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ORIGINAL ARTICLE

Characterization of the procoagulant phenotype of amniotic fluid across gestation in rhesus macaques and humans

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Abstract

Background: Amniotic fluid (AF) plays a key role in fetal development, yet the evolving composition of AF and its effects on hemostasis and thrombosis are poorly understood.

Objectives: To characterize the procoagulant properties of AF as a function of gestation in humans and nonhuman primates.

Methods: We analyzed the proteomes, lipidomes, and procoagulant properties of AF obtained by amniocentesis from rhesus macaque and human pregnancies at gestational age-matched time points.

Results: When added to human plasma, both rhesus and human AF accelerated clotting time and fibrin generation. We identified proteomic modules associated with clotting time and enriched for coagulation-related pathways. Proteins known to be involved in hemostasis were highly correlated with each other, and their intensity of expression varied across gestation in both rhesus and humans. Inhibition of the contact pathway did not affect the procoagulant effect of AF. Blocking tissue factor pathway inhibitor

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reversed the ability of AF to block the generation of activated factor X. The prothrombinase activity of AF was inhibited by phospholipid inhibitors. The levels of phosphatidylserine in AF were inversely correlated with clotting time. AF promoted platelet activation and secretion in plasma.

Conclusion: Overall, our findings reveal that the addition of AF to plasma enhances coagulation in a manner dependent on phospholipids as well as the presence of proteases and other proteins that directly regulate coagulation. We describe a correlation between clotting time and expression of coagulation proteins and phosphatidylserine in both rhesus and human AF, supporting the use of rhesus models for future studies of AF biology.

KEYWORDS
amniotic fluid, coagulation factors, hemostasis, pregnancy, rhesus macaque

Essentials

- AF is important to maternal coagulation, but the mechanisms are elusive.
- AF proteins involved in coagulation change throughout pregnancy in human and nonhuman primates.
- AF increases blood clotting by phospholipids and other proteins that help regulate coagulation.
- Our data support the use of nonhuman primate models for future studies of AF biology.

1 | INTRODUCTION

Amniotic fluid (AF) is comprised of a complex and dynamic milieu that serves to support fetal development throughout gestation. Despite the ubiquitous presence of AF in gestation, the dynamic role of AF in physiology remains largely undefined. It has long been suspected that the molecular constituents of AF facilitate delivery and newborn survival by contributing to hemostasis during birth [1]. This hypothesis gains support from the hemostatic challenge posed by delivery. Birth injuries can occur during labor or delivery when the baby passes through the birth canal, affecting about 31 in every 1000 hospital births in the United States [2]. Thus, it would be natural to assume that evolution favors such a styptic.

Diametrically opposed to the benefits of limiting birth injury, AF-induced coagulation is surmised to be the main driver of consumptive coagulopathy and mortality associated with maternal AF embolism [3]. *In vitro* experiments have confirmed the procoagulant nature of AF to some extent, affirming that AF accelerates thrombin production, shortens clotting time, and activates platelet aggregation when exposed to blood [4,5]. AF has also been shown to contain procoagulant extracellular vesicles and increasing quantities of tissue factor (TF) over gestation, with data suggesting that extrinsic pathway proteins in AF play a role in regulating barrier function in fetal skin during development [6–8].

Beyond these initial observations, however, the nature and content of AF hemostatic proteins, how they may change throughout gestation, and their potential role in processes such as hemostasis, birth injury prevention, and the prothrombotic phenotype of pregnancy remain poorly understood. There may be limited transfer of proteins from AF to the maternal circulation, in particular during vaginal delivery [9], but how this might contribute to thrombosis in pregnancy remains undefined, especially considering that thrombosis is the leading cause of maternal mortality in developed nations [9–11]. Similarly, the role in which AF may safeguard against fetal loss and prevent subchorionic bleeding, when blood pools between the uterine wall and the chorionic membrane of the amniotic sac, is unknown.

The study of potential biological roles of human AF has been impeded by the practical challenges associated with obtaining AF from human pregnancies, especially longitudinal samples throughout gestation, and the lack of a close and suitable animal model for AF research. We have recently shown that the rhesus AF proteome is very similar to the human AF proteome throughout pregnancy and have established a reference AF proteome across gestation [12]. This work established the nonhuman primate (rhesus macaques – *Macaca mulatta*) as a relevant model of human AF biology.

In this study, we examine the molecular profile of AF in both rhesus macaque and human samples in order to better understand the procoagulant effects of AF across gestation [13,14]. We establish parallels between nonhuman primate and human AF on coagulation and, for the first time, employ analysis of the defined AF proteome to quantify shifts in AF coagulation protein levels over the course of gestation. Ultimately, we align these findings with *in vitro* studies directed at furthering our understanding of the physiology of AF during pregnancy.

2 | METHODS

2.1 | Reagents

A list of reagents is included in the Supplementary Methods.

2.2 | Experimental design

All methods were carried out in accordance with relevant guidelines and regulations. Protocols were approved by the Oregon Health & Science University Institutional Review Board for human AF (#20623) and the Oregon National Primate Research Center Institutional Animal Care and Use Committee for nonhuman primate AF (IP0001389). Nonhuman primate methods are reported in accordance with the Animal Research: Reporting of In Vivo Experiments guidelines (https://arriveguidelines.org) [15]. This study used indoor-housed, reproductive-age female nonhuman primates (n = 7), *Macaca mulatta*, maintained on a standard chow diet (TestDiet). Human AF samples were obtained from the Oregon Health & Science University Knight Diagnostic Laboratory, where informed consent was previously obtained to utilize the samples for research purposes.

