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THE RELATIONSHIP OF CIRCULATING PROTEINS IN EARLY PREGNANCY WITH PRETERM BIRTH

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Abstract

BACKGROUND—Preterm birth (PTB) (<37 completed weeks gestation) is a pathological outcome of pregnancy and a major global health problem. Babies born preterm have an elevated risk for long term adverse medical and neurodevelopmental sequelae. Substantial evidence implicates intrauterine infection and/or inflammation in PTB. However, these are often relatively late findings in the process, when PTB is inevitable. Identification of earlier markers of PTB may make successful intervention possible. Although select proteins, notably, those related to the inflammatory pathways, have been associated with PTB, there has been a lack of research into the role of other protein pathways in the development of PTB. The purpose of this study was to investigate, using a previously described biomarker discovery approach, a subset of circulating proteins and their association with PTB focusing on samples from early pregnancy.

OBJECTIVES—1) To perform a large-scale biomarker discovery, utilizing an innovative platform to identify proteins associated with preterm birth in plasma taken between 10 and 15 weeks' gestation and, 2) To determine which protein pathways are most strongly associated with preterm birth. To address these aims we measured 1129 proteins in a plasma sample from early pregnancy using a multiplexed aptamer–based proteomic technology developed in Colorado by SomaLogic.

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STUDY DESIGN—Using a nested case-control approach, we measured proteins at a single time point in early pregnancy in 41 women who subsequently delivered preterm and 88 women who had term uncomplicated deliveries. We measured 1129 proteins using a multiplexed aptamer–based proteomic technology developed by SomaLogic. Logistic regressions and random forests were used to compare protein levels.

RESULTS—The complement factors B and H and the coagulation factors IX and IX ab were the highest ranking proteins distinguishing cases of preterm birth from term controls. The top 3 pathways associated with preterm birth were the complement cascade, the immune system and the clotting cascade.

CONCLUSIONS—Using a discovery approach, these data provide further confirmation that there is an association of immune and coagulation–related events in early pregnancy with preterm birth. Thus, plasma protein profiles at 10–15 weeks of gestation are related to the development of preterm birth later in pregnancy.

Keywords

Complement system; coagulation cascade; early pregnancy; preterm birth

Introduction

Preterm birth (PTB) (<37 completed weeks gestation) is a pathological[1] outcome of pregnancy and a major global health problem.[2] Babies born preterm have an elevated risk for long term adverse medical and neurodevelopmental sequelae.[3] The pathological mechanisms leading to PTB are complex with multiple pathways involved.[3,4] Substantial evidence implicates intrauterine infection and/or inflammation in PTB.[3] However, these are often relatively late findings in the process, when PTB is inevitable. Identification of earlier markers of PTB may make successful intervention possible. Although select proteins, notably, those related to the inflammatory pathways, have been associated with PTB, there has been a lack of research into the role of other protein pathways in the development of PTB.[3]

The purpose of this study was to investigate, using a previously described biomarker discovery approach,[5,6] a subset of circulating proteins and their association with PTB focusing on samples from early pregnancy. The goals of this study, using a broad-based proteomic screening platform were to: 1) identify top ranked proteins associated with PTB in early (10 to 15 weeks' gestation) pregnancy and, 2) determine the top ranked pathways associated with PTB. To address these aims we measured 1129 proteins in a plasma sample from early pregnancy using a multiplexed aptamer–based proteomic technology developed in Colorado by SomaLogic.[5–7]

MATERIAL AND METHODS

This is an analysis of data and samples collected as part of the Denver Complement Study.[8–10] This prospective cohort study was approved by the Colorado Multiple Institutional Review Board. In brief, 1287 women were recruited in the first half of pregnancy from the University of Colorado Hospital prenatal clinics and two affiliated sites.

Informed consent was obtained, and an additional EDTA-plasma tube was obtained with the routine prenatal labs. Data were gathered on the maternal medical and obstetrical history. After delivery, outcome data were collected and the gestational age at blood draw was assigned based on the best overall obstetrical estimate, incorporating assessment at the first visit and in the great majority, on early ultrasound examination.

