OBSTETRICS

Circulating microparticle proteins obtained in the late first trimester predict spontaneous preterm birth at less than 35 weeks' gestation: a panel validation with specific characterization by parity

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BACKGROUND: We have previously shown that protein biomarkers associated with circulating microparticles proteins (CMPs) obtained at the end of the first trimester may detect physiologic changes in maternal—fetal interaction such that the risk of spontaneous preterm delivery ≤35 weeks can be stratified.

OBJECTIVES: We present here a study extension and validation of the CMP protein multiplex concept using a larger sample set from a multicenter population that allows for model derivation in a training set and characterization in a separate testing set.

MATERIALS AND METHODS: Ethylenediaminetetraacetic acid (EDTA) plasma was obtained from 3 established biobanks (Seattle, Boston, and Pittsburgh). Samples were from patients at a median of 10—12 weeks' gestation, and the CMPs were isolated via size-exclusion chromatography followed by protein identification via targeted protein analysis using liquid chromatography—multiple reaction monitoring-mass (LC-MRM) spectrometry. A total of 87 women delivered at ≤35 weeks, and 174 women who delivered at term were matched by maternal age (±2 years) and gestational age at sample draw (±2 weeks). From our prior work, the CMP protein multiplex comprising F13A, FBLN1, IC1, ITIH2, and LCAT was selected for validation.

RESULTS: For delivery at ≤35 weeks, the receiver operating characteristic (ROC) curve for a panel of CMP proteins (F13A, FBLN1, IC1, ITIH2, and LCAT) revealed an associated area under the ROC curve (AUC) of 0.74 (95% CI, 0.63—0.81). A separate panel of markers (IC1, LCAT, TRFE, and ITIH4), which stratified risk among mothers with a parity of 0, showed an AUC of 0.77 (95% CI, 0.61—0.90).

CONCLUSION: We have identified a set of CMP proteins that provide, at 10—12 weeks gestation, a clinically useful AUC in an independent test population. Furthermore, we determined that parity is pertinent to the diagnostic testing performance of the biomarkers for risk stratification.

Key words: exosome, microparticle, parity, proteomics, spontaneous preterm birth

Classically, the great obstetric diseases preterm labor (PTL), preterm premature rupture of membranes (pPROM), and preeclampsia (PE) have been categorized based on their clinical presentation. The presentation of PE is distinct from, and would never be clinically confused with, that of PTL, although the presentations of pPROM and PTL are more similar as examples of spontaneous preterm birth (sPTB).[1] However, reproductive scientists have gradually come to appreciate that these great diseases of obstetrics, in many cases, share common pathogenic mechanisms rooted in aberrant placentation at the end of the first trimester.[2] Typically, cytotrophoblasts invade a receptive decidua and go on to physiologically transform the small diameter spiral arteries into larger-caliber and lower-resistance perfusion conduits.[3] In cases of sPTB, up to 30% of placental specimens will exhibit evidence of hypoperfusion and failed vascular remodeling.[4] Although this aberration is considered canonical in the setting of PE, it suggests that sPTB may also have its antecedents at the end of the first trimester. Considering the growing appreciation of the unique receptive and permissive nature of the human decidua in early pregnancy,[5] it is likely that an exploration of tissue interactions within the maternal system at this early and preclinical gestational age could be rewarding.

Circulating microparticles (CMPs) are made up of nanosized extracellular vesicles comprising lipid bilayers, proteins, nucleic acids, and metabolites. The vesicles include nanosized particles of cellular origin imbued with specific intercellular communication functions.[6] The circulating vesicles can be divided, largely based on size, into 3 primary subcategories; exosomes (50—150 nm), microvesicles (100 nm—1μm), and apoptotic bodies (200 nm—5 μm).[7] CMPs contain important mediators of biological function including nucleic acids, lipids, and proteins.[8] Although originally considered cellular detritus, or a means of biomolecule waste management, some CMPs have since been more fully understood to represent a fundamental means of intercellular communication and a powerful, minimally invasive means to "biopsy" the state of cells and tissues at the physiological or pathological moments in time of CMP secretion.[8,9] Across studied species, the high degree of evolutional conservation regarding CMP function and expression attests to their significant role in biology.[10,11]

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AJOG at a Glance

Why was this study performed?

Our objective was to perform a larger multicenter study to validate previous findings that a multiplex set of circulating microparticles (CMPs) obtained at the end of the first trimester could differentiate spontaneous preterm birth (sPTB) occurring ≤35 weeks' gestation from term deliveries.

Key findings

We identified a 5-plex combination of CMP protein analytes (F13A, FBLN1, IC1, ITIH2, and LCAT) in a training set that provide an area under the curve (AUC) of 0.74 (95% confidence interval [CI], 0.63−0.81) in a separate testing set that delineates sPTB.

What does this add to what is known?

In addition to confirming our previous findings, data from this study provides evidence that CMP protein analytes may predict sPTB before the end of 35 weeks' gestation among nullipara more robustly, with an observed AUC of 0.77 (95% CI, 0.61−0.90).

