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The human plasma proteome during normal pregnancy

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Abstract

The human plasma proteome is underexplored, despite its potential value for monitoring health and disease. Herein, using a recently developed aptamer-based platform, we profiled 7,288 proteins in 528 plasma samples from 91 normal pregnancies (Gene Expression Omnibus identifier GSE206454). The coefficient of variation was <20% for 93% of analytes (median 7%), and cross-platform correlation for selected key angiogenic and anti-angiogenic proteins was significant. Gestational age was associated with changes in 953 proteins, including highly modulated placenta- and decidua-specific proteins, and they were enriched in biological processes including regulation of growth, angiogenesis, immunity, and inflammation. The abundance of proteins corresponding to RNAs specific to populations of cells previously described by single-cell RNA-Seq analysis of the placenta was highly modulated throughout gestation. Furthermore, machine learning-based prediction of gestational age and of time from sampling to a term delivery compared favorably with transcriptomic models (mean absolute error of 2 weeks). These results suggested that the plasma proteome may provide a non-invasive readout of placental cellular dynamics and serve as blueprint for investigating obstetrical disease.

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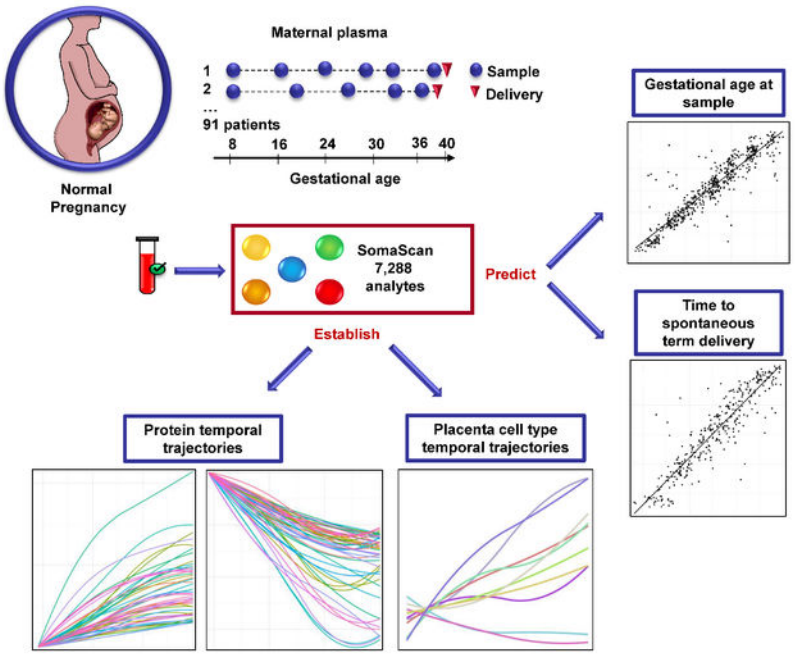
The authors declare no conflicts of interest.

Graphical Abstract



Keywords

aptamer; biomarker; machine learning; proteomic standards; single-cell RNA signature

INTRODUCTION

Prediction, prevention, and treatment of obstetrical diseases such as preterm labor, preeclampsia, small for gestational age (SGA), and fetal death are currently sub-optimal due to the syndromic nature and multiple etiologies of these conditions. Therefore, a personalized medicine approach is required to avoid dependence on non-specific clinical symptoms and signs. The success of such an approach depends on the accuracy, practicality, and low cost of generating patient-specific molecular readouts from non-invasive samples, such as the maternal blood.

High-throughput molecular studies of the maternal blood were proposed based on the analysis of whole-blood (cellular) RNA[1–5] or cell-free RNA[6–9], plasma or serum proteome[10–12], and metabolome[13–14], among other techniques. Often, in such studies, gestational age at venipuncture was used as a physiologic endpoint to assess the reliability of the omics platforms and to gauge their suitability prior to attempting the prediction of pathology such as preterm birth and preeclampsia[5, 7–8, 10, 15–17]. While the optimal blood omics platform to use in pregnancy is still a subject of research and may depend on the condition of interest, our earlier work suggests that plasma proteomics may have an advantage over cellular RNA for predicting spontaneous preterm birth[5].

Blood proteins were shown to be comprehensive indicators of human health as they are purposefully secreted as effectors of biological processes or they leak into circulation upon cell damage or death [18–19]. In pregnancy, in particular, proteins can also enter into the circulation from gestational tissues and therefore may reflect maternal adaptations to the developing fetus. For instance, human chorionic gonadotrophin (hCG) in human blood has allowed the early detection of pregnancy[20–30], while maternal alpha fetoprotein[31–34] is being used for biochemical screening of congenital anomalies[35–42]. Proteins with high modulation in the maternal circulation during normal pregnancy, including pro-angiogenic placental growth factor (PlGF) and anti-angiogenic vascular endothelial growth factor receptor (VEGFR)-1, also known as soluble fms-like tyrosine kinase-1 (sFlt-1), were shown to be dysregulated in preeclampsia[43–54], fetal death[55–56], SGA[57], and maternal floor infarction[52, 58–59]. Increasing sensitivity and specificity of prediction of such pregnancy complications would however require identification of additional biomarkers.

To enable highly multiplexed profiling of the human proteome, an aptamer-based platform was developed[60–61] and utilized in obstetrics by our group and others for proteomic profiling of the maternal plasma[5, 10–12, 16] and amniotic fluid[62], among many other proteomic studies in pregnancy[63–64]. The recently expanded version of the SomaScan® platform v4.1, which allows simultaneous profiling of 7,288 proteins, i.e. over one third of the human proteome[65], has not been applied in obstetrics. Therefore, we sought to 1) evaluate this high-throughput proteomic platform in pregnancy and define the expected protein values for gestational age and maternal characteristics, and 2) to determine the accuracy of the proteomic profiles for prediction of gestational age and time from venipuncture to spontaneous term delivery. We believe that such contribution has the potential to enable the development and implementation of predictive models in obstetrics.

MATERIALS AND METHODS

Study Design

Based on a prospective longitudinal biomarker study [54, 66], we conducted a retrospective analysis of 528 plasma samples collected from 91 women who had a normal pregnancy. Only singleton pregnancies without major medical or surgical complications, who delivered an appropriate-for-gestational-age infant, with a birthweight between the 10th and 90th percentiles, without major congenital anomalies were included in the study. Patients were enrolled at the Center for Advanced Obstetrical Care and Research of the Perinatology Research Branch, NICHD, the Detroit Medical Center, and Wayne State University. For each of the 91 women, 3 to 7 plasma samples were obtained from the first trimester up to two days before the spontaneous onset of term labor [median number of samples=6, interquartile range (IQR)=5–6]. Blood samples were collected in tubes containing EDTA and plasma was separated by centrifugation (1300 × g, 10 min). Plasma samples were immediately stored at −80 °C until proteomic analysis. Maternal plasma protein abundance was determined by using the SomaScan® platform v4.1 and its reagents.

All patients provided written informed consent, and the use of biological specimens and clinical and ultrasound data for research purposes was approved by the Institutional Review Boards of Wayne State University and NICHD.

Proteomics Techniques

Maternal plasma protein abundance was determined by using the SomaScan® platform v4.1, which is based on SOMAmer® (Slow Off-rate Modified Aptamer) reagents. This platform allowed multiplexed quantification of 7,288 analytes corresponding to 6,596 unique human protein targets in maternal plasma samples[60–61, 67]. Of these, 88.7% of proteins had one single assay, 10.4% had two, and less than 1% of proteins had 3 to 9 assays present on the platform. The experiments were run in batches of up to 85 samples per plate.

The plasma samples were diluted and then incubated with the respective SOMAmer® mixes pre-immobilized onto streptavidin-coated beads. The beads were washed to remove all non-specifically bound proteins and other matrix constituents. Proteins that remained specifically bound to their cognate SOMAmer® reagents were tagged by using an NHS-biotin reagent. After the labeling reaction, the beads were exposed to an anionic competitor solution that prevents non-specific interactions from reforming after dissociating.

