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Preterm birth prediction in asymptomatic women at mid-gestation using a panel of novel protein biomarkers: the Prediction of PreTerm Labor (PPeTaL) study

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BACKGROUND: Accurate prediction of spontaneous preterm labor/preterm birth in asymptomatic women remains an elusive clinical challenge because of the multi-etiological nature of preterm birth.

OBJECTIVE: The aim of this study was to develop and validate an immunoassay-based, multi-biomarker test to predict spontaneous preterm birth.

MATERIALS AND METHODS: This was an observational cohort study of women delivering from December 2017 to February 2019 at 2 maternity hospitals in Melbourne, Australia. Cervicovaginal fluid samples were collected from asymptomatic women at gestational week 16^{+0}–24^{+0}, and biomarker concentrations were quantified by enzyme-linked immunosorbent assay. Women were assigned to a training cohort (n = 136) and a validation cohort (n = 150) based on chronological delivery dates.

RESULTS: Seven candidate biomarkers representing key pathways in utero-cervical remodeling were discovered by high-throughput bioinformatic search, and their significance in both *in vivo* and *in vitro* studies was assessed. Using a combination of the biomarkers for the first 136 women allocated to the training cohort, we developed an algorithm to stratify term birth (n = 124) and spontaneous preterm birth (n = 12) samples with a sensitivity of 100% (95% confidence interval, 76–100%) and a specificity of 74% (95% confidence interval, 66–81%). The algorithm was further validated in a subsequent cohort of 150 women (n = 139 term birth and n = 11 preterm birth), achieving a sensitivity of 91% (95% confidence interval, 62–100%) and a specificity of 78% (95% confidence interval, 70–84%).

CONCLUSION: We have identified a panel of biomarkers that yield clinically useful diagnostic values when combined in a multiplex algorithm. The early identification of asymptomatic women at risk for preterm birth would allow women to be triaged to specialist clinics for further assessment and appropriate preventive treatment.

Key Words: biomarker, cervical remodeling, cervicovaginal fluid, predictive test, pregnancy, prognostic test, protein biomarker, spontaneous preterm birth

Spontaneous preterm labor/preterm birth (PTL/B) is a leading cause of perinatal morbidity and mortality throughout the world. Despite various measures implemented to reduce preterm birth, the average global rate has increased by 0.83% between 2000 and 2014.[1] The impact of premature infants on the healthcare system is immense, which largely derives from the short- and long-term morbidities associated with prematurity. Strategies to reduce the rate of spontaneous preterm birth (PTB) have been proposed, ranging from public health measures such as smoking cessation to universal cervical length screening combined with preventive treatments such as progestins or cerclage.[2] The ability to accurately identify and target treatment to women who are at high risk for spontaneous PTB would improve the efficiency and cost-effectiveness of the proposed prevention strategies if such a test were available.

There are various studies that report on the use of a single biomarker found in the cervicovaginal fluid (CVF) to predict spontaneous PTB in asymptomatic women, such as fetal fibronectin and phosphorylated insulin-like growth factor–binding protein 1 (phIGFBP1).[3–6] However, a systematic literature review and meta-analysis performed by Conde-Agudelo et al (2011) and Kekki et al (2001) revealed that no single biomarker could reliably predict PTB.[5,7] The limited predictive utility of single biomarkers might be attributable to the complex etiology of spontaneous PTB. In recent years, there have been several attempts to improve the prediction of PTB by combining several biomarkers, which promises to be a key breakthrough in the field of preterm birth prediction.[8–11] Here, we propose an innovative methodology for the discovery of novel CVF biomarkers that may complement classical diagnostic approaches toward preterm birth prediction. By leveraging pre-existing data from the public domain through bioinformatics approaches, we have identified novel biomarkers associated with PTB based on the process of cervical softening, a reliable indicator of labor. These protein biomarkers were validated by *in vitro* cell line and *in vivo* murine model studies to ensure

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AJOG MFM at a Glance

Why was this study conducted?

This study aimed to identify and validate novel biomarkers that can accurately predict the risk of preterm birth in an asymptomatic cohort of women at mid-gestation.

Key findings

Bioinformatics analysis revealed 7 novel biomarkers that have been associated in the literature with cervical remodeling processes. Combinatorial biomarker analysis of cervicovaginal fluid samples provided an accurate prediction of women at risk of preterm labor.

What does this add to what is known?

The combinatorial biomarker algorithm provides a superior alternative to single biomarker tests, which have been used to predict preterm labor in asymptomatic women with poor sensitivity.

biological relevance. Validation of these biomarkers was also performed in a prospective clinical trial. This methodology, combined with known biomarkers in the field, allowed us to achieve a robust panel of biomarkers that accurately detects the risk of spontaneous PTB in asymptomatic women, during the window of 16^{+0}–24^{+0} weeks' gestation.[12,13]

Materials and Methods

Clinical study design

The Predicting PreTerm Labor (PPeTaL) Study was conducted as a prospective cohort study of asymptomatic women. Institutional review board approval was obtained from the Human Research Ethics Committee from both the Mercy Hospital for Women (ID 2017-027, Heidelberg, Victoria, Australia) and the Royal Women's Hospital (ID 16/27, Parkville, Victoria, Australia). All participants were recruited from a cohort of pregnant women attending Antenatal Clinic by research midwives from each hospital. Informed and signed consent was provided by all participants. Inclusion criteria were asymptomatic women of at least 18 years of age and 16^{+0}–24^{+0} weeks' gestational age. A CVF sample was collected from each woman. A CVF sample was not collected if the woman had the following: fetal membrane rupture before sampling; active vaginal bleeding; or digital vaginal examination or internal ultrasound <6 hours before sampling. A total of 301 women were included in the study. Participants with indicated preterm birth were excluded by a panel of clinicians (MDQ, PS, SPB) who were blinded to the prediction results.

Cervicovaginal fluid collection and processing

The cervix was visualized using a sterile speculum and a sterile double-tipped swab (Medical Wire & Equipment Co. Ltd.) was inserted into the posterior vaginal fornix for 30 sec. The swab was placed into a 5-mL polystyrene tube containing 1 mL of CVF extraction buffer (100 mM Tris pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.1% Triton, 150 mM NaCl, 1 mM AEBSF; bioWorld, Dublin, OH) followed by a brief vortex. The tube containing the swab was centrifuged, and the supernatant was collected and stored at −80°C.

Protein quantification

Total protein of the CVF samples was quantified using the Pierce BCA Protein Assay Kit (23225, ThermoFisher, Rockford, Illinois, USA) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assays

The concentration of protein biomarkers in human CVF samples was quantified using in-house developed monoclonal antibody enzyme-linked immunosorbent assay (ELISA) or commercially available ELISA kits. Human interleukin−1 receptor antagonist protein (IL-1RA), γ-glutamyl hydrolase (GGH), extracellular matrix protein 1 (ECM1), and vitamin D−binding protein (VDBP) concentrations were quantified with in-house−developed anti-human monoclonal antibody ELISA. Metalloproteinase inhibitor 1 (TIMP-1) was quantified using human TIMP-1 DuoSet ELISA kit (DY970, R&D Systems, Minneapolis, Minnesota, USA), human laminin subunit gamma-2 (LAMC2) was quantified using LAMC2 ELISA kit (SEC083Hu, Cloud-clone, Wuhan, China), and pigment epithelium-derived factor (PEDF) was quantified using human SERPINF1/PEDF DuoSet ELISA kit (DY1177-05, R&D Systems).

