

OBSTETRICS

New biomarkers for the detection of fetal death derived from large-scale proteomic analysis of maternal plasma



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BACKGROUND: Normal pregnancy involves the modulation of thousands of maternal plasma proteins, and protein values not within the normal range may indicate the development of adverse pregnancy outcomes. A decrease in placental growth factor and an increase in soluble fms-like tyrosine kinase 1 in maternal plasma were shown to be associated with fetal death at the time of diagnosis and to predict this devastating pregnancy outcome at 24 to 28 weeks of gestation. However, these proteomic dysregulations are also present in other obstetrical syndromes, and more specific and sensitive biomarkers are needed to implement preventive strategies.

OBJECTIVE: This study aimed to identify candidate protein biomarkers that can improve the prediction of fetal death relative to placental growth factor and soluble fms-like tyrosine kinase 1.

STUDY DESIGN: This retrospective case-control study included 38 patients who experienced fetal death (cases) and 23 patients with uncomplicated pregnancies (controls). Plasma samples were collected at the time of diagnosis (20–41 weeks of gestation) from cases and during routine care from gestational age-matched controls. An aptamer-based multiplex assay was used to measure the abundance of >7000 protein analytes. Differential protein abundance was assessed using linear models with adjustment for gestational age at sample collection. Significance was inferred using a moderated *t* test adjusted *P* value of <.1 and a fold change of >1.25. Hypergeometric tests were performed to identify gene ontology biological processes enriched among proteins with significant changes in abundance. Random forest models were trained and evaluated via cross-validation to distinguish between fetal death cases and controls and to pinpoint the most salient predictors.

RESULTS: Among the 7146 protein assays tested, 97 assays (1.4%) corresponding to 87 unique proteins differed significantly in abundance between fetal death cases and controls: 63 of 87 proteins (72%) were less abundant in fetal death cases, and 24 of 87 proteins (26%) were more abundant in fetal death cases. Dysregulated proteins were involved in pregnancy-related processes, such as angiogenesis and lactation. Random forest models effectively differentiated fetal death cases from

controls, achieving an area under the receiver operating characteristic curve of 72% for the combination of placental growth factor and soluble fms-like tyrosine kinase 1, which increased to 86% when up to 50 additional proteins were included in the models (DeLong test: *P*=.004). In addition, the point estimate of sensitivity increased from 53% to 74% (false-positive rate of approximately 10% for both). Glycoprotein hormones alpha chain (CGA), DnaJ homolog subfamily B member 9 (DNAJB9), and DNA-directed RNA polymerase III subunit RPC10 (POLR3K) emerged as the top 3 candidates to improve discrimination relative to placental growth factor and soluble fms-like tyrosine kinase 1. The significant proteomic changes in a subset of fetal death cases diagnosed first with preeclampsia relative to controls were highly correlated (*r*=0.78; *P*<.001) with those reported in late preeclampsia cases leading to live births. On average, for each 2-fold change in protein abundance in late preeclampsia leading to live birth, there was an 8.6-fold change in preeclampsia leading to fetal death. Despite this overall correlation, transcobalamin 2, glucose-6-phosphate 1-dehydrogenase, and hepcidin, among others, demonstrated dysregulation only in preeclampsia leading to fetal death, suggesting both shared and distinct pathways perturbed in the 2 syndromes.

CONCLUSION: Our findings suggest that new maternal plasma proteins improve the discrimination of fetal death from controls relative to known biomarkers and that, although the signatures of fetal death and of preeclampsia are correlated, fetal death not only represents a much heightened disease state but also involves distinct perturbed pathways. Future studies are needed to determine whether the biomarkers can predict fetal death.

Key words: angiogenesis, aptamer, biomarker, blood proteins, DNAJB9 protein, fetal death, glucose-6-phosphate 1-dehydrogenase, glycoprotein hormones alpha chain, hepcidin, high-throughput screening assay, placental growth factor, proteome, RNA polymerase III subunit C10, soluble fms-like tyrosine kinase 1, transcobalamin 2, vascular endothelial growth factor receptor-1

Introduction

Fetal death is a major public health problem with profound medical, emotional, and societal consequences. In

the United States (US), approximately 57 fetal deaths occur each day, affecting more than 21,000 families annually.¹ Worldwide, an estimated 2 million fetal deaths occur every year, representing a substantial and persistent global burden.² The prevention and prediction of fetal death have been identified as national healthcare priorities. In July 2024, the US Congress enacted the Maternal and Child Health Stillbirth Prevention Act,

reflecting the need to improve risk identification and prevention strategies.³

Fetal death is generally defined as the cessation of fetal cardiac activity at or after 20 weeks of gestation or when the fetus weighs at least 350 g, although definitions vary according to reporting requirements across states within the US and among countries.⁴ Despite advances in obstetrical care, the fetal mortality rate in the US has remained largely

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AJOG at a Glance

Why was this study conducted?

This study aimed to identify candidate biomarkers that can improve the prediction of fetal death beyond the currently known biomarkers (placental growth factor [PLGF] and soluble fms-like tyrosine kinase 1 [sFlt-1]).

Key findings

A total of 87 proteins differed in abundance between fetal death cases and gestational age (GA)-matched controls. New candidate biomarkers increased the area under the receiver operating characteristic curve for fetal death classification from 72% (for PLGF and sFlt-1) to 86%. Proteomic changes in a subset of fetal death cases diagnosed first with preeclampsia were, on average, 8.6-fold for each 2-fold change that was reported in preeclampsia that resulted in live births.

What does this add to what is known?

PLGF and sFlt-1 are dysregulated in maternal plasma at the time of the diagnosis of fetal death and predict this syndrome at 24 to 28 weeks of gestation. However, additional biomarkers are needed. This study identified new proteins that can add information relative to PLGF and sFlt-1, paving the way for future studies to assess the risk of fetal death in samples collected before diagnosis.

unchanged over the past 2 decades.¹ Substantial racial and ethnic disparities persist, with Black women experiencing significantly higher rates of fetal death than other groups.^{1,5} These observations underscore the limitations of current clinical approaches and the need for improved biologically informed methods of risk assessment.

Fetal death fulfills the criteria of a great obstetrical syndrome^{6–10} in that it is characterized by (1) multiple etiologies; (2) the presence of a preclinical phase during which pathologic processes are active but clinically silent; (3) direct fetal involvement; (4) an adaptive nature, in which fetal demise may represent a response to an adverse intrauterine environment; and (5) modification of its occurrence by genetic and environmental factors.^{7,8,11} Therefore, fetal death represents a final common pathway rather than a single disease entity. Documented etiologies include infections (eg, syphilis,^{12,13} listeriosis,^{14–16} group B streptococcus,^{17,18} other bacterial infections,¹⁹ causing fetal sepsis,²⁰ and viral infections, including parvovirus B19^{21–23} and cytomegalovirus),^{24,25} placental vascular insufficiency^{26–32} (also known as ischemic placental disease),^{33–36} maternal antifetal rejection,^{37–41}

umbilical cord pathology (eg, true knots,^{42–46} vasa previa,^{47–49} and others^{44,50}), genetic anomalies,^{51–54} fetal cardiac arrhythmias,^{55–59} maternal metabolic disorders (such as diabetes mellitus),^{60,61} and placental senescence,^{62,63} among other pathologic processes.⁶⁴ This framework provides a biologically coherent basis for understanding heterogeneity in pathogenesis.

Mechanistic investigations conducted by our group have demonstrated that hypoxia secondary to placental dysfunction accounts for most fetal deaths (88%) among structurally normal fetuses.⁶⁵ These studies further showed that fetal death is frequently preceded by fetal growth restriction^{29,66–73} and evidence of myocardial injury, brain injury, or both, indicating that death commonly results from progressive pathophysiological processes rather than sudden, unpredictable events.^{37,65} These findings support the existence of a clinically silent preclinical phase during which biomarkers may identify pregnancies at increased risk.

Placental vascular disorders represent the most frequently identified pathologic etiology in fetal death.^{26,74,75} Lesions consistent with maternal vascular malperfusion are commonly observed

on placental examination, and fetal vascular malperfusion has also been implicated.^{26,50,74–79} In addition, placental lesions associated with maternal antifetal rejection, including chronic chorioamnionitis, villitis of unknown etiology, and chronic deciduitis, have been described in stillbirth.^{26,37,39,80,81}

Biomarkers reflecting placental vascular dysfunction, such as placental growth factor (PLGF) and soluble fms-like tyrosine kinase 1 (sFlt-1), have demonstrated potential in identifying pregnancies at increased risk.^{31,82–88} Nonetheless, a proportion of fetal deaths occur in the absence of abnormal angiogenic profiles, indicating that crucial pathogenic mechanisms remain unidentified. Therefore, improving risk stratification requires the interrogation of biological pathways beyond maternal vascular malperfusion. Maternal blood provides a unique window into the integrated biology of the placenta, fetus, and mother and may be conceptualized as a liquid biopsy of pregnancy.⁸ Systematic evaluation of changes in the maternal plasma proteome offers the opportunity to identify biomarkers reflective of diverse pathogenic processes and to complement existing tools (eg, biomarkers, such as alpha-fetoprotein^{89,90} and pregnancy-associated plasma protein A⁹¹).