We conducted a nested-case control study within this longitudinal cohort. We made an *a priori* decision to concentrate the analysis on women who had a blood sample taken between 10 and 15 weeks gestation (n = 856) to reduce the potential confounding effect of gestational age at blood draw and to consider early predictors of PTB. Only the first delivery of women who had greater than one delivery during the study period was included (6 records deleted) in the analysis. The following records were also removed: multiple births (n = 34), congenital or chromosomal anomalies or deliveries less than 20 weeks' gestation (n = 24), lost to follow-up (n = 32), chronic medical disease (cardiac disease, chronic hypertension, coagulation disorders, uterine anomalies, type 1 diabetes, and autoimmune disease, n = 111), consent not provided to research outside of the primary study (n = 22), and, deviation in study protocol (missing or inadequate sample, n = 24). Following these exclusions 603 records remained in the analytic dataset (41 cases of PTB and 562 term controls). As we were interested in the pathogenic mechanisms of PTB, we excluded (n = 474) from the term deliveries records of patients with preeclampsia, gestational hypertension, disorders of placentation, intrauterine growth restriction, induction of labor or cesarean delivery. Therefore, all term controls had an uncomplicated spontaneous vaginal delivery. The exclusion of these comorbidities allowed a more systematic comparison with PTB thereby increasing power to find differences attributed to PTB without being obscured by additional complications. Ultimately, 41 cases of PTB and 88 term controls were included in the analytic dataset.

The primary outcome was PTB (between 20 and less than 37 completed weeks gestation) resulting from spontaneous PTB [spontaneous preterm labor (SPTL) or preterm premature rupture of the membranes (PPROM), n = 22] or from a medical indication (n = 19). Ten of the medically indicated PTB resulted from hypertensive diseases of pregnancy, 5 from placental problems and 4 from intrauterine growth restriction.

Sample preparation

Each sample was centrifuged within an average time of 9 minutes from phlebotomy (median = 6 minutes, range = 1 to 38 minutes).. The supernatant was removed, aliquotted, placed on dry ice and placed in a freezer at −80°C. All samples had one freeze thaw prior to proteomic analysis in this study.[10]

SomaLogic Technology:[5]

The SOMAscan proteomic assay is supported by a new generation of protein-capture Slow Off-rate Modified Aptamer (SOMAmer™) reagents. SOMAmers bind to pre-selected targets including proteins and peptides with high affinity and specificity. The SOMAscan multiplex assay consists of 1129 individual affinity molecules SOMAmer reagents (described in detail elsewhere[5,6]). In brief, a biological sample in each well of a 96 well plate is incubated with a

mixture of the 1129 SOMAmer reagents. Two sequential bead-based immobilization and washing steps eliminate unbound or non-specifically bound proteins and the unbound SOMAmer reagents, leaving only protein target-bound SOMAmer reagents. These remaining SOMAmer reagents are isolated, and each reagent is quantified simultaneously on a custom Agilent hybridization array. The amount of each SOMAmer measured is quantitatively proportional to the protein concentration in the original sample.

Statistical Analysis—Descriptive statistics were calculated and compared across groups using t-tests or chi-squared tests. Concentrations for each of 1129 proteins were log (base 2) transformed and compared between PTB and term groups using a logistic regression. P-values were adjusted for multiple comparisons using the False Discovery Rate.[11] Proteins that were significant in this analysis were evaluated across the three subgroups (term, medically indicated and spontaneous PTB) using an Analysis of Variance. Random forests consisting of 5,000 classification trees were used to multivariately evaluate proteins, the mean decrease in the Gini index was used to rank proteins and the out-of-bag error rate was used to describe classification error.[12] Proteins were classified into pathways using Reactome[13] and were ranked using Fisher's combined probability test which assesses the association for the pathway by combining test statistics from the individual proteins contained within that pathway.

RESULTS

Baseline statistics on select variables (maternal age, race, parity, and gestational age at the first prenatal visit) from the 41 cases of preterm birth (22 spontaneous and 19 medically indicated) and 88 uncomplicated term deliveries from the nested case control study show comparable demographics between groups, with the exception of race (Table 1).

The SOMAmer proteins were compared in women with PTB and term delivery. The significance ($-\log_{10}$ p-value) and the magnitude (OR) for all the SOMAmer proteins are displayed graphically (Figure 1). Proteins with the largest magnitude of difference between groups and smallest p-value are named in the figure and appear in the upper left and upper right hand corners of the plot. Several proteins related to the clotting (coagulation factors IX and IX ab) and complement pathways (factor B, factor H and ficolin-3) were higher in PTB cases compared with controls. In addition, we found links between platelet endothelial cell adhesion molecule (PECAM-1), serum amyloid P-component (SAP), vascular endothelial growth factor receptor-2 (VEGF SR2), cathepsin Z (CATZ), growth hormone receptor (GHR) with PTB. Other proteins such as protein jagged-1 (JAG1), matrix metalloproteinase-2 (MMP-2), neurogenic locus notch homolog protein 3 (Notch 3), and angiopoietin-2 (ANGPT2), were lower in cases compared with controls. We demonstrate in Table 2 the magnitude of the association [(odds ratio (OR)] with 95% confidence intervals (CI) of the relationship of the top ranked proteins (n=34) with PTB along with the unadjusted p-value and the p-value adjusted for multiple comparisons. The coagulation factors IX, IX ab and SAP remained significantly related to PTB following adjustment for multiple comparisons, as did leptin. Factor B, GHR and insulin-like growth factor-binding protein-1 (IGFBP1) were of borderline significance after multiple comparisons adjustment.