We have previously observed changes in the CMP-associated proteins at the end of the first trimester among women who deliver prior to 35 weeks gestation.[12] We identified proteins from pathways with multiple, relevant biological functions including fibrinolysis, coagulation, immune modulation, and complement activity. All such functions could reasonably be conjectured to play important roles in early uteroplacental accommodation and development. We further identified a subset of these proteins as clinically predictive markers of sPTB. Using a bootstrapping algorithm with a fixed sensitivity of 80%, we modeled and cross-validated a specificity of 83% and an area under the curve (AUC) of 0.89. We did not, however, have a sufficient sample size to partition data into training and testing sets or to examine patterns of CMP-associated proteins within specific subpopulations. Specifically, the subpopulation of greatest interest to us is that of nulliparas. This group of patients represents a management challenge, as they lack standard clinical risk factors for sPTB, and the development of putative markers would represent an improvement in care. The present analysis uses samples obtained from 3 separate populations to address these limitations of our prior work.

Materials and Methods

Clinical Specimen Collection

Maternal ethylenediaminetetraacetic acid (EDTA) plasma samples (median 10.2 weeks' gestation) were obtained from Brigham and Women's Hospital (BWH), Boston MA; the Magee-Women's Research Institute, Pittsburgh PA; and the Global Alliance to Prevent Prematurity and Stillbirth (GAPPS), Seattle WA. Eligibility criteria included patients who were ≥18 years of age, initiated their prenatal care at ≤15 weeks' gestation, and planned on delivering at the respective institutions. Exclusion criteria included preexisting medical disorders (preexisting diabetes, current cancer diagnosis, HIV, and hepatitis), and fetal anomalies. The analysis was restricted to singleton gestations. Maternal race was determined by self-identification. Gestational age of pregnancy was confirmed by ultrasound scanning at ≤12 weeks' gestation. If consistent with last menstrual period (LMP) dating, the LMP was used to determine the due date; if not consistent, then the due date was set by the earliest available ultrasound at ≤12 weeks' gestation. Full-term birth was defined as birth at ≥37 weeks' gestation, and preterm birth, for the purposes of this investigation, was defined as sPTB at ≤35 weeks. The lower limit of the gestational age considered for this analysis was set at 22 weeks. We chose to concentrate on pregnancies ending at ≤35 weeks' gestation for 2 reasons: first, the phenotype of sPTB is more homogeneous in this gestational age range and therefore is more likely to be associated with a more uniform set of antecedent pathological processes; and second, the burden of neonatal morbidity is higher in this gestational interval and it therefore represents a higher-yield target for future prevention.

Patient cases at each center were independently reviewed and validated by physician investigators from the respective centers. The 68 cases of sPTB from Boston, the 9 cases from Magee, and the 10 cases from GAPPS were each randomly matched to 2 control term from the same center. At each center, cases were matched by maternal age (±2 years) and gestational age of sampling (±2 weeks). Our final sample size consisted of 87 cases and 174 controls, which included a new collection of 62 cases and 124 controls as well as 25 cases and 50 controls from our prior analysis[12] For the current study, freshly aliquoted plasma of samples from our previous work were reanalyzed together with the newly acquired samples under a uniform assay protocol to ensure consistence and to minimize potential batch effects. The study protocol was approved by the institutional review board at each institution, and written informed consent was obtained from all participating women.

CMP Enrichment

Plasma samples from Magee and GAPPS were shipped on dry ice to BWH and then randomly arranged by laboratory personnel blinded to the case/control status. All 261 samples were then shipped on dry ice to the David H. Murdock Research Institute (DHMRI, Kannapolis, NC) where CMPs were enriched by size exclusion chromatography (SEC) and isocratically eluted using the NeXosome Elution Reagent. Briefly, PD-10 columns (GE Healthcare Life Sciences, Pittsburgh, PA) were packed with 10 mL of Sepharose 2B Agarose Bead Standard (from a 2% stock solution) purchased from GE Healthcare Bio-Sciences

Corporation (Marlborough, MA). Columns were washed with Elution Reagent and stored at 4C° for a minimum of 24 hours and no longer than 3 days prior to use. On the day of use, the columns were again washed with Elution Reagent, and 1 mL of thawed plasma sample was applied to the column. The CMPs were captured in the column void volume and resolved from the high abundant protein peak.[13] To minimize variability between processing, the handling of individual samples was carried out in random batches. An aliquot of the pooled CMP column fraction from each clinical specimen, containing 200 μg of total protein (determined by the bicinchoninic acid protein assay reactions), was transferred to a 2-mL microcentrifuge tube (VWR, Radnor, PA) and shipped on dry ice to Biognosys (Zurich, Switzerland) for proteomic analysis.

Liquid Chromatography–Mass Spectrometry

Quantitative proteomic liquid chromatography–mass spectrometry (LC-MS) analysis was performed by Biognosys AG. Briefly, for each sample, a total of 20 μg of protein was lyophilized and then denatured with 8 M of urea, reduced using dithiothreitol, alkylated with the Biognosys alkylation solution, and digested overnight with trypsin (Promega, Madison, WI) as previously described.[13] The resulting sample peptides were dried using a SpeedVac system and re-dissolved in 45 μL of the Biognosys LC solvent and mixed and then with the Biognosys PlasmaDive (extended version 2.0) stable isotope-labeled reference peptide mix–containing Biognosys iRT kit.

Next, 1 μg of total protein was injected into an in-house packed C18 column (75-μm inner diameter and 10-cm column length; New Objective, Woburn, MA); the column material was Magic AQ, 3-μm particle size, with a 200-Å pore size (Michrom, Auburn, CA). This column was used in a Thermo Scientific (Waltham, MA) Easy nLC nano-liquid chromatography system. LC–multiple reaction monitoring (MRM) assays were measured on a Thermo Scientific TSQ Vantage, triple quadrupole mass spectrometer equipped with a standard nano-electrospray source. The LC gradient for running the LC-MRM was a 5–35% gradient of solvent B (97% acetonitrile in water with 0.1% formic acid), over 30 minutes, followed by 35-100% gradient of solvent B over 2 minutes and then 100% of solvent B for 8 minutes (the total gradient length, 40 minutes).

