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Authors

Laurent, Louise C
Saade, George R
Burchard, Julja
et al.

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Development and validation of a proteomic biomarker risk predictor for preterm preeclampsia in asymptomatic women

Louise C. Laurent, MD, PhD[1], George R. Saade, MD[2], Julja Burchard, MS[3], Angela C. Fox, MS[3], Ashoka D. Polpitiya, D Sc[3], Md. Bahadur Badsha, PhD[3], Tracey C. Fleischer, PhD[3], Thomas J. Garite, MD[3], J. Jay Boniface, PhD[3], Paul E. Kearney, PhD[3]

[1]Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Diego, La Jolla, CA, USA

[2]Department of Obstetrics and Gynecology, University of Texas Medical Branch, Galveston, TX, USA

[3]Sera Prognostics, Inc., Salt Lake City, UT, USA

***Corresponding author**

Paul E. KEARNEY, PhD

Sera Prognostics, Inc.

2749 East Parleys Way, Suite 200

Salt Lake City, UT 84109

(801) 990-6605

pkearney@seraprognostics.com

Short title

A proteomic biomarker risk predictor for preterm preeclampsia

Keywords

Preeclampsia, preterm preeclampsia, protein biomarker risk predictor, hypertension, diabetes mellitus

Abstract

Background: Clinical risk factors for preeclampsia (PE), including previous PE, chronic hypertension, and pregestational diabetes, are poorly predictive of PE. Preterm PE, defined as diagnosis of PE with delivery prior to 37 weeks' gestational age (GA), is more likely to be associated with serious morbidities and difficult clinical decision making. Therefore, there remains an urgent clinical need to develop a safe, feasible, and accurate predictor of preterm PE that integrates molecular biomarkers and relevant clinical factors into a single risk assessment score that can be used to guide clinical management.

Objective(s): To discover, verify, and validate a mid-trimester proteomic biomarker risk predictor for preterm PE, comprised of a composite clinical variable and a small number of maternal serum analytes.

Study Design: This was a secondary analysis of data from two large clinical trials (PAPR, NCT02787213; TREETOP, NCT01371019). PAPR subjects' eligibility was limited to those who had consented to research into preterm birth and pregnancy complications and who had blood drawn between $18^{0/7}$ – $22^{6/7}$ weeks' gestation. TREETOP subjects were limited to those who had blood drawn between $18^{0/7}$ – $20^{6/7}$weeks' gestation. PAPR subjects were assigned to a discovery cohort, and TREETOP subjects were randomly assigned to a first-phase cohort for verification (comprised of one-third of eligible subjects) and to a separate second-phase cohort for validation (comprised of the remaining two-thirds of eligible subjects). Peptides were analyzed by liquid chromatography-multiple reaction monitoring mass spectrometry measuring 77 pregnancy-related proteins and quality control proteins. Models were limited to a

maximum of one additional protein ratio and a composite clinical variable, referred to as Clin3, which was deemed positive if any of three factors was true for the subject: prior PE; pre-existing hypertension; and/or pregestational diabetes. Overall classifier performance was assessed via area under the receiver operating characteristic curve (AUC).

Results: Verification yielded nine multi-component classifier models for prediction of preterm PE, all of which were subsequently validated. Classifiers exhibited greater predictive performance than clinical factors alone. Example performance metrics across a range of classifier score thresholds and GA at birth cutoffs of 37, 34 and 32 weeks for the Clin3 + inhibin subunit beta c (INHBC)/SHBG classifier, which showed the highest AUC, demonstrating a sensitivity of 89% at a specificity of 75% for prediction of early-onset preeclampsia (<34 weeks' GA).

Conclusion(s): Here, we report on discovery, verification, and validation of models for prediction of preterm PE. The log ratio of INHBC/SHBG along with any one of three clinical risk factors demonstrated high sensitivity and specificity. This combination of protein biomarkers and clinical factors has the potential to be used in the mid-trimester of pregnancy to guide clinical management to avoid both unnecessary medical procedures and the most serious complications of early-onset PE.

Introduction

Preeclampsia (PE) is a common and clinically impactful complication of pregnancy, occurring in 4-7% of pregnancies[1,2] and implicated in 14% of maternal deaths[3] and 10-25% of perinatal deaths.[4,5] PE is also associated with significant increases in the risk of severe maternal morbidity, and accounts for approximately 25% of all preterm births (PTB) and 50% of medically indicated PTB.[6] The only curative treatment for PE is delivery, the timing of which involves balancing the maternal and perinatal risks of continuing the pregnancy versus delivery. Additional management decisions include administration of antihypertensive medications to prevent end-organ damage from severe hypertension, magnesium sulfate to prevent seizures (eclampsia), and antenatal steroids to decrease the risk of fetal complications from prematurity if appropriate. Because PE-associated perinatal morbidity and mortality, as well as healthcare costs, are strongly correlated with prematurity,[7-9] the GA of clinical PE onset is the most important factor in determining its clinical and economic impact. Therefore, accurate prediction of preterm PE, particularly early-onset PE at <34 weeks' GA, is of more clinical value than the prediction of term or late-onset PE.

Until recently, PE prediction has largely been based on clinical factors. Conditions that increase the risk of PE include a history of PE in a prior pregnancy; maternal comorbidities such as diabetes, chronic hypertension, renal disease, and autoimmune conditions (especially systemic lupus erythematosus and antiphospholipid antibody syndrome), and obstetrical characteristics such as multiple gestation and use of assisted reproductive technologies. An analysis of data from a Danish cohort of 597,492 pregnancies showed that using clinical predictors included in the National Institute of Health and Care Excellence (NICE) guidelines would have identified 47% of cases of PE at a screen-positive rate of 8.1%, while use of those included in the American College of Obstetricians and Gynecologists (ACOG) criteria would have identified 60.5% of PE cases at a screen-positive rate of 18.2%.[10] Hypertensive disease during a previous pregnancy is one of the most consistent risk factors in

these clinical criteria, which limits their utility in nulliparous patients. An important application of improved PE prediction would be identification of patients who would benefit from enhanced surveillance and/or prophylactic medical therapies for early diagnosis and prevention of complications of PE, respectively. Moreover, with a sufficiently high negative predictive value, a PE predictor could be used to identify low-risk patients who could safety avoid unnecessary surveillance and therapies.