Using this approach, pure cognate-SOMAmer® complexes and unbound (free) SOMAmer® reagents are released from the streptavidin beads using ultraviolet light that cleaves the photo-cleavable linker. The photo-cleavage eluate, which contains excess anionic competitor and all SOMAmer® reagents (some bound to a biotin-labeled protein and some free), was separated from the beads and then incubated with a second streptavidin-coated bead that binds the biotin-labeled proteins and the biotin-labeled protein-SOMAmer® complexes. The free SOMAmer® reagents were then removed during subsequent washing steps. In the final elution step, protein-bound SOMAmer reagents were released from their cognate proteins, using denaturing conditions. These SOMAmer® reagents were then hybridized to custom DNA microarrays. The Cyanine-3 signal from the SOMAmer® reagent was detected and measured on microarrays[60–61, 67]. Proteomics profiling was performed by SomaLogic, Inc. (Boulder, CO). In addition to the SomaScan® platform data generated herein, data for PlGF, sFlt-1 and soluble endoglin (sEng) were previously determined by immunoassays (R&D Systems, Minneapolis, MN, USA).[48] The inter- and intra-assay coefficients of variation of the assays were 1.4% and 3.9% for sFlt-1, 2.3% and 4.6% for sEng, and 6.02% and 4.8% for PlGF, respectively. The sensitivity of assays were 16.97 pg/ml for sFlt-1, 0.08 ng/ml for sEng, and 9.52 pg/ml for PlGF. Sample collection methods, biospecimen processing, and validation of the assays used were previously reported in greater detail [54, 68].

Statistical Analysis

Data reproducibility: The proteomic data preprocessing, including an adaptive normalization by maximum likelihood (ANML) step and a calibration step, were performed by SomaLogic, Inc. The goal of these steps was to make data comparable across samples by calculating plate-specific and analyte-specific scale factors. Based on such scale factors, a quality control flag was assigned to each sample and each analyte[67]. Using preprocessed data for samples and analytes that passed quality controls, the Spearman's correlation coefficient and coefficient of variation for each protein were determined based on 14 samples collected from 2009–2010 and profiled in duplicates in different batches. These samples spanned the full range of gestational ages considered (10.4–39.4 weeks), capturing gestational age-related variability in the proteome, and hence provided an opportunity to

observe correlations between duplicate values. The coefficient of variation from duplicates was determined by a method that accounted for the measurement error being potentially dependent on the mean protein abundance[69]. Proteomic data and sample annotation is available from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE206454).

Determining sources of variability in the meta-proteome: The proteome data for all 519 samples and 6,277 analytes that passed quality checks were analyzed using principal components (PC) analysis to reduce the dimensionality of the proteins to a few PCs (meta-proteins) using the *pca* function in the *PCAtools* R package. Next, the *eigencorplot* function from the same package was used to determine Pearson's correlation coefficients between each PC and maternal characteristics and gestational age. A p-value <0.05 was considered a significant result.

Differential abundance analysis: Protein abundance expressed as relative fluorescence units (RFU) was log (base 2) transformed to improve normality. Linear mixed-effect models with quadratic splines (one knot) were used to model protein abundance as a function of gestational age. Briefly, such a model assumes that $\log_2$ protein data throughout one-half of the gestational age span can be adequately modeled using a quadratic function of gestational age. Maternal age, parity, body mass index (BMI), and smoking status were considered as covariates and retained if they improved the model fit for a given protein. Covariate selection was based on the significance ($p<0.05$) of likelihood ratio tests implemented in the *glmerselect* function available in the *StatisticalModels* package under the R statistical language and environment (www.r-project.org). The models included patient identifiers as random effects to account for the repeated and likely correlated measurements from the same patient. Protein abundance was considered to have changed significantly with gestational age if the fold change was >1.25 and false discovery rate (FDR)[70] adjusted p-value (q-value) was <0.1. Fold change was defined as the ratio of highest to lowest mean protein abundance across the 9 to 40 weeks gestational age span. Linear mixed-effects models were fit using the *lme4* package[71].

Clustering proteins by average profile: The expected protein abundances determined by linear mixed-effects models across the 9 to 40 weeks of gestational-age span were used to perform hierarchical clustering of protein trajectories. A correlation distance measure was used in the clustering so that proteins with a similar trend vs. gestation but possibly different magnitude of changes were clustered together. Clustering was performed with the *WGCNA* package[72].

Gene ontology enrichment analysis: Proteins were mapped to Entrez gene database[73] identifiers based on SomaLogic, Inc. protein annotation, and then to gene ontology[74]. Biological processes over-represented among a given list of proteins (e.g. those differentially expressed with gestational age) were identified by using Fisher's exact tests. Gene ontology terms with three or more hits and having an adjusted enrichment q-value <0.1 were considered significantly enriched. Enrichment analysis was performed with the *GOStats* package[75] in Bioconductor[76]. The reference list used in all enrichment analyses was the

list of genes corresponding to the proteins profiled in the study, which is in line with best practices in the field[77–78]. This ensures that the enrichment we attributed to differential abundance with advancing gestation is not confounded by any biases in the design of the SomaScan platform relative to the universe of all known human proteins.

Quantification of single cell RNA-seq signatures of the placenta: To quantify the expression of previously defined signatures of cell sub-types identified by single cell RNA studies of the placenta[79], we have first selected the RNAs specific (most highly abundant 20 RNAs) to a given cell type that had a corresponding protein in our dataset. Then, the $\log_2$ protein abundance was transformed into a Z-scores based on the mean and standard deviation observed in samples at 8–16 weeks for each protein. Average Z-scores for each signature were used as a summary and further tested for association with gestational age at venipuncture using the same types of linear mixed-effects models described above for analysis of data of individual proteins.

Prediction of gestational age and time from sample to delivery: Predictive models for gestational age at venipuncture were based on all samples profiled, while prediction of time from venipuncture to delivery was limited to samples from patients who had a spontaneous term delivery. Prediction models were fit and evaluated using a leave-one-out cross-validation procedure. With this approach, a random forest model[80] was fit using data from all but one patient including all corresponding longitudinal samples, and it was then applied to the data of the patient left out during model training. Lasso regression[81], a procedure designed for fitting a continuous response variable (e.g. gestational age) using more predictors (i.e. proteins) than available samples, was utilized for multi-variate protein selection, and data for the selected proteins were used as input in random forest models. Prediction performance metrics were the Pearson correlation coefficient between actual and predicted values. The root mean squared error, i.e., the standard deviation of prediction errors (error=actual - predicted), and mean absolute error were also determined to enable direct comparison with previous reports. Lasso regression and random forest models fitting were implemented in *glmnet* and *randomforest* packages, respectively under the R statistical language and environment.

RESULTS AND DISCUSSION

Proteomic signals in the maternal blood are known to be correlated with both physiologic and pathologic endpoints in pregnancy. Large studies of maternal blood proteins were based on targeted profiling of specific angiogenic (PlGF) and anti-angiogenic factors (sFlt-1 and sEng)[52, 61, 82], or targeted profiling of pro-inflammatory proteins, cytokines, and chemokines[83–85]. Given the sub-optimal performance of current biomarkers for early prediction of obstetrical complications, high-throughput discovery platforms have been proposed to identify novel candidate biomarkers. Using earlier versions of the SomaScan® platform, measuring up to 1,310 proteins (v2 and v3), physiologic changes with gestational age[12], as well as pathologic perturbations in preeclampsia[10–11, 16], placenta accreta spectrum[86], and spontaneous preterm birth[5], have been reported, hence spurring the interest in this omics platform. In the current study, we utilized the SomaScan® platform v4.1 to

generate data for 7,288 proteomic assays in blood samples collected longitudinally from pregnant women throughout gestation.

Characteristics of the Study Population

The study population included 91 pregnant women, 32% (29/91) of these were nulliparous, the median maternal age was 23 years (IQR: 20–26), and the median BMI was 25.8 kg/m^2 (IQR: 22.5–30.5). All patients delivered at term gestation [median gestational age of 39.6 (IQR: 38.8–40.7) weeks] appropriate-for-gestational-age[87] neonates with a median birth weight of 3,400 (IQR: 3,137.5–3,702.5) grams (see Supplementary Table 1). The generation of data in a majority (92%) African-American population is important given the higher rate of pregnancy complications in this group of women. However, a more diverse cohort would have been ideal.

Proteomic Data Reproducibility

Of the 528 samples profiled, data for 519 (98.3%) of the samples passed the quality control checks, while of the 7,288 human protein assays, 6,277 (86%) passed the calibration filter and were included in the analyses. Based on data collected from 14 duplicate samples, the median of the Spearman's correlation coefficients of protein abundance was 0.77 (IQR 0.64%-0.87%), and of the coefficients of variation was 7% (IQR 4.5%-11.4%). Of importance, the coefficients of variation were below 20% for 93% of the assays. The median coefficient of variation found herein was somewhat higher than the 5% previously reported for a lower level of multiplexing of 4,000 proteins[88]. This can be explained in part by the higher multiplexing, the longer storage duration of the blood samples prior to profiling, and perhaps the lower biological variability in the current study compared to that in the study of Tin et al. [88] which included both male and females of various ages. Longer storage time is expected to negatively affect the reproducibility, while higher biological variability is essential to put in perspective the magnitude of technical noise in the data relative to biological variability.