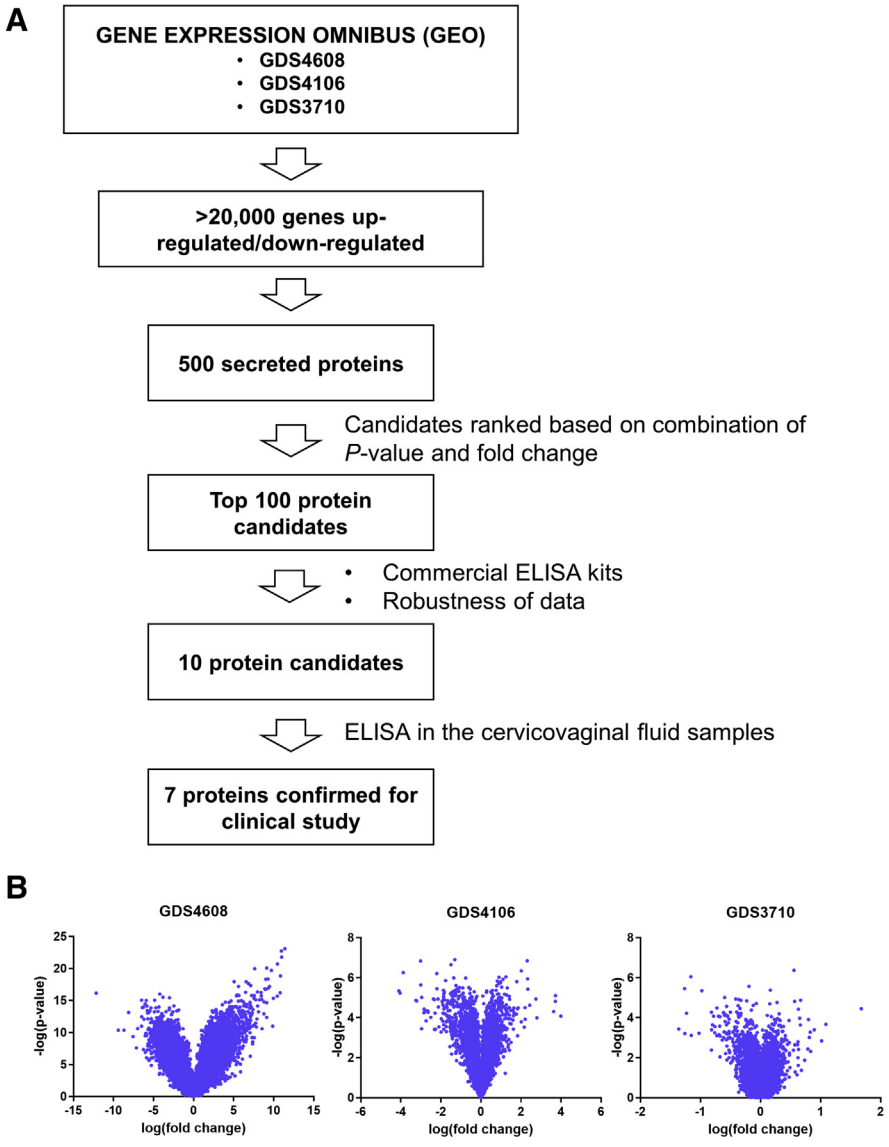
Gene Expression Omnibus databases

RNA expression data was collected from the Gene Expression Omnibus (GEO) database.[14] Three data sets were used for bioinformatics analysis: GDS4608 (Reference series: GSE30355),[15] GDS4106 (Reference series: GSE23952),[16] and GDS3710 (Reference series: GSE17708).[17]

Statistical analysis

Numerical data are presented as mean ± standard error of mean (SEM). Data were first assessed for normality with the Shapiro−Wilk test. *P* values for differences between 2 groups of continuous data were calculated using a 2-tailed *t* test where data were normally distributed, and by a 2-tailed Mann−Whitney *U* test where data were non−normally distributed. Comparisons of categorical data were performed by a 2-sided Fisher exact test. Volcano plots were generated by plotting the fold-change as a function of *P* value for the individual genes in the GEO data sets. Receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) were used to classify PTB and term birth cases based on biomarker thresholds. Significant differences were determined by $P < .05$. All statistical analyses were conducted with GraphPad Prism 8.0 (GraphPad Software, Inc, San Diego, CA).

FIGURE 1
Biomarker discovery through gene expression data. A, Schematic representation of the process used to discover and to identify potential biomarkers for preterm birth prediction using 3 Gene Expression Omnibus (GEO) datasets. B, Volcano plots showing the search results of 3 GEO datasets



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Results

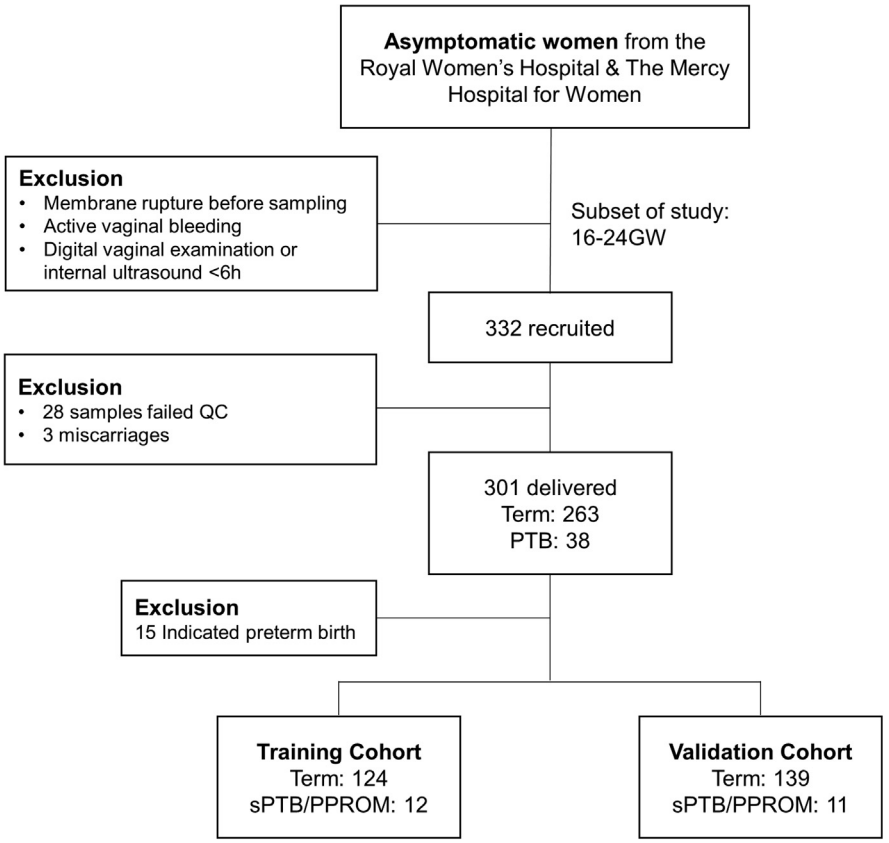
Biomarker discovery

To elucidate biomarkers that are associated with PTB, biological pathways that occur in the cervix during parturition were examined.[18–21] Bioinformatics analysis was performed on publicly available GEO sets based on the major pathways in cervical remodeling. GEO sets for these biological pathways were probed from an array of different tissues and disease indications unrelated to preterm birth, thus allowing for novel genes to be discovered. A total of 20,000 genes were found to be differentially expressed in the various pathways, out of which 500 secreted proteins were selected for downstream screening in human CVF samples (Figure 1A). Volcano plots for the GEO sets (GDS4608, GDS4106, and GDS3710) were derived for each gene, where the log-*P* value was plotted as a function of fold-change (Figure 1B). Based on the volcano plots, a cumulative score for each gene was computed across the various GEO sets. The genes were further filtered based on the assigned scores and practical considerations such as the availability of assays to assess the robustness of the genes. Of the 10 positively identified biomarkers, 7 were detectable in human CVF samples and were further assessed *in vitro* (human ectocervical cell line), *in vivo* (pregnant mouse cervices), and clinically (human CVF) (Supplementary Materials and Methods; Supplementary Figures 1 and 2). The biomarkers under investigation are GGH, LAMC2, ECM1, PEDF, IL-1RA, TIMP-1, and VDBP. The differential expression of the biomarkers in both *in vitro* and *in vivo* assays suggest a biological significance in the pathways leading to parturition.