2.3 | AF collection and proteomics

Rhesus and human AF samples were obtained as previously described [12]. Briefly, the rhesus sample group was comprised of 7 pregnant animals longitudinally sampled at 3 gestational time points (in days): gestational day (G)85, G110, and G135 (21 samples total, the term is approximately 168 days). These gestational time points were matched by percent completed gestation with 7 human samples at each time point (21 samples total). The comparable human gestational time point for G85 is approximately 20 weeks (G140); for G110, it is approximately 25.5 weeks (G175); and for G135, it is approximately 31.5 weeks (G210). Human samples were selected from pregnancies with the least pathologic fetal indications (eg, genetic testing with normal fetal anatomy on ultrasound). Determination of the overall AF proteomes at each gestational time point (rhesus G85, G110, and G135, and the comparable human gestational age [GA] time points: approximately G140, G175, and G210) was performed as previously described [12].

2.4 | Recalcified clotting time

Human venous blood was drawn in accordance with an institutional review board-approved protocol. Pooled normal plasma was prepared via centrifugation of citrated whole blood (in 0.32% w/v sodium citrate) from 4 separate donors at 2150 × g for 10 minutes twice. Pooled normal plasma or factor-depleted plasma (50 μL) was incubated with vehicle control or 1:10 AF samples followed by a 3-minute incubation with 50 μL of 25 mM 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid, 150 mM NaCl, pH 7.4 hepes buffered saline (HBS) in the absence or presence of selected inhibitors. Clot formation was initiated with $CaCl_2$ (50 μL, 8.3 mM final), and the time to clot formation was determined on a KC4 Coagulation Analyzer (Trinity Biotech). The clotting time measurement concluded when it reached 2000 seconds. Clotting time was measured using individual rhesus AF samples from G85, G110, and G135 gestational time points and individual human AF samples from equivalent gestational time points (G85, ∼20 weeks, G110, ∼25.5 weeks, and G135, ∼31.5 weeks).

2.5 | Fibrin generation

Pooled normal plasma (50 μL) was incubated with 1:10 AF at different gestational time points, followed by incubation with 50 μL of HBS for 3 minutes. $CaCl_2$ (50 μL, 8.3 mM final) was added to each plasma mixture. Fibrin generation was quantified by measuring changes in turbidity at an absorbance of 405 nm at 30-second intervals for 30 minutes using an Infinite M200 spectrophotometer (Tecan). The lag time and time to reach half of maximum turbidity were obtained.

2.6 | Factor XIIa generation

To measure the activation of factor (F)XII or prekallikrein in plasma, pooled normal plasma was incubated with pool samples of 1:10 AF at different gestational time points or 1:100 ellagic acid in the absence of $CaCl_2$ for 5 minutes. To measure the activation of FXII or prekallikrein in the AF, pool samples of 1:10 AF were incubated with 1:100 ellagic acid. The rate of hydrolysis of a FXIIa and kallikrein chromogenic substrate (2 mM Chromogenix S-2302) was measured at 405 nm.

2.7 | FXa generation

Innovin (1:100) was incubated with 50 pM FVIIa and 100 nM FX in the presence of 10 μL of pooled AF at different gestational time points in HBS containing 5 mM $CaCl_2$ and 0.3% bovine serum albumin for 15 minutes (final volume, 100 μL). In selected experiments, samples were incubated with the anti-TF pathway inhibitor (TFPI) K1 and K2 domain antibodies (10 μg/mL, respectively). A total of 10 μL of 100 mM EDTA was added to quench the reaction. The rate of substrate hydrolysis of 1 mM Chromogenix S-2765 was measured at 405 nm and then converted to FXa concentrations using a standard curve.

2.8 | Activation of prothrombin by prothrombinase complex

FVa (5 nM) and 0.2 nM FXa were incubated with 0.75 μM prothrombin in the presence of 10 μL of pooled AF at different gestational time points in HBS containing 5 mM $CaCl_2$ for 15 minutes (final volume, 100 μL). The reaction was stopped by adding 10 μL of 100 mM EDTA, and the rate of thrombin formation was determined by

measuring thrombin concentration through the rate of Chromogenix S-2238 hydrolysis. In separate experiments, annexin V (10 ng/mL), lactadherin (30 nM), or prothrombin substituted by γ-carboxyglutamic acid (GLA)-domainless prothrombin was added.

2.9 | Measurement of phosphatidylserine

Lipids in AF were dissolved using 1% peroxide-free Triton X-100 and subsequently extracted with methanol and methyl tert-butyl ether. The phosphatidylserine content within the lipid extract was quantified through fluorometric enzymatic assays following the manufacturer's instructions.