Moreover, the proteomic data from the SomaLogic SomaScan® Platform v4.1 was well correlated with enzyme-linked immunosorbent assay (ELISA)-based measurements for key biomarkers in pregnancy, including PlGF (ρ=0.87) and sFlt-1 (ρ=0.77), but only modestly for sEng (ρ=0.38) ($p<0.001$ for all Figure 1).

The high correlation of individual patient data between SomaScan and ELISA-derived data for PlGF and sFlt-1, currently used in screening to prevent preeclampsia[89–90], further supports the utility of this platform for research in obstetrics. The lower correlation between ELISA-based concentrations and SomaScan for sEng may be reflective of differences in epitopes being recognized between technologies and possible modifications such as misfolding, protein-protein interactions, and impact of genetic variants in protein structure. ELISA-based sEng was shown to add predictive value relative to PlGF and sFlt-1 when distinguishing between women with chronic hypertension from those who develop superimposed preeclampsia[54], and therefore, further studies on the value of SomaScan-derived sEng measurements in obstetrics is warranted.

Global Sources of Variation in the Maternal Plasma Proteome

Next, we aimed to assess the primary sources of variability in the plasma proteome by deriving PCs and correlating such meta-proteins with gestational age at venipuncture and maternal characteristics. We found a substantial modulation of proteomics data with advancing gestational age and, to a lesser extent, with maternal characteristics (Figure 2). Six of the top 10 PCs (ranked by % of variance explained), which explained 38% of the variance in the data (Figure 2A), were significantly correlated with gestational age at venipuncture ($p<0.05$ for all) (Figure 2B). As an example, the Pearson correlation of the PC4 and gestational age was $\rho=0.66$, and PC6 was $\rho=0.36$ (Figure 2 B, C, D). PCs derived from proteomics data were also correlated with maternal BMI (7/10 PCs), parity (5/10 PCs), and maternal age (2/10 PCs) ($p<0.05$ for all), although the magnitude of such correlations was lower than that observed for gestational age at venipuncture (e.g., Pearson $\rho=-0.24$ for PC3; $\rho=-0.22$ for PC4 for correlation with BMI) (Figure 2, B, E, F).

Protein Level Changes with Gestational Age

Subsequently, we analyzed the data from individual proteomic assays in relation to gestational age and maternal characteristics by fitting multivariate linear-mixed effects models. Of the 6,277 human protein targets that passed quality control filters, 953 (15.2%) changed in abundance as a function of gestational age while accounting for maternal characteristics (fold change >1.25 and q-value <0.1) (Figure 3a, Supplementary File 1 and Supplementary File 2).

The sizable fraction of the maternal plasma proteins modulated in abundance as a function of gestational age in normal pregnancy can be understood as protein abundance reflects both fetal development and maternal adaptations throughout gestation. This result is in line with the previously reported estimate (10% of proteins modulated during gestation), when considering that the later estimate was obtained using more conservative cut-offs ($q<0.1$, fold-change >1.5)[12]. The fraction of blood proteins modulated with advancing gestation that we report herein is about one order of magnitude higher than that in whole blood RNA (2.3%) determined in a similar population based on comparable sample size, modeling methods, and the same significance cut-offs[91]. This suggests that the plasma proteome could be a more abundant source of disease biomarkers relative to the cellular transcriptome, given the known association between modulation with advancing gestation in normal pregnancy and dysregulation in obstetrical disease, such as preeclampsia (odds ratio = 4.3)[11].

The $\log_2$ fold changes in protein abundance with gestational age were highly correlated with estimates derived from an independent set of patients profiled by using a lower throughput version (v3) of the SomaScan® platform[12]. This finding was based on all proteins with significant change with gestational age in the current study that were also measured in Romero et al. 2017[12] (N=255 proteins, $\rho=0.89$, $p<0.01$, see Figure 3B). Not only specific proteins were confirmed herein to be highly modulated with gestational age (>5-fold change), such as PlGF, Siglec-6, glypican-3, and Prolactin, but also the magnitude of changes were highly correlated between studies, hence providing an *in-silico* validation of the current results, despite a small fraction [9.4% (24/255)] of proteins with opposite direction of change between studies. However, the current study has identified almost 9-fold

as many proteins changing with gestational age than in the previous report[12]. Notably, several proteins assayed by the newly developed SomaScan® v4.1 platform, but not by the previous platform version, were highly modulated with gestational age and were placenta- and decidua-specific. These included: ABP1 (amiloride-sensitive amine oxidase, copper-containing), DLK1 (Protein delta homolog 1), EMBP (bone marrow proteoglycan), IGSF3 (immunoglobulin superfamily member 3), and SVEP1 (sushi, von Willebrand factor type A, EGF, and pentraxin domain-containing protein) (fold change >5 for all, see Supplementary File 1).

SVEP1, a protein highly expressed in the human placenta[92], has been characterized as an extracellular matrix protein important for cell adhesion[92] and plays a role in lymphangiogenesis[93–94], septic shock[95–96] and endotoxinemia[97], atherosclerosis, and coronary artery disease[98–100]. ABP1, also called diamine oxidase (DAO), is regulated by estrogens and is mainly localized in the decidua[101–102]. Maternal plasma ABP1 levels rise exponentially during the first 20 weeks of gestation and are potentially indicative of fetoplacental integrity[103–104]. IGSF3, also known as EWI-3, is widely expressed in the placenta, kidney, and lung. Although placental gene expression of IGSF3 has been described in a rat model of placental insufficiency[105], data in pregnant human subjects are lacking. Pregnancy-associated plasma protein A (PAPP-A) and the proform of eosinophil major basic protein (EMBP) are produced by the placenta.. EMBP has been implicated in placenta-mediated obstetrical syndromes such as preeclampsia[106], SGA[107], and preterm birth[108]. DLK1, also known as fetal antigen 1 and pre-adipocyte factor 1, is a transmembrane protein encoded by the DLK1 gene expressed in the placenta, yolk sac, fetal liver, adrenal cortex, and pancreas and in the beta cells of the islets of Langerhans in the adult pancreas[109]. In the placenta, DLK1 is specifically expressed by the stromal cells of the villi that are in close contact with the vasculature[109–110] and has been identified as a potential biomarker of fetal growth restriction[111–112].

Distinct Types of Longitudinal Trajectories and Functional Profiling

Given the complex patterns of protein abundance modulation during normal pregnancy (see examples in Supplementary File 2), we sought to identify clusters of proteins based on the similarity of their longitudinal trajectories. Figure 4 depicts the trajectories of the top 50 most highly modulated proteins in each of the three protein clusters identified in this analysis.

Cluster 1 includes 249 proteins with a trajectory characterized by a steady increase in abundance throughout gestation. Member proteins were specifically associated with regulation of growth, angiogenesis, immune (e.g., T-helper 1 type immune response) and inflammatory processes (e.g., regulation of macrophage cytokine production) (Figure 5, Supplementary File 3). Cluster 2 includes 151 proteins with trajectories that had an early decrease or remained unchanged early in pregnancy followed by an increase later in pregnancy. Cluster 2 proteins were involved in the regulation of blood vessel remodeling, response to steroid hormone, and metabolic and catabolic processes (Figure 5, Supplementary File 3). Cluster 3 includes 522 proteins that demonstrate an overall decrease in abundance throughout gestation and were associated with various processes ranging from

immune and defense response to hemostasis. The complexity of immune and inflammatory processes regulation is highlighted by the involvement of proteins with both increasing (Cluster 1 and 2) and decreasing (Cluster 3) trajectories (Figure 5).

