouse podocytes as a surrogate matrix. Conditionally immortalized mouse podocytes were a generous gift from Dr. Peter Mundel. Differentiated podocytes were used, after changing from permissive to non-permissive conditions, as previously described.[57] All digests contained the same volumes of frozen mouse podocytes that were thawed and solubilized in RapiGest™ buffer. **Figure 12** describes LC-MS/MS chromatograms specific to the human podocin tryptic peptide, QEAGPEPSGSGR,



Figures 12 A-B. *LC-MS/MS chromatograms*: MRM (multiple reaction monitoring) transition monitored for the tryptic peptide from podocin for a digest of immortalized mouse podocyte cell line digests without (Control: Figure 13A), and with recombinant human podocin added (Spike: Figure 13B)

digested in the mouse podocyte matrix. The chromatogram in **Figure 12A** indicates the control mouse podocyte digest without recombinant human podocin added prior to digestion, and **Figure**

12B shows the same mouse podocytes spiked with recombinant human podocin prior to digestion. **Figure 12B** clearly shows the highlighted peak representing the tryptic peptide, QEAGPEPSGSGR, from the recombinant human podocin spiked into the mouse podocytes. The response for this peptide is at the same retention time as the internal standard added to the sample after digestion, and before injection (chromatogram not shown). **Table 6** shows the peak areas for the tryptic peptide and the internal standard for each of the three replicates, along with their respective coefficient of variation values. The results suggest that this method is reproducible using the frozen mouse podocyte matrix.

Table 6. Peak areas for the tryptic peptide and the internal standard for each of the three replicates of the control mouse podocyte digest spiked with the recombinant human podocin.

Sample name	Analyte Peak Area (counts)	IS Peak Area (counts)	Area ratio
Mouse podocyte digest Spike 1	3,290	15,9000	.021
Mouse podocyte digest Spike 2	2,890	153,000	.019
Mouse podocyte digest Spike 3	3,210	155,000	.021
Average (mean)	3,130	155,667	.020
Standard deviation	212	3,055	.013
% Coefficient of variation	7%	2%	5%

All 15 urine samples obtained from preeclamptic patients demonstrated the podocin tryptic peptide of interest. **Figure 13** indicates a LC-MS/MS chromatogram representative of the results found using cells from a preeclamptic patient: the highlighted podocin tryptic peptide peak from the preeclampsia patient on the top, and the peak from the internal standard at the bottom. The figure demonstrates that the tryptic peptide derived from podocin in the patient sample has the same retention time as the synthetic stable isotope labeled internal standard peptide. In preeclamptic women, a positive correlation was present between the quantity of the podocin peptide in their urines, and both the amount of proteinuria in the respective urine samples (P=.03), and their systolic BPs at the time of urine collection (P=.001). The presence of podocytes in the respective urine samples at the time of delivery was further confirmed by the overnight podocyte culture method (**Figure 14**), indicating the presence of ≥ 0.85 podocin-positive cells/mg creatinine in all tested samples.[49] Podocyturia was absent at the 6-week postpartum visit in all women in whom it had been present at the time of delivery.

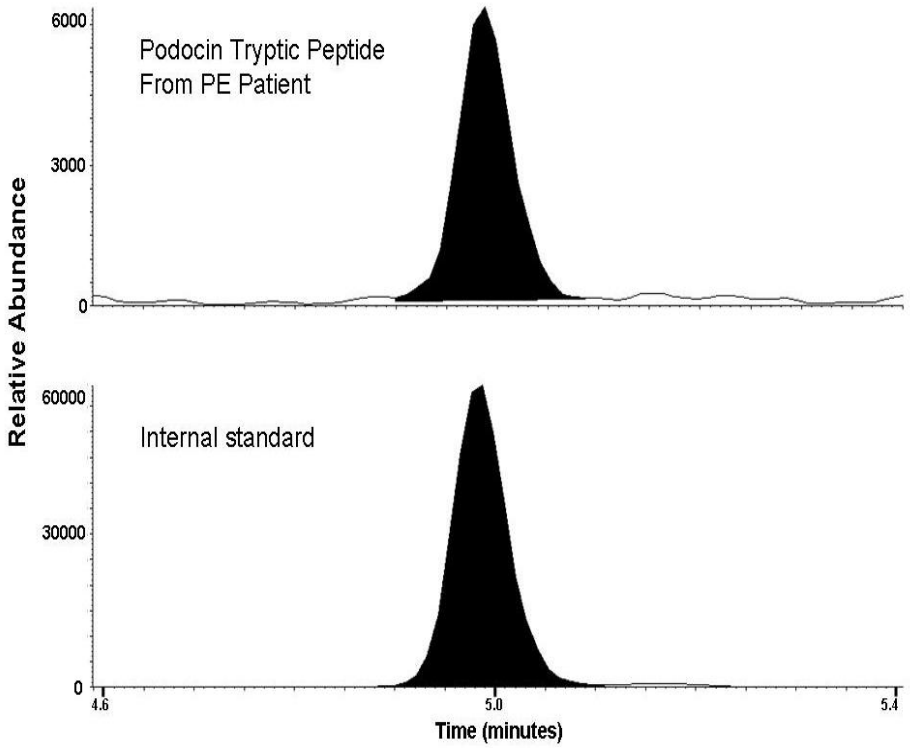


Figure 13. LC-MS/MS chromatograms: MRM transition monitored for the tryptic peptide from podocin for a digest of fixed cells from a patient with preeclampsia on the top (with a calculated tryptic peptide concentration of 3.4 fmol/mg creatinine), and the peak from the internal standard on the bottom. PE=preeclampsia

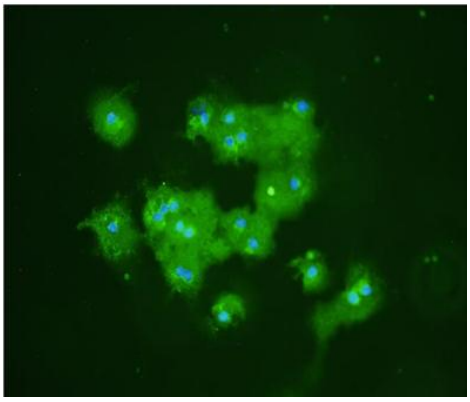


Figure 14. *Podocyturia assay*: Hoechst nuclear stain (blue); podocin antibody followed with a secondary, FITC-labeled antibody (green).