Prospective clinical study

To assess the potential of the putative biomarkers to stratify between term delivery and spontaneous PTB, a prospective clinical study was conducted at the Mercy Hospital for Women and the Royal Women's Hospital in Melbourne, Australia. Term delivery was defined as gestational age at delivery of $\geq$37 weeks, whereas preterm delivery was defined as gestational age at delivery of $<$37 weeks. As illustrated in Figure 2, a total of 332 asymptomatic women between 16^{+0} and 24^{+0} weeks' gestation and over the age of 18 years were recruited from an all-comers cohort of women attending Antenatal Clinic from both hospitals. Of these, 28 women (n = 3, PTB; and n = 25, term birth) were excluded, as their sample did not pass the quality control check (low total protein concentration, low volume, blood-stained sample, etc). The excluded women had a PTB rate of 10.7%, which approximates the natural prevalence, thereby indicating that the poor sample quality

FIGURE 2
Study design and analysis. Flow chart showing the enrollment, exclusion and inclusion criteria, and breakdown of subjects available for analysis. sPTB/PPROM, spontaneous preterm birth and spontaneous preterm, prelabor rupture of the fetal membranes (PPROM). QC, sample quality control check, gestational week (GW)



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does not have any association with birth outcome. Three women were further excluded because of spontaneous miscarriage at <20 weeks' gestation. Preterm birth cases included spontaneous preterm labor and spontaneous preterm prelabor rupture of the fetal membranes (PPROM), whereas all clinically indicated PTB cases (n = 15) were excluded from the study cohort. Term birth cases included spontaneous term labor, induction of labor, or cesarean delivery. In total, CVF samples from 257 women from the Royal Women's Hospital (n = 235, 91.4% term birth; and n = 22, 8.6% PTB) and 29 women from the Mercy Hospital for Women (n = 28, 96.6% term birth; and n = 1, 3.4% PTB) were analyzed. Segregation of the PPeTaL Study cohort into a training cohort and a validation cohort was based on chronological delivery dates. The first 136 women with delivery outcomes were included in the training cohort for biomarker quantification and algorithm design. The subsequent 150 women were categorized as the validation cohort. Table 1 summarizes the demographic and obstetric characteristics of women in the 2 cohorts.

Training cohort

A total of 124 term birth and 12 spontaneous PTB cases were included in the training cohort to establish the combinatorial biomarker algorithm to predict spontaneous PTB. Higher expression of all biomarkers was observed in spontaneous PTB samples compared to the term delivery samples, with a statistically significant increase in the expression of VDBP, GGH, and LAMC2 (Supplementary Figure 3). A strong degree of correlation was found between biomarker expression in CVF samples (Supplementary Figure 4A). Thus, we deduced that combining pairs of biomarkers could potentially strengthen the stratification between samples from women delivering both at term and preterm and could consequently enhance the biomarkers' predictive power.

Based on this concept, an algorithm was developed using all 7 candidate biomarkers to improve the term birth and spontaneous PTB stratification. With this algorithm, stratification of term birth and spontaneous PTB samples with an AUC of 0.86 was achieved ($P < .0001$) (Figure 3A). This unique algorithm accurately identified 12 of 12 preterm births (100% sensitivity), with a specificity of 74% (Table 2). Notably, the diagnostic performance of the combinatorial biomarker algorithm was significantly improved compared to the individual biomarkers (Table 3).

Validation cohort

To verify the robustness of the predictive algorithm established in the training cohort, the same algorithm was applied to the following 150 patients in the validation cohort (Table 1). In concordance with the training cohort, the expression of the biomarkers in the validation cohort was increased in spontaneous PTB samples compared to the term birth samples (Supplementary Figure 5). Furthermore, Spearman rank-order correlation of the biomarker pairs showed a similar correlation trend of biomarker pairs as compared to that in the training cohort (Supplementary Figure 4B). By applying the same algorithm to the validation cohort, statistically significant stratification of samples from term birth and spontaneous PTB ($P < .0001$) with AUC of 0.88 (Figure 3B) was achieved. Using the

TABLE 1
Demographic characteristics of study participants

Characteristic	Training cohort		Validation cohort	
	Term (n = 124)	Preterm (n = 12)	Term (n = 139)	Preterm (n = 11)
Maternal age, y[a]	33.42 ± 4.14	33.92 ± 3.47	33.07 ± 4.33	33.68 ± 3.46
Maternal BMI, kg/m^2[a]	25.09 ± 6.52	24.38 ± 5.43	25.11 ± 5.44	23.79 ± 4.80
Gravidity, n (%)				
1	39 (31.5%)	1 (8.3%)	44 (31.7%)	2 (18.2%)
2 or 3	67 (54.0%)	6 (50.0%)	68 (48.9%)	7 (63.6%)
≥4	18 (14.5%)	5 (41.7%)	27 (19.4%)	2 (18.2%)
Parity, n (%)				
Nulliparous	59 (47.6%)	3 (25.0%)	69 (49.6%)	2 (18.2%)
1	50 (40.3%)	5 (41.7%)	48 (34.5%)	5 (45.5%)
2 or 3	15 (12.1%)	4 (33.3%)	19 (13.7%)	3 (27.3%)
≥4	0 (0.0%)	0 (0.0%)	3 (2.2%)	1 (9.1%)
Preterm birth cases				
Extreme PTB, <28 wk	0 (0.0%)	3 (25.0%)	0 (0.0%)	0 (0.0%)
Very PTB, 28 to <32 wk	0 (0.0%)	2 (16.7%)	0 (0.0%)	3 (27.3%)
Late PTB, 32 to <37 wk	0 (0.0%)	7 (58.3%)	0 (0.0%)	8 (72.7%)
Current smoker, n (%)	2 (1.7%)	1 (8.3%)	9 (6.5%)	0 (0.0%)
Fertility-assisted pregnancy, n (%)	9 (7.3%)	3 (25.0%)	12 (8.6%)	2 (18.2%)
Singleton pregnancy	124 (100%)	11 (91.7%)	138 (99.3%)	11 (100%)
Multiple pregnancy	0 (0%)	1 (8.3%)	1 (0.7%)	0 (0%)
Delivery gestation, wk[a]	39.23 ± 1.10	32.37 ± 4.18	39.37 ± 1.21	33.56 ± 3.00
Birth weight, g[a]	3408 ± 448	1957 ± 751	3410 ± 487	2204 ± 618
Previous preterm birth(s), n (%)				
0	102 (82.3%)	3 (25.0%)	120 (86.3%)	2 (18.2%)
1	18 (14.5%)	9 (75.0%)	16 (11.5%)	8 (72.7%)
2	4 (3.2%)	0 (0.0%)	3 (2.2%)	1 (9.1%)

BMI, body mass index.