ACOG has recommended daily low-dose (81 mg) aspirin (LDASA) starting between 12-28 weeks' GA (preferably before 16 weeks' GA) for pregnant women with chronic hypertension using a diagnostic threshold of BP >140/90 mm Hg,[11] while the widely-used U.S. Preventive Services Task Force (USPSTF) guideline recommends daily LDASA for those with ≥1 high-risk factors (defined as those associated with a ≥8% risk of PE) or ≥2 moderate-risk factors.[12] A recent analysis of 2019 birth certificate data from the U.S. National Center for Health Statistics showed that at least 50.4% of pregnancies were eligible for LDASA based on USPSTF recommendations.[13]

Protein, metabolite, and nucleic acid biomarkers associated with PE and FGR have been reported. Use of some biomarkers, including soluble fms-like tyrosine kinase 1 (sFlt-1) and placental growth factor (PlGF), in patients with clinically suspected PE has been shown to enable earlier diagnosis of PE and/or modestly decrease adverse maternal adverse outcomes.[14-17] However, no molecular biomarker has been demonstrated to adequately predict PE in pre-symptomatic pregnancies and enable pre-symptomatic clinical management changes that result in improved outcomes.

In obstetrics, multi-analyte assays have long been used for assessment of fetal aneuploidy and neural tube defect risk. These assays also consider some clinical factors, such as maternal age, diabetes and smoking status, and some integrate fetal ultrasound findings such as nuchal translucency. The complexity of such approaches can increase the post-test counseling burden and subjectivity and raise difficult management decisions, as patients and clinical providers struggle to weigh the relative importance of serum screening vs ultrasound findings. Strategies for PE prediction using various

combinations of patient history, serum analytes, blood pressure, and uterine artery pulsatility index have been developed. However, such methods are not widely used, as the most accurate approaches are difficult to implement, requiring high-end ultrasound equipment with highly trained sonographers in addition to serum analyte measurements.[18-21]

Until now, most published studies of biochemical – especially proteomic – predictors of PE have either focused on late-onset PE, included only cases conforming to classical definitions of PE, or combined PE at all gestational ages or severity into one group. In recent years, it has been increasingly recognized that PE is a highly heterogeneous condition,[22-25] and that at a minimum, cases should be divided into early- and late-onset disease.[7,26,27] From the perspectives of risk of related severe fetal and maternal complications and morbidities, and impact on clinical management, it is likely that prediction of early-onset PE (i.e., <34 weeks' GA) is most impactful compared with prediction of late-onset PE or all PE given that the latter is mostly late-onset also. Specifically, aspirin prophylaxis is far more effective in prevention of early-onset PE than late-onset PE. Moreover, severe maternal morbidity, fetal growth restriction and stillbirth, and neonatal morbidity and mortality from premature delivery, are more strongly correlated with early-onset PE.

Therefore, there remains an urgent clinical need to develop a safe, practical, and accurate predictor of preterm PE that integrates molecular biomarkers and relevant clinical factors into a single risk assessment score that can be used to guide clinical management. Here, we have developed and validated a predictor of preterm PE across demographically, geographically, and temporally diverse and representative populations,[28,29] integrating a mid-trimester maternal blood-based proteomic signature with established clinical risk factors. Based on the results of the current study, this predictor is poised for clinical studies that will evaluate its clinical utility and cost effectiveness across diverse practice settings.

Materials and Methods

Study design

The approach to biomarker development was based on U.S. National Academy of Medicine guidelines.[30] Data from two large cohort studies aimed at developing a serum biomarker for preterm delivery risk stratification were used in these secondary analyses to discover, verify and validate predictors of preterm PE, defined as diagnosis with delivery prior to 37 weeks' GA. The Proteomic Assessment of Preterm Risk (PAPR; NCT02787213)[28] and the Multicenter Assessment of a Spontaneous Preterm Birth Risk Predictor (TREETOP; NCT01371019)[29] trials were prospective, observational studies conducted at 11 and 18 sites across the United States, respectively. The PAPR study was conducted from April 2011 through December 2014, while the TREETOP study was conducted from August 2016 through June 2019. The original study protocols as well as these secondary analyses were approved by the sites' representative IRBs, and participating subjects provided voluntary, informed consent. Discovery, verification, and validation cohorts were independent samples of the intended use population and are summarized in **Figure 1**. Discovery was performed in a cohort derived from the PAPR study and was fully separate from the TREETOP-derived cohorts used for verification and validation. The discovery and validation cohorts were demographically, geographically, and temporally diverse and distinct from each other. The verification cohort included one site not used in validation, and the validation cohort included four study sites that were not present in the verification cohort. Additionally, the verification and validation cohorts were temporally offset (**Figure 1**). Overfitting was mitigated through redundant feature selection methods and model simplicity.

Participants

The PAPR and TREETOP studies enrolled women who on average had lower risk for preterm birth, 18 years of age and older with singleton pregnancies experiencing no symptoms of preterm labor or

membrane rupture. In TREETOP women with planned delivery before $37^{0/7}$ weeks' gestation, major anomalies or chromosomal disorders, planned cervical cerclage or progesterone use after $13^{6/7}$ weeks' gestation were excluded. In PAPR, women were enrolled from $17^{0/7}$ to $28^{6/7}$ weeks' gestation, with GA determined using the first day of last menstrual period confirmed by earliest ultrasound, or by using ultrasound alone. In TREETOP, women were enrolled from $17^{0/7}$ to $21^{6/7}$ weeks' gestation, with GA determined using ACOG guidelines in place at the time of the study.[31]

Body mass index (BMI) was derived from height and pre-pregnancy self-reported weight. Pertinent information regarding subject demographic characteristics, past medical and pregnancy history, current pregnancy history, and concurrent medications was collected and entered in an electronic case report form. Following delivery, data were collected for maternal and infant outcomes and complications. All deliveries were classified by the study sites as term (≥$37^{0/7}$ weeks' GA), spontaneous preterm (including preterm premature rupture of membranes), or medically indicated preterm births. Classification of preterm deliveries in the PAPR study was subsequently adjudicated by the chief medical officer at Sera Prognostics, Inc., who was blinded to results from the laboratory analysis. In TREETOP, preterm delivery classification was conducted by a blinded Endpoint Adjudication Committee. Discrepancies were clarified with the principal investigator at the study site. The adjudication occurred prior to locking down the validation database and conducting laboratory and statistical analysis. PE cases in TREETOP were required to meet ACOG 2013 hypertension guidelines[31] without adjudication.