The results from the random forest reiterate those from the univariate logistic regressions; the coagulation Factors IX and IXab, Factor B and H were the highest ranking factors distinguishing cases of PTB from term controls (Figure 2). The random forest resulted in a classification error rate (ability of the random forest to accurately classify women into PTB/full term based only on their protein profiles) of 31%, which was reduced to 26.4% (17% for full term and 46% for PTB) after dimension reduction.

We show in Table 3 the pathways that are potentially involved in PTB. Proteins can be classified into multiple pathways and pathways are hierarchical, resulting in pathways that are not mutually exclusive. Smaller more specific pathways are nested into larger more general pathways (please see legend for Table 3). In this interactive context, the complement system ranked highest, followed by the immune system and the clotting cascade.

As a follow-up analysis, we explored the differences in the top ranking complement and coagulation proteins across the 3 categories of PTB. Of note, we found a significant difference in factor B, factor H, coagulation factors IX and IX ab in the medically indicated (n=19) and spontaneous groups (n=22) compared with the term group (Figure 3). In addition to evaluating the important term/PTB dichotomous outcome, we also considered gestational age as a continuous outcome. This analysis revealed similar top ranked proteins from the random forest including factor B, FN1–3, Coagulation Factor IX and Fibronectin but also indicated PECAM-1 and EG-VEGF increased in importance.

COMMENT

In this nested case-control study we used the innovative SOMAscan proteomic assay technology[5] to measure proteins in a plasma sample taken at a single time point in early (10 to 15 weeks' gestation) pregnancy. The coagulation factors IX and IX ab and the complement factors B and H were the highest ranking proteins distinguishing cases of PTB from term controls. The top 3 pathways associated with PTB were the complement cascade, the immune system and the clotting cascade. These data suggest that there is a contribution of immune-and coagulation–related events in early pregnancy to PTB. Of particular note, these potentially associated plasma protein profiles, evident very early in pregnancy, at 10–15 weeks of gestation, are related to the development of PTB several months later highlighting the possibility of developing early interventions to prevent these later gestational complications

We propose that activation of the complement cascade (described elsewhere[14]) at this time in early pregnancy is triggered by events linked with inflammation that could be infectious or non-infectious in origin. Subclinical microbial invasion of the amniotic cavity in early pregnancy[3,15] is an event that has been implicated in PTBs occurring specifically at earlier gestational ages.[16] We suggest that placental-derived injured or apoptotic cells[17,18] may be trigger for a non-microbial activation of the complement cascade. This finding would be in keeping with recognition by the complement pathway of danger-associated molecular patterns (DAMPS) of not only pathogens but also of injured or apoptotic cells.[18] Factors B and H were the leading complement factors related to PTB. Complement factor B is a member of the alternative complement pathway. In our previous research we demonstrated that elevated levels of the factor B-derived Bb activation fragment in early pregnancy were

related to PTB at less than 34 weeks' gestation.[8] In the current study using a broad-based screening approach we have again demonstrated a role for enhanced involvement of the proteins related to the alternative complement pathway in PTB. We also observed upregulation of the regulatory complement protein, Factor H in early pregnancy in women who subsequently had a PTB. The role of this complement regulator[19] is to prevent uncontrolled activation of the complement system, as dysregulation of control can lead to a severe inflammatory response with collateral damage to host tissue as seen in other human diseases.[20] We suggest that Factor H is elevated in an effort to self-regulate as a response to inflammatory events originating in the placenta in early pregnancy. It is noteworthy that Ingram et al. recently suggested that elevated complement factor H is a biomarker of multiple sclerosis disease state.[21]