Placental Single-Cell RNA Signatures are Modulated with Advancing Gestation

We have next evaluated whether the meta-proteome corresponding to RNAs specific to previously described placental-derived cell types changes with advancing gestation and hence can provide a non-invasive molecular readout of their activity in the placenta. Figure 6 shows that the activity of placental cell types undergoes a complex modulation as captured by the corresponding plasma protein abundance. Among the top ten single-cell signatures most strongly associated with gestational age, the signatures of B cells, cytotrophoblasts, dendritic macrophages, extravillous trophoblast, and stromal cells were increased, while the signatures of hematopoietic stem cell and monocytes were decreased ($q<0.1$). The decidual cell signature displayed a complex pattern of modulation culminating to an increase at term gestation (Figure 6). Tracking placental single cell signatures throughout gestation was previously shown using amniotic fluid cell free RNA[113] and maternal blood cell free[6] and cellular RNA[114]. Here we show that maternal plasma proteome also captures placenta cell population activity during gestation.

Prediction of Gestational Age at Venipuncture and of Time-from-Sample-to-Delivery

Given the strong modulation of maternal plasma proteins during normal pregnancy, we sought to determine whether gestational age could be ascertained by using machine learning methods based solely on proteomic profiles of pregnant women. Indeed, random forest models, trained using proteins selected by lasso regression and evaluated via cross-validation, significantly predicted the gestational ages at venipuncture in patients not included during protein selection and model training (test sets). The Pearson correlation between actual and predicted gestational ages was 0.92 ($p<0.001$), the root mean squared error (RMSE) of predictions was 3.1 weeks, and the mean absolute error (MAE) was 1.99 weeks (Figure 7A). Of note, the samples with the largest (top 10%) gestational age prediction errors, among those shown in Figure 7A, did not tend to cluster by patient, nor by specific maternal characteristics, suggesting lack of systematic biases.

We next used the proteomics data to predict the time from venipuncture to delivery using the data form 334 samples collected from the subset of 61/91 women included in the study who had a spontaneous term delivery, hence excluding the cases for whom the pregnancy was truncated by a selective cesarean section. The accuracy of the random forest model for this analysis was similar to that of the model for prediction of gestational age (Pearson correlation 0.92, $p<0.001$; RMSE=3.0 weeks; MAE=2.15 weeks, Figure 7B). This suggests that eventual biases in the gold standard of gestational age, defined by last menstrual period (LMP) and ultrasound, were minimal. The fraction of term deliveries predicted within one week of the actual delivery was 26%, 33%, and 43% based on samples collected in the first, second, and third trimesters, respectively, while LMP and ultrasound-based accuracy was 57%, almost identical to the 55.1% reported in the literature[115].

Finally, when 42 of the best protein predictors of time from venipuncture to a term delivery identified herein, that were also profiled in Romero et al. 2017[12], were used to predict time from sample to delivery in the Romero et al. 2017 dataset, the cross-validated prediction was still high (Pearson correlation 0.95, RMSE=2.99 weeks; MAE=2.35 weeks), suggesting external validity of the protein signature predicting time from sample to delivery in the absence of obstetrical disease. The correlation between the plasma proteome and the time from venipuncture to delivery assessed via multi-variate models (Pearson correlation of predicted vs. actual, ρ=0.91) was stronger compared to estimates obtained using an unbiased analysis of whole blood transcriptome via microarrays (ρ=0.83)[5]. This is in line with previous evidence of higher accuracy for the plasma proteome compared to whole blood transcriptome for prediction of subsequent spontaneous preterm birth based on analyses of the same blood samples[5]. SomaScan-based prediction of time from sampling to term delivery, however, was similar to estimates reported based on more than 100 placenta-, immune-, and fetal liver-specific cell-free RNAs profiled by real-time polymerase chain reaction in maternal circulation (ρ=0.89)[7]. The stronger prediction reported in the studies that used cell-free RNA compared to those using cellular transcriptomics can be explained by the fact that cell-free transcripts are more likely to be derived from the placenta, fetus, and maternal reproductive tissues[116], and also by the targeting of analysis based on biological plausibility. Indeed, when only proteins found herein to be predictive of time from sampling to delivery were used to narrow the pool of candidate predictor proteins in the Romero et al. 2017 dataset[12], the accuracy was slightly better (Pearson correlation 0.95) compared to the estimate (Pearson correlation 0.92) obtained using an unbiased analysis. Other approaches to narrow the search of biomarkers based on omics profiles leverage single cell-derived signatures of the placenta[6, 79, 113–114, 117–118].

The Effect of Maternal Characteristics on Protein Abundance

Among all the maternal characteristics and obstetrical history covariates considered, the maternal BMI had the strongest effect on the plasma protein abundance. Indeed, of the 6,277 human protein targets that passed quality control filters, 211 (3.4%) were significantly associated with BMI. These included fatty acid-binding protein (FABP), leptin, insulin-like growth factor-binding protein 1 (IGFBP-1), PlGF, and matrix metalloproteinase (MMP)-7 ($q<0.1$, Supplementary File 4). The top two proteins in this analysis, FABP3 and LEP (Supplementary File 4), were also among the top three most important predictors the percentage body fat out of 4000 proteins profiled with the SomaScan® platform in an independent study of 6,000 individuals[18]. Leptin, an appetite and metabolism regulator[18], was described also as a marker of placental function[119], and was implicated in several pathologies in pregnancy such as gestational age diabetes[120] and preterm delivery[121]. FABP3, expressed in adipocytes, is strongly linked to metabolic and inflammatory pathways[18]. Biological processes associated with BMI-modulated proteins included eating behavior, response to nutrient levels, regulation of growth, and inflammatory-related processes. In multivariate models, nulliparity was associated with an increase in Trefoil factor 3 (TFF3) and Serine/threonine-protein kinase (DCAK1) and with a decrease in Mesoderm development candidate 1 (MESD1). Moreover, maternal age was associated with a decrease in Collagen alpha-2(XI) chain (COL11A2) and with an increase in Spectrin alpha chain, non-erythrocytic 1 (SPTA2). These results suggest that among maternal

characteristics, maternal BMI, in particular, needs to be accounted for in analyses involving pathology to avoid confounding of effects.

Proteomic Standards to Enable the Discovery of Disease-Related Proteomic Perturbations

Last, given the current and potential use of maternal plasma protein dysregulation in predicting obstetrical complications, we sought to generate proteomic standards that would allow the comparison of data across studies and enable the discovery of new disease-related protein perturbations. To this end, we fitted the protein relative fluorescence data ($\log_2$ thereof) as a function of gestational age and maternal characteristics so that the expected levels could be determined and used to derive multiple of the mean values. The approach to convert raw protein concentration data into multiples of the mean or median for gestational age and maternal characteristics and obstetrical history is currently being used in obstetrics as a way to overcome assay- and population-specific biases[122–125]. For instance, for preeclampsia screening, the *a priori* risk of disease can be derived from maternal characteristics and obstetrical history (prior risk factors). This risk estimate, is then combined with biomarker-based risk scores that need to be independent of prior risk factors so that the two probabilities can be combined into a posterior risk of developing the disease[54, 82, 126].

The proteomics standards proposed herein were implemented and made available as an *R* software package called *SomaPreg*, which is available from the author's website at http://bioinformaticsprb.med.wayne.edu/software/ and also as Supplementary File 5. The functionality of the software package is illustrated in Figure 8 and Supplementary File 6. Blood sample annotation data, paired with normalized proteomic data (RFU) are used as inputs (Figure 8A). The expected proteomic abundance is determined from prediction models stored in the software package. Such models also allow exploring the effect of maternal characteristics (Figure 8B) and calculating multiple of the mean values for gestational age and maternal characteristics (Figure 8C). This functionality encapsulated in the *SomaPreg* package is expected to facilitate the discovery of proteomic disease signatures and the implementation of risk prediction models by removing physiologic variability from proteomic signals.

Previous work suggested that the normalization of RFUs via internal control samples present on the SomaScan® platform already allows for a significant prediction of gestational age at venipuncture across batches and cohorts[16]. However, prediction of preeclampsia across cohorts was not feasible, likely due to the heterogeneity of the disease and much weaker within-cohort proteomic dysregulation with preeclampsia than that with gestational age. Improving cross-cohort prediction of disease based on biomarkers requires transforming the data into multiple of the mean (MoM) values to account not only for gestational age but also maternal characteristics that affect such measurements in control pregnancies. Risk models in pregnancy based on MoM-transformed biophysical and biochemical data were previously described[54, 82, 90, 127].