Selection of cases and controls

All subjects from the PAPR and TREETOP studies who consented to research on preterm birth and pregnancy complications and who fulfilled blood draw requirements were included in the current study. Discovery was limited to PAPR samples with blood drawn in weeks $18^{0/7}$ – $22^{6/7}$ of gestation, and for

TREETOP samples drawn in weeks $18^{0/7}$ – $20^{6/7}$. Features of the classification model were assessed and selected in the discovery cohort, and model parameters were confirmed and tuned in the verification cohort to account for temporal changes in care and population. Cases were defined as subjects who had a PE diagnosis and delivery <37 weeks' GA, and controls were defined as all subjects who were not cases.

In a prespecified analysis, TREETOP subjects were randomly assigned by an independent third-party statistician to the first-phase cohort representing one-third of eligible subjects, here employed for verification, and a separate, second-phase cohort used for validation.[29] Fully prespecified selected classifiers, inclusive of risk thresholds, were validated on this blinded cohort by an independent third-party statistician. For prediction of PE with delivery at various GA cutoffs (e.g., <37, <34, <32 weeks), controls were defined as non-cases, and performance was reported without gapping.[32] For example, measurement of predictive performance for PE with delivery at less than 37 weeks, non-cases were those who delivered before 37 weeks without diagnosis of PE plus all patient who delivered at 37 weeks or later, including those with PE. This was done in order to ensure that the developed test would apply to the full intended-use population, and prevent inflating the predictive performance when gapping is allowed, as we have previously shown.[32] For each classifier, three risk thresholds were included for validation. These risk thresholds were predefined and selected based on performance observed in the discovery cohort to allow for trade-offs in sensitivity and specificity to allow the classifiers to be used in different clinical settings.

Sample processing

Maternal blood was collected and allowed to clot at room temperature for 10 minutes to 2 hours, followed by immediate refrigerated centrifugation or placement in an ice-water bath at 4°C until

centrifugation in PAPR or room temperature centrifugation in TREETOP. Serum was frozen on dry ice or at -80°C within 2.5 hours from collection, shipped on dry ice, and stored at -80°C until analyzed. Serum samples were assayed using a proteomic assay of pregnancy-related and placental proteins of relevance to PE pathways using a previously established and reported protocol.[28,33] Serum was depleted of the most abundant proteins using MARS14 (Agilent Technologies), reduced, alkylated, and digested with trypsin. Stable isotope standard (SIS) peptides were added post-digest. After desalting, peptides were analyzed by liquid chromatography-multiple reaction monitoring mass spectrometry measuring 77 biomarker and quality control proteins. Quantification was based on peptide response ratios, where the area of the endogenous peptide was divided by that of the SIS peptide.

Discovery and verification of risk predictors

Proteomic biomarkers measured by mass spectrometry that were selected for classifier model building were prefiltered based on criteria for analytical performance, including mass spectrometry peak area cutoffs, coefficients of variance from replicate quality control samples and preanalytical stability. Classifiers included as features no more than one novel two-protein ratio, one to three clinical factors as described below, and, optionally, the ratio of insulin-like growth factor-binding protein 4 (IBP4) to sex hormone-binding globulin (SHBG). Based on strong predictive performance in nulliparous women during the discovery phase, the log ratio of IBP4/SHBG was included in the classifier models unless SHBG was included in the other ratio. Discovery models were ranked by a combination of AUC and correlation with GA at birth amongst preeclamptic subjects, and further filtered for specificity at 75% sensitivity in all women, nulliparous women alone and multiparous women alone, and for significance of contribution of features to the model. Based on discovery performance, three clinical factors, including prior PE, pre-existing hypertension and/or pregestational diabetes mellitus, were combined into one variable (Clin3), which was deemed positive if any one of the factors was true for the subject. The selection of clinical

factors was based on PE risk assessment guidelines (ACOG[34], International Society for the Study of Hypertension in Pregnancy; NICE; Society of Obstetric Medicine of Australia and New Zealand;[35] Society of Obstetricians and Gynaecologists of Canada;[36] World Health Organization) and showed improved prediction in discovery over individual clinical factors. This fixed structure reduced overfitting, emphasized pregnancy biomarkers, and incorporated known clinical associations of PE. This strategy resulted in several potential classifiers which could be categorized into one of two models:

1. log(IBP4/SHBG) + Clin3 + log(X/Y)
2. Clin3 + log (X/SHBG)

where X and Y are protein analytes.

Data Analysis

Detailed analysis protocols, including study design, analysis plans, selected classifiers, hypothesis testing endpoints and a blinding protocol, were pre-specified. Laboratory and data analysis personnel were blinded to all clinical data, including case, control, and GA at birth in the validation cohort. Case, control, and GA data linked to the predictor scores were provided to an independent third-party statistician for analysis. Type I error was controlled by fixed sequence hypothesis testing.[37,38]

Classifiers' clinical validity was assessed for statistically significant stratification of preterm PE subjects above vs below preestablished score thresholds. Secondarily, overall prediction was by AUC and PE severity prediction was assessed using the correlation of classifier scores with gestational age at birth (GAB) amongst PE subjects. Following validation, additional clinically relevant performance metrics, including sensitivity and specificity, were assessed for select predictors, for outcomes such as PE with delivery <37, <34, and <32 weeks' GA.

All statistical tests were 2-tailed at significance of 0.05 and performed in R (3.5.1 or higher; Microsoft R Application Network[39]). Count differences in categorical variables were assessed using the

chi-squared test, and median differences in continuous variables were assessed with the Wilcoxon test.[40] Comparison among the discovery, verification and validation cohorts was performed using the Kruskal Wallis test. Overall classifier performance was assessed via area under the receiver operating characteristic curve (AUC) with direction of effect prespecified, significance assessed with a 1-sided Wilcoxon test, and confidence intervals (CI) calculated by the DeLong method.