We demonstrated a role for the clotting cascade (Table 3) and, specifically thrombosis-related coagulation factor IX (Christmas factor)[22] in PTB. This finding is aligned with the results from other authors (reviewed in[3]) who described a role for defective decidual hemostasis/vaginal bleeding in PTB. Indeed, Mackenzie et al. demonstrated that thrombin resulting from placental abruption promotes PTB by mediating fetal membrane extracellular matrix degradation via enhanced decidual cell matrix mettaloproteinase-3 expression.[23] This finding is in agreement from other investigators who have observed crosstalk between the coagulation and the complement system.[24,25]

We also found other proteins that were higher in PTB cases compared with term controls. These proteins include: PECAM-1, a cell adhesion and signaling receptor that is expressed on hematopoietic and endothelial cells that has a role in the inflammatory process[26]; SAP, a protein found in amyloid deposits that binds to apoptotic cells and the nuclear component of necrotic cells[27] and has links with the complement system;[28] VEGF sR2, a soluble receptor involved in vascular endothelial cell development[29]; cathepsin Z which has links with protein turnover in cells and tumor progression[30]; the GHR, that mediates the actions of growth hormone which promotes cell division, regeneration and growth[31]; ficolin-3, a protein that is related to the complement system[32] and mediates the clearance of apoptotic cells,[33] and leptin, an adipocyte-derived hormone linked with obesity and preeclampsia.[34] In contrast, other proteins were lower in cases of PTB compared with term controls and included: JAG1, a protein linked with the notch signaling pathway,[35] MMP-2, a metalloproteinase involved in the breakdown of extracellular matrix that has links with tumor progression[36] notch-3, shown to have a role in fetal-maternal communication during implantation and placentation,[37] and, angiopoietin-2, which has a role in the regulation of endothelial cell survival and vascular maturation.[38] We suggest that alterations in levels of these proteins in early pregnancy are potential markers of subsequent PTB.

Regarding the pathway analysis, we found that in addition to the complement, clotting and immune system pathways, top ranked pathways included metabolism of proteins (synthesis and catabolism of proteins), signal transduction (activation of cell-specific receptors by an extracellular signal), and post translational protein modification (a component of protein biosynthesis) in PTB. These findings could reflect the high turnover/remodeling of cells in the developing placenta at this time of pregnancy and the alterations due to the underlying pathogenic process promoting future PTB.[17] A role for regulation of IGF activity by Insulin-

like Growth Factor (IGF) Binding Proteins was also demonstrated. This finding in early gestation may be important as this family of proteins has significant connections with intrauterine growth restriction.[39] Finally, we found the innate immunity and diabetes pathways to be among the top ranked pathways related to PTB.

Notwithstanding the important results from this study, there are some limitations. Validation[5] of the findings and this technology is required in a much larger PTB cohort. Of relevance to this suggestion are the results from a recent study where this biomarker discovery platform was used to investigate protein profiles in two independent cohorts of patients with Duchenne muscular dystrophy and age-matched control. Forty-two proteins showed significant differences that were consistent in both cohorts suggesting the utility of this approach to biomarker discovery.[40] An additional limitation was the heterogeneous group of women studied and the small sample size. Careful stratification of the cases by the major antecedent of PTB is required. We view as important to also stratify by gestational age at delivery in the analysis of future pregnancy cohorts. The protein profiles may be different in early pregnancy in women who have babies born at the lower extremities of PTB, as different pathways may be involved.[3] Despite these limitations, our cohort was very well characterized, with measurement of a tremendous range of protein markers very early in the PTB causal pathway.

In summary, we have used an innovative technology to uncover new proteins along several coalescing pathways related to the development of PTB. Moreover, using this approach, we have validated our previous findings of a role for the complement system in PTB[8] and prior studies of the coagulation pathway.[23] Moving forward, investigators should focus attention on the role of the complement and clotting cascades in PTB in the early pathogenesis of PTB. Ultimately, identification of protein(s) that can be targeted for therapeutic intervention, particularly early in the course of pregnancy, as described here, could provide an improved prognosis for the many women and their babies who suffer the consequences of PTB.