In the final step, urine samples were collected from 4 patients with normotensive pregnancies and 4 patients with preeclampsia, and each whole urine sample was used for the detection and

quantification of the podocyte tryptic peptide of interest. The quantification of the podocin peptide was performed by a single point calibration, using a known amount of the internal standard (expressed in fmol of units) that was added to each sample, and expressed in fmol of podocin/mg of creatinine in the respective samples. Podocin tryptic peptide from fixed cells taken from normotensive pregnant patients' urines, as compared to preeclampsia patients' urines, showed a significantly lower response : 0.4 ± 0.04 vs 4.6 ± 2.3 fmol /mg creatinine, respectively, P=.01 (**Figure 15**). The reproducibility of fixed cell digests was evaluated by using solubilized fixed cells from a single patient, splitting the sample three ways, then performing the digestion and LC-MS/MS analysis. The results, presented in **Table 7**, demonstrate that the method is reproducible for fixed cells derived from patient urine.

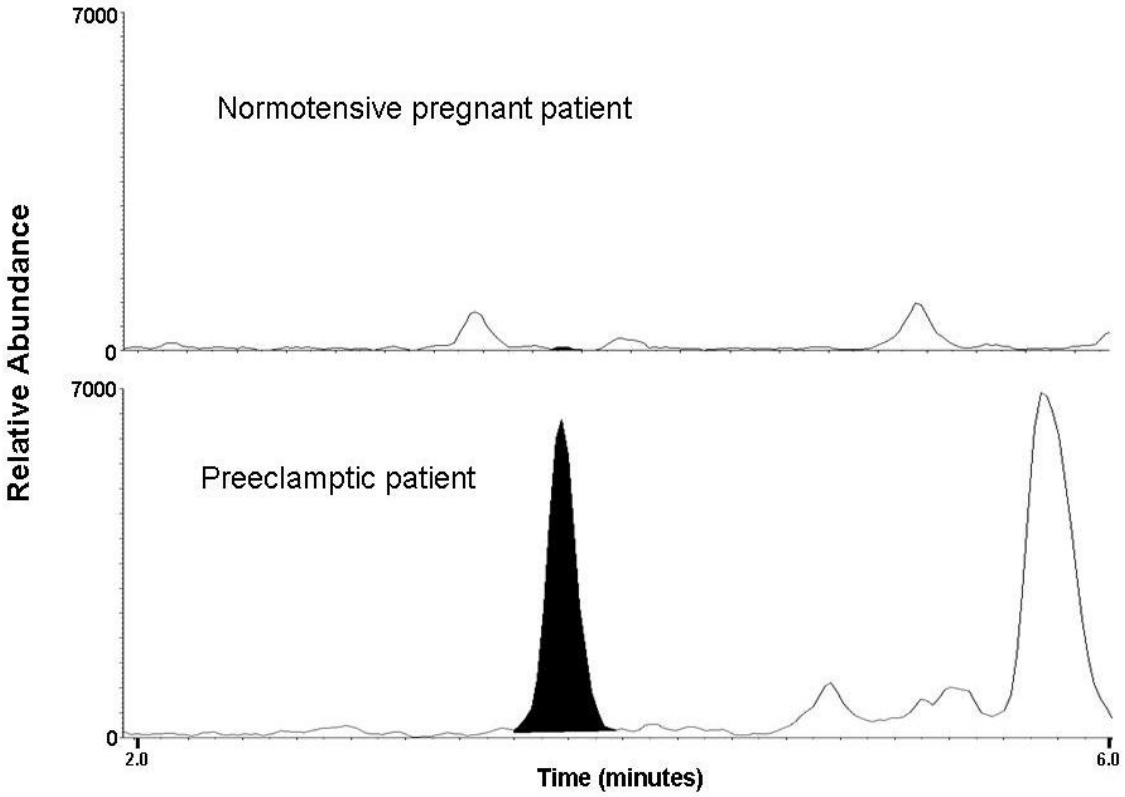


Figure 15. LC-MS/MS chromatograms from fixed cells: normotensive pregnant patient (top), preeclamptic patient (bottom), with a calculated tryptic peptide concentration of 0.42 versus 5.4 fmol/mg creatinine, respectively.

Table 7. Peak areas for the tryptic peptide for each of the fixed cells derived from the urine of a preeclamptic patient

Sample name	Analyte Peak Area (counts)	IS Peak Area (counts)	Area ratio
Patient fixed cell digest 1	921	22,0000	4.2E-03
Patient fixed cell digest 2	777	173,000	4.5E-03
Patient fixed cell digest 3	947	201,000	4.7E-03
Average	882	198,000	4.46E-03
Standard deviation	92	23,643	0.25E-03
% Coefficient of variation	10	12	6

4.6. Sample description and characteristics

A total of 4,782 women from 2,443 sibships participated in the second FBPP study visit between 2000 and 2004. Demographic characteristics measured at the second FBPP visit, are shown by network in **Table 8**.

Table 8. The Family Blood Pressure Program (FBPP) Demographics by Network (n=4,782).