Results

The PAPR study contributed 45 cases of preterm PE and 1307 controls (including 57 controls with term PE). The TREETOP study contributed 96 cases and 3540 controls (including 167 controls with term PE), which were randomly divided, with one-third assigned to the verification cohort, and two-thirds assigned to the validation cohort (**Figure 1**, **Table 1**). The demographic and clinical characteristics of preterm PE case and control subjects for the discovery, verification, and validation cohorts are summarized in **Table 1**. Subjects delivering with preterm PE in all three cohorts were more likely to have chronic hypertension, pregestational diabetes mellitus, prior PE, and deliver neonates with longer hospital stay (**Table 1**). Subjects with preterm PE in all three cohorts had significantly higher BMI (**Table 1**). Other demographic and clinical characteristics did not differ between the cases and controls.

The discovery, verification, and validation cohorts did not differ significantly in the proportions of two clinical risk factors for preterm PE; chronic hypertension and pregestational diabetes but differed in their proportions of prior PE, which was increased in controls in the validation cohort (**Table 2**). Several other characteristics were significantly different among these cohorts, including maternal race, ethnicity, parity and education level, with the discovery cohort having a higher percentage of parous individuals, a higher percentage of White and a lower percentage of Hispanic participants, and a higher percentage of “No high school graduation” and lower percentage of “College degree.” There were also small but statistically significant differences among the three cohorts for several other parameters,

including maternal age and BMI, and GA at birth, which were not likely to be of clinical consequence. The difference in gestational age at blood draw was expected, as the discovery cohort intentionally included a broader blood draw range to increase sample numbers. Maternal age and BMI were examined and determined not to contribute independently to risk of preterm PE. The verification and validation cohorts were not significantly different for any characteristic.

Nine predictors developed in the discovery cohort showed acceptable preterm PE predictive performance in the verification cohort, exhibiting significant stratification of cases above a probability score threshold corresponding to a sensitivity of 75% for preterm PE with GA at birth <37 weeks. Six of the nine predictors were of the form log(IBP4/SHBG) + Clin3 + log(X/Y), and three were of the form Clin3 + log(X/SHBG), where X and Y are protein biomarkers.

These nine predictors were independently tested for clinical validity in the validation cohort. The results of validation are shown in **Supplemental Table 1**. All nine predictors passed validation by exhibiting statistically significant stratification of preterm PE cases < 37 weeks GA at three prespecified thresholds, a significant Pearson correlation of predictor score with the gestational age at birth (GAB) amongst PE subjects and significant AUC. AUCs ranged from 0.72-0.78 for prediction of preterm PE <37 weeks' GA, and all were highly significant. In contrast, Clin3 alone exhibited an AUC of 0.68, which falls below the lower 95% CI of seven of the nine validated classifiers and is therefore statistically inferior to these biomarker-containing classifiers (**Supplemental Table 1**).

The receiver operating characteristic curve for the Clin3 + inhibin subunit beta c [INHBC]/SHBG) predictor is shown in **Figure 2**. As an example, its performance metrics for different clinically relevant testing scenarios are shown in **Table 3**. For prediction of preterm PE <37 weeks' GA, the sensitivity and specificity values were 76% and 75%, respectively. Lowering the GA cutoff to 34 weeks allowed for improved sensitivity (89%) but left specificity (75%) unchanged, while using a GA cutoff of 32 weeks enabled improved specificity with sensitivity remaining as high as 50%. Restricting the analysis to

nulliparas improved the performance of the classifier; at a 34-week cutoff, we observed excellent sensitivity (100%) and specificity (83%).

Discussion

Principal Findings

Here we report on discovery, verification, and validation of nine multi-component classifier models for prediction of preterm PE, each of which is comprised of one or two bivariate maternal serum protein biomarkers with a composite clinical variable.

Results

Initial discovery was performed on maternal serum samples on the large multicenter PAPR study, and verification and validation were performed on samples randomly assigned in a 1:2 ratio from the subsequent large multicenter TREETOP study. The higher representation of Black (17.8 – 19.0%) and Hispanic (37.3 – 41.2%) women in these respective cohorts, a reflection of study site patient population, compared with the U.S. population as a whole (13.6% Black and 18.9% Hispanic[41]) ensures that the developed preterm PE predictors adequately address racial and ethnic disparities. By requiring that the multi-component classifiers be simple (containing no more than two bivariate protein biomarkers and one composite clinical variable) and using large, diverse and divergent cohorts comprised of non-overlapping sets of pregnancies, we mitigated the effects of over-fitting, resulting in all nine classifiers validating in an independent population. Of the clinical features that were significantly different in cases and controls in our cohorts and could be ascertained in the mid trimester, three (chronic hypertension, pre-existing diabetes mellitus and prior PE) were components of the composite clinical variable. A fourth, BMI, was not included in the composite clinical variable because it was found in our discovery analyses not to independently contribute to the risk of PE. This may be due to the strong associations

between BMI and both diabetes and SHBG.[42,43] Emphasizing strong predictive performance in both nulliparous and multiparous pregnancies in the discovery process ensured broad applicability of the validated classifiers, inclusive of women who have not yet developed obstetric risk factors.

Elevated Inhibin A levels were initially reported to correlate with the presence of hypertension and PE[44,45] and were later shown to precede development of PE symptoms.[46] However, Inhibin A on its own or in combination with other serum biomarkers developed for aneuploidy and fetal anomaly screening demonstrates insufficient predictive performance to be clinically useful.[47,48] INHBC is a less well-characterized member of the inhibin family. After the work reported here was performed, INHBC was reported to act through the activin receptor-like kinase 7 (ALK7).[49] ALK7 is a member of the transforming growth factor beta (TGFβ) pathway that participates in angiogenesis, cytokine activity, pituitary and gonadal hormone signaling and embryonic development. Interestingly, ALK7 is expressed in villous and extravillous trophoblasts and is reported to be dysregulated in placentas from patients with PE[50] and involved in hypoxia-induced impairment of trophoblast invasion.[51] These observations, in retrospect, may explain the ability of INHBC to predict preterm PE reported here and may implicate it as an additional angiogenic factor involved in PE pathogenesis.