CONCLUSIONS

Herein, we have presented the most comprehensive characterization of the maternal plasma proteome in normal pregnancy. The proteome undergoes dramatic modulation with advancing gestation and is substantially affected by maternal body mass index. The models we have proposed and implemented in freely available software may enable the discovery of disease-related perturbations and the implementation of disease-prediction models in obstetrics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ANML	adaptive normalization by maximum likelihood
APB1	amiloride-binding protein 1
BMI	body mass index
DAO	diamine oxidase
DLK1	delta-like homolog 1
ELISA	enzyme-linked immunosorbent assay
EMBP	bone marrow proteoglycan
FABP	fatty acid-binding protein
FDR	false discovery rate
hCG	human chorionic gonadotrophin
IGSF3	immunoglobulin superfamily member 3
IQR	interquartile range
LMP	last menstrual period

MAE	mean absolute error
MMP	matrix metalloproteinase
MoM	multiple of the mean
PAPP-A	pregnancy-associated plasma protein A
PC	principal component
PlGF	placental growth factor
RFU	relative fluorescence unit
RMSE	root mean squared error
sFlt-1	soluble fms-like tyrosine kinase-1
sEng	soluble endoglin
SGA	small for gestational age
SOMAmer®	slow off-rate modified aptamer
SVEP1	sushi, von Willebrand factor type A, EGF, and pentraxin domain-containing protein 1
VEGFR	vascular endothelial growth factor receptor

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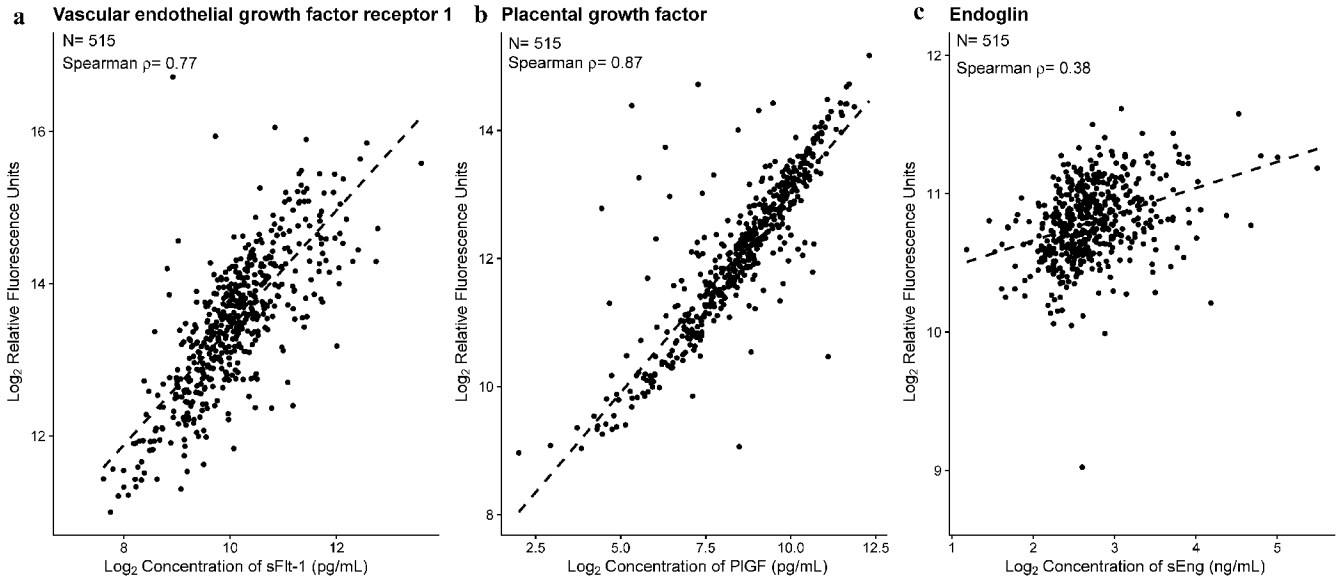


Figure 1: Agreement between ELISA and SomaScan® measurements for key angiogenic and anti-angiogenic proteins.

Protein abundance for 515 samples are shown, with one dot for each sample. The SomaScan relative fluorescence units in (log, base 2) (y-axis) is shown vs. ELISA based concentrations (log, base 2) (x-axis) for sFlt-1 (A), PlGF (B) and sEng (C). ρ is the Spearman's correlation coefficient. Correlation test $p<0.001$ for all three proteins.

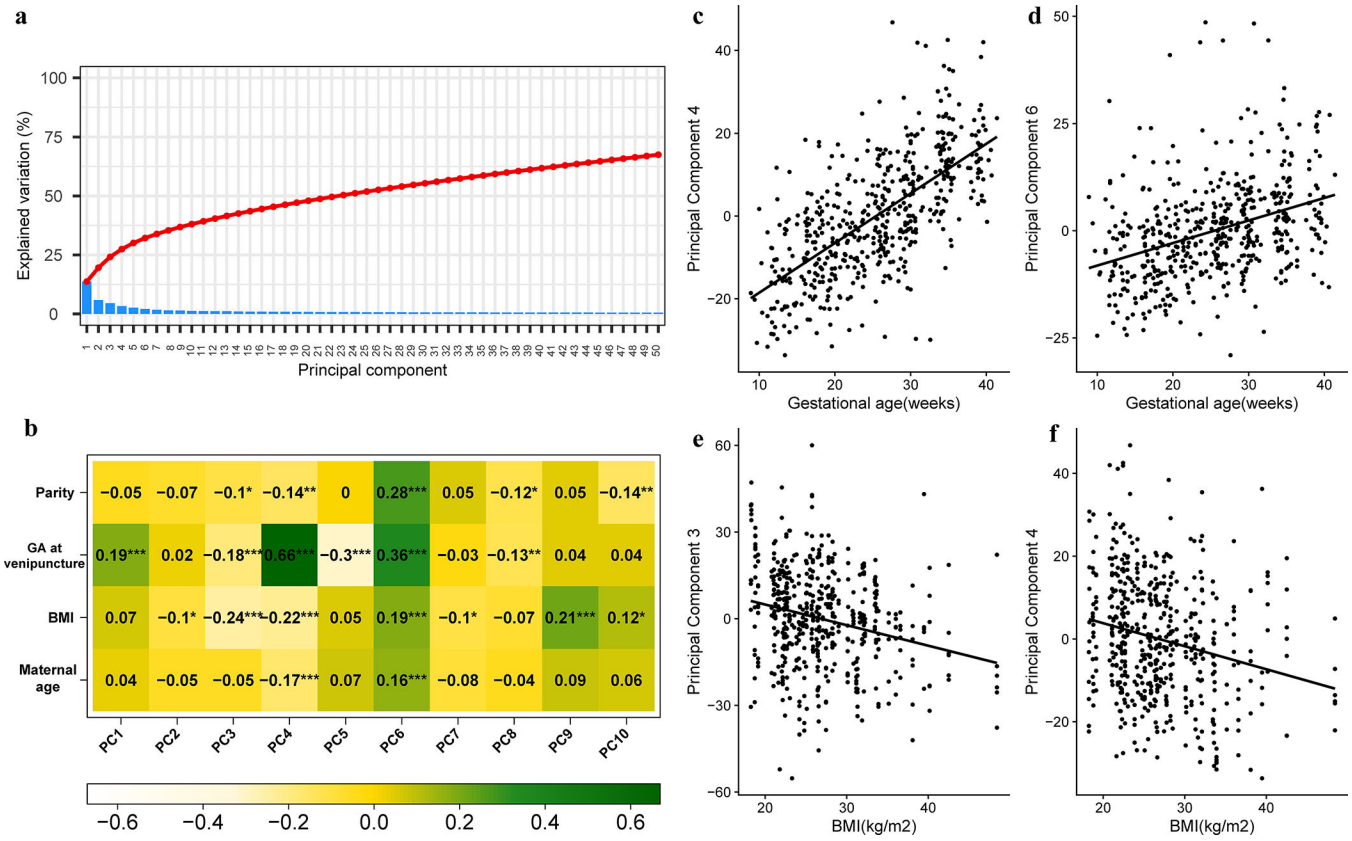


Figure 2: Principal component analysis of 6,277 proteins and correlation with gestational age and maternal characteristics.

Protein abundance in relative fluorescence units (log, base 2) was analyzed using principal components (PC) analysis. The resulting principal components can be understood as meta-proteins. A) The % of variance explained by each principal component is shown as a scree plot. (B) The top 10 principal components were correlated with maternal age, parity, gestational age (GA) and body mass index (BMI). The heatmap shows Pearson correlation coefficients between PC and covariates (significance levels: *<0.05, **<0.01, ***<0.001). The correlation between PC4 (C) and PC6 (D) with gestational age is also shown, with each dot representing one sample. Similar correlations are shown for PC3 (E) and PC4 (F) in relation with BMI.