2.10 | Flow cytometry and analysis

Washed platelets (2×10^7/mL final concentration) were incubated in recalcified fibrinogen-depleted plasma containing 1:10 AF for 10 minutes. In selected experiments, rivaroxaban (10 μM), hirudin (25 U/mL), or annexin V (10 nM) was added. Alternatively, washed platelets (2×10^7/mL) were preincubated with 1:10 AF or vehicle (modified 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid/Tyrode buffer) for 10 minutes. FITC PAC-1 (1:50) or antigen presenting cell CD62P (1:50) were added to stain for activated integrin $\alpha_{IIb}\beta_3$ and P-selectin, respectively. In selected experiments, the platelet mixtures were then stimulated with cross-linked collagen-related peptide (10 μg/mL), ADP (adenosine diphosphate, 30 μM), AYPGKF (250 μM), or U46619 (2 μM) for 20 minutes. The reactions were stopped by adding 2% paraformaldehyde. Platelet activation was measured using a BD FACS Canto II flow cytometer (BD Biosciences), and analyses were performed on FlowJo software (version 10.8.1). Platelets were identified by logarithmic signal amplification for forward and side scatter characteristics and were collected for 1 minute at a medium flow rate.

3 | RESULTS

3.1 | The procoagulant effect of AF varies over GAs in humans and rhesus

We first evaluated the coagulability of AF in human plasma. AF accelerated clotting time and fibrin generation in both species. Clotting time in recalcified human plasma at baseline was ~570 seconds. The addition of human AF significantly reduced clotting time: G140 = 326.4 ± 43.1 seconds, G175 = 262.1 ± 43.8 seconds, and G210 = 294.2 ± 34.9 seconds (Figure 1A). Concurrently, human AF decreased the lag time for fibrin generation as well as the time to reach half-maximal in a dose-dependent manner (Figure 1B, Supplementary Figure S1A). The addition of rhesus AF also significantly decreased clotting time with a less prominent effect observed in AF collected at later gestational time points: G85 = 156.9 ± 43.1 seconds, G110 = 224.6 ± 43.8 seconds, and G135 = 294.9 ± 34.9 seconds. Rhesus AF also shortened the lag time and the time to reach half-maximal for fibrin generation in a similar manner to human AF (Figure 1C, D, Supplementary Figure S1B).

3.2 | Human and rhesus AF consensus coexpression networks

We previously compared the human and rhesus AF proteomes using quantitative liquid chromatography-mass spectrometry and found a 95.5% overlap between species with a high correlation of mean protein intensities [12]. In this study, we used a weighted gene correlation network analysis approach to identify protein consensus modules using as input 1090 unique orthologs that were identified in AF from both species. Protein intensities were log normalized within each dataset (species) prior to performing consensus network analysis. We identified 7 consensus modules that were subsequently correlated with biological traits and analyzed at the pathway level to characterize the associated biology (Figure 2A). The modules were enriched for a variety of biological processes, Kyoto Encyclopedia of Genes and Genomes pathways, and protein families but were most frequently associated with terms related to extracellular matrix, adhesion, neutrophil-mediated immunity, and complement or coagulation cascades.

3.3 | Protein modules associated with clotting time

Focusing on the coagulation-related pathways, the MEred3 module contained 59 proteins and was enriched for proteins involved in endopeptidase activity, proteolysis, and blood coagulation biological processes. Additionally, the MEred3 module had the highest correlation between the module eigenvalue and clotting time for individual human AF samples (Figure 2A). The majority of module eigenvalues in rhesus AF samples were correlated with clotting time due to the strong association between GA of rhesus AF and clotting time (Figure 2A) in these longitudinally collected samples. While the MEgrey module eigenvalue was also correlated with clotting time, this module, by default, contains proteins that were not highly coexpressed and, therefore, were unassigned to other modules. Analysis of the distributions of individual protein correlations with clotting times within each module confirms that the MEred3 module proteins were individually negatively correlated with clotting time (left-skewed distribution), while the MEgrey module proteins were not (Figure 2B). A STRINGdb protein-protein interaction network of the MEred proteins highlights proteins involved in complement activation (red), negative regulation of endopeptidase activity (yellow), blood coagulation (green), response to stress (pink), and the phosphatidylcholine metabolic process (blue; Figure 2C).