Previous investigations of maternal circulating proteins in fetal death have primarily used targeted approaches, focusing on a limited number of analytes.^{31,82–86,92–96} High-throughput technologies permit unbiased characterization of protein alterations associated with gestational age (GA) and obstetrical complications before and at the time of diagnosis.^{97–101} To date, a comprehensive large-scale evaluation of the maternal plasma proteome in fetal death has not been performed. Therefore, here, we used an aptamer-based proteomic platform to quantify more than 7000 protein analytes in maternal plasma samples obtained at the time of diagnosis of fetal death.¹⁰² Aptamers are chemically synthesized DNA/RNA molecules that bind protein targets with high affinity and specificity. The reliability and reproducibility of this

platform have been established in maternal plasma, with low coefficients of variation and strong correlation with enzyme-linked immunosorbent assay measurements for PlGF and sFlt-1.^{8,98,101} Moreover, an earlier version of this platform was used to identify placenta-specific proteins released into the maternal circulation, supporting its suitability for the current investigation.¹⁰³

The primary objective of this study was to identify maternal plasma proteins that improve discrimination beyond PlGF and sFlt-1 in fetal death cases compared with GA-matched controls. We hypothesized that this would be possible by screening more than 7000 plasma proteins, including some proteins that were never assayed in the plasma of women who experienced fetal death. The identification of such markers is clinically relevant, given the evidence that Great Obstetrical Syndromes molecular abnormalities present at the time of diagnosis may also be detectable earlier in pregnancy.^{8,83,97,100,104} A secondary objective was to compare proteomic alterations in cases of fetal death associated with preeclampsia with those previously reported in late-onset preeclampsia resulting in live birth using the same proteomic platform in the same population.¹⁰⁵

Materials and methods

Study design and population

Pregnant women seeking care at the Center for Advanced Obstetrical Care and Research of the Pregnancy Research Branch, Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Institutes of Health, US Department of Health and Human Services in the Detroit Medical Center and Wayne State University were enrolled in a prospective study between 2010 and 2018. From this cohort, a retrospective case-control study was designed to include 38 women with fetal death (cases) and 23 controls. The lower number of controls than cases was decided based on the availability of a longitudinal proteomics dataset in normal pregnancy (n=91) obtained by the investigators in the same population using the same version of the SomaScan

platform (SomaScan, Boulder, CO).^{101,105} Controls were women who were enrolled in a longitudinal study in the same period as the cases (to avoid differences in sample storage time); had a pregnancy without major medical, surgical, or obstetrical complications; delivered an appropriate-for-gestational-age (AGA) neonate at term; and had one or more blood samples available in the 20- to 41-week interval. The blood samples from controls were selected so that they matched the distribution of cases in terms of GA at sampling. Women with a multiple pregnancy or with fetal chromosomal abnormalities and congenital anomalies were excluded from the study.

The collection of the clinical data and biological specimens from these women for research was approved by the institutional review boards of Wayne State University and the NICHD under the protocols entitled “Biological markers of disease in the prediction of preterm delivery, preeclampsia and intra-uterine growth restriction: a longitudinal study” (approval number: 110605MP2F) and “Establishment of a clinical perinatal database and bank of biological materials” (approval number: 082403 MP2F). All patients provided written informed consent before the collection of data and biospecimens.

Clinical definitions

GA was determined by the self-reported last menstrual period and confirmed using ultrasound. If there was a discrepancy between the menstrual dating and the ultrasound findings, GA was determined solely by ultrasound examination.¹⁰⁶ Fetal death was defined as fetal demise diagnosed at or after 20 weeks of gestation, as confirmed by ultrasound examination before the onset of labor.¹⁰⁷ Preeclampsia was defined as the onset of elevated blood pressure in the presence of proteinuria after 20 weeks of gestation, following the diagnostic criteria of the American College of Obstetricians and Gynecologists.¹⁰⁸ A small-for-gestational-age (SGA) neonate was defined as a neonate with a birthweight of <10th percentile for their specific GA at delivery using the standard

of Alexander et al.¹⁰⁹ An AGA neonate was defined as a neonate with a birthweight between 10th and 90th percentiles for their specific GA at delivery using the standard of Alexander et al.¹⁰⁹

Placental pathology

After delivery, placental tissues were collected and subjected to thorough examination by perinatal pathologists blinded to the clinical diagnoses. The evaluation followed standardized protocols to ensure consistency and accuracy as we previously described.^{26,39,80,110–122} Placental lesions, including acute and chronic inflammatory lesions and lesions consistent with maternal and fetal vascular malperfusions, were diagnosed based on established criteria set by the Perinatal Section of the Society for Pediatric Pathology.^{26,112–124}

Maternal plasma collection

Blood samples were collected in tubes containing ethylenediaminetetraacetic acid, and plasma was separated by centrifugation (1300 g for 10 minutes). Plasma samples were immediately stored at -80°C until proteomic analysis. For all cases with a fetal death, the samples were collected after diagnosis.

Large-scale proteomic analysis of maternal plasma

The study used the SomaScan assay (version 4.1; SomaLogic), an aptamer-based platform, to measure the abundance of maternal plasma proteins.^{102,125,126} The platform allows for the simultaneous quantification of 7288 analytes, representing 6596 unique human protein targets, of which 7146 analytes (representing 6288 unique proteins) passed quality control (QC) and were retained for downstream analysis. The full list of proteins analyzed in this study is available from the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>; platform identifier: GPL32354). The proteomic profiling was performed by SomaLogic. We previously reported that the median coefficient of variation of the protein analytes was 7.0% (interquartile range [IQR], 4.5%–11.4%), and the coefficient of variation was <20% for 93% of the assays.¹⁰¹ Our group has previously

used the SomaScan platform to report on maternal plasma changes with advancing pregnancy during normal pregnancy,^{98,101} term parturition,¹²⁷ and pathologic processes, such as early¹⁰⁰ and late preeclampsia⁹⁷ and SARS-CoV-2 infection.¹²⁸ Proteomic changes detected with different versions of the SomaScan platform were shown to be replicated across diverse cohorts.^{105,129}

SomaScan assay

Plasma samples undergo 3 sequential dilutions at ratios of 1:5, 1:200, and 1:20,000. These diluted samples are incubated with specific SOMAmer mixes attached to streptavidin-coated beads, enabling the binding of proteins to their respective SOMAmer reagents. Subsequent washing removes nonspecifically bound proteins and other matrix constituents from the beads. An N-hydroxysuccinimide-biotin reagent is used to label the proteins bound to the SOMAmer reagents. After the labeling reaction, an anionic competitor solution is introduced to the beads, preventing nonspecific interactions from reforming after dissociation. Pure cognate SOMAmer complexes and unbound SOMAmer reagents are separated from the streptavidin beads using ultraviolet light, which cleaves the photocleavable linker present in the SOMAmer reagents. The resulting eluate contains excess anionic competitors and all SOMAmer reagents, including those bound to biotin-labeled proteins and free proteins. Subsequently, this eluate is incubated with a second set of streptavidin-coated beads that specifically bind to biotin-labeled proteins and biotin-labeled protein-SOMAmer complexes. The free SOMAmer reagents are removed during the additional washing steps. Under denaturing conditions, the SOMAmer reagents bound to their cognate proteins are released and then hybridized to custom DNA microarrays. The resulting cyanine-3 signal from the SOMAmer reagents is detected and quantified on the microarrays. The proteomics profiling was performed by SomaLogic in July 2022 as a paid service.

Data standardization

The SOMAScan assay uses rigorous data standardization steps to ensure accurate and consistent results.^{125,126} First, hybridization normalization adjusts for variations in microarray binding. Next, intraplate signal normalization is applied using both calibrator and buffer (no protein) replicates, addressing plate-specific fluctuations. Next, plate scale standardization is combined with calibration using a global calibrator reference. The signals of QC replicates are normalized using a global reference, followed by a QC check that contrasts the median of these replicates with a specific global QC standard for the pooled QC lot present on the plate. Finally, individual sample signals are standardized with a universal reference. Collectively, these procedures are designed to mitigate systematic biases emerging after microarray feature aggregation.

Demographics data analysis

Clinical characteristics and demographics of the study participants were presented as median and interquartile range for continuous variables and as proportions and percentages for categorical variables. The Welch *t* test and Fisher exact test were used to compare continuous and categorical variables between the groups. A *P* value of $<.05$ was considered statistically significant. The analysis was performed using the R statistical language and environment (www.r-project.org).

Principal components analysis for proteomics data visualization

To visualize the proteomic profiles and detect eventual outlier samples, we used principal component (PC) analysis to project the high-dimensional proteomic data onto the first 2 PCs, which can be seen as independent (uncorrelated) variables (meta-proteins) derived as a weighted combination of different subsets of proteins. The PCATools package in R was used for this analysis.

Differential protein abundance analysis

The abundance of proteins, in relative fluorescence units, was log₂ transformed

and compared between the groups (ie, all fetal death cases vs controls, fetal death with preeclampsia cases vs controls, and fetal death without preeclampsia cases vs controls) by fitting linear models implemented in the *limma* R/Bioconductor¹³⁰ package while adjusting for GA at sampling. A fold change of ≥ 1.25 and a false discovery rate (FDR)-adjusted *P* value (*q* value) of $<.1$ were used as thresholds to infer significance. Selecting proteins as significant based on $q <.1$ alone would mean that, on average, 10% of the proteins identified as associated with fetal death are false discoveries, which is commonly accepted in omics studies.^{127,128,131} In addition, requiring that the fold change is at least 1.25-fold further reduces the FDR and increases the chance that the effect is biologically meaningful. The results of differential expression analysis were summarized and visualized with volcano plots and heatmaps using the R/Bioconductor packages *EnhancedVolcano* and *pheatmap*, respectively.

In addition to the primary differential abundance analysis (38 fetal death cases vs 23 controls), we performed and expanded the analysis to include 367 additional samples collected at 20 to 41 weeks of gestation from 91 patients with normal pregnancy initially reported in Tarca et al¹⁰¹ and later contrasted with preeclampsia cases reported by Andresen et al.¹⁰⁵ The *dupcor* method from the *limma* package was used to account for longitudinal samples from the same control patients. Before analysis, data were transformed into multiple of the mean (MoM) values (log₂ thereof) as previously described.¹⁰⁵ The goal of this secondary differential protein abundance analysis was to provide additional confidence in the primary analysis by capturing additional variability in the proteomes of normal pregnancy and better accounting for the effect of GA on proteome trajectories via longitudinal sampling.

Gene ontology enrichment analysis

To investigate the functional roles of proteins with altered abundance in fetal

death cases compared with controls, we performed gene ontology¹³² enrichment analysis. Each protein was mapped to its corresponding gene identifier in the Entrez gene database¹³³ according to the annotation provided by SomaLogic. To identify biological processes over-represented among the proteins significantly changing in abundance in fetal death cases vs controls, we used the hypergeometric test implemented in the R/Bioconductor package, *clusterProfiler*. Biological processes with at least 3 hits and an adjusted *P* value of $<.05$ were deemed statistically significant.