Variable *	GenNET† (N=1,073)	GENOA (N=2,501)	HyperGEN (N=449)	SAPPHIRe (N=759)
Age at Clinic Visit, Median (Q1, Q3)	40.0 (28.9, 50.0)	61.0 (53.0, 67.0)	38.0 (31.0, 43.0)	57.0 (51.0, 61.0)
Race, No. (%)				
Non-Hispanic White	412 (38%)	704 (28%)	280 (62%)	0 (0%)
Hispanic	521 (49%)	747 (30%)	0 (0 %)	0 (0 %)
Non-Hispanic Black	140 (13%)	1,050 (42%)	169 (38%)	0 (0 %)
Japanese	0 (0%)	0 (0 %)	0 (0%)	759 (100 %)
High school education or higher, No. (%)	662 (62%)	1,607 (64%)	430 (96 %)	751 (99 %)
Pregnancy status, No. (%)				
Nulliparous, 718 (15%)	235 (22 %)	220 (9 %)	98 (22 %)	165 (22 %)
No history of hypertension in pregnancy, 3421 (72%)	715 (67 %)	1,870 (75 %)	290 (65 %)	546 (72 %)
History of hypertension in pregnancy, 643 (13%)	123 (11 %)	411 (16 %)	61 (14 %)	48 (6 %)

*All variables are expressed as percentages, with the exception of age, which was expressed as median age in years (interquartile range).
† 3 subjects from GenNet were missing education information

At least one pregnancy that lasted more than 6 months was reported by 4,064 women (85%), overall. Of these women, 643 (13%) reported hypertension in at least one of their pregnancies. Among those, 209 (4.4% of the whole cohort) reported a history of preeclampsia. The percentages of women reporting hypertension in pregnancy did not differ significantly among

non-Hispanic blacks (232, 18.95%), non-Hispanic whites (201, 17.14%), and Hispanic whites (162, 15.10%); however, the percentage was significantly lower in Asians (48, 8.08%; P <.001). Education had no significant effect on the percentage of women reporting hypertension in pregnancy, i.e., the percentage did not differ significantly between those who did or did not complete high school. All subsequent analyses were adjusted for age, network, education, and race, as major differences were present across the networks.

We also compared traditional risk factors among the pregnancy groups, measured at the time of the Phase 2 FBPP examination, after controlling for differences in age, network, and race (**Table 9**). Nulliparous women had significantly lower BMIs, a higher prevalence of a current diagnosis of diabetes mellitus and hypertension, and a lower prevalence of positive family histories of hypertension and CHD, compared to women with histories of normotensive pregnancies.

Table 9. The Family Blood Pressure Program Risk Factor Data by Pregnancy Group

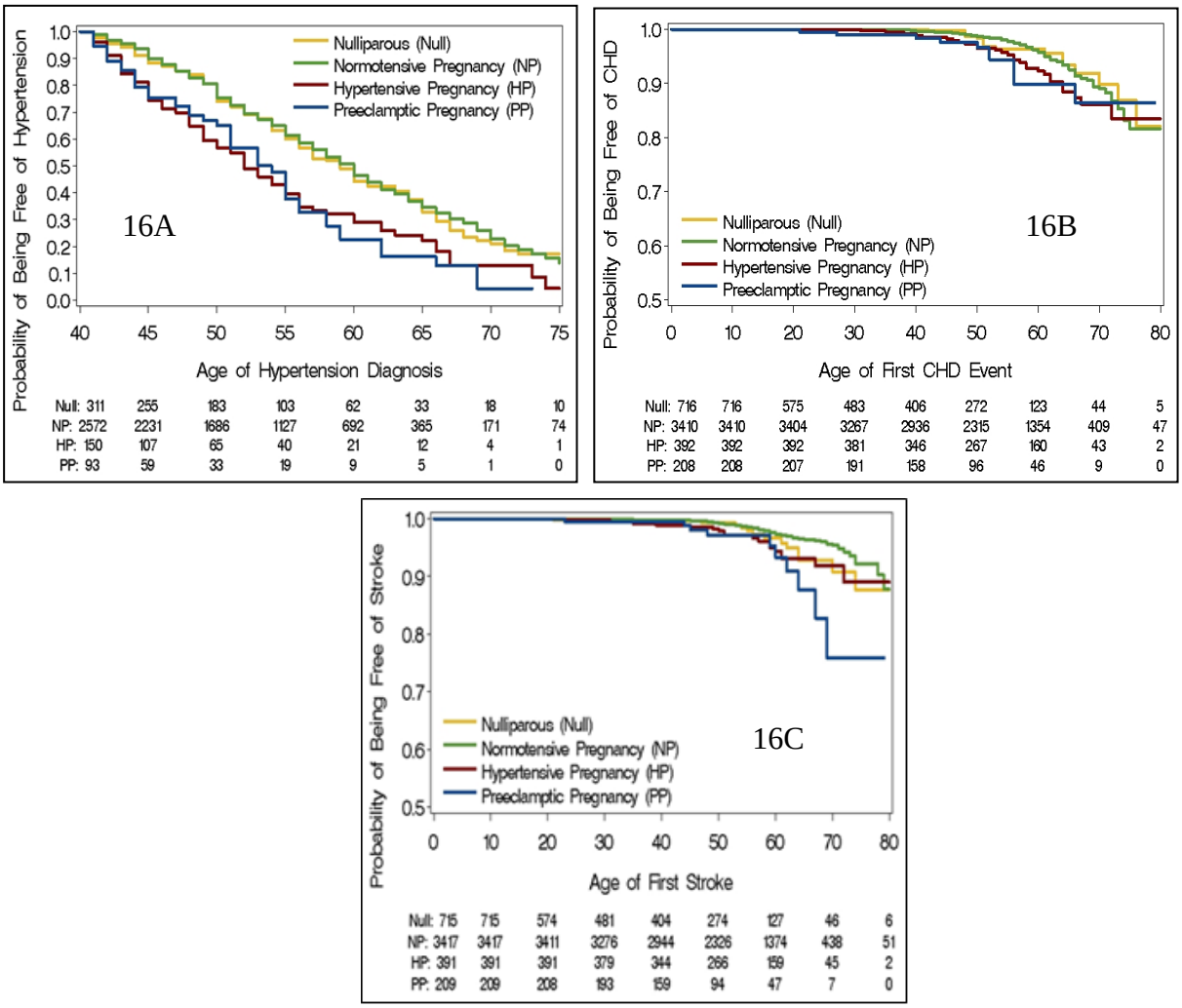
Variable [a]	Nulliparous (N=718)	Normotensive Pregnancy (N=3,421)	Hypertensive Pregnancy (N=6430)	Normotensive vs. Nulliparous *P* value	Hypertensive vs. Normotensive *P* value
Smoking history [b]	24.7	31.5	29.6	<.001	.336
BMI [c]	27.94	28.50	31.19	--	--
Log BMI [d]	3.33 ± 0.25	3.35 ± 0.20	3.44 ± 0.21	.028	<.001
Diabetes [e]	21.1	18.0	25.5	.037	<.001
Dyslipidemia [f]	81.9	77.0	72.0	.193	.069
Family history of stroke	23.0	24.7	29.6	.281	.004
Family history of CHD	30.2	33.7	38.6	.041	.004
Current HTN [g]	49.3	39.8	57.6	<.001	<.001
Family history of HTN	67.8	72.3	79.8	.018	<.001