SHBG, which regulates the levels of biologically active free steroid hormones, increases 5- to 10-fold in maternal circulation during pregnancy.[52] Extrahepatic expression, including in placental trophoblasts, has been demonstrated.[53] SHBG abundance is inversely correlated with triglyceride levels, insulin resistance, and BMI, and its transcription is suppressed by pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNFα).[54] Inflammation has been associated with the development of PE, as have increased placental[55] and circulating levels of TNFα.[56,57] Dysregulation of maternal circulating SHBG in PE has been reported by some[58] and disputed by others,[59-61] and in cases where it has been described, its predictive performance is poor.[58] Discrepancies could be due to known pathophysiological differences in disease phenotypes (PE vs early-onset PE), or the gestational age of blood sampling. Low

levels of SHBG have been associated with insulin resistance and Type 2 diabetes,[62] as well as endothelial dysfunction[63] in non-pregnant adults. Interestingly, in a small study, gravidas with low levels of both SHBG and PLGF in the first trimesters had the highest risk of developing PE, leading the authors to conclude that dysregulated angiogenesis and insulin resistance had additive effects on the risk of PE. [64] The results in our study suggest that SHBG serves as an independent biomarker for PE due to potential roles in insulin resistance and endothelial dysfunction, both of which are associated with increased risks for PE, but clinically meaningful predictive performance occurs in concert with INHBC as an angiogenic factor.

Clinical Implications

These classifiers have the potential to enable clinical risk assessment at 18-20 weeks and to individualize surveillance, both for low-risk and high-risk patients. For those at high-risk, increased surveillance would be expected to detect the disease earlier and thus provide the opportunity to diagnose impending serious complications and avoid their sequelae. Since for a given pregnancy, we are able to provide separate assessments for the risk of delivery for preterm PE before 32, 34, and 37 weeks' GA, which differ between nulliparas and multiparas, our classifiers can be used to develop customized management plans even among the broader category of high-risk patients. As exemplified with the Clin3 + INHBC/SHBG classifier, we succeeded in identifying biomarkers capable of excellent stratification of subjects at elevated risk for early-onset PE (<34 weeks), an outcome with a large clinical burden and unmet need. Coincidentally, this is an important gestational age landmark, as ACOG recommends delivery for all above 34 weeks' GA and expectant management with close observation for those below this GA.[34] When newer pharmacologic or other effective interventions become developed, they might be applied earlier and more accurately to those destined to develop the disease and its complications. To determine whether the classifiers are clinically useful for decision-making regarding

LDASA prophylaxis, future studies are needed to establish the efficacy of LDASA in patients in which the USPSTF criteria and this classifier produce discordant risk assessments.

Research Implications

The validated classifiers in this study may also be useful for selection of potential of pregnancies at high risk for preterm PE for interventional studies for novel therapies for prophylaxis or early treatment of preterm PE. The protein biomarkers, or the biochemical pathways that they play roles in, may be potential therapeutic targets. It will also be of interest to determine whether preventive or therapeutic interventions alter the levels of these protein biomarkers in the maternal serum. Clinical application of a screening test is a two-step process. The initial step is to validate that the test is predictive of the outcome, which we have accomplished. The second step, finding clinical utility, requires additional research.

Strengths and Limitations

The strengths of this study include three large, diverse, and non-overlapping datasets that were used for discovery, verification, and validation. Moreover, the studies from which the discovery (PAPR) and verification/validation (TREETOP) cohorts were derived were temporally distinct. The classifiers developed in this study are comprised of a composite of three readily ascertained clinical factors, and measurement of two or four proteins, with the candidate pool of protein biomarkers being prefiltered for assay performance and stability with ambient temperature transport. These properties ensure that the classifiers are practical for clinical use, especially for those very early-onset PE cases that pose the highest opportunity to avoid or ameliorate major complications. Limitations of the validation approach are generalizability across populations. The size and diversity of the cohorts increases the likelihood that the validated classifiers will be generalizable, but all performance measurements are biased in that only

a sample of the intended-use population was tested, and samples will overrepresent some population characteristics while underrepresenting others.

Conclusions

In conclusion, we have discovered, verified, and validated multiple classifiers for mid-trimester prediction of preterm PE comprised of a composite of three easily ascertained clinical factors combined with measurements of two or four serum proteins. These classifiers show performance superior to that of clinical factors alone. The protein biomarkers identified in this study and their underlying molecular pathways may serve as potential drug targets. Interventional studies will be needed to determine whether these classifiers identify patients who will benefit from LDASA prophylaxis, or from therapies that will be developed in the future.

Conflict of interest

J.B, A.C.F., M.M.B., T.C.F., T.J.G., J.J.B., and P.E.K. are employees of Sera Prognostics, Inc., or were employees at the time of this work, and receive(d) salary and stock options. A.D.P. is a paid consultant to Sera Prognostics, Inc. L.C.L. and G.R.S have no conflict of interest to declare.

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Author contributions

Conceptualization, J.B., T.J.G., J.J.B., and P.E.K.; data curation, A.D.P., M.M.B, A.C.F., and T.C.F.; formal analysis, J.B., A.D.P., M.M.B, and P.E.K.; investigation, L.C.L., G.R.S. J.B., A.C.F., and T.C.F.; methodology, J.B., T.C.F., T.J.G., J.J.B., and P.E.K.; project administration, J.B., J.J.B., and P.E.K.; resources, L.C.L., and

G.R.S..; supervision, P.E.K.; validation, J.B., J.J.B., and P.E.K.; visualization, J.B.; writing—original draft, L.C.L., J.B., T.J.G., and P.E.K.; writing—review and editing, L.C.L., G.R.S., J.B., A.C.F., T.C.F., T.J.G., J.J.B., and P.E.K. All authors have read and agreed to the published version of the manuscript.