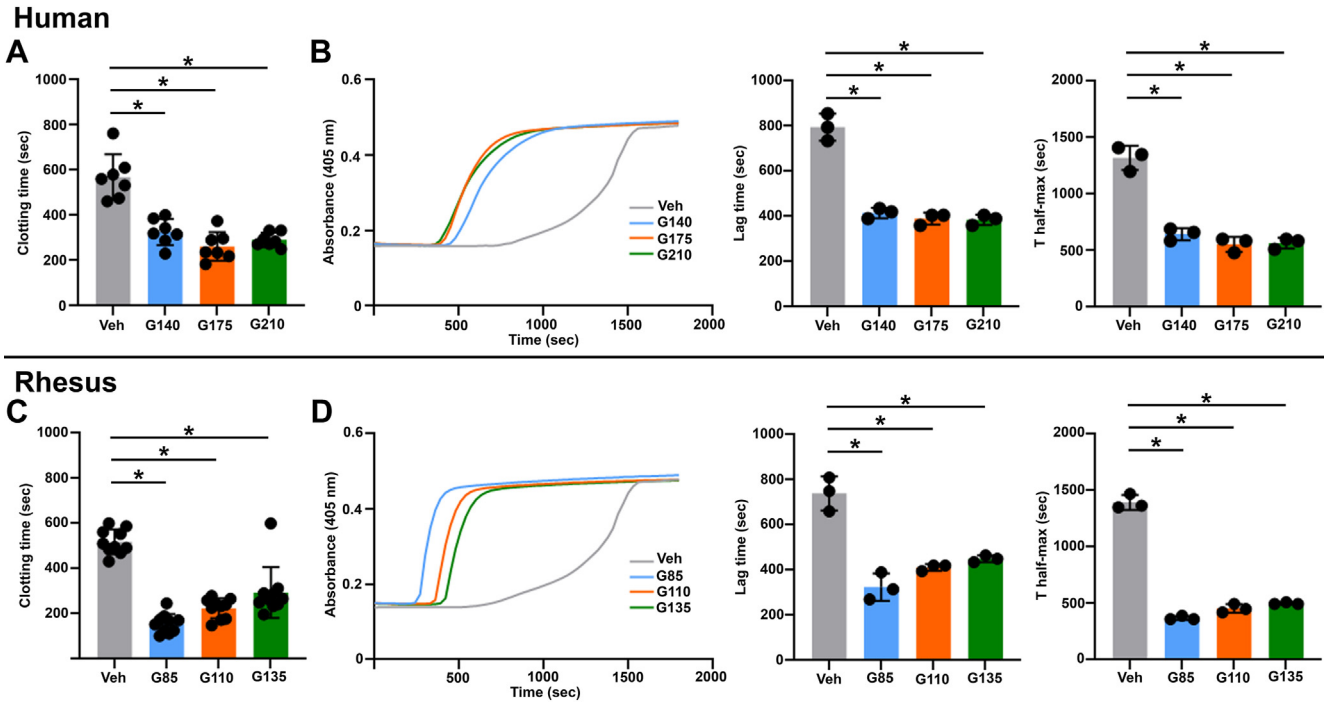


FIGURE 1 The procoagulant effect of amniotic fluid (AF) at different gestational time points. (A, C) A clotting assay was performed following the addition of human or rhesus AF at various gestational time points in a ratio of 1:10 to recalcified plasma. (B, D) Fibrin generation assay was measured in recalcified plasma preincubated with 10% v/v human or rhesus AF at different gestational time points. Time for fibrin generation initiation (lag time) and reaching half-maximum ($T_{half\text{-}max}$) were quantified. Data are mean ± SD (n = 7 in the clotting time assay and n = 3 in the fibrin generation assay). The Mann–Whitney U-test was used for statistical comparisons. G, gestational day. Veh, vehicle.

3.4 | Individual proteins associated with clotting time are enriched for complement and coagulation cascades

In addition to performing weighted gene correlation network analysis-based consensus network analysis to identify clusters of proteins correlated with AF effects on clotting time, we also performed proteome-wide association analysis to identify individual proteins associated with clotting time in each species. In human AF, we detected and analyzed 1381 proteins, of which 1 protein (HEG1) was associated with clotting time at false discovery rate $P < .05$ and 131 at nominal significance. In rhesus AF, out of 1381 proteins measured, a total of 415 were associated with clotting time at false discovery rate significance (205 up; 210 down). In both species, the top significant proteins were highly enriched for the Kyoto Encyclopedia of Genes and Genomes pathway "complement and coagulation cascades" (Supplementary Figure S2).

3.5 | AF coagulation proteins are highly correlated with each other

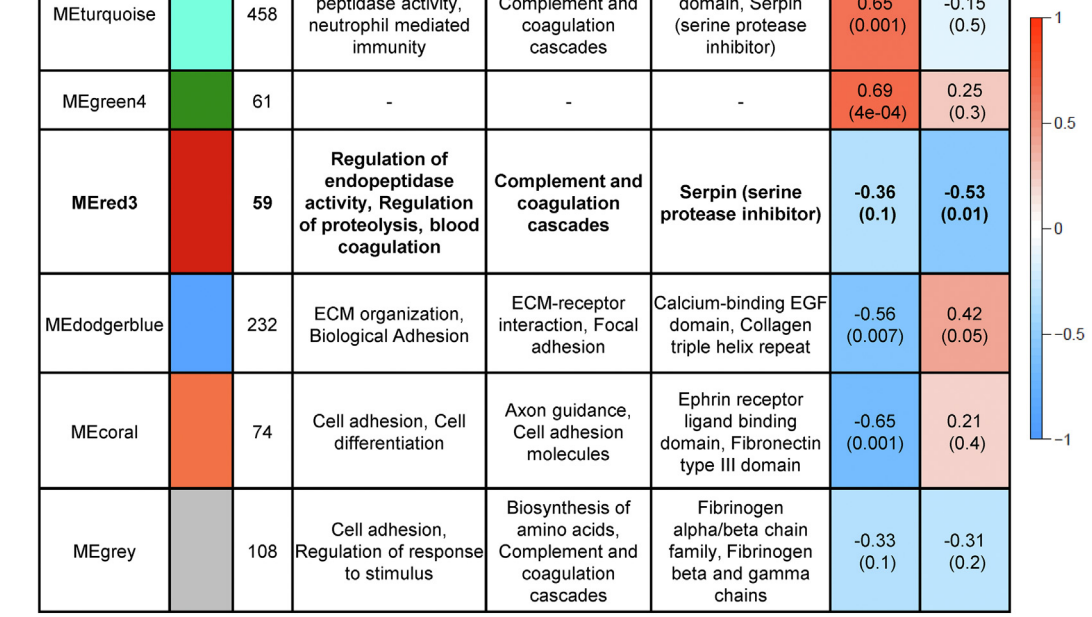
We next performed *a priori* analysis of candidate proteins known to be involved in hemostatic regulation. Our lookup table consisted of 7 categories: "Complement" (n = 37 proteins), "Contact Pathway" (n = 2), "Fibrinolysis" (n = 7), "Regulators of Coagulation" (n = 4), "Primary Hemostasis" (n = 5), "Secondary Hemostasis" (n = 14), and "Iron Metabolism" (n = 6). Out of 75 proteins in the lookup table, 51 were identified in rhesus AF and 55 in human AF. We next examined the correlation between individual proteins within each species and visualized proteins with a correlation above .7 in Figure 3A for humans and rhesus separately. Out of the 55 candidate proteins identified in human AF, 35 were significantly correlated (correlation > .7) with 1 or more candidate proteins. In human AF, complement component C6 had the largest number of significant correlations (n = 11), followed by antithrombin (SERPINC1; n = 10) and plasminogen (PLG; n = 8). In rhesus AF, there were 35 unique proteins with 1 or more correlations among candidate proteins; transferrin had the highest number of significant correlations (n = 12), followed by complement FI, PLG, and SERPINC1 (n = 11). Most correlations between candidate proteins were positive apart from TFPI, which was negatively correlated with SERPINC1, FV (F5), and transferrin, and a negative correlation between glycoprotein IV (CD36) and FXII (F12) in rhesus. In human AF, the only negative correlation was observed between FXIII (F13A) and protein homologous restriction factor (CD59).