For quantification of the peptides across samples, the TSQ Vantage was operated in a scheduled MRM mode with an acquisition window length of 3.25 minutes. The LC eluent was electrosprayed at 1.9 kV, and the Q1 quadrupole was operated at unit resolution (0.7 Da). Signal processing and data analysis were carried out using SpectroDive, Biognosys' proprietary software, for multiplexed MRM data analysis. A Q-value filter of 1% was applied. Protein concentration was determined based on the normalized 1 μg of protein injected into the LC-MS/MS instrument.

TABLE 1
Baseline characteristics of sPTB vs term control pregnancies

Characteristic	sPTB (n = 87) n (%) or Mean (SD)	Controls (n = 174) n (%) or Mean (SD)	*P* value[a]
Center			.98
BWH	68 (78.2)	136 (78.2)	
Magee	9 (10.3)	18 (10.3)	
GAPPS	10 (11.5)	20 (11.5)	
Maternal age, y	31.2 (6.2)	30.7 (5.6)	.66
Race			.82
African American	20 (23.0)	38 (21.8)	
Not African American	67 (77.0)	136 (78.2)	
Maternal BMI (kg/m^2)	28.7 (7.7)	27.5 (7.2)	.18
Private insurance[b]	46 (67.7)	97 (67.8)	.54
Maternal smoking during pregnancy	9 (10.3)	18 (10.3)	.99
Parity	1.1 (1.3)	1.0 (1.2)	.90
Gestational age at sample collection	10.9 (2.7)	10.9 (2.5)	.99
Gestational age at delivery	31.7 (3.3)	39.4 (1.0)	<.0001
Male fetal sex	42 (48.8)	86 (49.1)	.96

BMI, body mass index; *BWH*, Brigham and Women's Hospital; *GAPPS*, Global Alliance to Prevent Prematurity and Stillbirth; *sPTb*, spontaneous preterm birth.

[a] *P* values calculated with Wilcoxon rank sum test, χ^2 test, Fisher exact test, or analysis of variance where appropriate; [b] n = 59 missing.

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Statistical Analysis

Prior to statistical analysis, the protein quantitation data from the LC-MS/MS MRM assays were normalized into *z* scores. The data were then split into a training set and a testing set. The training set consisted of all samples that were involved in our prior analysis[12] as well as 60 samples from the new collection selected through block randomization. The remaining new collection samples were used as the testing set. The use of block randomization preserved the case:control ratio in the training and testing sets. The test set was then set aside until step 3 (below) of the analysis.

Univariate Analysis (Step 1)

Within the training set, the candidate set of protein analytes were first subjected to univariate selection for their ability to differentiate sPTB from term deliveries. Briefly, for each protein, receiver operating characteristic (ROC) analysis was repeatedly performed 10 times on

bootstrapped samples with replacement of the training data. The mean and standard deviation (SD) of the areas under the curves (AUCs) from bootstrap ROC analysis were used as measures of the level and statistical stability of the performance, respectively, to rank the putative analytes for their ability to distinguish sPTBs from term deliveries.[14,15] To establish an objective selection criterion for the analytes and to minimize false discoveries due to random chances, the exact same bootstrap ROC analysis procedure was applied to the training data set again with the sample labels (ie, sPTB vs control) permuted and randomly shuffled. This permutation analysis procedure functionally models the effect of random chance and serves as a "negative control" in selecting candidate protein markers. Using the same cutoffs on the mean AUCs and SDs, the relative ratio of the number of analytes selected from permutation analysis over that from "real label" analysis allowed us to estimate the false discovery rate while controlling for the effect of multiple comparisons.[16]

Multivariate Analysis (Step 2)

The top performing candidate analytes (ie, with highest mean AUCs and relatively low SDs) from the univariate analysis were then assessed for their complementary values as part of multivariate panels for the prediction of sPTB risk within the training set. To do this, we evaluated all possible combinations of 5-analyte panels using a multivariate classification model with 10 times repeated within-training set cross-validation (each time the model was derived using randomly selected 60% training samples and evaluated on the remaining 40% training samples). Each panel is assessed by 3 performance metrics: mean AUC, mean sensitivity at a fixed 70% specificity, and mean specificity at a fixed 70% sensitivity, all from within-training cross-validation. We then computed the frequencies of individual analytes being a member of the top performing 1% panels of each of the 3 performance metrics. These estimated frequencies served as measures of the ability of the protein analytes to complement one another with regard to differentiating sPTBs from term deliveries and as objective criteria to further reduce the number of candidate biomarkers. The choice of evaluating only 5-analyte panels exhaustively and the use of a particular conservative multivariate model type was based on both our previous experience of a minimally sufficient number of biomarkers to reveal multivariate relationships in analytes for sPTB risk[12] and our desire not to over-fit the data, as well as the practical constraints of computational complexity. Specifically, the conservative model structure is a support-vector machine (SVM) with radial-basis function kernel. The radius was chosen to be twice the SDs of the analytes. The resulting SVM was therefore heavily constrained and behaved similarly to an SVM with linear kernel.

With the number of candidate analytes and their associated panels significantly reduced, we were then able to use computationally sophisticated approaches to fine-tune the parameters of the machine-learning algorithms, and to afford much more extensive within-training data resampling/cross-validation to finalize and select the top-performing marker panel and associated multivariate predictive model.