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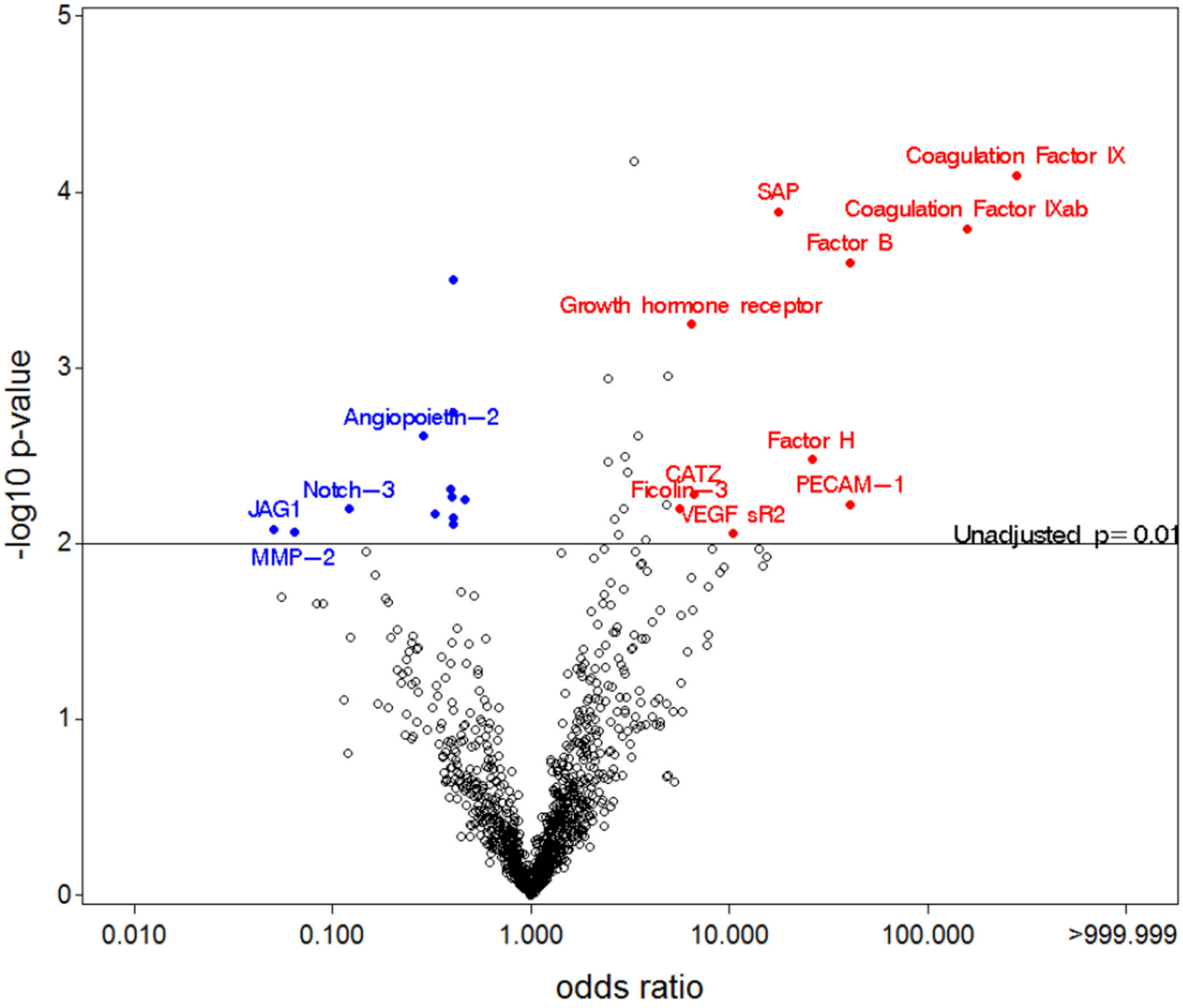

Figure 1. Significance and magnitude for association between PTB and all proteins

Volcano plot displaying the -log p-values (statistical significance) versus the odds ratio (magnitude of difference) for each individual protein. Those proteins which were significant (unadjusted $p < 0.01$ indicated by the horizontal line) and had an OR > 5 (red) or < 0.5 (blue) are distinguished by color.