3.6 | AF coagulation proteins are dynamic across gestation

We previously determined that GA at AF collection was a large source of variability in AF protein composition, with 31.9% of human proteins differentially expressed with GA and >50% of rhesus AF proteins

A

Name	Color	Size	Biological Process	KEGG	Pfam	Rhesus clotting	Human clotting
MEblack		98	Cell adhesion, neutrophil degranulation	Galactose metabolism, Metabolic pathways	-	-0.32 (0.2)	-0.18 (0.4)
MEturquoise		458	Regulation of peptidase activity, neutrophil mediated immunity	Lysosome, Complement and coagulation cascades	Calcium-binding EGF domain, Serpin (serine protease inhibitor)	0.65 (0.001)	-0.15 (0.5)
MEgreen4		61	-	-	-	0.69 (4e-04)	0.25 (0.3)
MEred3		**59**	**Regulation of endopeptidase activity, Regulation of proteolysis, blood coagulation**	**Complement and coagulation cascades**	**Serpin (serine protease inhibitor)**	**-0.36 (0.1)**	**-0.53 (0.01)**
MEdodgerblue		232	ECM organization, Biological Adhesion	ECM-receptor interaction, Focal adhesion	Calcium-binding EGF domain, Collagen triple helix repeat	-0.56 (0.007)	0.42 (0.05)
MEcoral		74	Cell adhesion, Cell differentiation	Axon guidance, Cell adhesion molecules	Ephrin receptor ligand binding domain, Fibronectin type III domain	-0.65 (0.001)	0.21 (0.4)
MEgrey		108	Cell adhesion, Regulation of response to stimulus	Biosynthesis of amino acids, Complement and coagulation cascades	Fibrinogen alpha/beta chain family, Fibrinogen beta and gamma chains	-0.33 (0.1)	-0.31 (0.2)