[a] All variables were adjusted for age, network, and race, and expressed as percentages, with the exception of body mass index (BMI), which was expressed as a mean. *P* values calculated from a generalized linear regression model using generalized estimating equations to account for sibling relationships.
[b] 2 normotensive subjects missing smoking history
[c] 2 nulliparous, 11 normotensives, and 1 hypertensive subject missing BMI data
[d] Due to skewness of distribution, log BMI (mean ± SD) was used for comparison between the groups
[e] 2 nulliparous and 5 normotensives missing diabetes data
[f] Genetic Epidemiology Network of Arteriopathy (GENOA) participants only, N=149, 1,303, and 293, respectively
[g] 2 normotensives and 2 hypertensive subjects missing current hypertension status

Those with histories of hypertension in pregnancy had higher BMIs at the FBPP study visit and a higher prevalence of current diabetes mellitus, hypertension, and family histories of stroke, CHD, and hypertension, compared to women with histories of normotensive pregnancies.

4.7. Cardiovascular Events

No significant differences in cardiovascular outcomes were observed between nulliparous women and women with a history of normotension in pregnancy (**Figure 16A**). Women



Figures 16A, 16B, 16C. Kaplan-Meier plots of the cumulative probability of being free of hypertension (Fig.16A), CHD (Fig.16B), and stroke (Fig16C), as a function of age among nulliparous women, those with a history of normotensive pregnancies, and women with a history of either hypertensive or preeclamptic pregnancies. Numbers along the x-axis show the number of women at risk in each age group over time.

reporting hypertension in pregnancy compared to those without this history in pregnancy had increased unadjusted risks for hypertension diagnosed after the age of 40 (50% hypertensive at the age 52 vs 60, *P*<.001), CHD (14% estimated event rate vs 11% at 70 years, *P*=.049), and stroke (8% estimated event rate vs 5% at 70 years, *P*=.009) (**Figure 16B**). Similarly, women reporting preeclampsia in pregnancy, compared to those with no history of hypertension in pregnancy, had increased unadjusted risks for hypertension diagnosed after the age of 40 (50% hypertensive at the age 54 vs 60, *P*<.001), CHD (14% estimated event rate vs 11% at 70 years, *P*=.045), and stroke (24% estimated event rate vs 5% at 70 years, *P*<.001). The prevalence of these outcomes was similar in women with a history of hypertension only and those with a history of preeclampsia (**Figure 16C**). When stroke and CHD were analyzed as a combined endpoint, there was a trend for an elevated risk for women with a history of preeclampsia, although this was not statistically significant (*P*=.1).

We used Cox proportional hazard models to model the age at diagnosis of hypertension (after age 40 years) and the occurrences of CHD and stroke in each pregnancy group. As the Kaplan-Meier curves showed no significant difference in cardiovascular outcomes between women with a history of hypertension in pregnancy versus those with a history of preeclampsia (**Figure 16 A-C**), these 2 subsets of women were grouped together under "hypertensive pregnancy" for these analyses. The HRs for each event type were contrasted in nulliparous women relative to women with normotension in pregnancy, and in women with a history of normotension in pregnancy relative to those with hypertensive pregnancies (**Table 10**), after adjusting for the potentially confounding variables that differed among the pregnancy groups (**Table 9**).

Among FBPP participants (**Table 10**), the HR for hypertension after age 40 years (Model A) in nulliparous women relative to those with a history of normotensive pregnancies did not differ significantly from 1.0. The HRs for CHD and stroke (Model B) were also not significantly different from 1.0, although the estimated HR for stroke was 0.55 (95% CI 0.31 – 1.00, *P*=.05), suggesting a lower hazard of stroke in those with a history of normotensive pregnancy compared to nulliparous women.

Those with a history of hypertensive pregnancies had an increased HR of 1.55 (95% CI 1.26 – 1.89, *P*<.001) for the diagnosis of hypertension after age 40 (Model A), compared to women with a history of normotensive pregnancies. After controlling for a diagnosis of hypertension after age 40 (Model B), the hazard for stroke remained significantly increased in women with a

history of hypertensive pregnancies relative to those with a history of normotensive pregnancies (HR = 1.86, 95% CI 1.16 – 2.98, *P*=.01). The hazard for CHD was no longer significantly increased (HR: 1.14, 95% CI 0.78 – 1.68, *P*=.50).

In the GENOA subset of FBPP participants (**Table 10**), in which we were able to also control

Table 10. Adjusted Hazard Ratios for Hypertension after Age 40, Coronary Heart Disease, and Stroke among Pregnancy Groups

	Group Contrasts	**Hypertension (after age 40) n=3,093[b]**			**Coronary Heart Disease n=4,720[b]**			**Stroke n=4,726[b]**		
FBPP Participants		HR [c]	95% CI	*P*	HR [d]	95% CI	*P*	HR [d]	95% CI	*P*
	Normotensive vs. Nulliparous	0.88	0.73 – 1.08	.22	1.02	0.60 – 1.75	.94	0.55	0.31 – 1.00	.050
	Hypertensive vs. Normotensive	1.55	1.26 – 1.89	<.001	1.14	0.78 – 1.68	.50	1.86	1.16 – 2.98	.010
	Group Contrasts	**Hypertension (after age 40) n=1,288[b]**			**Coronary Heart Disease n=1,741[b]**			**Stroke n=1,744[b]**		
GENOA Sub-sample		HR [e]	95% CI	*P*	HR [f]	95% CI	*P*	HR [b]	95% CI	*P*
	Normotensive vs. Nulliparous	0.78	0.59 – 1.04	.095	0.84	0.39 – 1.82	.67	0.61	0.27 – 1.40	.24
	Hypertensive vs. Normotensive	1.88	1.49 – 2.39	<.001	0.65	0.32 – 1.30	.22	2.10	1.19 – 3.71	.010