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Ethics statement

The ethics committees/IRBs of all institutions participating in the original published studies upon which this work is based gave ethical approval for this work. The following is a listing of PAPR (NCT01371019) IRBs: IRB Intermountain Healthcare; IRB for Human Research, Medical University of South Carolina; IRB The University of North Carolina at Chapel Hill; IRB Maricopa Integrated Health System; IRB Baystate Medical Center; IRB Oregon Health & Science University; IRB University of Texas Medical Branch; IRB Christiana Care; WCG IRB (previously Western IRB, used by The Ohio State University, San Diego Perinatal Center, and Regional Obstetrical Consultants). The following is a listing of TREETOP (NCT02787213) IRBs: IRB for Human Research, Medical University of South Carolina; IRB The University

of North Carolina at Chapel Hill; IRB Maricopa Integrated Health System; IRB Oregon Health & Science University; IRB University of Texas Medical Branch; IRB Boston Medical Center; IRB Ochsner Clinic Foundation; IRB University of California San Diego; Human Research Protections Program Northwestern University; IRB Indiana University; IRB Duke Medicine; IRB for Clinical Investigations, Greenville Health System; WCG IRB (previously Western IRB, used by Denver Health & Hospital Authority, University of Colorado-Denver, UC-Irvine, Thomas Jefferson University, Regional Obstetrical Consultants, and Baystate Medical Center).

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Table 1. Demographic and clinical characteristics of preterm preeclampsia (PE) cases and noncases in the discovery, verification, and validation cohorts.

	Discovery (PAPR)			Verification (TREETOP)			Validation (TREETOP)		
Cases and noncases, n (%)	**Preterm PE**	**No PE or PE ≥37 weeks' gestation**	***P* value**	**Preterm PE**	**No PE or PE ≥37 weeks' gestation**	***P* value**	**Preterm PE**	**No PE or PE ≥37 weeks' gestation**	***P* value**
Number of subjects	45	1307		34	1217		62	2227	
Maternal age			0.586			0.640			0.705
Median	26	27		28.5	30		30	29	
IQR	24-31	23-32		27-32	25-33		25-34	25-33	
Unknown (n)	0	0		0	0		0	0	
BMI			0.003			0.027			<0.001
Median	29.6	26.6		28.3	26.1		30.9	25.8	
IQR	26.17-38.0	22.7-32.4		25.7-32.9	22.6-31.0		25.9-35.5	22.3-31.1	
Unknown (n)	1	23		1	14		1	25	
Maternal race			0.114			0.838			0.579
Black	13 (28.9%)	224 (17.1%)		5 (14.7%)	233 (19.1%)		14 (22.6%)	393 (17.6%)	
White	29 (64.4%)	952 (72.8%)		21 (61.8%)	757 (62.2%)		34 (54.8%)	1411 (63.4%)	
Other	3 (6.7%)	131 (10.0%)		7 (20.6%)	205 (16.8%)		12 (19.4%)	370 (16.6%)	
Unknown (n)	0 (0.0%)	0 (0.0%)		1 (2.9%)	22 (1.8%)		2 (3.2%)	53 (2.4%)	
Maternal ethnicity			0.180			0.104			0.243
Hispanic	12 (26.7%)	492 (37.6%)		20 (58.8%)	495 (40.7%)		30 (48.4%)	895 (40.2%)	
Non-Hispanic	33 (73.3%)	815 (62.4%)		14 (41.2%)	720 (59.2%)		32 (51.6%)	1332 (59.8%)	
Unknown (n)	0 (0.0%)	0 (0.0%)		0 (0.0%)	2 (0.2%)		0 (0.0%)	0 (0.0%)	
Maternal education level			0.131			0.329			0.194
No high school graduation	17 (37.8%)	338 (25.9%)		4 (11.8%)	192 (15.8%)		10 (16.1%)	317 (14.2%)	
High school degree/GED	21 (46.7%)	635 (48.6%)		21 (61.8%)	558 (45.9%)		35 (56.5%)	1033 (46.4%)	
College degree	6 (13.3%)	323 (24.7%)		9 (26.5%)	463 (38.0%)		16 (25.8%)	861 (38.7%)	
Unknown (n)	1 (2.2%)	11 (0.8%)		0 (0.0%)	4 (0.3%)		1 (1.6%)	16 (0.7%)	
Parity			0.591			0.838			0.307
Nulliparous (*P*=0)	18 (40.0%)	457 (35.0%)		13 (38.2%)	505 (41.5%)		31 (50.0%)	950 (42.7%)	
Multiparous (*P*≥1)	27 (60.0%)	850 (65.0%)		21 (61.8%)	712 (58.5%)		31 (50.0%)	1277 (57.3%)	
Unknown	0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)	
Prior PE (preterm + term)			<0.001			<0.001			<0.001
No	13 (48.1%)	777 (91.4%)		11 (52.4%)	653 (91.7%)		18 (58.1%)	1102 (86.3%)	
Yes	14 (51.9%)	73 (8.6%)		10 (47.6%)	59 (8.3%)		13 (41.9%)	175 (13.7%)	
Unknown	0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)	
Chronic hypertension			<0.001			<0.001			<0.001
No	35 (77.8%)	1229 (94.0%)		26 (76.5%)	1154 (94.8%)		37 (59.7%)	2124 (95.4%)	
Yes	10 (22.2%)	78 (6.0%)		8 (23.5%)	63 (5.2%)		25 (40.3%)	103 (4.6%)	
Unknown (n)	0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)	
Pregestational diabetes mellitus			<0.001			0.001			<0.001