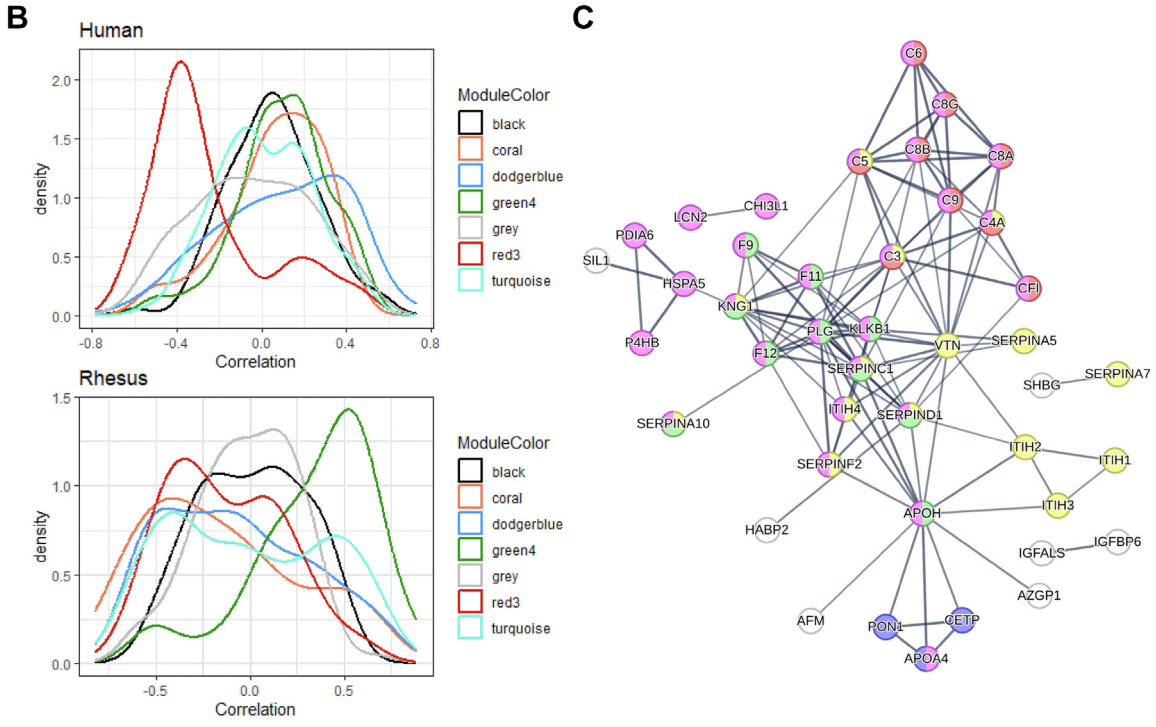


FIGURE 2 Untargeted proteomics identified modules associated with clotting time and enriched for pathways related to coagulation. We used consensus-weighted gene correlation network analysis to cluster all ortholog proteins into modules of correlated proteins. (A) The size of modules, biological enrichment summary, and association with clotting time. (B) We summarized the distribution of correlation values with clotting time for individual proteins in each module separately for each species. (C) Proteins identified in the MEred module are presented in a protein-protein interaction network and colored based on STRINGdb enrichment categories: complement activation (red), negative regulation of endopeptidase activity (yellow), blood coagulation (green), response to stress (pink), and the phosphatidylcholine metabolic process (blue). EGF, epidermal growth factor; ECM, extracellular matrix; KEGG, Kyoto Encyclopedia of Genes and Genomes.