[a] Expressed as hazard ratios (HR), with 95% confidence intervals (CI) from Cox proportional hazards models fit using custom software to account for potential correlation among women within sibships.
[b] Due to missing data, sample sizes are smaller than the total number of 4,782 and 1,754 women in the Family Blood Pressure Program (FBPP) and The Genetic Epidemiology Network of Arteriopathy (GENOA) cohorts, respectively.
[c] Model A adjusted for network, race, and family history of cardiovascular disease (CVD), smoking, body mass index (BMI), education, and diabetes (time-dependent)
[d] Model B adjusted for network, race, family history of CVD, smoking, BMI, education, diabetes mellitus (time-dependent), and hypertension (time-dependent)
[e] Model C adjusted for race, family history of CVD, smoking, dyslipidemia, and diabetes (time-dependent)
[f] Model D adjusted for race, family history of CVD, smoking, dyslipidemia, diabetes mellitus (time-dependent), and hypertension (time-dependent)

for dyslipidemia (Model C), the HR for diagnosis of hypertension after age 40 years was also significantly increased in women with a history of hypertension in pregnancy relative to those with normotension in pregnancy (HR: 1.88, 95% CI 1.49 – 2.39, *P*<.001).

In Model D, which included hypertension and dyslipidemia, and thus controlled for all traditional risk factors, the HR for stroke (HR: 2.10, 95% CI 1.19 – 3.71, *P*=.010), but not for

CHD (HR 0.65, 95% CI 0.32 – 1.30, *P*=.22), was significantly increased in women with a history of hypertension in pregnancy relative to those with normotension in pregnancy.

5. DISCUSSION

Proteinuria is the hallmark of preeclampsia that differentiates it from other hypertensive disorders of pregnancy, despite the controversy surrounding its usefulness in diagnosing preeclampsia. Proteinuria is thought to be due to endothelial cell swelling and disruption of fenestrae. Our research over the last 10 years has focused on derangements of podocytes and podocyte-specific proteins (such as nephrin, synaptopodin, podocin, and podocalyxin), and their roles in the mechanism(s) of proteinuria in preeclampsia.

Our study, to the best of our knowledge, was the first to report decreased glomerular expressions of nephrin and synaptopodin in renal tissue sections from women who died from preeclampsia compared to those of women with normal pregnancies who died from other causes.[58] Podocin expression, however, was relatively unchanged. Studies of human tissue that followed confirmed that the expressions of podocyte-specific proteins are severely affected by preeclampsia. A study comparing renal sections from women with preeclampsia, compared to those from women with either normotensive or chronic hypertensive pregnancies, reported reduced expressions of podocyte-associated proteins, nephrin, glomerular epithelial protein 1, GLEPP-1, and ezrin in their renal tissue sections.[59] The degree of podocyte dysfunction required for such dramatic changes in nephrin and synaptopodin expressions might be expected to cause changes in multiple other proteins important to the integrity of the glomerular filtration barrier and, possibly, podocyte attachment.

The detection of podocyte products and live podocytes in the urine (podocyturia) suggests that podocyte pathology is more severe than might be inferred from renal biopsy studies. Various methods have been developed to detect urinary podocyte products.[60] We employed the culturing of urinary podocytes that increases specificity by removing dead and non-specific cells, but this technique, although extremely valuable for identification of viable, detached cells, is difficult and time consuming. Cytospin techniques, while rapid and possibly more amenable to automation, suffer from low sensitivity and specificity due to the large amount of cellular debris. More sensitive techniques using reverse transcriptase-polymerase chain reaction (RT-PCR) and mass spectrometry remain in development. Studies utilizing these methods are outlined below and summarized in **Table 11**.

Table 11. Studies of podocyturia and urinary podocyte markers in preeclampsia

Author and year	Study groups	Time point(s)	Sample preparation	Podocyte detection method	Results
Garovic *et al* (2007)[61]	15 PE 16 NL	<24 hours before delivery	Podocyte culture	IF for podocin, nephrin, podocalyxin, and synaptopodin	Podocin staining present in 15/15 PE and absent in 16/16 NL. Other three less sensitive and specific
Aita *et al* (2009)[62]	11 PE 45 NL	35 weeks 4 days post 1 month post	Cytospin	IF for podocalyxin	Podocyturia at 35 weeks and four days post - in PE. Almost no podocyturia at 1 month post – delivery. Nine of 45 controls showed podocyturia four days post – delivery. Correlation between podocyturia and BP, but not proteinuria.
Zhao *et al* (2011)[63]	16 severe PE 3 mild PE 7 NL 7 NP with nephrotic syndrome	3rd trimester	Podocyte culture	IF for nephrin	Podocyturia present in all cases of severe PE and nephrotic syndrome. Podocyturia absent in all NL and all three mild PE cases.
Jim *et al* (2012)[64]	29 PE 9 GHTN and HTN 9 NL	<24 hours before delivery	Cytospin	IF for synaptopodin	Podocyturia in 11 of 29 (38%) of PE, three of nine (33%) with HTN, and 0 of 9 NL Sensitivity = 38%, Specificity = 70%
Facca *et al* (2012)[65]	25 NL 14 PE	3rd trimester	Cytospin	IF for nephrin	Mean total number of podocytes 0.9 ± 1.6 for NL vs 9.3 ± 16.8 for PE (*P*=.212)
Kelder *et al* (2012)[66]	35 PE 5 GHTN 34 NL 12 NP	31 to 36 weeks gestation	Urine centrifugation TRIzol RNA isolation	RT-PCR for nephrin, podocin, VEGF	Elevated mRNA for nephrin, podocin, VEGF in PE compared to NL and NP. Positive correlation between nephrin and VEGF mRNA in PE (r=0.82, *P*<.0001)
Wang *et al* (2012)[67]	20 PE 6 HTN 8 NL	3rd trimester	ELISA of frozen urine supernatant	ELISA for nephrin, podocalyxin, Big-h3, and	Urinary nephrin, podocalyxin, and βig-h3 levels are increased in PE.