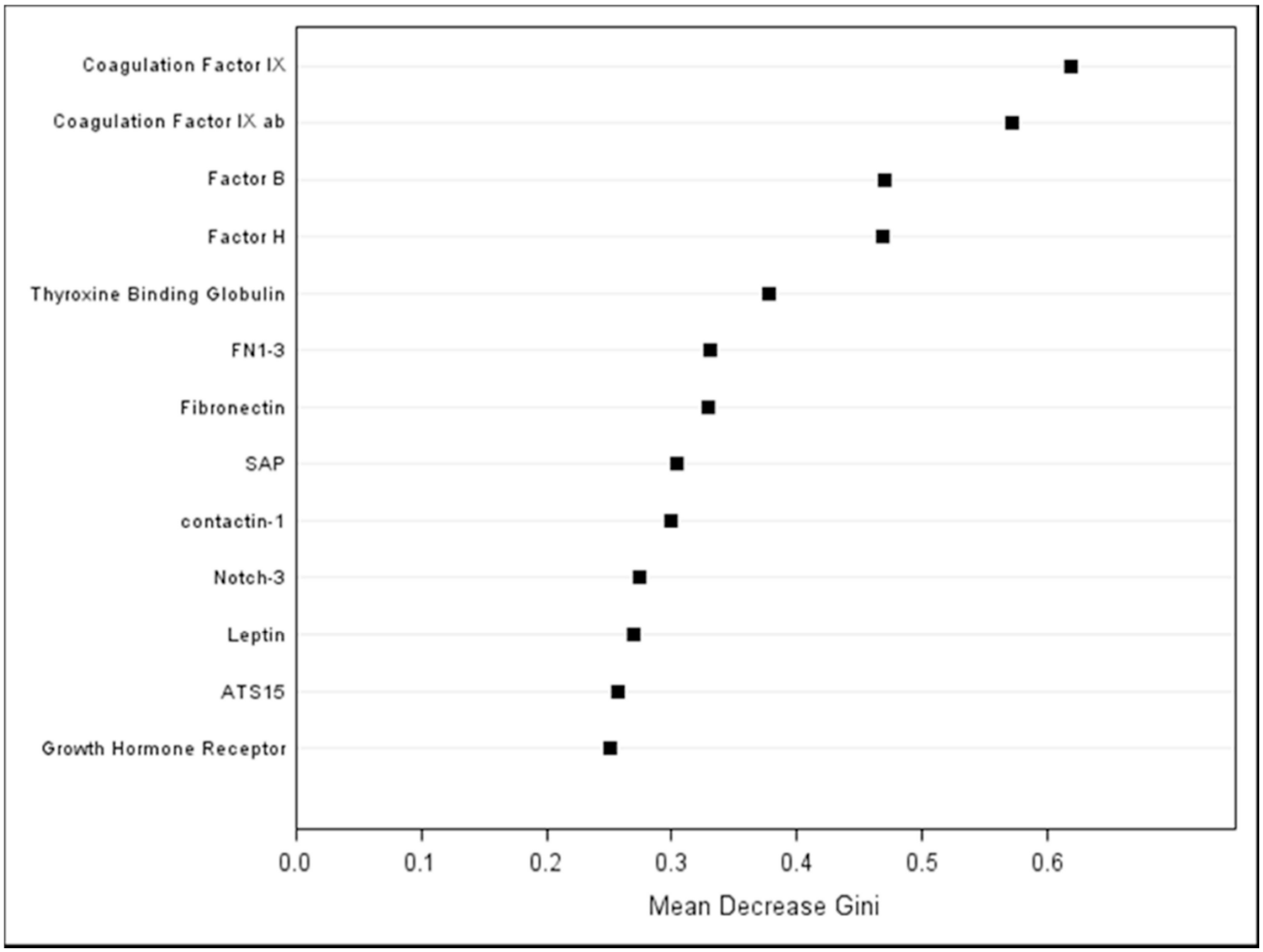

Figure 2. Variable importance plot from random forest

A variable importance plot is useful for visualizing the rank importance of variables. Mean Decrease Gini index identifies features that are ranked as more important for distinguishing women who had a PTB from women who had a term delivery. Features with a larger value indicate variables that contribute more information compared to random noise, the separation in the indices between predictor variables degrades as the features become less useful for distinguishing between groups.