Evaluation in the Testing Set (Step 3)

In the third portion of this analysis, the top-performing model was evaluated on the data from the testing set and reported in terms of AUC with associated estimated confidence intervals, sensitivity, and specificity.

Evaluation of Parity 0 Subset

To evaluate the utility of these analytes in the parity 0 population, the training and testing sets were restricted to primipara mothers. The procedures described above were reapplied. Given the sample size restrictions imposed by this stratification, we targeted a 4-analyte panel. As noted, this was to reduce the risk of overfitting the data. In addition to ROC analysis, the 4-analyte model output was used to divide the subjects into high- and low-risk groups. The 2 groups were compared using Kaplan–Meier curve by week of gestation. Since the test set represents a case-control sample set, the purpose of the comparison was to graphically demonstrate the noticeable differences in, rather than the actual shape of, the individual Kaplan–Meier curves.

Statistical and model development calculations were carried out in the R 3.2.4 statistical computational environment[17] and using Matlab R2017b (Mathworks, Natick, MA).

Results

The clinical and demographic characteristic of the cases and controls in the entire multicenter cohort are presented in Table 1. Their baseline continuous variables of maternal age, parity, and prepregnancy body mass index (BMI) had similar means. Maternal categorical variables of race, insurance type, smoking, and fetal sex did not differ between cases and controls. Given the design, there were expected differences between gestational age at delivery ($P < .0001$) and birthweight ($P < .0001$). Importantly, there were no differences between the mean gestational age at sample collection time between the cases or controls.

As noted, the total sample set of 261 was split randomly into training and testing sets. A total of 45 cases of sPTB and 90 term controls comprised the training set, and the remaining 42 cases of sPTB and 84 term controls made up the testing set. The characteristics of the new training and testing sets are compared in Table 2.

An initial inclusion of 36 protein analytes was based on discriminatory performance in our prior analysis.[12] Within the training set, these 36 analytes were further subselected as described above through multivariate analysis for their complementary contribution in top-performing panels. Figure 1 displays the frequency with which individual analytes were members of the top 1% of performing panels with respect to ROC-AUC analysis among all possible 376,992 combinations of 5-analyte panels, with specificities determined at a fixed sensitivity of 70%, and sensitivities

TABLE 2
Characteristics of the secondary validation and training set

Variable	Training set			Test set			
	sPTB (n = 45)	Control (n = 90)		SPTB (n = 42)	Control (n = 84)		
	Mean (SD) or n (%)	Mean (SD) or n (%)	*P* value[a] (SPTB vs control)	Mean (SD) or n (%)	Mean (SD) or n (%)	*P* value[a] (SPTB vs control)	*P* value[a] (SPTB in training vs validation)
Maternal age, y	32.4 (6.6)	31.5 (5.6)	.49	30.0 (5.6)	29.9 (5.6)	.93	.09
Race							
African American	7 (15.6%)	13 (14.4%)	.86	13 (30.9%)	25 (29.4%)	.86	.09
Not African American	38 (84.4%)	77 (85.6%)		29 (69.1%)	59 (70.6%)		
Prepregnancy BMI	29.3 (7.7)	27.4 (6.9)	.16	28.0 (7.7)	27.5 (7.6)	.67	.41
Parity	1.2 (1.4)	1.1 (1.9)	.74	1.0 (1.3)	1.1 (1.2)	.86	.62
Smoked in pregnancy	3 (6.7%)	8 (8.9%)	.75	6 (14.3%)	10 (11.8%)	.69	.30
Past history of PTB	12 (26.7%)	7 (7.8%)	.007	19 (45.2%)	35 (41.2%)	.71	.08
Gestational diabetes	4 (8.9%)	4 (4.4%)	.44	2 (4.8%)	7 (8.2%)	.47	.68
Male fetus	23 (51.1%)	46 (51.1%)	.99	19 (46.3)	40 (47.1%)	.94	.67
Birthweight	1889 (679)	3488 (467)	<.0001	1656 (611)	3318 (467)	<.0001	.24
Gestational at sampling	11.0 (2.8)	11.1 (2.6)	.78	10.9 (2.6)	10.7 (2.4)	.71	.89

BMI, body mass index; PTB, preterm birth; *sPTb*, spontaneous preterm birth.

[a] *P* values calculated with Wilcoxon rank sum test, χ^2 test, or Fisher exact test where appropriate.