Proteomic classifier for fetal death

Random forest models were used to classify maternal plasma proteomic profiles between patients with a fetal death and controls. Random forests are an ensemble technique that creates multiple decision trees for classification and regression.¹³⁴ Each tree functions as a flowchart, assigning samples to groups based on protein abundance cutoffs of specific proteins. By aggregating outcomes from all trees, the random forest method enhances predictive accuracy and minimizes biases inherent in single trees. Moreover, this approach provides a robust measure of multivariate feature importance score, enabling the identification of key features affecting the overall decision-making.¹³⁵ Feature importance is estimated by evaluating how a protein's absence reduces the model's accuracy.

A 20-fold cross-validation framework was used to assess the accuracy of 2 distinct types of models and, hence, provide internal validation of the findings. The first model used the angiogenic factor PlGF and the antiangiogenic factor sFlt-1 for classification. This choice was based on their well-established value in predicting and distinguishing between fetal death cases and controls, thereby serving as a reference model.^{31,82–88} For the second model, in addition to PlGF and sFlt-1, we incorporated 50 additional proteins. These proteins were selected on the basis of their feature importance scores, which were derived from a random forest

analysis that considered all available proteins.

The 20-fold cross-validation process started by randomly splitting the patients into 20 folds. Next, in each iteration, the samples in one of the folds were set aside as a test set, and the remaining samples were used to train an initial random forest classifier. From this preliminary model, the top 50 features (excluding PlGF and sFlt-1), ranked by their importance, were identified. These features, together with PlGF and sFlt-1, were used to fit the final model that was used in the test set. The model performance was evaluated by calculating the area under the receiver operating characteristic (ROC) curve (AUC) by concatenating classification probabilities derived from each test set. The Delong method for paired ROC curves was used to compare the 2 models.¹³⁶ The ROC was plotted using the R package, *pROC*, and the R packages, *randomForest* and *ranger*, were used to fit the random forest models.

Cross-study validation

As there was no other publicly available dataset in fetal death obtained using the same large-scale proteomics platform, we sought to assess the reliability of findings herein based on the results that we previously reported in late preeclampsia based on samples from patients enrolled in the same clinical center.¹⁰⁵ Differential protein analysis in a subset of fetal death cases first diagnosed with preeclampsia ($n=9$) and all controls was performed as described above for the main analysis. Log₂ fold changes of resulting significant proteins were correlated to those found in plasma samples collected between 30 and 41 weeks of gestation from 80 patients who developed late preeclampsia and 88 of 91 controls who had an available sample in this window of pregnancy. Of the 80 preeclampsia cases, 49 were already diagnosed at the time of blood collection, whereas 31 were diagnosed later in pregnancy. The Pearson correlation coefficient and the slope of the regression line between the 2 studies were calculated.

To further assess the external validity of the generated data, proteins were ranked by variable importance on the entire fetal death and control datasets, and the 50 top-ranked proteins were used to fit a random forest model. This model was applied to data from the 80 patients diagnosed with late preeclampsia and 88 controls from Andresen et al¹⁰⁵ to estimate the posterior probability of fetal death for each sample. ROC curves and AUC statistics with 95% confidence intervals (CIs) were obtained. Protein abundances in both datasets were first converted to GA-adjusted MoMs using the same procedure that we previously described in cross-cohort studies.^{105,129}

Results

Demographics and clinical characteristics

The study included plasma samples from 38 women with a fetal death and 23 controls sampled in the same window of gestation. Among women with a fetal death, individual or combined pregnancy complications were noted: 9 (24.0%) were diagnosed with preeclampsia, 15 (39.0%) were diagnosed with an SGA neonate, 4 (10.5%) were diagnosed with both preeclampsia and SGA, 3 (8.0%) were diagnosed with clinical chorioamnionitis, and 1 (3.0%) had placental abruption. Additional underlying diseases in the fetal death group included diabetes mellitus ($n=1$ [3%]), anemia ($n=4$ [11%]), and asthma ($n=4$ [11%]). Table 1 displays the demographics and clinical characteristics of the study participants between the 2 groups. GA at delivery, birthweight, and birthweight percentiles in the fetal death group were significantly lower than those in the control group ($P<.001$ for all). Although a reduction in growth velocity is known to precede a subset of cases destined to experience fetal death,²⁹ the 16.0% median birthweight percentile for cases is lower than the 21.5% estimate that we have previously reported based on third-trimester ultrasound²⁹ and can be explained in part by the delay between fetal demise and delivery. Women with a fetal death were

TABLE 1
Demographic and clinical characteristics of the study population

Characteristics	Controls	Fetal death	P value
Maternal age (y)	23 (19–32)	24 (17–41)	.023
BMI (kg/m ²)	25.2 (16.1–39.0)	27.1 (18.1–50.5)	.11
Race (African American)	20/23 (87.0%)	34/38 (89.5%)	1
Smoking	1/23 (4.3%)	11/38 (28.9%)	.022
Alcohol consumption	0/23 (0%)	1/38 (2.6%)	1
Nulliparous	6/23 (26.1%)	18/38 (47.4%)	.11
GA at blood collection (wk)	28.9 (20.6–40.1)	26.8 (20.1–40.9)	.54
GA at delivery (wk)	38.9 (37.7–41.1)	27.0 (20.1–41.3)	<.001
Birthweight (g)	3350 (2855–3960)	709 (48–3235)	<.001
Birthweight percentile	45.3 (25.0–80.5)	16.0 (1.0–62.0)	<.001
Fetal sex (male)	11/23 (47.8%)	26/37 (70.3%)	.10

Continuous variables are presented as median (interquartile range) and were compared using the Welch *t* test. Categorical variables are presented as number (percentage) and were analyzed using the Fisher exact test.

BMI, body mass index; GA, gestational age.

Romero. Plasma protein biomarkers for fetal death. *Am J Obstet Gynecol* 2026.

significantly older ($P=.023$) and more likely to be smokers ($P=.022$) than controls. By study design, the median GA at sampling did not differ between the groups. Other variables, including body mass index (BMI), nulliparity, and ethnicity, also by design did not differ significantly between the groups.

Table 2 presents the placenta histopathologic results of the study population. Placental weight was lower in cases than in controls ($P<.001$). The prevalences of chronic inflammatory lesions in the placenta and placental lesions consistent with maternal and fetal vascular malperfusions were significantly higher in women with a fetal death than in controls ($P<.001$ for all). Supplemental Table 1 provides patient-level clinical and obstetrical characteristics and the placental histology of women with fetal death.

Unsupervised analysis of the plasma proteome in cases of fetal death and controls

Of the 7288 assays profiled in plasma samples, 7146 (representing 6288 unique proteins) passed QC and were retained for all downstream analyses. Next, PC analysis was performed to examine the relationship between the maternal plasma

metaproteome and fetal death diagnosis. Although PC1 and PC2, accounting for 21% and 8% of the variance, respectively, did not demonstrate a significant correlation with group status (fetal deaths cases vs controls; $P>.05$ for both), PC3 and PC5, which explained 7% and 4% of the variance, respectively, showed a significant association with fetal death ($P<.05$). Figure 1 shows the separation between the samples from women with fetal death and controls. This unsupervised analysis suggests differences in protein abundance between the 2 groups.

Differential protein abundance

Comparing 7146 maternal plasma protein assays between women with a fetal death and controls, we observed a significant change in abundance for 97 SOMAmer targets (1.4%), corresponding to 87 unique proteins (Supplemental Table 2). All 10 duplicate SOMAmers targeting the same significant proteins were consistent in terms of direction of change and had similar magnitude of change (Supplemental Table 2). Of these 87 proteins, 24 were more abundant in the fetal death group, whereas 63 were less abundant in the fetal death group. This differential abundance is visualized in a volcano plot (Figure 2), contrasting

$\log_2$ fold changes against significant q values for all proteins and highlighting the significant ones. In addition, Figure 3 presents a heatmap showing the $\log_2$ -transformed abundance (in relative fluorescence units) of these differentially abundant proteins across all samples, using a color gradient. When expanding the control group to include data from 91 additional patients, the resulting protein changes were highly correlated (Pearson $r=0.95$; $P<.001$), the direction of change remained the same for all 97 SOMAmer targets reported in the main analysis, and additional significant proteins were identified. We preferred not to focus on the expanded analysis results due to potential biases that can be introduced when combining the data from the current study with the dataset of Andresen et al,¹⁰⁵ which did not include fetal death cases.

Adjusting for BMI did not alter results. The direction of protein abundance changes remained consistent for all 97 differentially abundant targets, and the fold changes exhibited a perfect Pearson correlation of 1, regardless of BMI adjustment. Furthermore, the changes in abundance of the 97 SOMAmer targets obtained in the primary analysis were consistent and highly correlated, regardless of the diagnosis of preeclampsia among the fetal death cases (Pearson $r=0.92$; $P<.001$) (Supplemental Figure). The 1 notable exception was glucose-6-phosphate 1-dehydrogenase (G6PD), which was highly decreased in fetal death with preeclampsia cases relative to controls (4.3-fold) but did not change in fetal death without preeclampsia cases (Supplemental Figure).

Differential protein abundance in women who experienced fetal death was associated with perturbation of 53 biological processes, including angiogenesis, female pregnancy, lactation, and chemotaxis (FDR of 5%) (Table 3).