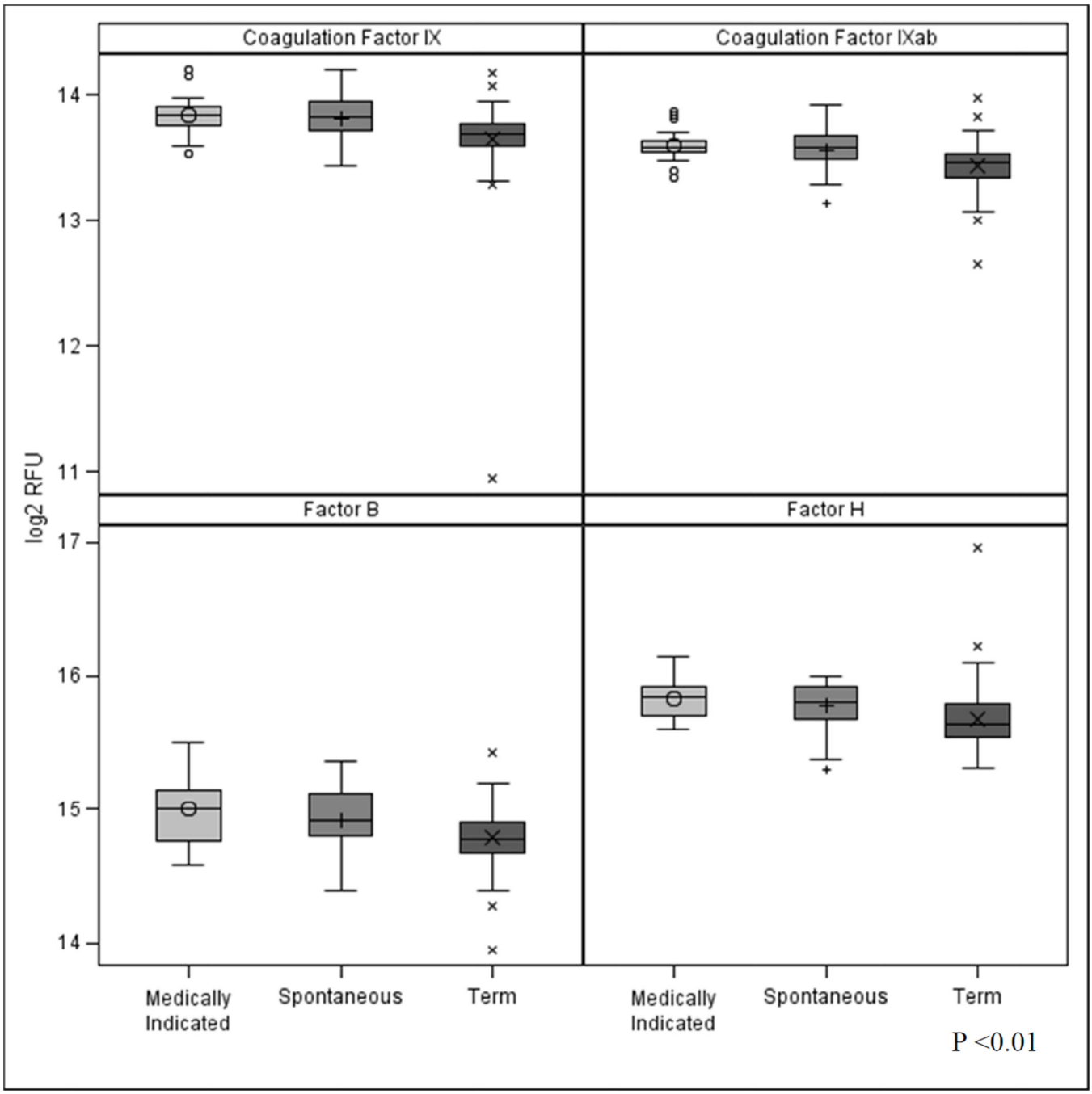

Figure 3.

Distribution of coagulation factors IX and IX ab and complement factor B and factor H across medically indicated PTB (n = 19), spontaneous PTB (n = 22) and term deliveries (n = 88) ($p<0.01$ for all four proteins). The box indicates the interquartile range (IQR: 25^{th} to 75^{th} percentile), the median and mean are indicated by the lines and points, respectively. The whiskers indicate data within 1.5 times the IQR and points are data outside 1.5 times the IQR. RFU = relative fluorescence units.

Table 1

Characteristics of Women with Preterm Birth and Term Deliveries

	Term Deliveries n = 88	All PTB n = 41	P Value
Mean maternal age ±SD (years)	34.2 (4.0)	33.7 (7.2)	0.60
Mean Gestational age at blood draw ±SD (weeks)	12.4 (1.0)	12.3 (1.1)	0.62
Race/ethnicity			
Non-Hispanic White	78 (89%)	24 (59%)	
Hispanic	7 (8%)	12 (29%)	
African American	1 (1%)	3 (7%)	
Asian and other race/ethnicities	2 (2%)	2 (5%)	< 0.01
Nulliparous	43 (49%)	16 (39%)	0.30

Table 2

The Unadjusted and Adjusted[1] Association of Proteins[2] Measured in Early Pregnancy with PTB