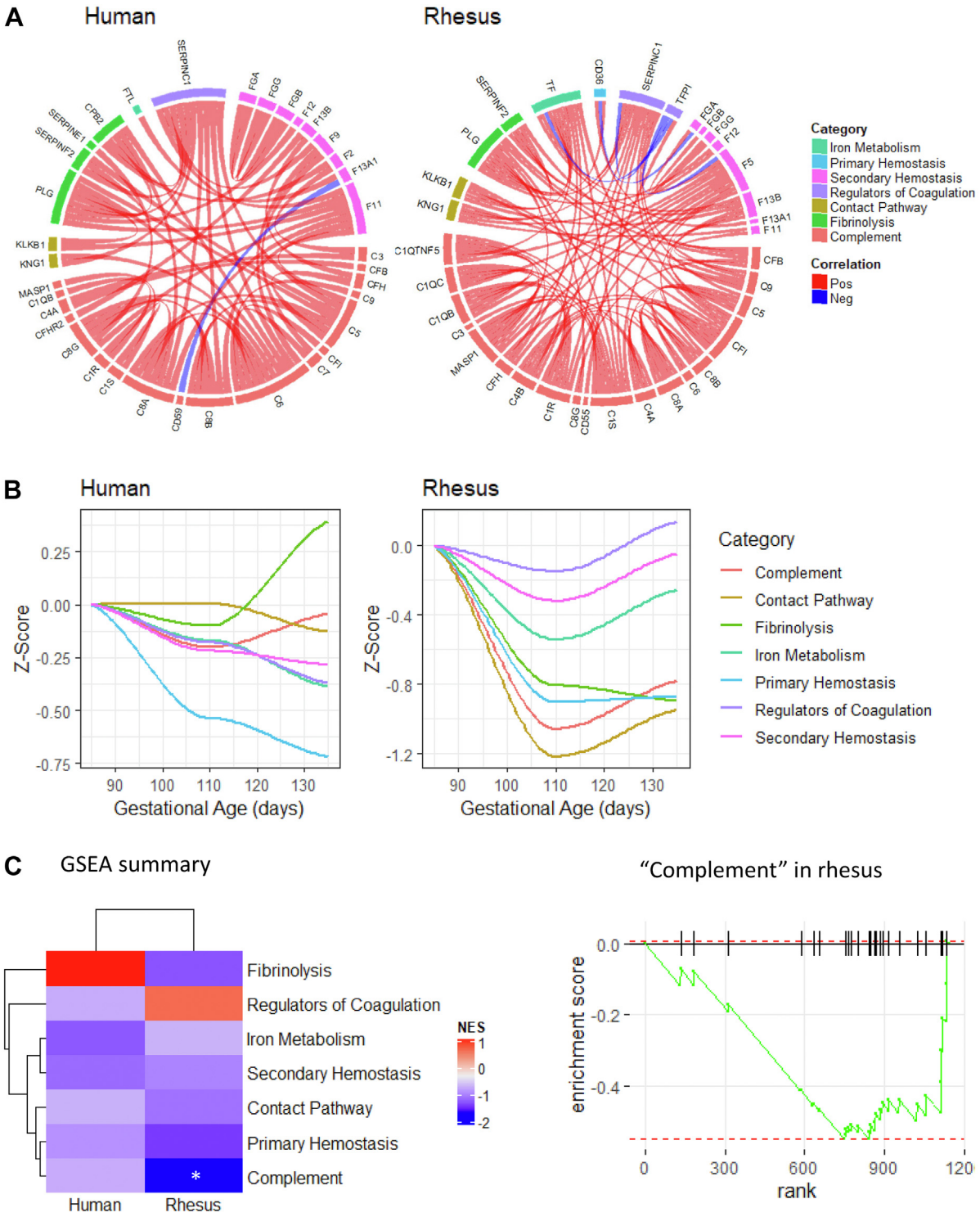


FIGURE 3 Proteins related to coagulation and hemostatic regulation change across gestation. Untargeted proteomic data were filtered for candidate proteins, and we summarized significant correlations (absolute value of correlation > .7) between individual proteins within each species, with positive (Pos) correlations shown in red and negative (Neg) in blue. (A) Individual candidate proteins are annotated to human ortholog names and colored according to functional categories. (B) For each species, we normalized the expression of each candidate protein to the reference group (~gestational day 85 nonhuman primate equivalent) and summarized within each category using Z-scores in order to visualize the longitudinal profile across gestation. (C) Gene set enrichment analysis (GSEA) was performed to determine whether *a priori* categories are enriched among proteins associated with gestational age. NES, normalized enrichment score.

changing with GA [12]. As described above, we also observed a strong relationship between clotting time and GA in rhesus AF. Therefore, we next examined whether the *a priori*-defined hemostatic protein candidates (Supplementary Table) were associated with GA. We first visually inspected the relative mean abundance of proteins in each category normalized to G85 rhesus equivalent AF (Figure 3B). In human AF, proteins involved in fibrinolysis increased while regulators of primary hemostasis categories either decreased gradually with increasing GA or remained stable. In rhesus AF, the trajectory of mean protein expression within each category exhibited 2 main patterns, with regulators of coagulation, secondary hemostasis, and iron metabolism decreasing slightly compared with G85, while proteins in the contact and complement pathways, fibrinolysis, and primary hemostasis categories decreasing more dramatically between G85 and G110 before plateauing or gradually increasing between G110 and G135. We next used gene set enrichment analysis to determine whether the *a priori*-defined protein categories described above are randomly distributed among ranked lists of proteins according to association with GA from our previous study [12] or whether they are enriched among top-up or downregulated proteins with GA [16]. We found no significant enrichment scores for candidate pathways among the proteins associated with GA in human AF. However, we did observe significant enrichment of the "Complement" candidate proteins among proteins associated with GA in rhesus AF (ES [enrichment score] = −0.55; NES [normalized enrichment score] = −1.72; adjusted *P* value = .03; Figure 3C).

3.7 | The procoagulant effect of AF is independent of the contact pathway of coagulation

To assess whether the procoagulant effect of the AF involves the activation of the contact pathway of coagulation, we performed experiments using human plasma deficient in FXII, FXI, FIX, or prekallikrein. We found that both human and rhesus AF effectively shortened the clotting time of plasma depleted in FXII, FXI, FIX, and prekallikrein (Figure 4A–D, H–K). To differentiate whether AF itself contained abundant coagulation factors or its procoagulant effect was not associated with the contact pathway, we employed pharmacologic inhibition of the contact pathway with corn trypsin inhibitor. Corn trypsin inhibitor was unable to preclude the ability of AF to reduce the clotting time of normal plasma (Figure 4E, L). These results separated the procoagulant effects of AF from the contact pathway of coagulation. The incubation of normal pooled plasma with AF did not result in significant FXII or pyruvate kinase activation (Figure 4F, M). In contrast, the incubation of ellagic acid with AF, which contained a certain amount of FXII and pyruvate kinase zymogen, could generate FXIIa or kallikrein (Figure 4G, N).

3.8 | FXa generation is regulated by TFPI in AF

We next assessed whether AF affected FXa generation initiated by the TF-FVIIa complex in a purified system. Surprisingly, the addition of human and rhesus AF led to a moderate reduction in FXa generation (Figure 5A, E). Previous research has reported that AF contains TFPI, an anticoagulant protein that inhibits the TF-FVIIa complex [17]. Therefore, we used blocking anti-TFPI K1 and K2 domain antibodies to determine whether TFPI present in AF was responsible for impeding FXa generation. We observed that the blocking anti-TFPI antibodies restored FXa generation in the presence of AF (Figure 5B, F). These results suggest that the procoagulant effect of AF was modulated by TFPI. The incubation of AF with FX and FVIIa in the absence of TF did not induce FX activation (data not shown). Proteomics of AF confirmed the existence and expression level of TFPI at different gestational time points (Figure 5C, G). The amount of TFPI present in the rhesus AF was significantly higher at later gestational time points (Figure 5G) and correlated with the clotting time of plasma with the addition of rhesus AF (Figure 5H).