	No	31 (68.9%)	1250 (95.6%)		28 (82.4%)	1166 (95.8%)		47 (75.8%)	2118 (95.1%)	
	Yes	14 (31.1%)	57 (4.4%)		6 (17.6%)	51 (4.2%)		15 (24.2%)	109 (4.9%)	
	Unknown (n)	0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)	
Gestational age at blood draw (d)				0.233			0.854			0.600
	Median	137	140		137	136		136.5	137	
	IQR	133-143	132-148		134-140	132-141		130-141	133-141	
	Unknown (n)	0	0		0	0		0	0	
Gestational age at birth (d)				<0.001			<0.001			<0.001
	Median	248	274		243.5	275		240	275	
	IQR	236-254	267-279		224-253	269-281		226-252	270-280	
	Unknown (n)	0	0		0	0		0	0	
Neonatal length of hospital stay (d)				<0.001			<0.001			<0.001
	Median	5	3		13.5	2		11	2	
	IQR	3-13	2-3		6-28	2-3		4-28	2-3	
	Unknown (n)	0	12		0	6		2	7	
Neonatal gender				0.920			0.809			0.676
	Female	23 (51.1%)	643 (49.2%)		15 (44.1%)	603 (49.5%)		34 (54.8%)	1100 (49.4%)	
	Male	22 (48.9%)	664 (50.8%)		19 (55.9%)	613 (50.4%)		28 (45.2%)	1124 (50.5%)	
	Ambiguous	0 (0.0%)	0 (0.0%)		0 (0.0%)	1 (0.1%)		0 (0.0%)	3 (0.1%)	
	Unknown	0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)		0 (0.0%)	3 (0.1%)	

Categorical variables are shown with counts and percentages, and continuous variables are shown with medians and interquartile ranges.

Comparisons between cases (PE < 37 weeks' gestation) and noncases (no PE or PE ≥37 weeks' gestation) were performed using Wilcoxon or chi squared tests as appropriate. Collection of neonatal hospital stay was capped at 28 days according to the PAPR[28] and TREETOP[29] study protocols.

BMI, body mass index; IQR, interquartile range; GED, general education diploma; NA, not applicable; PE, preeclampsia; PTB, preterm birth.

Table 2. Comparison of demographic and clinical characteristics across the discovery, verification, and validation cohorts.

		Discovery (PAPR)	Verification (TREETOP)	Validation (TREETOP)	*P* value
Number of subjects		1352	1251	2289	
Maternal age					<0.001
	Median	27	30	29	
	IQR	23-32	25-33	25-33	
	Unknown (n)	0	0	0	
BMI					0.005
	Median	26.6	26.3	26.0	
	IQR	22.7-32.6	22.6-31.0	22.3-31.2	
	Unknown (n)	24	15	26	
Maternal race					<0.001
	Black	237 (17.5%)	238 (19.0%)	407 (17.8%)	
	White	981 (72.6%)	778 (62.2%)	1445 (63.1%)	
	Other	134 (9.9%)	212 (16.9%)	382 (16.7%)	
	Unknown (n)	0 (0.0%)	23 (1.8%)	55 (2.4%)	
Maternal ethnicity					0.028
	Hispanic	504 (37.3%)	515 (41.2%)	925 (40.4%)	
	Non-Hispanic	848 (62.7%)	734 (58.7%)	1364 (59.6%)	
	Unknown (n)	0 (0.0%)	2 (0.2 %)	0 (0.0%)	
Maternal education level					<0.001
	No high school graduation	355 (26.3%)	196 (15.7%)	327 (14.3%)	
	High school degree/GED	656 (48.5%)	579 (46.3%)	1068 (46.7%)	
	College degree	329 (24.3%)	472 (37.7%)	877 (38.3%)	
	Unknown (n)	12 (0.9%)	4 (0.3%)	17 (0.7%)	
Parity					<0.001
	Nulliparous (*P*=0)	475 (35.1%)	518 (41.4%)	981 (42.9%	
	Multiparous (*P*≥1)	877 (64.9%)	733 (58.6%)	1308 (57.1%)	
	Unknown	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Prior PE (preterm + term)					<0.001
	No	790 (90.1%)	664 (90.6%)	1120 (85.6%)	
	Yes	87 (9.9%)	69 (9.4%)	188 (14.4%)	
	Unknown	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Chronic hypertension					0.495
	No	1264 (93.5%)	1180 (94.3%)	2161 (94.4%))	
	Yes	88 (6.5%)	71 (5.7%)	128 (5.6%)	
	Unknown (n)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Pregestational diabetes mellitus					0.531
	No	1281 (94.7%)	1194 (95.4%)	2165 (94.6%)	
	Yes	71 (5.3%)	57 (4.6%)	124 (5.4%)	
	Unknown (n)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Gestational age at blood draw (d)					<0.001
	Median	140	136	137	
	IQR	132-147	132-141	133-141	
	Unknown (n)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Gestational age at birth (d)					<0.001
	Median	273	275	274	
	IQR	266-278	268-281	269-280	
	Unknown (n)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Neonatal length of hospital stay (d)					<0.001
	Median	3	2	2	
	IQR	2-3	2-3	2-3	
	Unknown (n)	12	6	9	
Neonatal gender					0.384
	Female	666 (49.3%)	618 (49.4%)	1134 (49.5%)	
	Male	686 (50.7%)	632 (50.5%)	1152 (50.3%)	
	Ambiguous	0 (0.0%)	1 (0.1%)	0 (0.0%)	
	Unknown	0 (0.0%)	0 (0.0 %)	3 (0.1%)	

Categorical variables are shown with counts and percentages, and continuous variables are shown with medians and interquartile ranges. Comparison among the three cohorts was performed using the Kruskal Wallis test. Collection of neonatal hospital stay was capped at 28 days according to the PAPR[28] and TREETOP[29] study protocols.