The maternal plasma proteome can discriminate between fetal deaths and controls

We observed the differential abundance of several proteins not previously

TABLE 2

Placenta pathology in fetal death and in the control group

Characteristics	Controls	Cases of fetal death	P value
Placenta weight (g)	543 (414–817)	197 (17–670)	<.001
Birthweight—to—placental weight ratio	6.4 (4.1–7.2)	3.7 (1.1–30.6)	.07
Acute inflammatory lesions	4/23 (17.4%)	12/38 (31.6%)	.25
Maternal inflammatory lesions	3/23 (13.0%)	10/38 (26.3%)	.34
Fetal inflammatory lesions	4/23 (17.4%)	3/38 (7.9%)	.41
Chronic inflammatory lesions	1/23 (4.3%)	25/38 (65.8%)	<.001
Chronic chorioamnionitis	0/23 (0%)	10/38 (26.3%)	.01
Chronic villitis	0/23 (0%)	2/38 (5.3%)	.52
Chronic deciduitis	1/23 (4.3%)	24/38 (63.2%)	<.001
Maternal vascular malperfusion	2/23 (8.7%)	26/38 (68.4%)	<.001
Fetal vascular malperfusion	0/23 (0%)	24/38 (63.2%)	<.001

Continuous variables are presented as median (interquartile range) and were compared using the Welch *t* test. Categorical variables are presented as number (percentage) and were analyzed using the Fisher exact test.

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reported in the plasma of women with a fetal death. Based on these findings, we postulated that integrating these unstudied proteins into proteomic models might enhance the accuracy in distinguishing fetal death cases from controls. To test this hypothesis, we trained random forest classification models with maternal plasma protein concentrations and evaluated their discriminative power using 20-fold cross-validation. As a benchmark, we used a model based on the plasma abundance of PlGF and sFlt-1 (model 1) to determine the potential improvement upon incorporation of additional proteins (model 2).

The 20-fold cross-validated AUC that differentiated between fetal death cases and controls using model 1 was 0.72 (95% CI, 0.59–0.84) (Figure 4, A), reflecting moderate accuracy. With a 10% false-positive rate (FPR), the sensitivity of model 1 was 52.6% (95% CI, 31.6–71.1). After incorporating 50 additional proteins (model 2), the AUC increased to 0.86 (95% CI, 0.77–0.95) (Figure 4, A). The increase in model 2's AUC was deemed statistically significant by a 1-sided comparison of correlated ROC curves via the DeLong method ($P=.004$).¹³⁶ In addition, model

2 displayed an enhanced sensitivity of 74% (57%–87%) at an 8.7% FPR corresponding to a positive likelihood ratio of 8.47 (2.22–32.29) and a negative likelihood ratio of 0.29 (0.17–0.50). The addition of previous risk factors (maternal age, nulliparity, BMI, history of preterm birth, history of preeclampsia, and chronic hypertension) in the proteomics model only increased the AUC for the prediction of fetal death from 0.86 to 0.87 (0.78–0.95) and outperformed previous risk factors alone (AUC, 0.70 [95% CI, 0.55–0.84]; DeLong test: $P=.044$).

The protein importance in the random forest, as assessed by the mean decrease in accuracy, for the top 50 proteins is presented in Figure 4, B. Proteins, such as the glycoprotein hormones alpha chain, DnaJ homolog subfamily B member 9, DNA-directed RNA polymerase III subunit RPC10, vascular noninflammatory molecule 2 (VNN2), and H/ACA ribonucleoprotein complex subunit 2 (NHP2), emerged as the most significant contributors to the model's discriminatory capacity (Figure 4, B). The latter 2 candidates did not meet the protein-level differential abundance criteria (VNN2: $q=0.17$; fold change=1.27; NHP2:

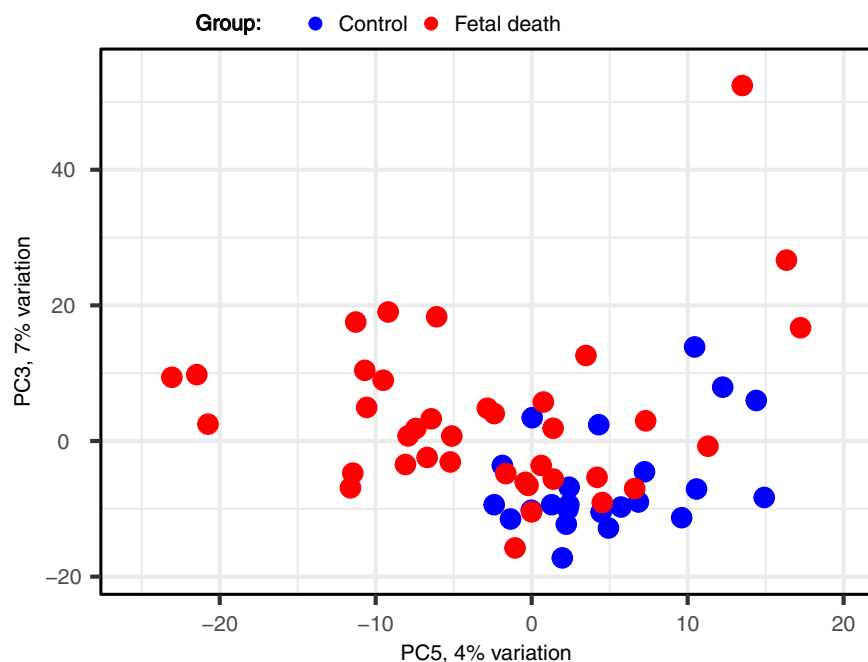
$q=0.016$; fold change=1.22) and, hence, are not presented in Supplemental Table 2.

Overall, these findings suggest that maternal plasma proteomic models have the potential to distinguish patients who have experienced a fetal death from those who have an uncomplicated pregnancy. The enhanced overall accuracy and sensitivity at 10% FPR achieved by incorporating proteins previously unstudied in the maternal plasma of patients with a fetal death suggest that such proteins may also improve prediction based on data collected from asymptomatic patients.

Cross-study validation results

The significant protein abundance changes in the 9 cases of fetal death diagnosed first with preeclampsia in this study were highly correlated with those we previously determined in patients with late preeclampsia leading to live births (Pearson $r=0.78$; $P<.001$), yet their magnitude of change was larger. For each 2-fold change in abundance in preeclampsia leading to live birth, there was, on average, an 8.6-fold change in preeclampsia leading to fetal death (log₂ linear slope of 3.11 in Figure 5, A). For instance, placenta growth factor (PGF) and glutaredoxin-3 (GLRX3) were decreased, whereas fatty-acid amide hydrolase 2 (FAAH2) and sialic acid-binding immunoglobulin-like lectin 6 (SIGLEC6) were increased in preeclampsia, leading to either fetal death (this study) or live birth (Andersen et al¹⁰⁵), among other proteins with shared dysregulation (Supplemental Table 3 and Figure 5, A). In contrast, transcobalamin 2 (TCN2) and G6PD were decreased, and hepcidin (hepcidin antimicrobial peptide) was increased only in preeclampsia leading to fetal death. Supplemental Table 3 and Figure 5, A, present additional examples of shared or fetal death-specific protein dysregulation.

Given the shared component of protein dysregulation between preeclampsia and fetal death, a 50-protein classifier trained to discriminate fetal death cases from controls using the data from this

FIGURE 1**The maternal plasma proteome separates fetal death from control pregnancies**

All samples are depicted based on two principal components derived from the maternal plasma protein abundance. The proportion of variance explained by each principal component is shown along the axis.

Romero. Plasma protein biomarkers for fetal death. *Am J Obstet Gynecol* 2026.

study significantly discriminated late preeclampsia cases from controls in the dataset reported by Andresen et al¹⁰⁵ (AUC, 0.72 [95% CI, 0.65–0.80]) (Figure 5, B). Overall, these cross-study analyses suggest that the protein changes that we reported herein are likely to be reproduced in other similar cohorts using the same proteomics platform.

Comment

Principal findings of the study

This study represents the most comprehensive analysis of the maternal plasma proteome in patients who experienced a fetal death. Specifically, (1) a significant difference in the abundance of 97 proteins, including 72 decreased and 25 increased proteins, representing approximately 1.4% of the measured maternal plasma proteome, was observed between fetal death cases and controls; (2) proteomic models incorporating proteins not previously explored in this context

effectively distinguished fetal death cases from controls, achieving an AUC of 86%, which represents a substantial improvement over a model based on PlGF and sFlt-1 alone (AUC, 72%); (3) protein dysregulation in fetal death was associated with biological processes fundamental to pregnancy, including angiogenesis, growth, and lactation related proteins; and (4) in the subset of cases in which fetal death was first diagnosed in the context of preeclampsia, protein changes were, on average, 8.6-fold for each 2-fold change that we previously reported in late preeclampsia with live birth, and fetal death-specific patterns of dysregulation were also identified.

Results in the context of what is known

Our findings are consistent with and extend previous work demonstrating that fetal death is frequently associated with an antiangiogenic profile in maternal circulation.^{31,82–86,92–96} Down-regulation

of angiogenesis, a biological process essential for placental development and fetal growth, may reflect disruption of normal placental vascular development.^{111,137–140}

This disruption is corroborated by placental histologic examination showing an increased prevalence and burden of lesions consistent with maternal vascular malperfusion in fetal deaths.^{26,74–77} In a previous study from our group, placental lesions consistent with maternal vascular malperfusion were present in 108 of 143 fetal death cases (75.5%), compared with 337 of 944 controls (35.7%).²⁶ The difference was even more pronounced when considering lesion burden, defined as the presence of ≥ 2 pathologic features, which occurred in 71 of 143 fetal death cases (49.7%) vs 70 of 944 controls (7.4%).²⁶