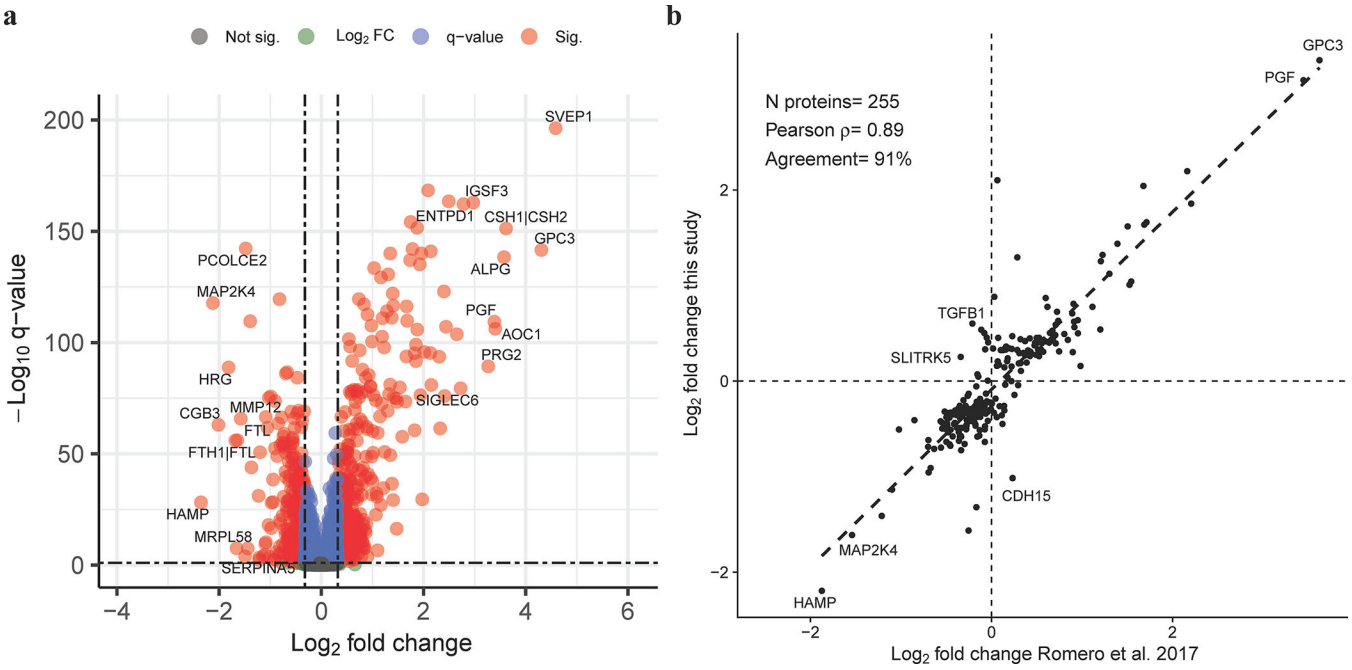


Figure 3: Proteomic changes with advancing gestation in normal pregnancy.

The volcano plot (A) shows the significance (y-axis) vs. magnitude of change (x-axis) for each protein. Protein with significant modulation (adjusted p-value, q <0.1 and fold change >1.25, N=953 proteins) are shown in red. The names of a select set of most significant proteins are also displayed. The correlation between fold changes (log, base 2) from 10 weeks to 32 weeks of gestation and similar results based on Romero et al. 2017 study is shown for 255 proteins deemed significant in this study and profiled in Romero et al. 2017 using SomaScan® platform v3. The gene symbols of the top increased and decreased proteins with concordant direction of change between studies are listed in the figure. The same is true for top three proteins with the most discordant fold change between studies.

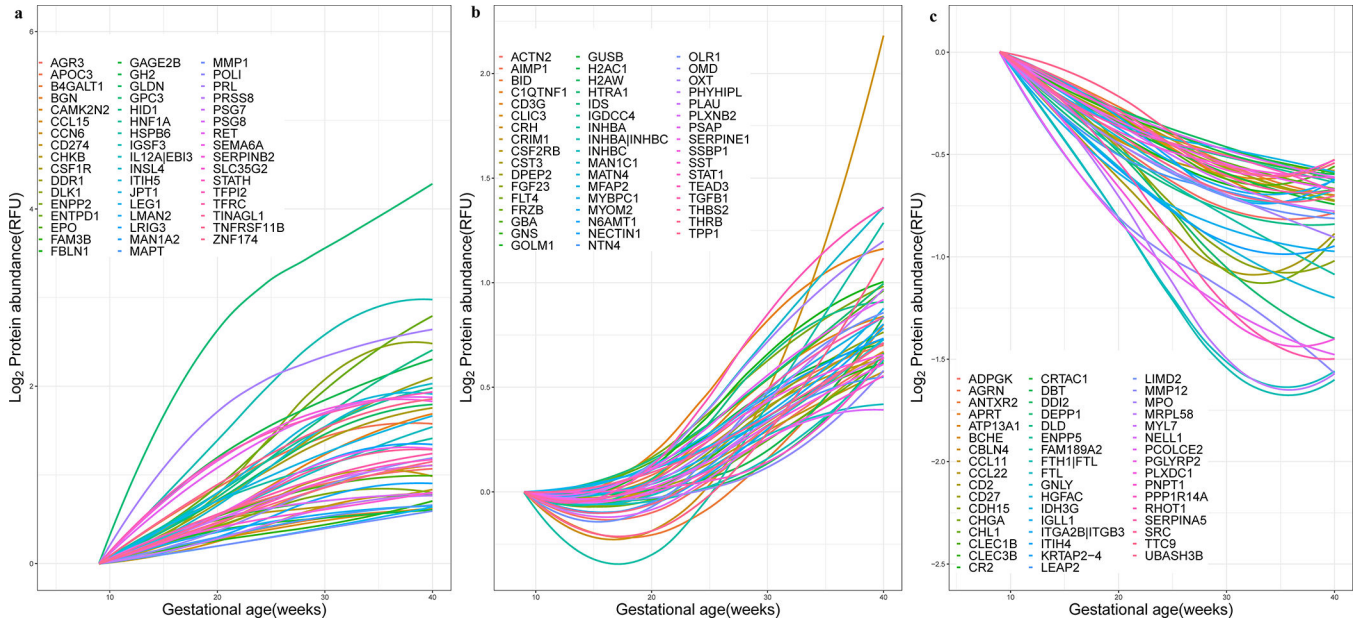


Figure 4: Maternal plasma proteomic trajectories throughout gestation.

The figure shows three clusters of proteins with steady increase (A), slow increase or decrease early in pregnancy followed by an increase later in pregnancy (B), and overall decreasing trend (C). The 50 proteins most representative of each cluster are shown, with names displayed representing corresponding gene symbols. See Supplementary File 2 for a depiction of the raw data used to derive the protein trajectories for selected proteins.