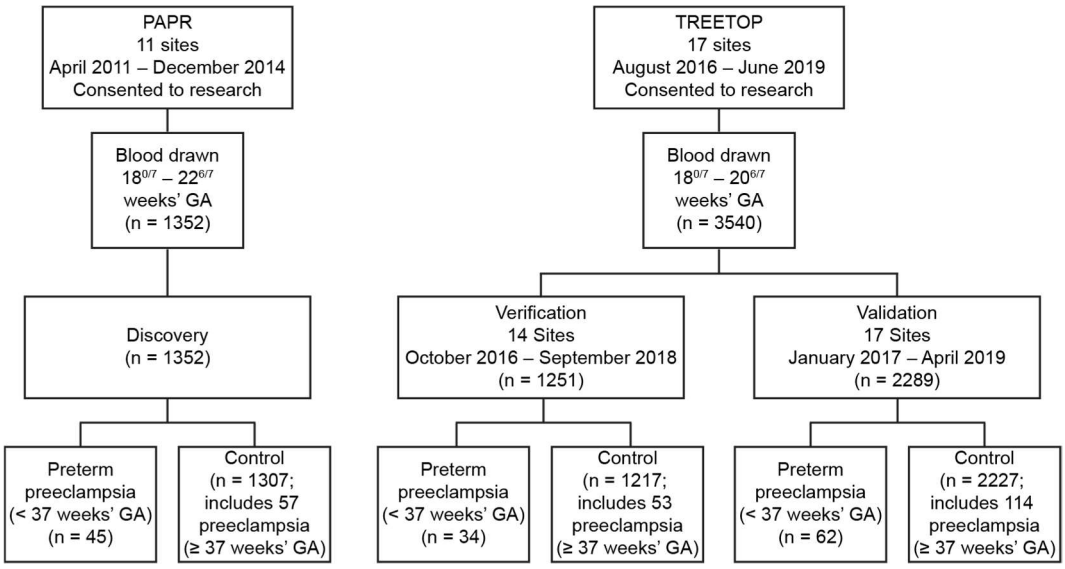
BMI, body mass index; IQR, interquartile range; GED, general education diploma; NA, not applicable; PE, preeclampsia; PTB, preterm birth.

Table 3. Performance of the Clin3 + INHBC/SHBG classifier model for prediction of PE by GA cutoff, risk score threshold, and parity in the validation cohort.

Testing scenario	Tested population	Risk score threshold	Preterm preeclampsia GA (weeks)	Sensitivity	Specificity
1	All women	.075	<37	76%	75%
2	All women	.075	<34	89%	75%
3	All women	.16	<32	75%	88%
4	All women	.32	<32	50%	99%
5	Restricted to nulliparous women	.05	<37	90%	59%
6	Restricted to nulliparous women	.10	<37	53%	92%
7	Restricted to nulliparous women	.075	<34	100%	83%

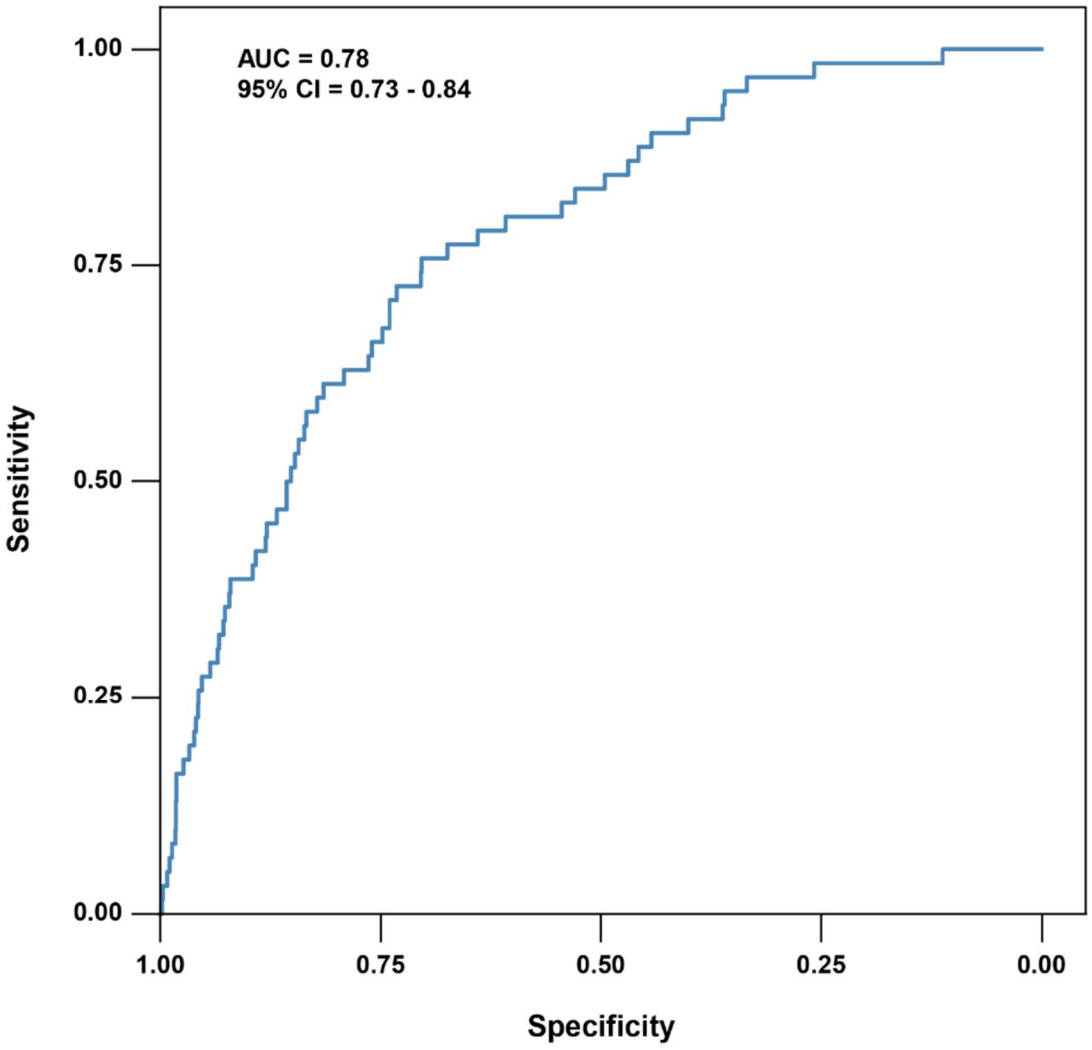
GA, gestational age.

Figure 1. Distribution of subjects in the discovery, verification, and validation cohorts.



GA, gestational age.

Figure 2. Receiver operating characteristic performance for the preterm preeclampsia (PE) classifier Clin3 + INHBC/SHBG in the validation cohort. The plot graphs sensitivity (true-positive rate) vs specificity (true-negative rate), where preterm PE cases (n=62) are defined as PE <37 weeks' gestational age.



AUC, area under the receiver operating characteristic curve; CI, confidence interval.