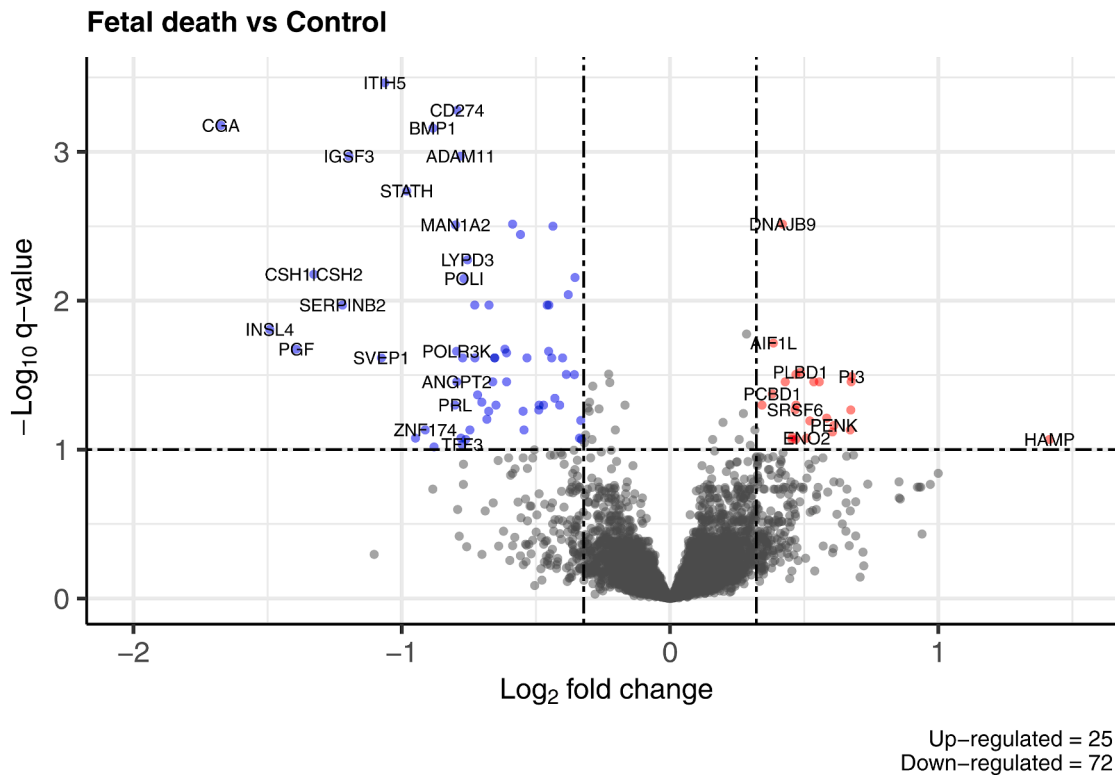
The maternal circulation mirrors this placental antiangiogenic state through reduced concentrations of angiogenic factors, such as PlGF, and increased levels of antiangiogenic components, such as sFlt-1.^{8,141} This pattern has been detected as early as 20 weeks of gestation in fetal deaths.⁸⁶ Although such alterations allow the prediction of fetal death,⁸³ the antiangiogenic profile is not specific to this condition and can result in false-positive findings.^{8,31,83} In the current study, we observed decreased abundance of multiple angiogenesis-related proteins, including prolactin, endothelial monocyte-activating polypeptide 2, PlGF, vascular endothelial growth factor A, and angiopoietin-2, consistent with previous observations.^{31,82–86}

The placental growth factor-to-soluble fms-like tyrosine kinase 1 ratio as an index for the prediction of fetal death

In addition, the proteomic model built herein using aptamer-based measurements of PlGF and sFlt-1 resulted in a significant discrimination of cases from controls (AUC, 72% [95% CI, 59%–84%]). We did not observe a significant difference in the maternal plasma abundance of sFlt-1 between fetal death cases and controls, which diverges from previous research

FIGURE 2

Differences in the maternal plasma protein abundance between fetal death and control pregnancies described in a volcano plot



The figure shows a volcano plot of $\log_{10}$ -transformed—adjusted P values against $\log_2$ -transformed fold changes of maternal plasma proteins (identified by corresponding gene symbols) significantly changing in abundance between fetal death cases and controls. The R/Bioconductor package, *EnhancedVolcano*, was used to generate the plot.

Romero. Plasma protein biomarkers for fetal death. *Am J Obstet Gynecol* 2026.

findings. The SomaScan assay used herein uses 2 specific SOMAmer reagents targeting Flt-1. However, neither of these reagents indicated a significant difference between the groups. This outcome is unexpected, considering the well-documented increases in maternal plasma concentrations of sFlt-1 associated with fetal death and other adverse pregnancy outcomes, as independently reported by various studies.^{8,84–86,142–144} Platform differences and the limited number of control patients may explain the divergence from previous findings for sFlt-1.

Here, we broadened the search for candidate fetal death biomarkers beyond traditional angiogenesis and inflammatory marker categories by conducting a large-scale, unbiased

analysis of the maternal plasma proteome. Through functional analysis of differentially abundant proteins, we uncovered various biological processes that were dysregulated in fetal death cases, including vasculature development, signaling pathways, cell migration and motility, growth and development, reproductive functions, bone and tissue remodeling, and immune response. Several proteins related to immune regulation and immune-placental communication, including CD274 (PD-L1), CSF1R, IL22RA2, and EBI3, were dysregulated in fetal death cases, supporting the concept that altered immune tolerance and immune signaling contribute to this syndrome.^{39,145} These findings suggest a complex landscape of biological disruptions associated with fetal death, implicating processes

essential for fetal development, the immune system, and structural integrity. This underscores the value of broadening the scope of biomarker discovery to improve disease prediction.

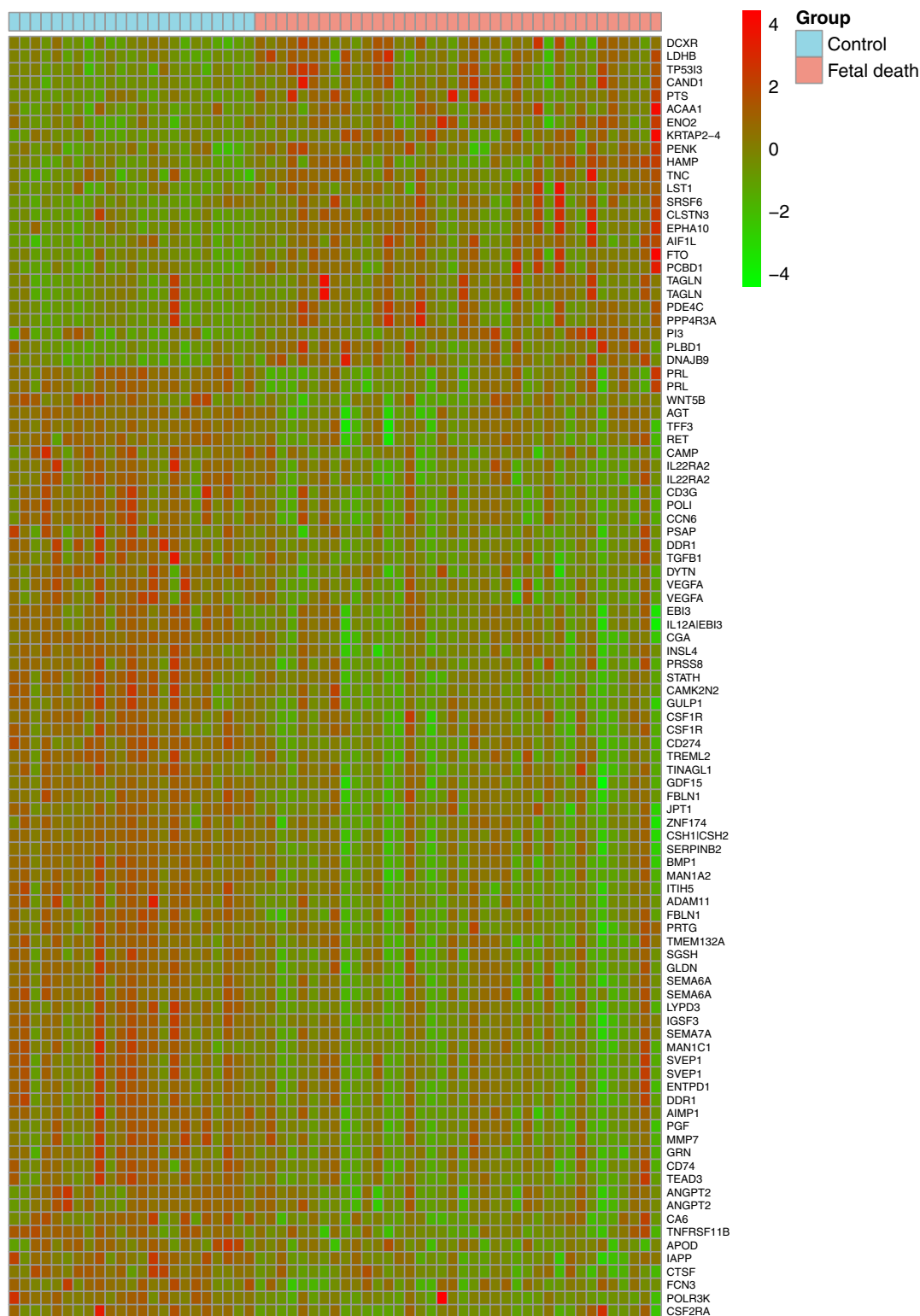
The integration of additional proteins into our molecular classifier, alongside PlGF and sFlt-1, increased the AUC from 72% to 86%. The proteins glycoprotein hormones alpha chain, DnaJ homolog subfamily B member 9, and DNA-directed RNA polymerase III subunit RPC10 emerged as the top 3 most important candidates to improve discrimination beyond PlGF and sFlt-1.

New biomarkers and biological processes implicated in fetal death

Glycoprotein hormones alpha chain, also known as chorionic gonadotropin subunit alpha, is the shared alpha subunit of

FIGURE 3

Maternal plasma profiles of differentially abundant proteins in fetal death and control pregnancies displayed as a heatmap



Heatmap depicting proteins, identified by their corresponding gene symbols, that showed significant differences in abundance between pregnancies complicated by fetal death and gestational age—matched controls. The heatmap was generated using the R/Bioconductor package, *pheatmap*.

Romero. Plasma protein biomarkers for fetal death. *Am J Obstet Gynecol* 2026.