[a] Data are represented as mean ± standard error of mean.

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same optimal cut-off value established from the training cohort, the algorithm accurately identified 10 of 11 spontaneous PTB samples (91% sensitivity) with a specificity of 78% in the validation cohort. Table 2 summarizes and compares the diagnostic performance of the predictive algorithm in the training and validation cohorts. The consistency in diagnostic performance between the 2 cohorts further validates the robustness of the algorithm for the prediction of spontaneous preterm birth.

Comment

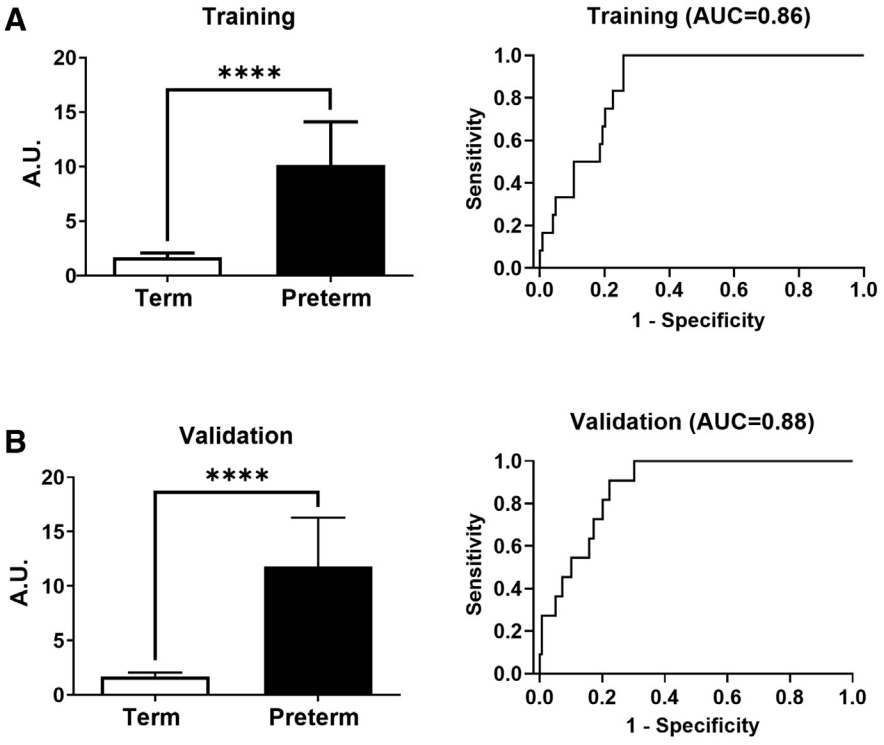
Principal findings

The objective of this study is to discover novel protein biomarkers that can accurately predict the risk of preterm birth. Through a bioinformatics search, 7 biomarkers associated with the biological mechanism leading to labor were determined and subsequently verified by *in vitro* and *in vivo* assays. Clinically, in an all-comers cohort of women at mid-gestation, the combinatorial biomarker panel identified women at risk for PTB with high sensitivity and specificity.

Results

Despite various advances in the area, the prediction of PTB remains a challenge. The lack of accurate clinical prognosis of PTB can be attributed to its multifactorial etiology. Well-established epidemiological risk factors for PTB include maternal risk factors, pregnancy history, and pregnancy characteristics.[22] However, the most accurate epidemiological predictor of PTB is a history of previous PTB, which is not helpful for the prediction of PTB in nulliparas. There

FIGURE 3
Preterm birth prediction with biomarker combination. Bar graph and receiver operating characteristic (ROC) curves for biomarker combination algorithm designed to identify preterm birth samples from term birth samples for A, training and B, validation cohort. ****$P < .0001$



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are multiple underlying biochemical mechanisms that lead to PTB. A meta-analysis performed by Menon (2008) has summarized these pathways to include maternal or fetal hypothalamic pituitary axis (stress), inflammation, decidual hemorrhage, and pathologic distension of the myometrium.[23] Based on these pathways, several single biomarkers such as interleukin-6,[24–27] interleukin-8,[27,28] pIGFBP-1,[29] and C-reactive protein[30–32] have been reported to predict PTB with varying degrees of success, but none have been widely accepted in clinical practice in the context of prediction in asymptomatic women. Clinically, fetal fibronectin[33,34] and phIGFBP-1[35,36] are used as negative predictors of PTB in both asymptomatic and symptomatic women, but their usefulness is limited by the biomarkers' poor sensitivity, as they do not reflect the multi-etiological pathways leading to PTB.

In view of the complexity of PTB pathology, there has been a shift of focus from a single biomarker approach to using combinatorial approaches for PTB prediction. It has been shown that odds ratios and/or predictive efficiency for PTB increases when 2 or more biomarkers are combined compared to single biomarkers alone.[37,38] Our study supports these findings, as we observed that combining the candidate biomarkers into a single algorithm significantly improved the sensitivity and AUC in PTB prediction. Development of a panel of key biomarkers that reflect the various pathways involved in PTB development at an earlier stage of pregnancy would allow the triage of asymptomatic women into different models of care and for interventions to be performed.

TABLE 2
Diagnostic performance of biomarker combination

Outcome	Training	Validation
n	136	150
AUC[a]	0.86 (0.79–0.93)	0.88 (0.81–0.95)
Sensitivity[a]	1.00 (0.76–1.00)	0.91 (0.62–1.00)
Specificity[a]	0.74 (0.66–0.81)	0.78 (0.70–0.84)
PPV[a]	0.27 (0.16–0.42)	0.24 (0.14–0.39)
NPV[a]	1.00 (0.96–1.00)	0.99 (0.95–1.00)
Likelihood ratio	3.875	4.076
P value[b]	<.0001	<.0001

AUC, area under the curve; *NPV*, negative predictive value; *PPV*, positive predictive value.

[a] Values in parentheses are 95% confidence intervals; [b] *P* values are calculated from 2-sided Fisher exact test.

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Clinical implications

Cervical remodeling is a dynamic process that involves substantial changes in protein expression profiles in the local microenvironment interlaced with up- and down-regulation of progesterone and estrogen hormonal action.[39] In recent years, considerable evidence has suggested that cervical remodeling constitutes one of the major mechanisms leading to PTB.[40–42] In this study, we have outlined 5 key molecular pathways in the functional network of cervical remodeling for further investigation: namely, inflammation, stress (oxidative stress or stress-related), hormonal regulation, matrix

TABLE 3
Diagnostic performance of individual candidate biomarkers and biomarker combination

Cohort	Biomarker	AUC	Sensitivity[a]	Specificity[a]	*P* value[b]
Training[c]	IL-1RA	0.61	0.50 (0.25–0.75)	0.74 (0.66–0.81)	.0942
	VDBP	0.72	0.58 (0.32–0.81)	0.74 (0.66–0.81)	.0385
	TIMP-1	0.62	0.42 (0.19–0.68)	0.74 (0.66–0.81)	.3073
	PEDF	0.58	0.42 (0.19–0.68)	0.74 (0.66–0.81)	.3073
	GGH	0.67	0.42 (0.19–0.68)	0.74 (0.66–0.81)	.3073
	LAMC2	0.75	0.50 (0.25–0.75)	0.74 (0.66–0.81)	.0942
	ECM1	0.61	0.50 (0.25–0.75)	0.74 (0.66–0.81)	.0942
	Combination	0.86	1.00 (0.76–1.00)	0.74 (0.66–0.81)	<.0001
Validation[d]	Combination	0.88	0.91 (0.62–1.00)	0.78 (0.70–0.84)	<.0001

AUC, area under the curve.