Author and year	Study groups	Time point(s)	Sample preparation	Podocyte detection method	Results
				VEGF	Urinary Big-h3 levels correlate with levels of nephrin and podocalyxin.
Chen *et al* (2013)[68]	14 PE 14 GHTN 13 NL	<1 week before delivery	Cytospin	IF for podocalyxin	Number of podocytes was higher in PE compared to GHTN (P<.05) and NL (P<.001)
Son *et al* (2013)[69]	43 Severe PE 30 NL	<24 hours before delivery	ELISA of frozen urine supernatant	ELISA for nephrin	Urine nephrin higher in severe PE than in NL. Urine nephrin correlated with proteinuria, diastolic BP, and renal dysfunction.
Craici *et al* (2013)[70]	15 PE 15 GHTN 44 NL	Late second trimester (median 27 weeks)	Podocyte culture	IF for podocin	Podocyturia present in 15/15 PE, absent in 15/15 GHTN, and absent in 44/44 NL. Podocyturia in second trimester was more sensitive and specific for later PE than any combination of angiogenic factors.
Garovic *et al* (2013)[71]	13 PE 6 PE/HELLP 4 NL	<24 hours before delivery	Trypsin digestion of the urinary sediment	LC-MS/MS	Podocin-specific tryptic peptide significantly higher in PE/HELLP compared to NL.

Abbreviations: BP, blood pressure; GHTN, gestational hypertension; HTN, hypertension; IF, immunofluorescence; NL, normal pregnancy; NP, not pregnant; PE, preeclampsia; VEGF, vascular endothelial growth factor; HELLP, hemolysis, elevated liver enzymes, low platelet count; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry.

Using staining for podocin to detect live podocytes, we have shown 100% sensitivity and specificity in diagnosing preeclampsia at the time of delivery.[49] Synaptopodin, nephrin, and podocalyxin were also useful markers for urinary podocytes, but lacked sensitivity and specificity compared to podocin. This is in agreement with autopsy studies showing that podocin expression is preserved in podocytes from preeclamptic patients compared with nephrin and synaptopodin.[58] Our study that followed indicated that podocyturia appears before the onset of proteinuria, and the number of podocytes positively correlates with the degree of proteinuria,

suggesting a cause-effect relationship between ongoing podocyte loss and the onset and severity of proteinuria,[70] i.e., that these are mechanistically related.

Several lines of evidence support the associations among dysregulated pro-angiogenic factors, hypertension, and podocyte injury. Most convincing is the observation that bevacizumab, an anti-VEGF antibody that decreases VEGF signaling in a manner similar to sFlt-1, causes hypertension and proteinuria in non-pregnant individuals. The renal findings of endotheliosis and thrombotic microangiopathy[72] in patients treated with bevacizumab are similar to those found in preeclampsia and its severe form, HELLP syndrome. Podocyturia is also seen in patients treated with bevacizumab, although less consistently than with preeclampsia.[73]

The usefulness of podocyturia for the early diagnosis of preeclampsia remains an active research topic. Other groups have confirmed that podocyturia is specific to the diagnosis of preeclampsia, using both podocalyxin and nephrin staining.[44,46] A recent study using synaptopodin staining of urinary cytospins questioned the usefulness of this technique, finding only 38% sensitivity and 70% specificity for diagnosing preeclampsia.[64] Previous research indicates, however, that synaptopodin is a marker of intact, well-differentiated podocytes, and that its expression may be altered in proteinuric diseases.[24,30] Urinary sediments obtained from cytospin techniques are also contaminated with non-specific cellular and non-cellular debris that may significantly affect the performance characteristics of the test.[74] Reduced sensitivity and specificity in this case may, therefore, be the result of technical aspects of podocyte detection rather than the actual presence or absence of podocyturia.

Urine and serum measurements of circulating angiogenic proteins using current techniques have not proven to be reliable screening tools for preeclampsia.[75] Compared to normotensive pregnancies, significant differences in increases in sFlt-1[76] and decreases in both serum[76] and urinary PlGF[77] were observed during the second and third trimesters in preeclamptic pregnancies, that were further complicated by either early onset preeclampsia or intrauterine growth retardation. A significant overlap in PlGF and sFlt-1 values was observed, however, between mild forms of preeclampsia and normotensive pregnancies, leading to both false positive and false negative screening test results. A systematic review of the published studies concluded that, short of prospective studies employing rigorous laboratory and study design criteria, the evidence is insufficient to recommend that these markers be used for screening.[75] Our comparative study of preeclamptic women versus normotensive pregnant controls for

angiogenic factors and podocyturia showed a greater positive predictive value for podocyturia than any of the measured angiogenic markers.[49]

While podocyturia demonstrates excellent performance characteristics in different clinical settings, its use in clinical practice is limited by the complexity of the methodology based on sediment culturing and staining. Emerging novel methods for detecting urinary podocytes and their products look promising for the early diagnosis of preeclampsia. Mass spectrometry for the detection of podocyte products offers a reproducible technique. [78] This method is operator-independent and may facilitate large-scale studies that determine the clinical utility of podocyturia in larger patient populations that are more broadly representative of pregnant women. Critical clinical questions that remain unanswered include, the ability of podocyturia to differentiate between preeclampsia, other complications of pregnancy (such as gestational diabetes mellitus), and other proteinuric diseases that either predate pregnancy or occur during pregnancy.