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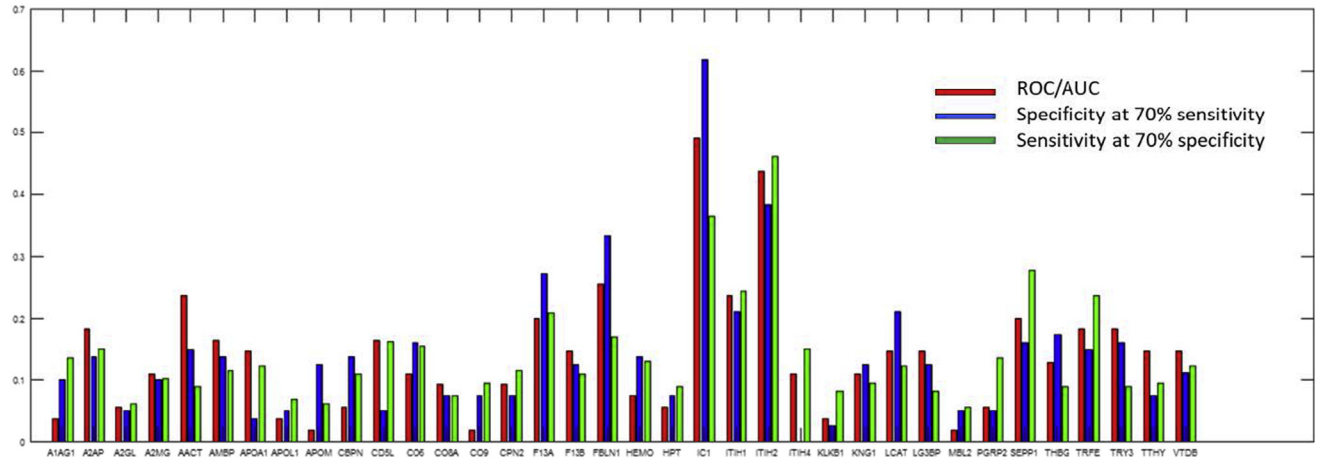
determined at a fixed specificity of 70%. Based on the results, panels of eligible analytes were cross-validated to form final panels. Taken as individual markers, the CMP-associated proteins encompassing F13A, FBLN1, IC1, ITIH2, and LCAT yielded the most stable performance based on repeated cross-validation evaluation within the training data.

Combining these individual markers and applying them to the test data as a multi-marker panel, the combination of F13A, FBLN1, IC1, ITIH2, and LCAT demonstrated an AUC of 0.74 (95% confidence interval [CI], 0.63−0.81) from ROC analysis (Figure 2). A cutoff of the score maximizing both sensitivity and specificity yields values of 0.70 and 0.81 respectively. The positive likelihood ratio would therefore be 2.70, with a negative likelihood ratio of 0.27. Assuming a hypothetical population of 1000, the 95% CIs would be respectively 2.29−3.19 and 0.15−0.48.

The same work flow was again used on the training set but now with the specific purpose of selecting analyte combinations to discern the risk of sPTB only among primipara mothers. The process described above, through cross-validation, on the training set resulted in the combination of the TRFE, IC1, ITIH4, and LCAT proteins as the highest performing multi-marker panel classifying primipara mothers. In the testing data, this 4-plex combination demonstrated an AUC of 0.77 (95% CI, 0.61−0.90), as displayed in Figure 3. At a specificity of 0.86, the corresponding sensitivity would be 0.63. The positive likelihood ratio would be 4.50, with a negative likelihood ratio of 0.43. Assuming a hypothetical population of 1000, the 95% confidence intervals would be respectively 3.45−5.87 and 0.30−0.63.

Using the multi-marker panel selected for the primipara mothers, and classifying the pregnancies into high- and low-risk strata across the test set, Figure 4 displays the Kaplan−Meier curves for pregnancy survival by week of gestation. The log-rank test indicates that the curves are significantly different ($P < .00001$) and demonstrates that a

FIGURE 1
Stability analysis for selection of protein analytes. Quantitation data of 36 protein analytes selected by univariate analysis were used. The plot shows the frequencies of a biomarker having contributed to the top 1% performing panels—based receiver operating characteristic (ROC)/area under the curve (AUC) (red), specificity at a preset 70% sensitivity (blue), and sensitivity at a preset 70% specificity (green)



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positive marker panel is associated with shorter gestation at all gestational ages, not only those ending at ≤35 weeks. Table 3 describes the functional categories for the protein analytes identified in this analysis.

Comment

We defined in a training set a 5-plex combination of CMP-associated protein analytes (F13A, FBLN1, IC1, ITIH2, and LCAT) with an AUC of 0.74 (95% CI, 0.63−0.81) in a testing set. Using Bayesian logic, given a generalized baseline risk (pretest probability) of 4.9% for delivery at ≤35 weeks within the United States, those that test positive at 10-12 weeks would now have a posttest risk (posttest probability) of 13%, whereas in those that test negative the risk would be reduced to 1% risk. We anticipate that with further refinement, including the addition of clinical risk scoring based upon maternal characteristics, multi-marker panels would improve these performance metrics.

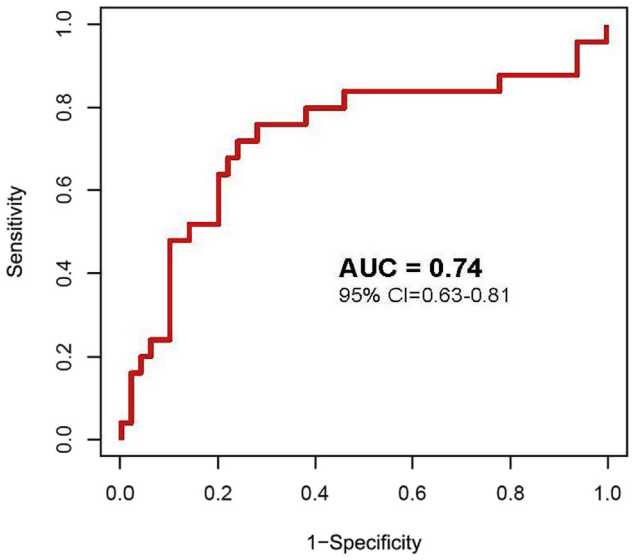
In addition, we were able to explore the predictive characteristics of CMP-associated protein analytes to predict spontaneous preterm birth before the end of 35 weeks' gestation among nulliparas. In this population, using a separate set of CMP protein markers, we observed an AUC of 0.77 (95% CI, 0.61−0.90). With a sensitivity of 0.63, this implies a specificity of 0.86. Again, framed as a Bayesian argument, a pretest probability of risk of 4.9% for delivery at ≤35 weeks implies a posttest probability of risk of 20% if positive and 2% if negative. In this population of patients in whom prior history is lacking, these results imply a potentially clinically useful stratification for the risk of sPTB before the end of 35 weeks.