[a] Values in parentheses are 95% confidence intervals; [b] *P* values were calculated from 2-sided Fisher exact test; [c] To make a direct comparison of the sensitivity between the combined training algorithm and the individual biomarkers, the specificity was kept constant to that of the combined algorithm; [d] Sensitivity and specificity are derived from the optimal cut-off determined in the training cohort.

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remodeling, and vascular function/regulation. With these pathways as the basis for biomarker discovery, an unbiased bioinformatics search was conducted on an array of genes from public GEO databases where genes previously not associated with pregnancy were evaluated regardless of their function(s). After screening more than 20,000 genes, we selected 7 candidate biomarkers that were found to be pivotal in modulating the 5 pathways that occur during cervical remodeling and were expressed in the human CVF. Table 4[43–66] categorizes the candidate biomarkers into the molecular pathways of cervical remodeling as well as corresponding literature reviews highlighting the role of these biomarkers in other disease modalities. To our knowledge, this is the first study to demonstrate the predictive functions of PEDF, GGH, LAMC2, and ECM1 in relation to preterm birth. IL-1RA,[67] VDBP,[68–70] and TIMP-1[71] have previously been established in our studies and others.

TABLE 4
Biological process associated with candidate biomarkers

Biological pathway	Candidate biomarker	Author, year, reference
Inflammation	IL-1RA	Arend et al, 1998[43]; Arend, 2002[44]
	VDBP	Petrini et al, 1984[45]; Perez, 1994[46]; Gomme and Bertolini, 2004[47];
Stress	IL-1RA	Lavieri et al, 2014[48]; Lavieri and Carta, 2016[49]
	VDBP	Ma et al, 2012[50]
	TIMP-1	Pentland and Welgus, 1995[51]
Hormonal regulation	TIMP-1	Leppert, 1992[52]; Imada et al, 1994[53]
	GGH	Shubbar et al, 2013[54]
Matrix remodeling	TIMP-1	Winkler et al, 1999[55]; Arpino and Gill, 2015[56]
	PEDF	Farnoodian et al, 2016[57]
	LAMC2	Koshikawa et al, 1999[58]; Hlubek et al, 2001[59]; Takahashi et al, 2002[60]; Garg et al, 2014[61]
	ECM1	Chan, 2004[62]; Oyama and Merregaert, 2014[63]; Chen et al, 2016[64];
Vascular	PEDF	Chandolu and Dass, 2012[65]
	LAMC2	Delgado-Bellido et al, 2017[66]

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To validate these biomarkers clinically, human CVF samples were collected in a prospective clinical study. The human CVF provides a rich source of locally secreted proteins from the gestational tissues, which reflects the various pathways that occur during cervical remodeling. As spontaneous PTB is a result of multi-etiological processes that cannot be reliably tested by a single biomarker, all 7 biomarkers were combined into a multiplex panel to develop a predictive algorithm to distinguish term and spontaneous PTB in the training cohort. As the biomarkers might be involved in one or more pathways of cervical remodeling (Table 4), combinations of these biomarkers allowed enrichment of spontaneous PTB cases of similar etiology as well as capturing spontaneous PTB cases resulting from different biological processes (eg, stress, inflammation, infection). Compared to the predictive values of each single biomarker, improved prognostic accuracy was observed with the multiplex panel (Table 3). In addition, the multiplex panel showed significant distinction between samples derived from term birth and spontaneous PTB in both the training and validation cohorts. It is worth noting that the single patient with spontaneous preterm labor that was incorrectly classified was known to have a uterine anomaly, and that this may explain the false-negative classification, as the mechanism of PTB likely relates to myometrial stretch rather than cervical mechanisms.[9]

Strengths and limitations

The main strength of this study lies in the study design, in which asymptomatic women were recruited from an all-comers cohort with a PTB rate that truly reflects the prevalence of PTB in a multi-ethnic Australian population. As such, the predictive power of the combinatorial biomarker algorithm could be easily applied to the general population. In addition, by using an electronic health record and active surveillance, there was no loss-to-follow-up of cases in this study, and all pregnancy outcomes could be tracked accurately and quickly.

Despite the relatively low PPV, the improved NPV (near 100%) suggests that the test can be used in a way similar to that of current tests, to exclude the likelihood of preterm birth and thus to provide reassurance and reduce unnecessary transfers to tertiary centers or specialist referrals. More accurate tests may also assist in further research by better defining a high-risk cohort for future trials.

For future work, it will be of interest to apply the algorithm to women from different geographies to validate the predictive value in different ethnicities. Furthermore, other confounding factors such as maternal characteristics and obstetric history were not included as covariables in the analyses. A combination of these factors with the multiplex algorithm might yield a greater predictive accuracy. In addition, it would be of interest to elucidate the mechanism(s) of action of the candidate biomarkers, thereby allowing tailored interventions based on the etiologies of PTB identified by these biomarkers.

Conclusion

We have developed a panel of 7 protein biomarkers that can provide an accurate prognostic test for the early (mid-gestation) prediction of PTB. The relatively noninvasive nature of CVF collection could be incorporated as a routine part of an obstetric visit. Furthermore, the biomarkers have shown the capability of identifying subgroups of spontaneous PTB, given their differential expression profile in the *in vitro* and *in vivo* studies when subjected to different stress and inflammation stimuli. The early identification of asymptomatic women at risk for PTB would allow women to be triaged to specialist clinics for further assessment and appropriate preventive treatment. Newly identified therapies with potential for prevention of PTB include low-dose aspirin[72] and omega-3 fatty acids,[73] which should be started early in the second trimester for best efficacy. This has the potential to greatly reduce the global incidence of spontaneous PTB, with consequent benefits to maternal healthcare and the economic burden associated with prematurity. ■

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Preliminary data relating to this project have been presented at the following conferences: Society for Maternal Fetal Medicine 39th Annual Pregnancy Meeting, Feb. 11–16, 2019, Las Vegas, NV, USA; 2nd World Congress on Maternal Fetal Neonatal Medicine, April 4–6, 2019, London, UK; 10th international Symposium on Diabetes, Hypertension, Metabolic Syndrome and Pregnancy, May 29–June 1, 2019, Florence, Italy; PREBIC-Australasia, June 18–20, 2019, Shenyang, China; and RANZCOG Annual Scientific Meeting, Oct. 13–16, Melbourne, VIC, Australia.

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Supplementary Material

Supplementary Materials and Methods

Cell line and culture

The human ectocervical Ect1/E6E7 cell line was obtained from ATCC (ATCC CRL-2614). Ect1 cells were cultured in keratinocyte serum free medium (17005-042, Gibco, Grand Island, NY), supplemented with 50 μg/mL bovine pituitary extract (Gibco), 0.1 ng/mL human recombinant epidermal growth factor (Gibco), 0.4 mM calcium chloride (Kanto chemical, Tokyo, Japan), and 1% penicillin/streptomycin (Gibco), at 37°C with 5% CO_2 in a humidified atmosphere.

Cell treatment

Ect1 cells were seeded at a density of 0.2×10^6 cells per well in a 6-well plate prior to treatment with either H_2O_2 (Sigma, St. Louis, MO) or lipopolysaccharide (L2630, Sigma) for 24 hours. The cells were treated with 200 μM and 400 μM of H_2O_2 ($n \geq 4$ replicates at each concentration) or with 10 μg/mL, 25 μg/mL, and 50 μg/mL of LPS ($n \geq 4$ replicates at each concentration). The cell culture conditioned medium and cells were collected 24 hours posttreatment.