UniProt[3]	Target	Odds Ratio	95% CI		Unadjusted p-value	FDR[4] p-value
P00740	Coagulation Factor IX	280.7	17.04	4623	0.00	0.05
P00740	Coagulation Factor IX ab	158.4	11.41	2201	0.00	0.05
P00751	Factor B	40.66	5.59	295.8	0.00	0.06
P16284	PECAM-1	40.41	2.89	564.7	0.01	0.29
P08603	Factor H	26.16	2.96	231.1	0.00	0.26
P02743	SAP	17.81	4.08	77.78	0.00	0.05
P35968	VEGF sR2	10.51	1.81	61.01	0.01	0.31
Q9UBR2	CATZ	6.61	1.76	24.84	0.01	0.29
P10912	Growth hormone receptor	6.42	2.23	18.47	0.00	0.09
O75636	Ficolin-3	5.64	1.63	19.52	0.01	0.29
Q8TE58	ATS15	4.93	1.89	12.86	0.00	0.15
P22223	P-Cadherin	4.80	1.57	14.73	0.01	0.29
P36507	MP2K2	3.82	1.39	10.52	0.01	0.32
P10619	Cathepsin A	3.47	1.55	7.77	0.00	0.23
P41159	Leptin	3.30	1.83	5.93	0.00	0.05
P01031	C5a	3.06	1.43	6.53	0.00	0.28
P01833	PIGR	2.98	1.44	6.17	0.00	0.26
P63098	Calcineurin B a	2.94	1.36	6.37	0.01	0.29
O00408	cGMP-stimulated PDE	2.77	1.29	5.95	0.01	0.31
O60603	TLR2	2.65	1.30	5.41	0.01	0.29
Q9UK85	Soggy-1	2.46	1.35	4.50	0.00	0.26
P07949	RET	2.44	1.43	4.19	0.00	0.15
P78504	JAG1	0.05	0.01	0.46	0.01	0.31
P08253	MMP-2	0.06	0.01	0.50	0.01	0.31
Q9UM47	Notch-3	0.12	0.03	0.55	0.01	0.29
O15123	Angiopoietin-2	0.29	0.13	0.64	0.00	0.23
Q16644	MAPKAPK3	0.33	0.14	0.73	0.01	0.29
Q13219	PAPP-A	0.39	0.21	0.75	0.00	0.29

UniProt[3]	Target	Odds Ratio	95% CI		Unadjusted p-value	FDR[4] p-value
P02751	FN1.3	0.40	0.21	0.76	0.01	0.29
P18065	IGFBP-2	0.40	0.21	0.78	0.01	0.29
P08833	IGFBP-1	0.41	0.25	0.66	0.00	0.06
P02751	Fibronectin	0.41	0.23	0.72	0.00	0.20
P45985	MP2K4	0.41	0.21	0.79	0.01	0.30
P02790	Hemopexin	0.47	0.27	0.80	0.01	0.29

[1] This table only includes the proteins significantly related to PTB from the univariate analysis

[2] Adjusted for multiple comparisons

[3] UniProt = Universal protein resource (UniProt). Protein specific ID from a well-curated, centralized and freely accessible database:

[4] FDR= false discovery rate

Table 3

Ranking of Protein Pathways[1]

Rank	Pathway	Number of proteins in pathway
1	Complement cascade	24
2	Immune System	222
3	Formation of Fibrin Clot (Clotting Cascade)	21
4	Metabolism of proteins	33
5	Signal Transduction	236
6	Post-translational protein modification	15
7	PTM: gamma carboxylation, hypusine formation and arylsulfatase activation	12
8	Regulation of Insulin-like Growth Factor (IGF) Activity by Insulin-like Growth Factor Binding Proteins (IGFBPs)	17
9	Innate Immune System	120
10	Diabetes pathways	25
11	Integrin cell surface interactions	25
12	Hemostasis	119
13	Transcriptional Regulation of White Adipocyte Differentiation	5
14	Signaling by NOTCH	16
15	Signaling by NOTCH3	5
16	Integrin alpha IIb beta3 signaling	12
17	Activation of Genes by ATF4	3
18	Disease	137
19	Platelet Aggregation (Plug Formation)	16
20	Prolactin receptor signaling	6
21	Signaling by NOTCH1	14
22	Cytokine Signaling in Immune system	64
23	Growth hormone receptor signaling	10
24	Cell surface interactions at the vascular wall	38
25	Amyloids	13

[1]Top 25 ranked pathways. The proteins were classified into known pathways using Reactome, these pathways can overlap with each other and are hierarchical (i.e., one pathway could be a subset of another pathway). The ranking of each pathway was determined by combining the individual p-values from the logistic regression for the proteins in the pathway.