These test performance characteristics should be understood in the context of the paucity of existing available risk stratification methods available at the end of the first trimester. Such methods amount primarily to an individual's pregnancy history. History has become the most important single metric for gauging a patient's potential for delivery.[18−22] Iams et al report a history of a prior preterm delivery has a 15% risk of delivery before 35 weeks in a subsequent pregnancy.[19] This is significantly higher than the baseline risk in the overall population of 5.1%.[18] Using the statistics from the Iams et al analysis, history alone implies a sensitivity of 0.67 and a specificity of 0.73 for delivery at <36 weeks (ie, ≤ 35 weeks).[1] In a separate study, Crane and Hutchens find similar statistics (sensitivity, 0.63; specificity, 0.77) for the combination of history of prior preterm delivery and a cervical length of <3 cm between 24 and 30 weeks. The characteristics of our initial panel of markers at 10−12 weeks for all patients, regardless of history, compares favorably. More importantly, however, the ability to gauge risk among mothers with 0 parity displays comparable if not superior statistics in a population in which the means of evaluating risk has been lacking. From a clinical perspective, the possibility of having a means to stratify risk among the primiparas is attractive.

With this analysis, we have demonstrated that CMP-associated protein analytes collected at the end of the first trimester have the potential to be predictive of the risk of birth at ≤35 weeks' gestation. Furthermore, we have explored the informational utility of these markers among nulliparas, a clinical situation in which tools for clinical

[1] Iams et al report that of a total sample of 1282 subjects, 378 had a history of delivery at >37 weeks and 904 did not. Of these, 14.55% had a history of delivery of <37 weeks and went on to deliver at ≤35 weeks, whereas 3% had no history of delivery of <37 weeks but did deliver at ≤35 weeks.

FIGURE 2
Receiver operating characteristic for multiplex protein panel of F13A, FBLN1, IC1, ITIH2, and LCAT predicting spontaneous preterm birth at ≤35 weeks' gestation in test set



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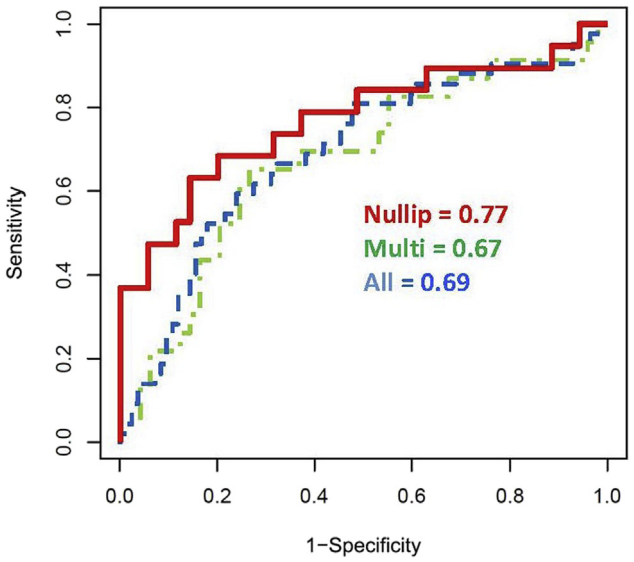
risk stratification have been challenging if not absent. Our findings here are intriguing, but not final. The proper derivation and validation of the potential of CMP-associated protein analytes to adequately risk stratify delivery prior to the end of the 35th week of gestation will require larger, prospectively collected, multi-center sample sizes. Additional work incorporating clinical cofactors such as body mass index, maternal age, and fertility status would likely, if anything, enhance the predictive potential of these markers. In this analysis, we have demonstrated that 1 clinical cofactor (ie, primiparity) alters and potentially improves the predictive potential of the CMP-associated protein analytes. Different combinations of analytes may inform and associate with different clinical characteristics. Looking back over the prior work from our group,[12,13] we observe a consistency in the CMP-associated proteins that are identified as discriminators. All members of the validated 5-plex panel described above, as well as all members of the validated 4-plex panel for parity 0 subjects, were identified as discriminatory for spontaneous preterm birth in our prior biomarker verification study.[12] In addition, 5 of the 7 markers comprising the 5-plex and 4-plex panels were described in our earlier discovery study,[13] despite population (European vs American) and sampling (week 15−17 vs week 10−12) differences.

Taking a step back from the predictive characteristics of CMP proteins and considering what is known regarding their functional characteristics, is intriguing, as it suggests nascent aberrations in pregnancy physiology that begin at the end of the first trimester but come to clinical fruition multiple weeks later. F13A (aka FXIIIA) is the clotting factor XIII A subunit. It represents the last zymogen to become activated at the level of the common pathway in the clotting cascade.[23] It catalyzes the crosslinking of fibrin, thereby stabilizing the forming of a clot. As such, F13A is involved in wound healing, tissue remodeling, and angiogenesis.[24] Not surprisingly, alterations in F13A levels are associated with reduced reproductive fitness. Congenital deficiency in F13A is associated with a tendency toward pregnancy loss both in mice[25] and in the first and early second trimesters in humans.[26−29] Although the exact mechanism of this tendency is unclear, evidence suggests that low F13A levels may be associated with aberrant formation of the early cytotrophoblastic shell.[30] Circumstantial implication of early interference with trophoblastic function can also be inferred from the observed association between F13A and an increased risk of preeclampsia[31]—a condition widely believed to have its origins in aberrant trophoblastic invasion.[32] Interestingly, F13A activity is only loosely correlated with the circulating level of F13A.[33] This observation suggests that changes in F13A functionality may be associated with relatively small changes in overall concentration. However, isolated CMPs may magnify these levels by providing a membrane-associated nidus of F13A activity.