Murine models

In vivo mouse experiments were approved by the Israel Board for Animal Experiments (Approval No.: IL-15-09-292). Two mouse models were used for this study: (1) an intrauterine inflammation model[1–5], and (2) noninfectious model that mimics progesterone withdrawal[6,7], as previously described. Briefly, CD-1 female mice were bred with CD-1 male mice and the appearance of a mucus plug represented day 1 of gestation. At gestation day 15 (GD15), mice were anesthetized with Carprofen (SML1713, Sigma), with 5 mg/kg administered subcutaneously. To establish an intrauterine inflammation mouse model of preterm birth, a small abdominal incision was made in each mouse and 30 μL of 5 μg/μL LPS (L2630, Sigma) was injected between the gestational sacs in the left uterine horn, or with sterile water injected as a sham surgical control. The mice were sacrificed at 6 hours, 12 hours, or at preterm birth to harvest the cervix ($n \geq 2$).

To establish a noninfectious preterm mouse model, exposure of GD15 mice to RU486 (Mifepristone, M8064, Sigma) was conducted.[6,7] RU486 was solubilized in ethanol and brought up in glyceryl trioleate (T7140, Sigma) and a dose of 0.5 mg/200 μL was injected subcutaneously in the interior left or right flank of the hind leg. A 50-μL quantity of ethanol in 150 μL of glyceryl trioleate was administered subcutaneously as a vehicle control. The mice were sacrificed at 6 hours, 12 hours, or at preterm birth to harvest the cervix ($n \geq 2$).

For both the LPS and RU486 mouse models of PTB, the cervix was isolated by transection at the utero-cervical junction, and all vaginal tissue was removed from the cervical tissue specimens. The cervix specimens were cryopreserved. Prior to analysis, tissues were homogenized in extraction buffer (50 mM HEPES, 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 1 mM ethylinediaminetetraacetic acid (EDTA) and were further subjected to enzyme-linked immunosorbent assay (ELISA) analysis for biomarker concentrations.

Enzyme-linked immunosorbent assays

The concentration of biomarkers for the *in vitro* Ect1/E6E7 cell line studies was quantified by ELISA. Human IL-1RA, GGH, ECM1, and VDBP concentrations were quantified with in-house–developed anti-human monoclonal antibodies. TIMP-1 was quantified using human TIMP-1 DuoSet ELISA kit (DY970, R&D Systems, Minneapolis, MN), human LAMC2 was quantified using LAMC2 ELISA kit (SEC083Hu, Cloud-clone, Wuhan, China), and PEDF was quantified using human SERPINF1/PEDF DuoSet ELISA kit (DY1177-05, R&D Systems).

The concentration of biomarkers for the *in vivo* mouse cervical tissues was quantified using commercially available ELISA kits. Mouse IL-1RA was quantified using IL-1RA DuoSet ELISA kit (DY480, R&D systems); mouse TIMP-1 was quantified using TIMP-1 DuoSet ELISA kit (DY980, R&D systems); mouse GGH was quantified using GGH ELISA kit (MBS9333356, MyBioSource, San Diego, CA); mouse LAMC2 was quantified using LAMC2 ELISA Kit (MBS355231, MyBioSource); mouse ECM1 was quantified using ECM1 ELISA Kit (LS-F23755, LS Bio, Seattle, WA); and mouse PEDF was quantified using SERPINF1/PEDF ELISA Kit (LS-F12302, LS Bio).

Supplementary References

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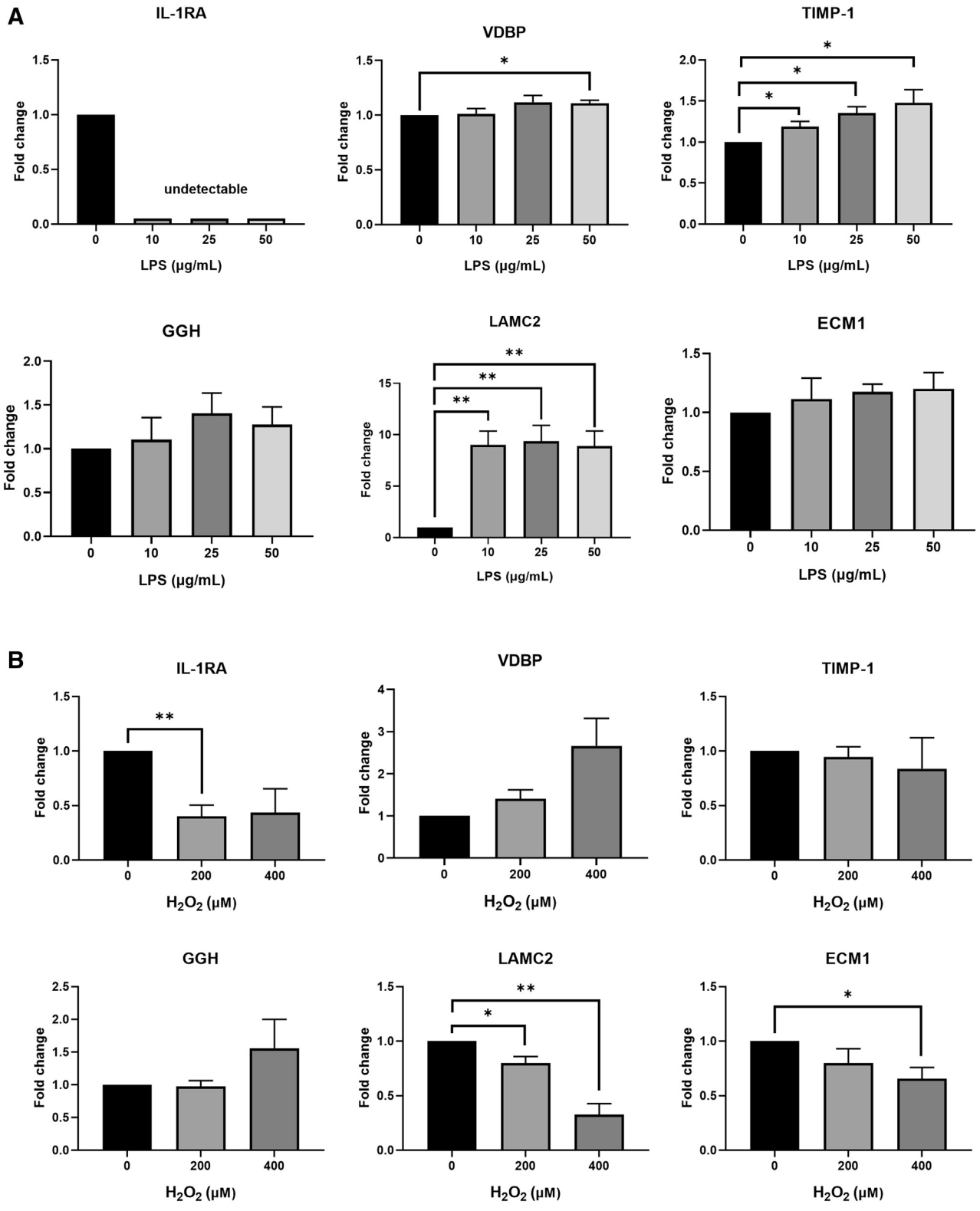
SUPPLEMENTARY FIGURE 1

In vitro **validation of biomarker expression. Biomarker fold changes in conditioned medium of Ect1 cells treated with A, LPS and B, H_2O_2. *$P < .05$, **$P < .01$**