TABLE 3

Biological processes associated with plasma proteins differentially abundant in fetal deaths vs controls

Biological process	No. of proteins	OR	<i>q</i> value	Decreased in fetal death	Increased in fetal death
Negative regulation of bone resorption	3	19.5	.016	IAPP, TNFRSF11B	HAMP
Negative regulation of tissue remodeling	4	17.5	.009	IAPP, TNFRSF11B, AGT	HAMP
Negative regulation of bone remodeling	3	16.2	.023	IAPP, TNFRSF11B	HAMP
Regulation of bone resorption	4	11.4	.016	IAPP, TNFRSF11B, CSF1R	HAMP
Regulation of kidney development	3	11.4	.041	VEGFA, AGT, RET	
Lactation	3	10.8	.042	DDR1, VEGFA, PRL	
Monocyte differentiation	3	10.8	.042	VEGFA, CSF1R, CD74	
Regulation of positive chemotaxis	3	10.8	.042	PGF, VEGFA, ANGPT2	
Positive regulation of morphogenesis of an epithelium	3	10.2	.042	VEGFA, WNT5B, AGT	
Vascular endothelial growth factor signaling pathway	4	9.7	.023	SEMA6A, PGF, VEGFA, IL12A EBI3	
Mmacrophage cytokine production	3	9.7	.043	SEMA7A, CD74, TGFB1	
Regulation of macrophage cytokine production	3	9.7	.043	SEMA7A, CD74, TGFB1	
Regulation of bone remodeling	4	9.0	.028	IAPP, TNFRSF11B, CSF1R	HAMP
Regulation of tissue remodeling	6	8.7	.009	IAPP, TNFRSF11B, AGT, CSF1R, TGFB1	HAMP
Bone resorption	4	7.9	.036	IAPP, TNFRSF11B, CSF1R	HAMP
Body fluid secretion	4	7.5	.041	STATH, DDR1, VEGFA, PRL	
Positive regulation of receptor signaling pathway via STAT	4	6.9	.042	CSH1 CSH2, AGT, PRL, TGFB1	
Cellular response to vascular endothelial growth factor stimulus	4	6.7	.042	SEMA6A, PGF, VEGFA, IL12A EBI3	
Bone remodeling	5	6.2	.035	IAPP, TNFRSF11B, CSF1R, TGFB1	HAMP
Receptor signaling pathway via STAT	10	6.1	.007	CSH1 CSH2, IL22RA2, CSF2RA, AGT, PRL, CSF1R, IL12A EBI3, TGFB1, RET	HAMP
Receptor signaling pathway via JAK-STAT	9	5.7	.009	CSH1 CSH2, IL22RA2, CSF2RA, AGT, PRL, CSF1R, IL12A EBI3, RET	HAMP
Female pregnancy	8	5.3	.014	DDR1, FBLN1, TEAD3, PGF, VEGFA, ANGPT2, AGT, PRL	
Negative regulation of angiogenesis	5	5.2	.042	SEMA6A, CCN6, ANGPT2, AGT, PRL	
Negative regulation of blood vessel morphogenesis	5	5.2	.042	SEMA6A, CCN6, ANGPT2, AGT, PRL	
Negative regulation of vasculature development	5	5.1	.043	SEMA6A, CCN6, ANGPT2, AGT, PRL	
Positive regulation of leukocyte chemotaxis	5	5.1	.043	PGF, VEGFA, CSF1R, CD74, IL12A EBI3	
Multiorganism reproductive process	8	4.9	.016	DDR1, FBLN1, TEAD3, PGF, VEGFA, ANGPT2, AGT, PRL	
Regulation of receptor signaling pathway via STAT	5	4.8	.049	CSH1 CSH2, AGT, PRL, TGFB1, RET	
Multi-multicellular organism process	8	4.7	.016	DDR1, FBLN1, TEAD3, PGF, VEGFA, ANGPT2, AGT, PRL	

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(continued)

TABLE 3

Biological processes associated with plasma proteins differentially abundant in fetal deaths vs controls (continued)

Biological process	No. of proteins	OR	<i>q</i> value	Decreased in fetal death	Increased in fetal death
Regulation of chemotaxis	9	4.5	.016	SEMA6A, PGF, VEGFA, ANGPT2, SEMA7A, CSF1R, CD74, IL12A EBI3, TGFB1	
Positive regulation of chemotaxis	6	4.2	.043	PGF, VEGFA, CSF1R, CD74, IL12A EBI3, TGFB1	
Tissue remodeling	6	4.1	.049	IAPP, TNFRSF11B, AGT, CSF1R, TGFB1	HAMP
Positive regulation of cell migration	15	3.6	.009	CD274, CGA, SEMA6A, PGF, VEGFA, SEMA7A, WNT5B, GRN, AGT, CSF1R, MMP7, CD74, IL12A EBI3, TGFB1, RET	
Regulation of angiogenesis	9	3.6	.034	SEMA6A, PGF, VEGFA, CCN6, ANGPT2, GRN, AGT, PRL, CAMP	
Regulation of vasculature development	9	3.6	.036	SEMA6A, PGF, VEGFA, CCN6, ANGPT2, GRN, AGT, PRL, CAMP	
Regulation of growth	14	3.5	.012	CGA, DDR1, CSH1 CSH2, SEMA6A, VEGFA, SEMA7A, AGT, PRL, GDF15, TGFB1	FTO, CLSTN3, TNC, HAMP
Regulation of developmental growth	8	3.5	.042	CGA, SEMA6A, VEGFA, SEMA7A, PRL, GDF15	FTO, HAMP
Positive regulation of cell motility	15	3.4	.010	CD274, CGA, SEMA6A, PGF, VEGFA, SEMA7A, WNT5B, GRN, AGT, CSF1R, MMP7, CD74, IL12A EBI3, TGFB1, RET	
Positive regulation of locomotion	15	3.4	.011	CD274, CGA, SEMA6A, PGF, VEGFA, SEMA7A, WNT5B, GRN, AGT, CSF1R, MMP7, CD74, IL12A EBI3, TGFB1, RET	
Gland development	10	3.3	.036	CGA, IGSF3, DDR1, VEGFA, PRL, CSF1R, TGFB1, PSAP	TNC, HAMP
Transmembrane receptor protein tyrosine kinase signaling pathway	15	3.0	.016	DDR1, CSH1 CSH2, SEMA6A, PGF, VEGFA, SVEP1, APOD, ANGPT2, AGT, CSF1R, GDF15, IL12A EBI3, TGFB1, RET	EPHA10
Peptidyl-tyrosine phosphorylation	11	3.0	.039	DDR1, CSH1 CSH2, VEGFA, IL22RA2, AGT, CSF1R, CD74, IL12A EBI3, TGFB1, RET	EPHA10
Peptidyl-tyrosine modification	11	3.0	.041	DDR1, CSH1 CSH2, VEGFA, IL22RA2, AGT, CSF1R, CD74, IL12A EBI3, TGFB1, RET	EPHA10
Negative regulation of developmental process	17	2.8	.016	CGA, STATH, FBLN1, IAPP, SEMA6A, VEGFA, TNFRSF11B, CCN6, ANGPT2, SEMA7A, PRTG, AGT, PRL, GDF15, CD74, TGFB1	SRSF6
Developmental growth	12	2.8	.042	CGA, DDR1, SEMA6A, VEGFA, APOD, SEMA7A, AGT, PRL, GDF15, PSAP	FTO, HAMP
Regulation of cell migration	18	2.7	.016	CD274, CGA, SEMA6A, PGF, VEGFA, APOD, ANGPT2, SEMA7A, WNT5B, GRN, AGT, CSF1R, MMP7, CD74, IL12A EBI3, TGFB1, RET	TNC
Regulation of anatomical structure morphogenesis	17	2.7	.022	FBLN1, SEMA6A, PGF, VEGFA, TNFRSF11B, CCN6, ANGPT2, SEMA7A, WNT5B, GRN, AGT, PRL, CSF1R, CAMP, TGFB1, RET	LST1
Growth	16	2.7	.023	CGA, DDR1, CSH1 CSH2, SEMA6A, VEGFA, APOD, SEMA7A, AGT, PRL, GDF15, TGFB1, PSAP	FTO, CLSTN3, TNC, HAMP
Tube morphogenesis	16	2.7	.027	DDR1, AIMP1, SEMA6A, PGF, VEGFA, APOD, CCN6, ANGPT2, GRN, AGT, PRL, CSF1R, CAMP, IL12A EBI3, TGFB1, RET	
Blood vessel morphogenesis	13	2.7	.042	AIMP1, SEMA6A, PGF, VEGFA, APOD, CCN6, ANGPT2, GRN, AGT, PRL, CAMP, IL12A EBI3, TGFB1	

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(continued)

TABLE 3

Biological processes associated with plasma proteins differentially abundant in fetal deaths vs controls (continued)

Biological process	No. of proteins	OR	q value	Decreased in fetal death	Increased in fetal death
Angiogenesis	12	2.7	.042	AIMP1, SEMA6A, PGF, VEGFA, APOD, CCN6, ANGPT2, GRN, AGT, PRL, CAMP, IL12A EBI3	
Positive regulation of cell population proliferation	17	2.5	.036	CD274, CGA, INSL4, PGF, VEGFA, CSF2RA, GRN, AGT, PRL, CSF1R, CD74, CAMP, IL12A EBI3, TGFB1	TNC, SRSF6
Vasculature development	14	2.5	.042	AIMP1, SEMA6A, PGF, VEGFA, SVEP1, APOD, CCN6, ANGPT2, GRN, AGT, PRL, CAMP, IL12A EBI3, TGFB1	

List of biological processes overrepresented among plasma proteins differentially abundant in fetal death cases compared with controls. For processes with at least 3 significant proteins, the ORs and Q values from a Fisher exact test are provided. In addition, the corresponding gene symbols for these proteins and their direction of change in the fetal death group compared with controls are included.

HAMP, hepcidin antimicrobial peptide; PGF, placenta growth factor; OR, odds ratio.