FBLN1 or fibulin 1 is a calcium-dependent glycoprotein found within the extracellular matrix. It is associated with basement membranes, matrix structural proteins, and elastic fibers, including fibronectins and elastins; increasingly, in cancer it appears to function as a tumor suppressor gene and plays a role in cell adhesion and migration. Given its ability to bind fibrinogen and associated proteins such as F13A, it may also play a role in trophoblastic development and hemostasis.[34] In the murine models, expression of the *FBLN1* gene is involved in placentation and is expressed only in mid gestation, and then only within the spongiotrophoblast.[35] In humans, its expression has been observed in the endometrium.[36]

The inhibitor of C1, IC1, is involved in the activation of the C1 complex and subsequent regulation of the complement cascade. During pregnancy, the maternal liver and, tellingly, the trophoblast are the major sources of IC1.[37] As the placenta displays paternal protein epitopes, regulation of complement activity is key to successful implantation and hemochorial placentation without the direct activation of innate immunity.[38,39] Hereditary angioedema, IC1 deficiency, represents a significant complication to pregnancy, although it is not necessarily involved with a risk of sPTB.[40] Derangements in other components of the complement

FIGURE 3
Receiver operating characteristic for 4-plex of TRFE, IC1, ITIH4, and LCAT in predicting birth at ≤35 weeks' gestation among primiparous mothers in test set



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system have been associated with an increased risk of sPTB. Coding variants in CR1 and aberrate elevations in CD5a and CD55 have all been associated with preterm labor.[41–44] Many of these effects, however, may be in the setting of intrauterine inflammation. The circulating levels of IC1 in the third trimester are lower in patients with preeclampsia than in healthy controls.[45] Although this is at a later point in gestation than that in our present investigation, this relationship suggests the potential for trophoblastic sensitivity to IC1 activity. Similar to our observation, Lynch et al report similar findings of complement derangements between 10 and 15 weeks' gestation and an increased risk of preterm birth[46]; this intimates a potential for an early window of susceptibility of gestational tissues.

Inter-alpha-trypsin inhibitor heavy chain 2 (ITIH2) is a member of a structurally related family of plasma serine protease inhibitors that are involved in extracellular matrix interactions and structural maintenance; it has been implicated in the prevention of tumor metastases.[47] There is not much in the contemporary literature associating ITIH2 with pregnancy outcomes, let alone associations with sPTB. However, in a porcine animal model, ITIH2 is present in the intrauterine environment and appears to be involved with the endometrial adhesion of the conceptus early in pregnancy.[48] ITIH2 expression levels can be reduced by estrogen treatment.[48] The apparent mechanism is through ITIH2-based support of the endothelial glycocalyx.[49] Although an analogous role in humans is unclear, in the context of the present investigation, embryonic attachment is less of a concern, but common mechanisms of extracellular matrix attachment, stabilization, and cellular migration may similarly apply.

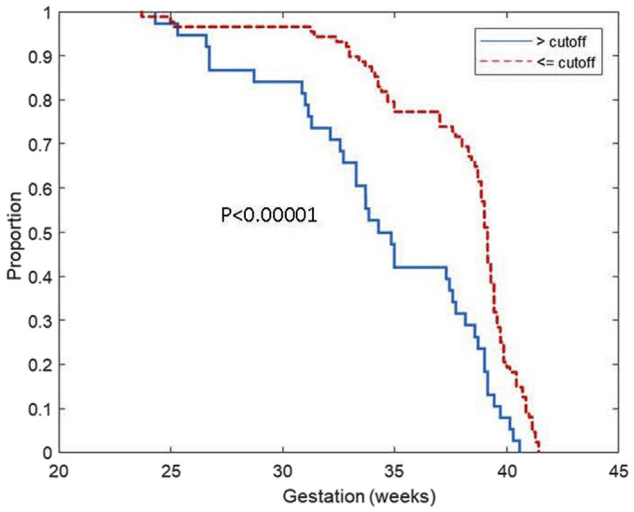
Lecithin-cholesterol acyltransferase (LCAT), is involved in the extracellular metabolism of cholesterol.[50] Serum lipid and lipoprotein levels increase systematically with the duration of pregnancy in concert with LCAT activity,[51] and preterm birth has been associated with variants in maternal and fetal genes that regulate the metabolism of serum cholesterol.[52] Although an underlying mechanism associating preterm labor and cholesterol metabolism is unclear, this indirect evidence suggests that it is not unreasonable to link LCAT levels and activity to the risk of sPTB.