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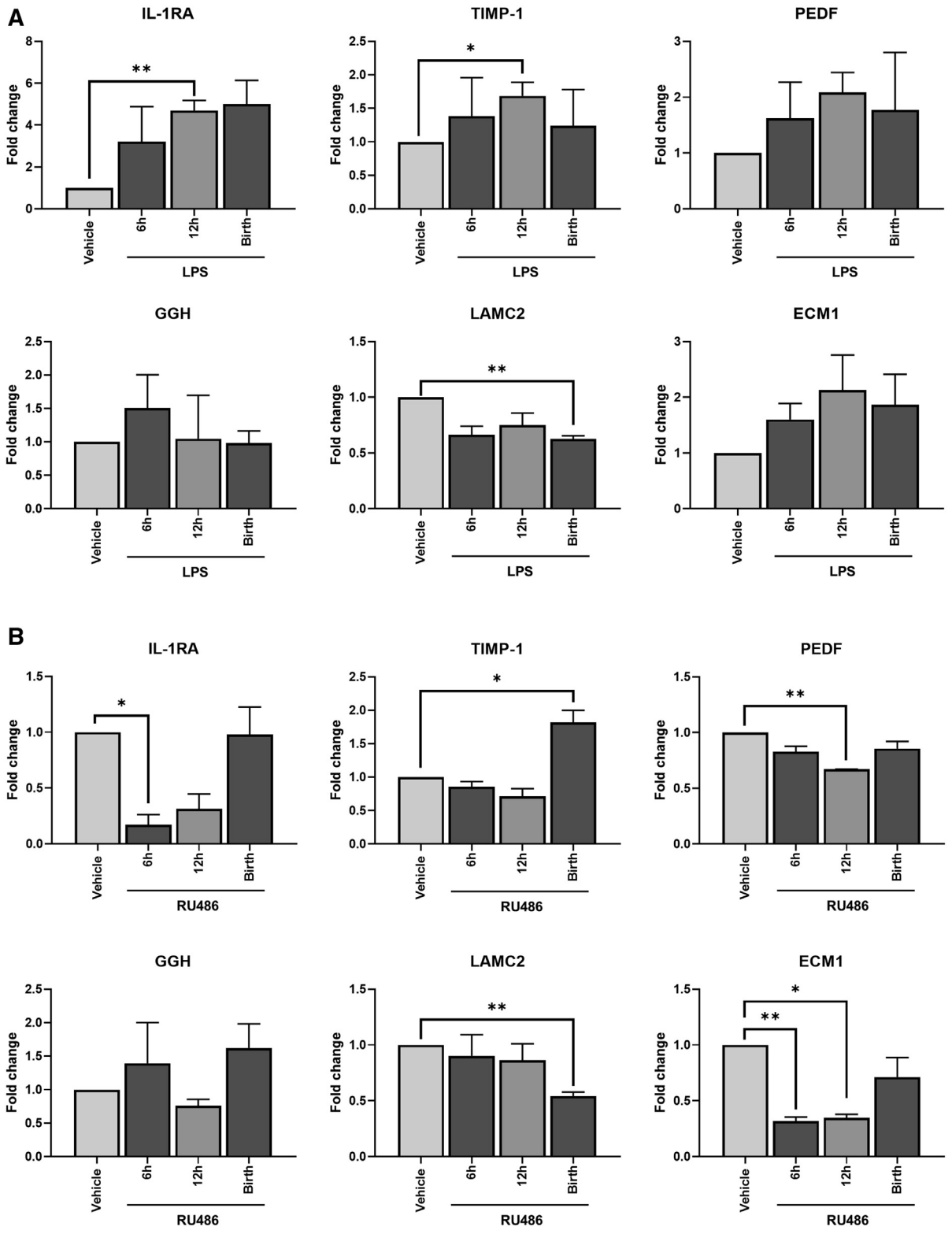
SUPPLEMENTARY FIGURE 2

***In vivo* mouse model. Biomarker expression fold changes in the cervix of pregnant CD-1 mice in A, inflammation (LPS treatment) and B, noninfectious (RU486) models of preterm birth (PTB). *$P < .05$, **$P < .01$**



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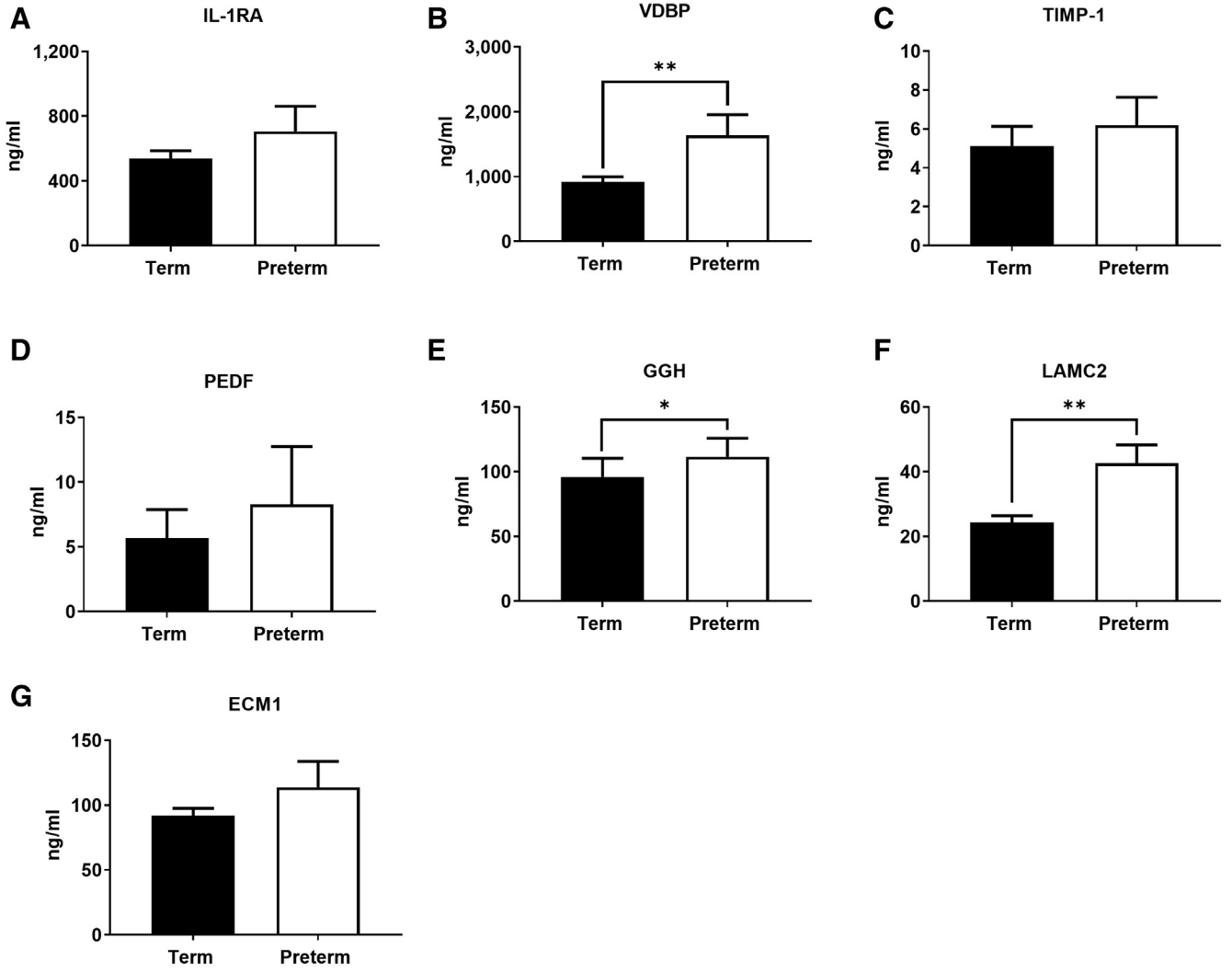
SUPPLEMENTARY FIGURE 3