Romero. Plasma protein biomarkers for fetal death. Am J Obstet Gynecol 2026.

human chorionic gonadotropin, luteinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone.¹⁴⁶ A recent meta-analysis demonstrated that low levels of human chorionic gonadotropin in the first trimester of pregnancy are associated with spontaneous abortion, whereas elevated levels of human chorionic gonadotropin in the second trimester of pregnancy are associated with both spontaneous abortion and stillbirth.¹⁴⁷ Here, glycoprotein hormones alpha chain abundance was significantly reduced in fetal death cases compared with controls. Similar reductions were observed for chorionic gonadotropin subunit beta 3 and intact human chorionic gonadotropin, although these did not remain significant after correction for multiple comparisons. These proteins are produced by placental syncytiotrophoblasts.^{148,149} In addition, our earlier study of the maternal plasma proteome in uncomplicated pregnancies showed a constitutive abundance of chorionic gonadotropin (CG)-alpha, followed by a reduction approaching term.¹⁰¹ CG-alpha and chorionic gonadotropin subunit beta 3 are produced by the placental syncytiotrophoblasts.¹⁵⁰ Consequently, the reduced concentrations observed in fetal death cases could indicate placental insufficiency.

DnaJ homolog subfamily B member 9, also known as endoplasmic reticulum

DnaJ homolog 4 (ERdj4), emerged as the second most informative biomarker after CG-alpha. ERdj4, a co-chaperone for the Hsp70 protein endoplasmic reticulum chaperone BiP, has been implicated in several biological processes, including epithelial-mesenchymal transition, cellular survival, B-cell development, and glucose metabolism.¹⁵¹ It assists in the clearance of misfolded proteins through the endoplasmic reticulum-associated protein degradation pathway.¹⁵² Elevated levels of ERdj4 have been reported in serum samples from individuals with fibrillary glomerulonephritis, highlighting its potential as a biomarker for this disease.¹⁵³ We previously noted a moderate increase in maternal plasma levels with gestation during normal pregnancy.¹⁰¹ To the best of our knowledge, although abnormal levels of heat shock proteins have been documented in adverse pregnancy outcomes,¹⁵⁴ the increase in circulating ERdj4 in fetal death is a new finding.

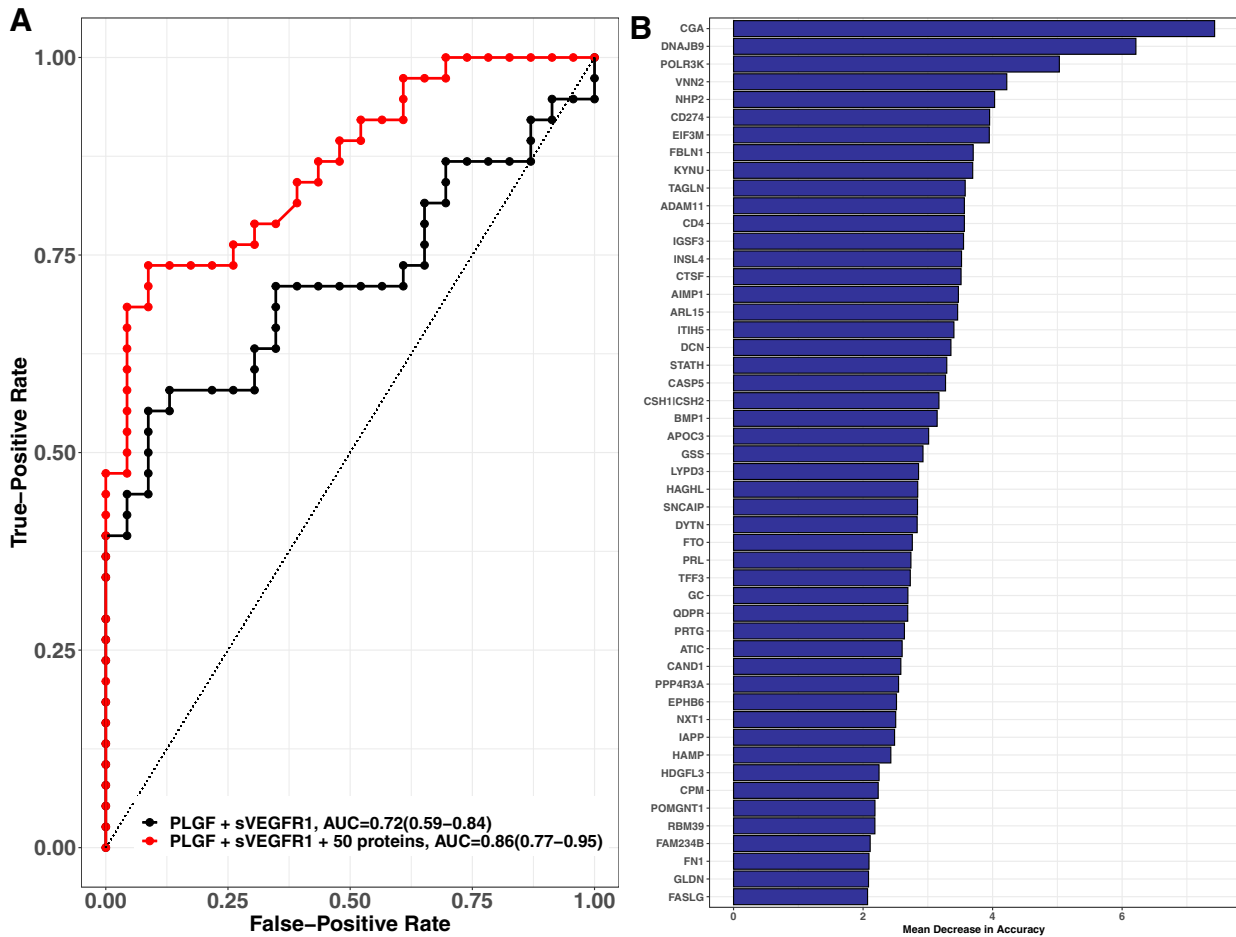
Furthermore, the association between abnormal soluble DNA-directed RNA polymerase III subunit RPC10 (RPC10) and adverse pregnancy outcomes seems to be a new finding. We previously observed an increase in the abundance of this protein in maternal plasma during uncomplicated pregnancies.¹⁰¹ Herein, we observed a significant decrease in the abundance of the RPC10 in fetal death

cases compared with controls. Moreover, this protein is ranked third among the most important biomarkers of fetal death. RPC10, a ubiquitously expressed intracellular protein, serves as a component of RNA polymerase III.^{150,155,156} It is integral to transcription termination, monitors transcription fidelity, and facilitates the correction of misincorporations.^{157,158} Mutations in polymerase III subunits, including RPC10, have been linked to a range of neurodegenerative diseases, including hypomyelinating leukodystrophy.^{159,160}

The fact that the 3 most informative proteins are among those modulated during normal pregnancy aligns with our previous observation that biomarkers of preeclampsia are enriched among proteins that change with advancing pregnancy.⁹⁷ In addition, the shared antiangiogenic profile and presence of maternal vascular malperfusion between fetal death and preeclampsia can explain why risk scores obtained with the fetal death proteomic classifier significantly discriminate patients diagnosed with late preeclampsia from GA-matched controls. The lower discrimination accuracy obtained for late preeclampsia in an independent dataset (AUC, 0.72) relative to the cross-validated estimate for fetal death (AUC, 0.86) is expected, given the lower frequency of maternal vascular

FIGURE 4

The maternal plasma proteome can discriminate fetal death from control pregnancies



A, Receiver operating characteristic curves for classifying maternal plasma samples into fetal death and controls. **B**, Bar plot displaying the relative importance of the top 50 proteomic predictors for distinguishing fetal death cases from controls. Glycoprotein hormones alpha chain (CGA), DnaJ homolog subfamily B member 9 (DNAJB9), and DNA-directed RNA polymerase III subunit RPC10 (POLR3K) emerged as the top 3 candidates to improve prediction relative to PLGF and soluble fms-like tyrosine kinase 1.

AUC, area under the receiver operating characteristic curve; PLGF, placental growth factor; sVEGFR1, soluble vascular endothelial growth factor receptor-1.

Romero. Plasma protein biomarkers for fetal death. *Am J Obstet Gynecol* 2026.

malperfusion and the more attenuated antiangiogenic profile in late pre-eclampsia than in fetal death.^{8,26,120}

New biological processes implicated in fetal death

Pathway analysis revealed dysregulation of biological processes not traditionally emphasized in the clinical evaluation of fetal death. These included immune activation, complement regulation, inflammatory signaling, cellular stress responses, extracellular matrix remodeling, and metabolic pathways. These findings

extend previous mechanistic work from our group, showing that fetal death in normally formed fetuses is frequently preceded by hypoxia with myocardial and brain injury.⁶⁵ The current data demonstrate that such fetal and placental processes are accompanied by measurable changes in maternal plasma composition.