Recent data suggest that endothelin-1, one of the most powerful human vasoconstrictors, may act through the endothelin type A (ET_A) receptor to provide a bridge between placental ischemia and the clinical signs of preeclampsia, both hypertension and podocyte damage/proteinuria. Endothelin-1 may act both in an autocrine and paracrine manner; therefore, systemic levels do not necessarily reflect local tissue expression or effects. Endothelin-1 mediates hypertension in pregnant rats after infusion of either TNFα[79] or AT1-AA,[80] whereas antagonism of the ET_A receptor has resulted in blood pressure improvement in animal models of preeclampsia.[81-83] There is strong *in vitro* evidence, with respect to podocytes, to support the role of endothelin-1 in podocyte dysfunction and subsequent proteinuria. Preeclamptic sera are not directly toxic to cultured podocytes. Endothelial cells exposed to sera from preeclamptic women, however, produce compounds that alter nephrin expression and cause extracellular nephrin cleavage in cultured podocytes.[84] These effects can be replicated with purified endothelin-1 and are prevented by ET_A blockade. These findings suggest that preeclamptic sera induce proteinuria by affecting the glomerular capillary endothelium, and that endothelin-1 may cause podocyte dysfunction via the ET_A receptor. This is further supported by both *in vivo* and *in vitro* studies indicating that i) endogenous endothelin contributes to glomerulosclerosis and proteinuria, as these changes are reversible by endothelin-1 inhibition, and ii) podocyte apoptosis and structural

damage, induced by puromycin, an aminoglycoside, may be reduced by blocking endothelin receptors.[85]

Podocyturia has been shown to decrease with blood pressure control and modulation of the renin-angiotensin-aldosterone system, by either angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor antagonists in proteinuric disorders, such as IgA nephropathy,[86] and in animal models of progressive proteinuric disease.[87] Both ACE inhibitors and angiotensin II receptor antagonists are contraindicated in pregnancy. As podocyte detachment in preeclampsia may represent an end-event where different dysregulated pathways converge, future studies focusing on the mechanism(s) of podocyte injury and detachment may identify novel therapeutic targets. With respect to endothelin as a possible therapeutic target, animal studies demonstrate fetal malformations in both ET_A receptor knockout mice[88] and with ET_A receptor blockade.[89] There may be safe "windows" for the use of ET_A blockade in mid and late gestation, where prolonging pregnancy for even a few weeks might reduce fetal morbidity and mortality.[89] Additional research is needed to determine whether maternal ET_A blockade in late gestation might be safe and efficacious, especially in view of recent clinical studies showing a significant improvement in proteinuria in diabetic nephropathy, but raising safety concerns due to an increased risk for cardiovascular events.[90]

In addition to its potential diagnostic utility, podocyte damage and shedding, as a marker of glomerular vascular capillary damage, may signal small vessel injury in general, affecting both cardiovascular health and renal function for years following preeclamptic pregnancies. We have shown recently that women with a history of hypertensive pregnancy disorders havean increased future risk of albuminuria,[91] a known risk factor for both CVD, and CKD, including ESRD. It is unclear whether the association between preeclampsia and CVD/CKD/ESRD is independent of risk factors that may be common to these conditions (**Figure 17**). A single episode of podocyte injury, in a mouse model of selective podocyte depletion using diphtheria toxin,[92] resulted in glomerular destabilization and persistent podocyte loss. Podocyturia is seen in patients with focal segmental glomerular sclerosis (FSGS),[93] which, in turn, has been identified as a dominant histopathological lesion in the renal biopsies from women with persistent proteinuria after

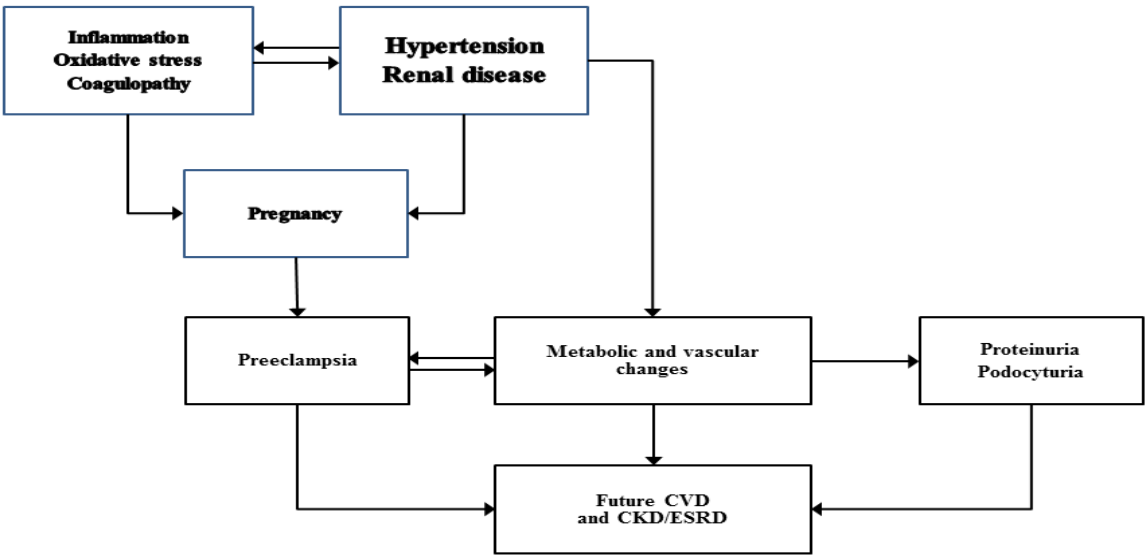


Figure 17. *Possible mechanisms of the association between preeclampsia and future cardiovascular and renal disease. Abbreviations:* CVD, cardiovascular disease; CKD, chronic kidney disease; ESRD, end-stage renal disease

preeclamptic pregnancies.[94] Our recent study additionally has shown that up to 30% of women with preeclampsia have ongoing podocyturia 5-8 weeks postpartum.[95] Taken together, these data raise a testable hypothesis that women with preeclamptic pregnancies and persistent proteinuria postpartum may have an underlying lesion of FSGS characterized by ongoing podocyte loss, which may contribute to their increased risks for proteinuria, CKD, and ESRD later in life. However, the cause-effect relationship between podocyturia and FSGS needs to be confirmed in appropriately designed longitudinal studies. With respect to future CVD, our study[96] unequivocally indicates that women with a history of hypertension in pregnancy, including preeclampsia, compared to those who were normotensive while pregnant, have an increased risk for developing hypertension after age 40, and develop it earlier in life. They were also more likely to have CHD, but adjusted risks were not significantly different, suggesting that the association between hypertension in pregnancy and CHD may be partially mediated by traditional risks factors, including a greater risk of hypertension. The increased risk for stroke in women who reported hypertension in pregnancy, in contrast, remained significantly elevated, even after controlling for traditional risk factors, including the greater risk of hypertension.