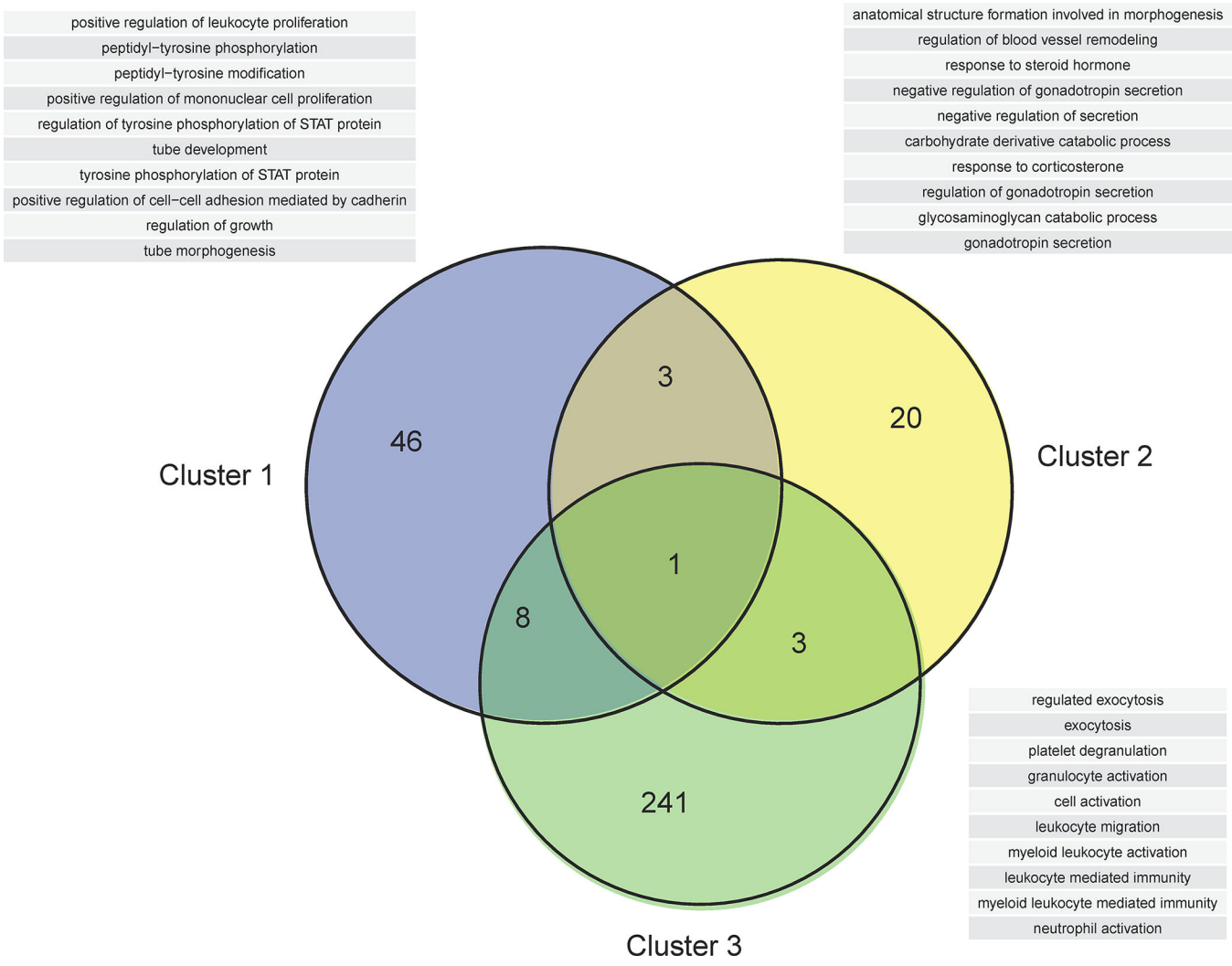


Figure 5: Biological processes associated with maternal plasma protein modulation with gestational age.

The Venn diagram shows the overlap in biological processes significantly associated with differentially modulated proteins for each cluster illustrated in Figure 4. See also Supplementary File 3 for the full list of biological processes associated with gestational age modulation. The list of the top 10 biological processes (ranked by enrichment p-value) for each Venn diagram category is shown in a table.

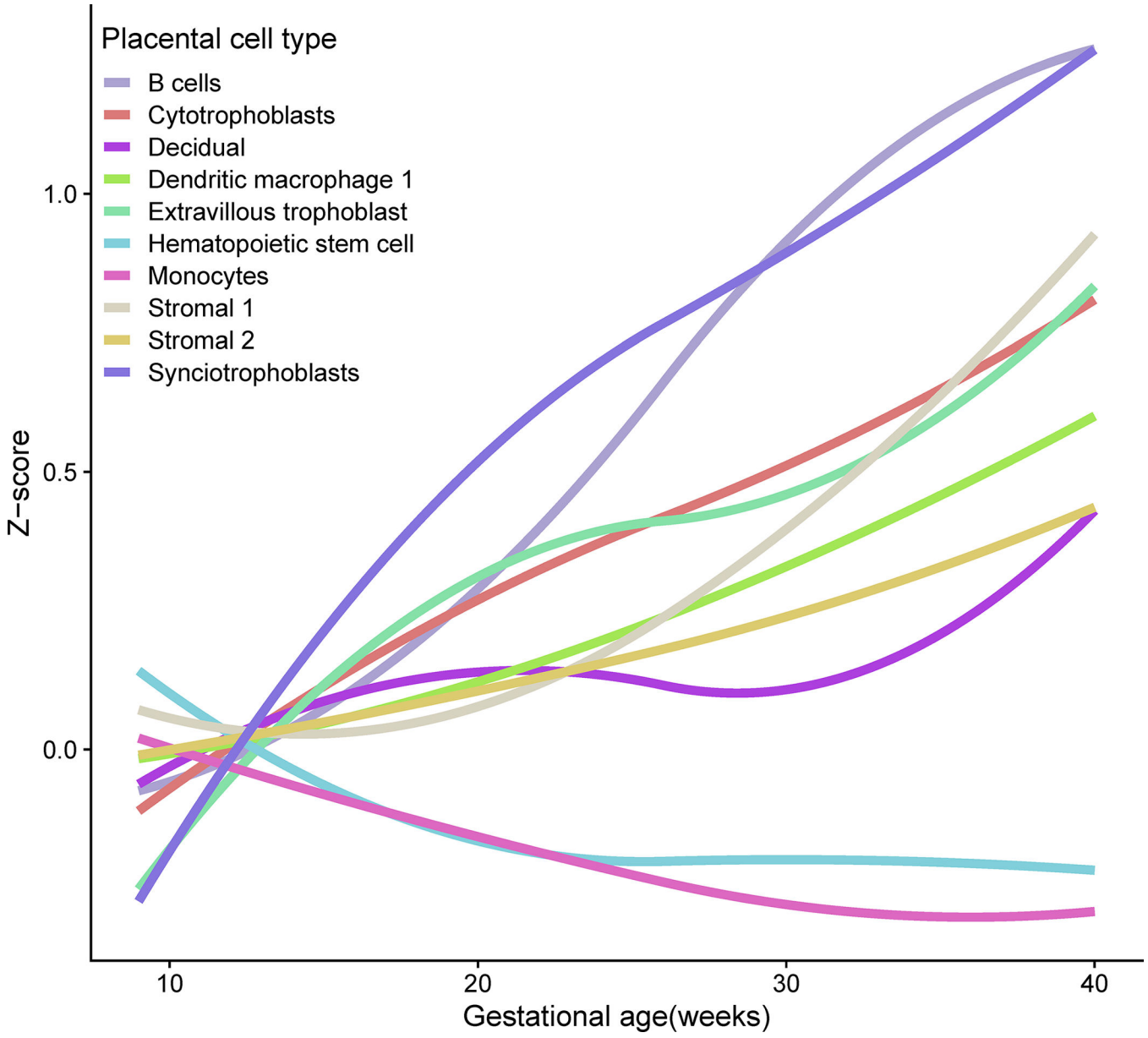


Figure 6: Maternal plasma proteomic trajectories of single-cell signatures throughout gestation.

For each placental single-cell signature the average Z-score of member proteins is shown as a function of gestational age. The gene symbols corresponding to each signature are: *Synciotrophoblasts* (KISS1, CSH1, TFPI2, CGA, GH2, PSG3, PSG2, PSG1, HOPX, CRH, GDF15, S100P, PSG11), *B cells* (CD79A, CD74, RPS5), *Extravillous trophoblast* (AOC1, PRG2, IGF2, NOTUM, FSTL3, FLT1, EBI3, PAPPA2, HPGD, HLA-G, PAPPA, ITM2B, KRT19, SERPINE2, MFAP5, HEXB, QSOX1, TPM1, TNFSF10), *Stromal 1* (TIMP1, DLK1, COL3A1, COL1A1, TGFBI, IL1RL1, COL6A2, IGFBP3, DCN, COMP, SERPINE2, COL6A1), *Stromal 2* (CXCL14, EGFL6, PTGDS, APOD, TCF21, DLK1, IGFBP3, COL3A1, PLA2G2A, COL1A1, C7, GPC3, LUM, CTHRC1, SERPINF1, RARRES2), *hematopoietic stem cell* (SPARCL1, ENPP2, EDN1, IGFBP7, CRIP2, A2M, SOCS3, ID1), *Monocytes* (S100A8, LYZ, S100A9, IL1B, S100A12, CXCL2,

BCL2A1, CCL3, CCL20, CXCL3, G0S2, PLAUR, FCN1, SOD2, C15orf48, EREG, IL1RN), *Decidual* (IGFBP1, LUM, DKK1, IGFBP2, DCN, RBP1, IGFBP4, PRL, IGFBP5, HSD11B1, IGFBP6, CD248, TIMP3, CFD), *Dendritic/Macrophage 1* (APOE, APOC1, CCL18, CD74, SPP1, C1QC, FTL, RNASE1, CXCL3, CTSZ), *Cytotrophoblasts* (PAGE4, CGA, TINAGL1, SPINT1, SPINT2, LDHB).