Among the primipara, TRFE and ITIH4 were markers associated with an increased risk of delivery at ≤35 weeks. TRFE, or transferrin, is an iron-binding glycoprotein, that plays an important role in iron metabolism, transporting iron in a nontoxic form to cells and tissues.[53] Although iron is an essential cofactor for a number of proteins involved in a host of central physiological processes, including the transport of oxygen and its role in cellular energy production, it may be toxic to cells because it can generate oxidative stress through the its ability to create free radicals. Oxidative stress is 1 of the prevailing pathological processes that we described in earlier characterizations of exosomes in patients at risk for sPTB.[12] Transferrin transports iron to both the fetal and maternal compartments, and was thought not to cross the placental barrier.[54] As transferrin is expressed by placental cells and is known to be involved in the moderation of oxidative stress through its binding of iron, its role in a possible placental cause of sPTB is interesting. The transferrin receptor has been found in exosomes isolated from human cerebrospinal fluid,[55] and this receptor binds transferrin and internalizes it through vesicle-dependent endocytosis.[53] That we are measuring transferrin in CMPs suggests that transferrin may be trafficking between the placenta and the maternal compartment and peripheral tissues; dysregulation in such communication could reflect pathology in placentation.

Inter-alpha-trypsin inhibitor heavy chain family member 4 (ITIH4) is coded for by a gene on chromosome 3 (3p21.1).[56] Kim et al have observed serum expression of ITIH4 to be associated with recurrent pregnancy losses prior to the third trimester.[57] It has similarly been observed as a serum marker for preeclampsia although in the third trimester[58] and a more general marker for preterm birth.[59]

Although none of these proteins, when considered alone, can be seen as a mechanistic driver of spontaneous preterm birth based on our current understanding of

FIGURE 4
Comparison of 2 Kaplan–Meier curves by gestation weeks for nullipara mothers. Test set samples were grouped according to the 4-plex model score (analytes: TRFE, IC1, ITIH4, and LCAT) at a cutoff corresponding to 80% specificity



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their functions, each is associated with plausible roles that could alter the early gestational environment. Comparison of the curves in Figure 4 indicates an increased risk of sPTB across all gestational ages as well as a tendency for earlier delivery among those delivering at term. This observation suggests that these markers may be associated with mechanisms for the overall shortening of gestation and early onset of the parturition process.

Early gestation is characterized by aggressive cytotrophoblastic invasion of the maternal decidua and transformation of the decidual spiral arteries to a low-resistance phenotype; as discussed above, many of the protein markers presented here are involved in migration, extracellular matrix remodeling, and the structural integrity of the extracellular matrix. Classically, failure of cytotrophoblastic decidual transformation has been associated with the early pathology of preeclampsia;[32] however, increasing evidence suggests that this may also be a generalizable aberration in trophoblastic function common to sPTB.[60] Vascular lesions suggestive of early underperfusion of the placental surface occur in a significant proportion of cases of sPTB.[61] In some studies, up to 48% of placentas from cases of sPTB have been characterized by these stigmata of early underperfusion.[4,62,63] Remarkably, as opposed to sPTB associated with infectious inflammatory lesions, placental evidence of maternal malperfusion tended to be more common in pregnancies ending after 28 weeks' gestation.[62,64]

From the early first trimester, the number of CMPs released into maternal circulation increases dramatically with gestation.[65] The potential to decipher these early signals being sent by these vesicles represents a new window into the circumstances of early gestation.[66] The functions of the CMPs proteins that we have identified here represent fundamental, and indispensable, biological processes (cholesterol metabolism, matrix adhesion, complement regulation, wound healing, etc). Slight imbalances in the normal functioning of these processes may not be sufficient to cause the catastrophic early loss of the pregnancy, but may be sufficient to signal perturbations in normal trophoblast function and adequate early placental development; these early events may set the stage for more pronounced dysregulation later in a pregnancy. Thus, with time, these perturbations may reverberate and amplify, like the functions of the proteins in common pathogenic mechanisms elsewhere in the body, to become significant enough pathologies to cause harm at the preterm end of a gestation.

The findings of this study should be viewed within the context of its design. Although our findings are consistent with our prior work, the present results should be considered preliminary regarding the ultimate clinical utility of these markers. Currently, we are relying on retrospectively collected and matched samples. Although we believe that our multiple layers of matching and randomization preclude center or batch effects, we understand that as-yet-unknown differences with respect to collection or processing may affect outcomes. We believe, however, that this is unlikely, as noted earlier, and the ultimate utility will be determined by a prospectively collected sample reflecting population distribution of delivery at ≤35 weeks' gestation. In addition, the

TABLE 3
Biological categories associated with multiplexed proteins

Analyte	Functional category
F13A, FBLN1	Coagulation/wound healing
IC1	Complement/adaptive immunity
ITIH2, ITIH4	Fibrinolysis/anticoagulation
LCAT	Lipid metabolism
TRFE	Inflammation/oxidative stress

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predictive potential of these proteins will likely be enhanced with the addition of clinical and demographic information. We have just reported that there are performance differences by primi- vs multi-parity status. With a larger, prospectively collected sample set, the interaction of maternal characteristics and test performance can be refined. At a minimum, we have demonstrated that there is useful clinical information derived from the markers presented here at 10−12 weeks gestation regarding the prognosis of pregnancies as they proceed. We believe that this information in large part is derived from CMPs originating within the intrauterine space.

Given the need to coordinate cellular-level interactions and tolerance between 2 individuals of differing genetic and proteomic backgrounds (ie, the fetus and the mother), it is not surprising that CMPs appear to play important roles in pregnancy homeostasis and pathogenesis.[67] These particles are key constituents of communication between the placenta and both proximal and remote maternal tissues across gestation. Because the placenta is the source of close to half of the exosomal CMPs present in the maternal circulation,[65,68] this communication is particularly essential at the end of the first trimester when, as noted above, establishing normal placental−uterine interactions are fundamental to the later health of a pregnancy.[66,67]

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