Our study has several limitations. The prevalence of hypertension in pregnancy among study subjects appeared to be higher than has been estimated for the general population, as high as 18% for GENOA participants versus 8%,[6] respectively. A possible reason may relate to our sampling scheme that preferentially recruited persons who either had hypertension or were at greater risk to become hypertensive (by virtue of higher blood pressure levels or positive family history of hypertension). Our analyses also were based on self-reported, physician-diagnosed hypertension in pregnancy and CVD events, and thus were subject to recall bias. Our results, however, not only extend previous reports by confirming this association in a large, multi-racial cohort, but provide new evidence for a positive association between hypertension in pregnancy and CVD later in life, after controlling for essentially all traditional risk factors, despite these limitations.

One possible mechanism underlying the association between hypertension in pregnancy and future CVD is that these two conditions share several common risk factors, including obesity, diabetes mellitus, and renal disease, which may lead to hypertension and hypertensive sequelae during the childbearing years, only when pregnancy is superimposed. Alternatively, hypertension in pregnancy may induce metabolic and vascular changes that may not resolve after pregnancy, thus increasing the risk for CVD later in life. Results from a study of neuroimaging abnormalities in women with eclamptic seizures may support this hypothesis.[97] One-fourth of the women studied (5 of 27), while clinically asymptomatic, had persistent imaging abnormalities 6 weeks postpartum, presumably caused by gliosis in response to infarction. Women who have had eclampsia additionally may experience impaired cognitive functioning, potentially due to permanent neurological damage.[98] The answer to the question as to whether or not hypertensive pregnancy disorders, and particularly preeclampsia, may cause CVD is dependent upon longitudinal studies of risk factors and CVD events before, during, and after hypertensive pregnancies. These may have significant influences, not only on the screening and primary prevention strategies for women, but also on the treatment of hypertensive pregnancy disorders. Pending results of such studies, we suggest that women who develop hypertension during pregnancy be informed of their increased risks for hypertension and other cardiovascular disease sequelae later in life; and that questions regarding not only pregnancy, but also hypertension in pregnancy become a routine part of their medical histories. Those women should have their blood pressures checked regularly, and be treated for modifiable risk factors.

With respect to future studies of the mechanisms of renal injury later in life, these may be limited by the fact that humans are the only species known to suffer from spontaneous preeclampsia. Although many animal models have been developed for the study of this disorder, renal endotheliosis, considered specific for preeclampsia, is absent in most of them. Two recently reported mouse models may prove useful. One is a matrix metalloproteinase-9 (MMP9) null mouse model: pregnant MMP9-null mice bearing null embryos exhibited clinical features of preeclampsia, including a reduced percentage of glomeruli with open capillaries (i.e., glomerular endotheliosis).[99]The second model used IL-$10^{-/-}$ mice injected with 100 µl of sera from preeclamptic women. They developed elevated BPs, proteinuria, intrauterine growth restriction, glomerular endotheliosis, increased levels of sFlt-1 and sEng, and spiral artery pathology.[100] These changes were absent when sera from normal pregnant women were injected, and they were specific to pregnancy, i.e., absent after injection of non-pregnant IL $10^{-/-}$ mice. These models closely mimic human disease, including the renal pathology, and may provide valuable insights into preeclampsia pathophysiology and treatment.

6. CONSLUSIONS

- Proteinuria in preeclampsia is associated with down-regulation of podocyte-specific proteins (nephrin, synaptopodin, podocin, and podocalyxin) in the renal tissue of the affected women.
- Urinary loss of viable podocytes, i.e., podocyturia is a mechanism that may contribute to proteinuria in preeclampsia.
- Podocyturia may serve as a highly sensitive and specific test for diagnosis of preeclampsia.
- Podocytes, in addition to the endothelium, present a critical focus for damage in the evolution of kidney injury in preeclampsia.
- Podocyte damage and shedding, as a marker glomerular vascular capillary damage, may signal small vessel injury in general, affecting both cardiovascular health and renal function for years following preeclamptic pregnancies.
- Women with a history of hypertension in pregnancy, including preeclampsia, compared to those who were normotensive while pregnant, have an increased risk for developing hypertension after age 40, and develop it earlier in life. The increased risk for stroke in women who reported hypertension in pregnancy remained significantly elevated, even after controlling for traditional risk factors, including the greater risk of hypertension.
- Preeclampsia is a hypertensive pregnancy disorder with both immediate and long-term adverse effects on women's health.

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CURRICULUM VITAE

Dr. Vesna Garovic graduated from the Medical School, University of Belgrade in 1980. Subsequently, she defended her Master Thesis in 1987 and completed her residency in Obstetrics and Gynecology in 1988. Upon moving to North America, she completed a Master Degree in Medical Genetics at McGill University, Montreal, Canada in 1992, training in the Internal Medicine in 1997,and subspecialty in Nephrology at Albert Einstein College of Medicine in New York City in 1999. Throughout her clinical training, she has had a continuos interest in research, particularly in the field of the pathophysiology of hypertensive pregnancy disorders, including preeclampsia.

Dr. Garovic joined Mayo Clinic, Rochester, MN in 1999 and started the Pregnancy-related Hypertension and Kidney Disease Clinic. Since 2005, she has been funded through highly competitive mechanisms of the National Institute of Health. Her interests in preeclampsia span several research areas: epidemiology, underlying molecular mechanisms, and epigenetics, with the long-term objective of identifying diagnostic biomarkers and potential new therapeutic targets in order to improve immediate and long-term outcomes of this enigmatic disease. Her basic research pioneered and evaluated glomerular epithelial cell (podocyte) injury in preeclampsia, and has led to a paradigm shift in that the podocyte is now regarded, in addition to the endothelium, as a critical focus for damage in the evolution of kidney injury in this disorder. Her research broadens the ramifications of hypertension in pregnancy well beyond reproductive age to future risks for renal and cardiovascular disease. To date, she published more than 100 peer-reviewed publications and is Professor of Internal Medicine since 2011.