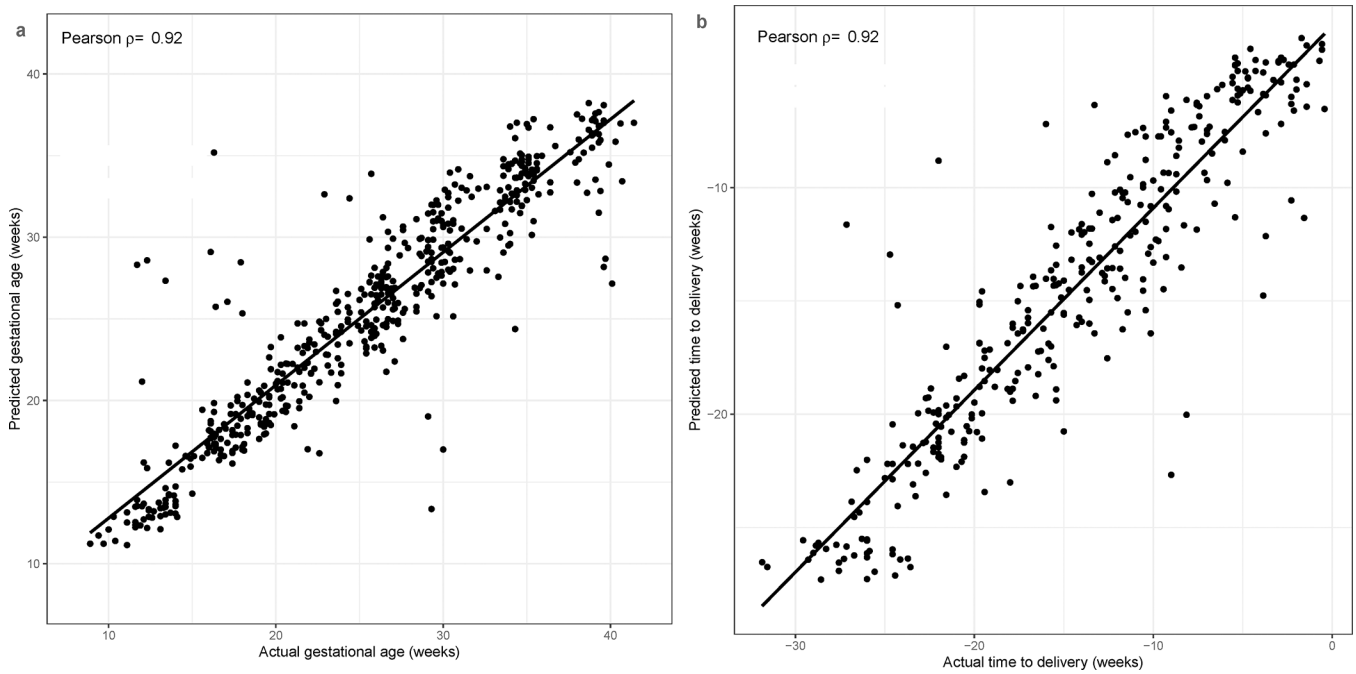
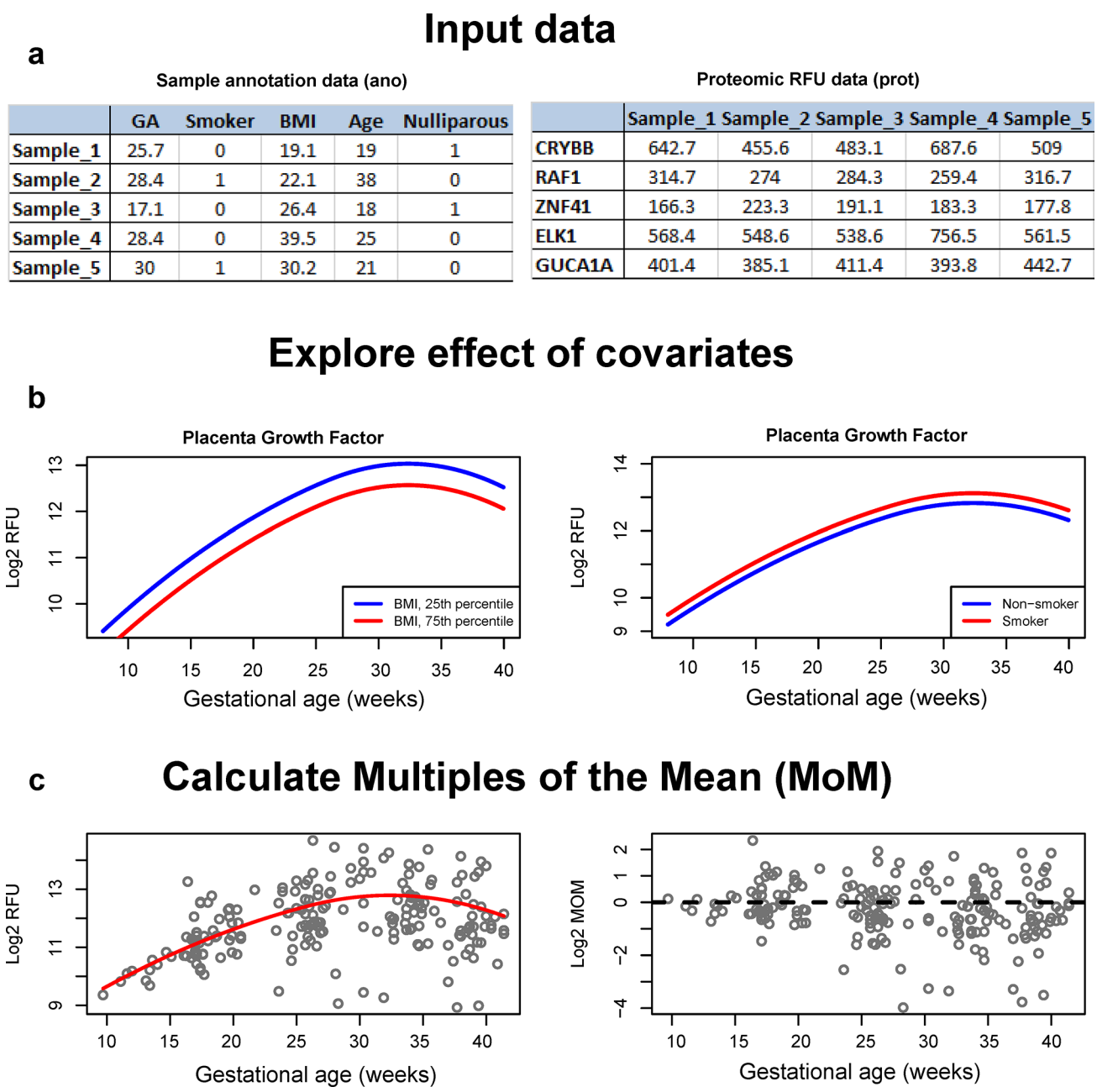


Figure 7: Prediction of gestational age and of time from sample to delivery using proteomic data.

Prediction of gestational age (A) and of time from sample to a spontaneous term delivery (B) is shown. Each dot corresponds to a sample. Random forest predictions are obtained via cross-validation, in which, data from all samples of a given patient are left out when selecting predictor proteins and training the model. ρ: correlation coefficient.



	GA	Smoker	BMI	Age	Nulliparous
Sample_1	25.7	0	19.1	19	1
Sample_2	28.4	1	22.1	38	0
Sample_3	17.1	0	26.4	18	1
Sample_4	28.4	0	39.5	25	0
Sample_5	30	1	30.2	21	0

	Sample_1	Sample_2	Sample_3	Sample_4	Sample_5
CRYBB	642.7	455.6	483.1	687.6	509
RAF1	314.7	274	284.3	259.4	316.7
ZNF41	166.3	223.3	191.1	183.3	177.8
ELK1	568.4	548.6	538.6	756.5	561.5
GUCA1A	401.4	385.1	411.4	393.8	442.7

Figure 8: Functionality implemented in the SomaPreg package.

Sample annotation data paired with proteomic data (RFU) (5 proteins of 7288 are shown) are used as input (A) to determine the expected proteomic abundance (B) and to calculate multiple of the mean values (MoM) for gestational age and maternal characteristics (C).