Biomarker expression in the training cohort. Biomarker expression was quantified by enzyme-linked immunosorbent assay (ELISA) in the term birth (n = 124 samples) and spontaneous preterm birth (PTB; n = 12 samples) cervicovaginal fluid (CVF) samples of the training cohort. *$P < .05$, **$P < .01$



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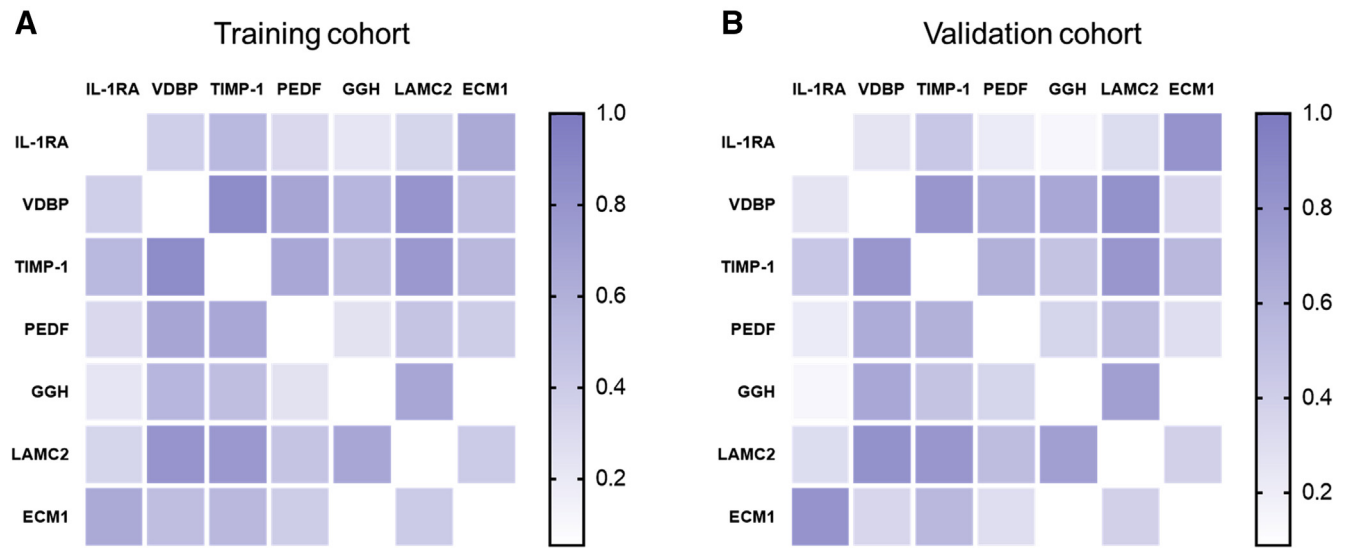
SUPPLEMENTARY FIGURE 4

Correlation between candidate biomarkers. Heat map of the Spearman correlation coefficient for candidate biomarkers in A, training and B, validation cohorts



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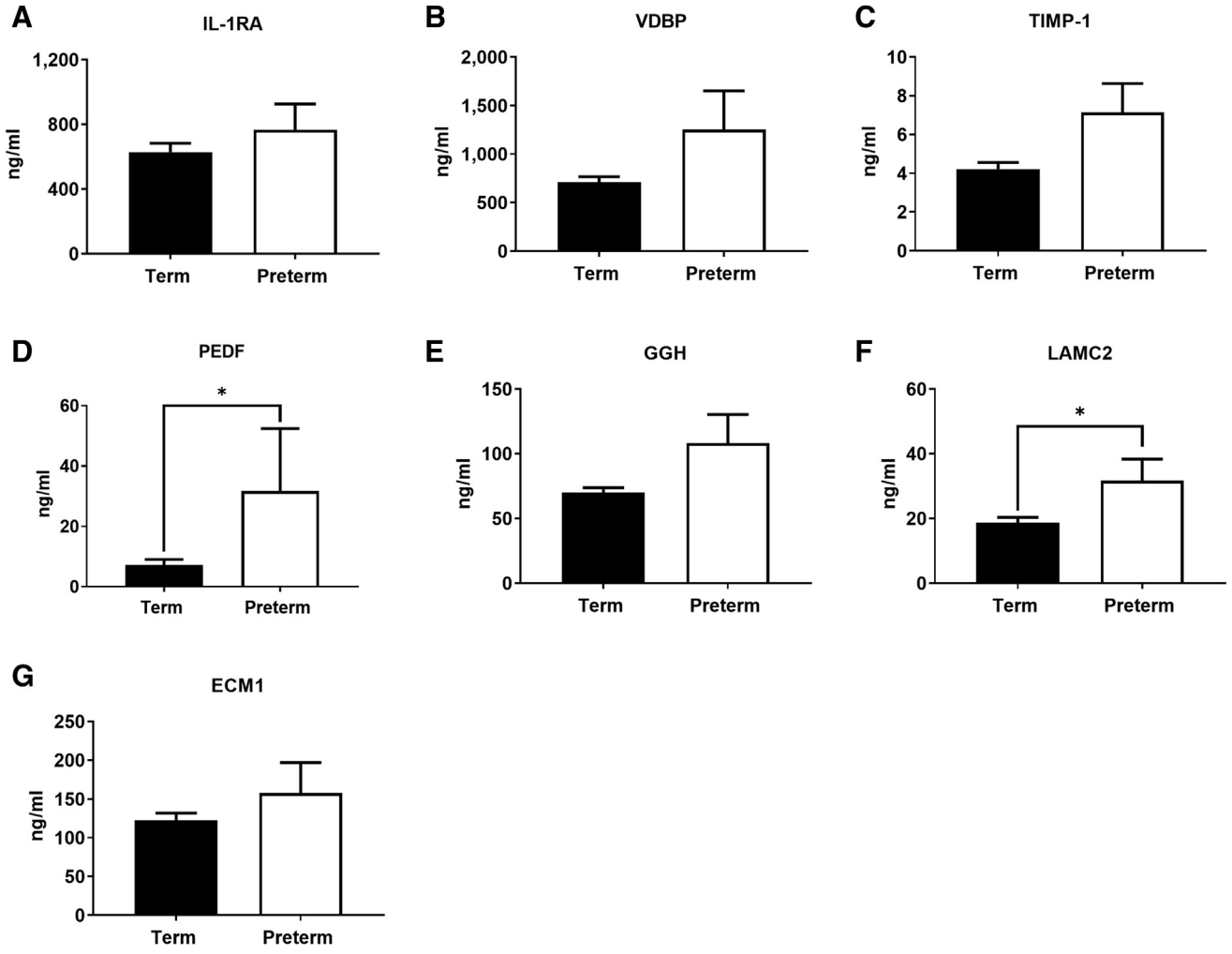
SUPPLEMENTARY FIGURE 5

Biomarker expression in the validation cohort. Biomarker expression was quantified by enzyme-linked immunosorbent assay (ELISA) in the term birth (n = 139 samples) and spontaneous preterm birth (PTB; n = 11 samples) cervicovaginal fluid samples of the validation cohort. *$P < .05$



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