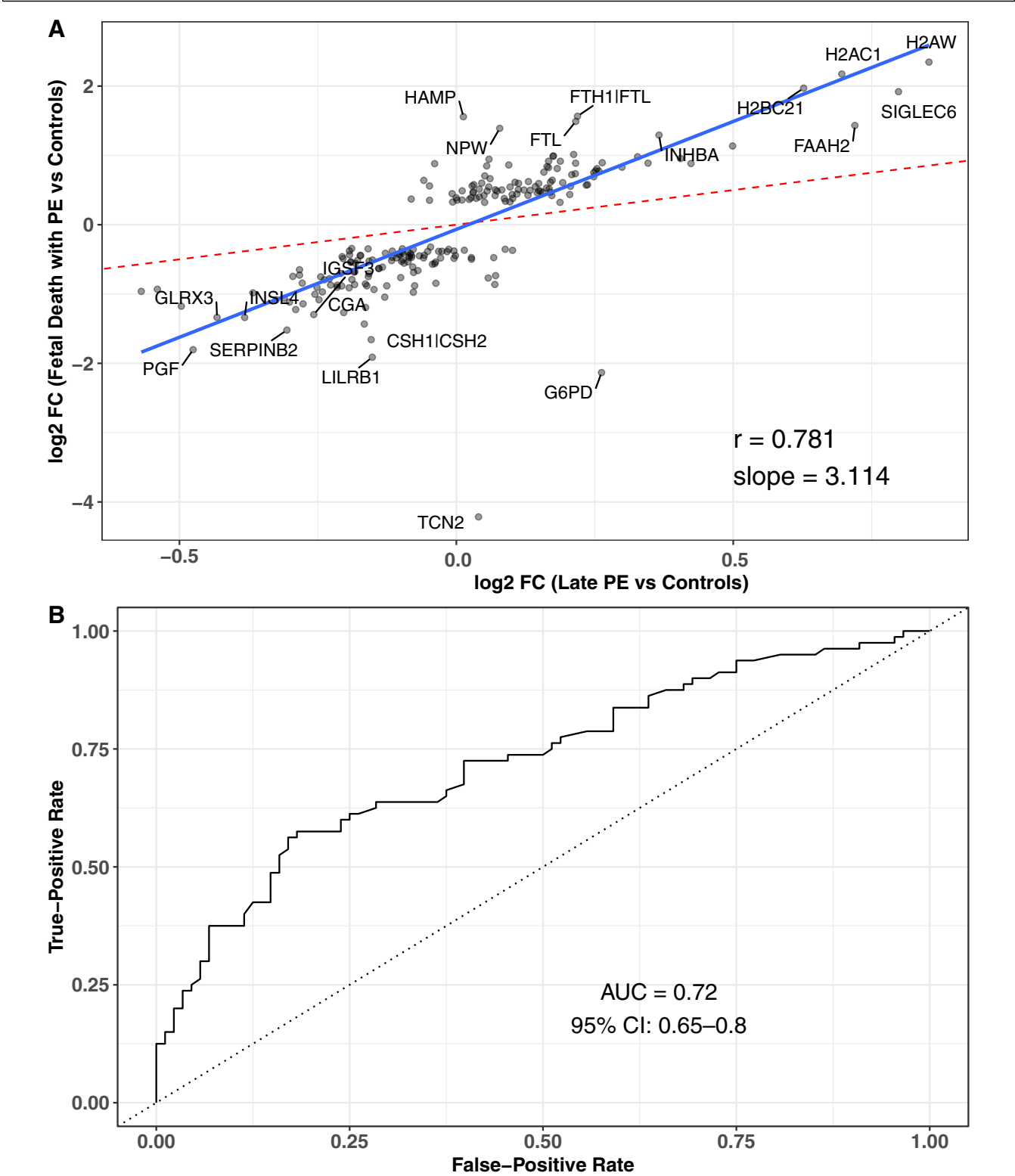
Therefore, maternal blood may be conceptualized as a liquid biopsy of the placenta, fetus, and mother, providing an integrated view of intrauterine pathophysiology. The systematic analysis of the maternal plasma proteome

captures biological signals arising from multiple pathogenic pathways and offers a powerful approach for interrogating complex obstetrical syndromes.

Clinical implications

This study represents a step forward in the investigation of fetal death by expanding molecular discovery at the time of diagnosis. By moving beyond targeted biomarker approaches and applying large-scale maternal plasma proteomics, we have demonstrated that fetal death is associated with a broad spectrum of

FIGURE 5
External validation of fetal death—associated proteomic changes



dysregulated biological processes that extend beyond angiogenic imbalance alone. Although these findings do not directly translate into immediate changes in clinical management, they provide an essential foundation for progress. Advances in the prediction and prevention of fetal death require a deeper understanding of the underlying biology, and discovery-driven studies of this nature are necessary to inform future translational efforts and the development of improved risk stratification strategies.^{141,161–167} There is considerable clinical interest in identifying the risk of fetal death across all pregnancies, particularly among patients with a history of stillbirth, who face a substantially increased recurrence risk and often seek individualized risk assessment and counseling. In a large multicountry cohort study, women with a stillbirth in their first pregnancy had more than double the risk of stillbirth in a subsequent pregnancy than those whose first pregnancy resulted in a live birth (adjusted hazard ratio, 2.25 [95% CI, 1.86–2.72]; absolute risks: 2.5% vs 0.5%, respectively), although stillbirth remains a relatively rare outcome overall.¹⁶⁸ These findings are consistent with other reports in third pregnancies of mothers with a stillbirth.¹¹ Angiogenic biomarkers, such as PlGF and sFlt-1, perform well in pregnancies characterized by maternal vascular malperfusion^{82–84,88,143,144,169} but may not capture risks arising from other pathogenic pathways. Fetal death is a syndrome, and therefore, biomarkers limited to angiogenic pathways will miss some cases. Discovery-based approaches, including large-scale proteomic profiling, a platform that has yielded encouraging results in identifying candidate biomarkers across the great obstetrical

syndromes,^{99,101,170–173} can help uncover additional pathways and expand the repertoire of potential risk markers beyond placental vascular dysfunction.

Research implications

The ability to accurately identify women at risk of fetal death will allow for optimal clinical management and the development of suitable treatment strategies. Proteins dysregulated at the time of diagnosis of fetal death, such as PlGF and sFlt-1, have been shown to have predictive value before diagnosis at 24 to 28 weeks of gestation.⁸³ Similar evidence of dysregulation in maternal plasma protein abundance at the time of diagnosis being preceded by changes earlier in pregnancy has been found for other obstetrical syndromes, such as preeclampsia and SGA.^{8,97,100,174} Moreover, the fact that this study identified proteins that are more differentially dysregulated at the time of diagnosis relative to the state-of-the-art biomarkers (the combination of PlGF and sFlt1) suggests their predictive value in samples collected before diagnosis. Therefore, integrating such novel proteomic markers with known risk factors for fetal death has the potential to improve the prediction of this syndrome. Although a cross-validated AUC of 86% was obtained using a highly multiplexed platform, it is reasonable to assume that more accurate targeted assays for the novel candidate proteins may lead to superior prediction accuracy.

Strengths and limitations

The strengths of this study include the profiling of more than 7000 protein analytes and the robust data analysis methods that include the control of the FDR and cross-validation. Cross-study

validation of changes in a subset of cases first diagnosed with preeclampsia is also a strength. The limitations of the study include the moderate sample size given the low prevalence of fetal death, and data collection from a single center located in an area with a high risk of preterm birth, possibly restricting the generalizability of the results to other populations. Therefore, replication of the study using larger and more diverse cohorts, eventually including multiple pregnancies, is warranted. Although the number of controls used in the primary analysis was lower (n=23) than the number of cases (n=38), the resulting proteomic changes reported were consistent with those obtained when data from 91 additional controls were included. Furthermore, although we demonstrated a significant correlation between protein log₂ fold changes in fetal death with preeclampsia and those reported in late preeclampsia in an independent study, the GA window at sampling in the late preeclampsia study was narrower (30–41 vs 20–41 weeks, respectively), and the fraction of cases already diagnosed with the disease at the time of blood collection was lower (61% vs 100%, respectively). It is also important to acknowledge that the samples were collected after diagnosis, which likely occurred up to several days after fetal demise. Future studies will be needed to determine whether the changes observed at the time of diagnosis are also present several weeks before diagnosis, leading to prediction of fetal death.

Conclusion

This study provides the most comprehensive investigation of the proteomic profiles of maternal plasma in pregnancies complicated by fetal death. The

A, Scatterplot of log₂ fold changes for proteins significantly dysregulated in fetal deaths with preeclampsia (this study) and log₂ fold changes in late-onset preeclampsia from an independent study (Andresen et al¹⁰⁵). As an example, PlGF and GLRX3 were decreased, whereas FAAH2 and SIGLEC6 were increased in preeclampsia leading to either fetal death (this study) or live birth (Andresen et al¹⁰⁵). In contrast, TCN2 and G6PD were decreased, and hepcidin (HAMP) was increased only in preeclampsia leading to fetal death. **B**, ROC curve showing the performance of the fetal death random forest classifier when used in the late-onset preeclampsia study by Andresen et al¹⁰⁵ (AUC, 0.72 [95% CI, 0.65–0.80]).

AUC, area under the receiver operating characteristic curve; CI, confidence interval; FAAH2, fatty acid amide hydrolase 2; G6PD, glucose-6-phosphate 1-dehydrogenase; GLRX3, glutaredoxin 3; HAMP, hepcidin antimicrobial peptide; PlGF, placenta growth factor; ROC, receiver operating characteristic curve; SIGLEC6, sialic acid-binding immunoglobulin-like lectin 6; TCN2, transcobalamin 2.

Romero. Plasma protein biomarkers for fetal death. *Am J Obstet Gynecol* 2026.

GLOSSARY

Area under the receiver operating characteristic curve: A summary measure of a model's ability to correctly distinguish cases from controls.

Cross-validation: A model evaluation approach in which the dataset is partitioned into multiple folds, with each fold used once as an independent test set while the remaining folds are used for training.

Decision tree: A simple predictive model that classifies samples by applying a series of sequential rules based on feature values, resembling a flowchart.

False discovery rate: The expected proportion of false-positive findings among all results declared statistically significant.

Feature importance: A measure of how much a specific protein contributes to a model's predictive accuracy.

Hypergeometric test: A statistical test used to identify biological processes that are overrepresented among proteins showing significant changes in abundance compared with what would be expected by chance.

Multiple of the mean: A normalized measure of protein abundance in which each value is expressed relative to the mean value at the same gestational age.

Multiple testing correction: A statistical adjustment applied when many hypotheses are tested simultaneously.

Principal component analysis: An unsupervised method that reduces high-dimensional data into a smaller number of components that capture the main sources of variation.

Random forest: A machine-learning method that combines many decision trees to improve predictive accuracy and robustness.

Receiver operating characteristic curve: A graphical summary of model performance that plots sensitivity against the false-positive rate.

Sensitivity: The proportion of true cases correctly identified by the model.

Specificity: The proportion of true controls correctly identified by the model.

q value: A false discovery rate—adjusted nominal *P* value that accounts for multiple testing.

differential abundance of specific proteins, coupled with functional analysis, highlights the dysregulation of pregnancy-related biological processes in fetal death. Furthermore, the superior accuracy of plasma proteomic models relative to state-of-the-art protein markers (PIGF and sFlt-1) supports the potential utility of the new candidate markers for this disease.

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