3.9 | Phospholipids in AF contribute to activation of prothrombin by prothrombinase complex

The final and most critical step in the convergence of the contact and extrinsic pathway of coagulation is the common pathway. In the common pathway, FXa, activated FV, and calcium form the prothrombinase complex, which, along with phospholipids, catalyze the proteolytic conversion of prothrombin to thrombin. As shown in Figure 6A, E, we found that human and rhesus AF substantially enhanced thrombin generation in the absence of additional phospholipids. To assess the role of phospholipids in AF in thrombin generation, we employed 2 phospholipid-targeting inhibitors, lactadherin or annexin V. Both of these phospholipid inhibitors significantly blocked the prothrombinase-activating action of AF by >80% (Figure 6B, F), in agreement with previous work [18]. We repeated these thrombin generation experiments using a recombinant prothrombin that lacked the GLA domain. The GLA domain is essential for the high-affinity binding of coagulation factors to phospholipids, and the absence of the GLA domain ablates binding to phospholipids, greatly reducing their activities. We found that the ability of AF to potentiate thrombin was significantly reduced when the GLA-domainless prothrombin was used in this system (Figure 6B, F), validating a role for phospholipids in AF in thrombin generation. Furthermore, we quantified the level of phosphatidylserine in AF using a fluorometric enzymatic assay. The phosphatidylserine level in human and rhesus AF was approximately 25 times more than that in plasma, measuring 730.4 ± 139.8 μM and 842 ± 307.8 μM in human and rhesus AF, respectively, compared with 31.3 ± 14.0 μM and 26.5 ± 11.3 μM in human and rhesus plasma, respectively (Figure 6C, G). Notably, the levels of phosphatidylserine (34:2) in AF lipidomics were inversely correlated with clotting time (Figure 6D, H) for both human and rhesus plasma (R = −.52 and −.91; p = .018 and 1.6E-8, respectively). This result suggests the indispensable role of phospholipids in the procoagulant effect of AF.

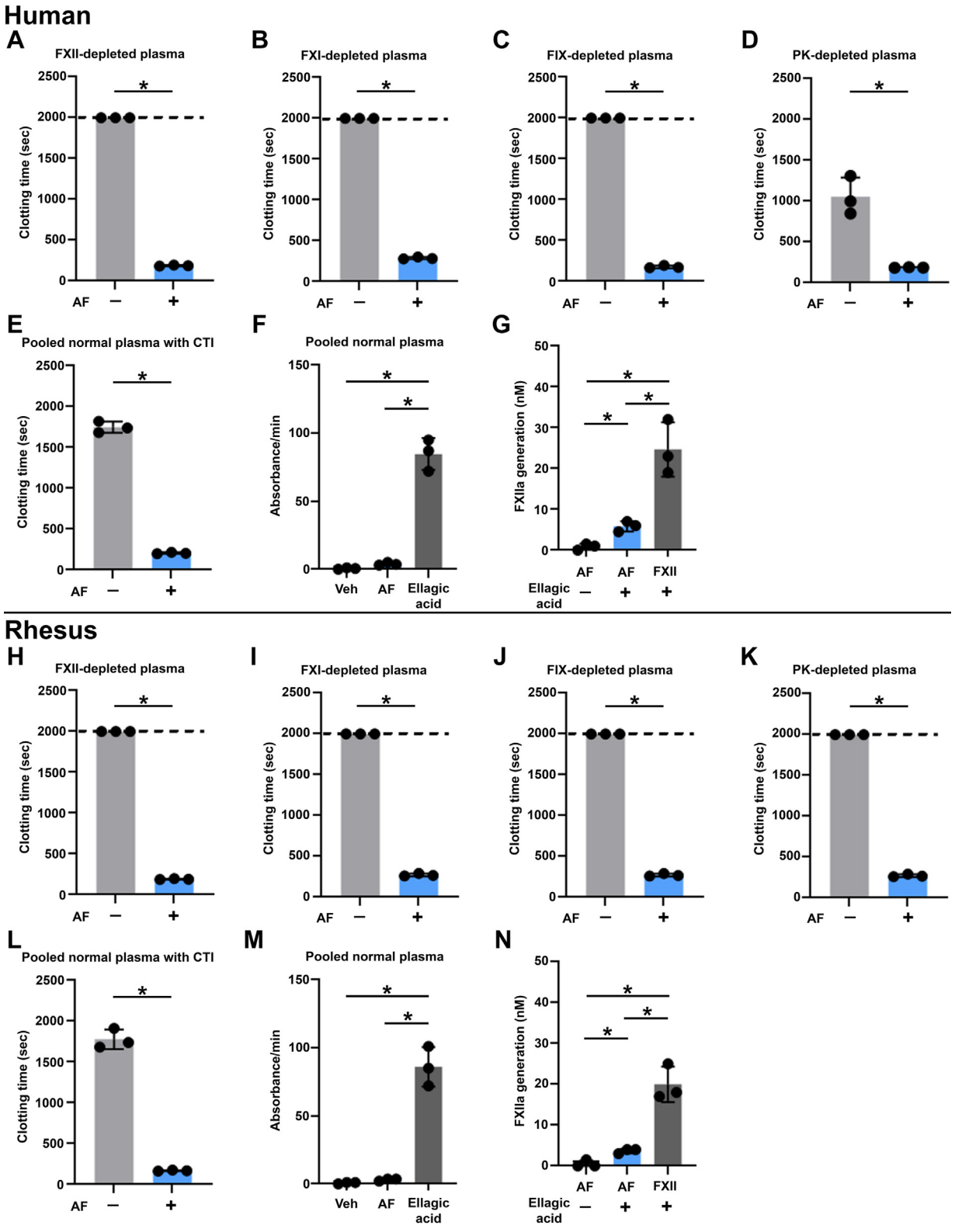


FIGURE 4 Contact pathway of coagulation and procoagulant effect of amniotic fluid (AF). A clotting assay was performed following the addition of human or rhesus AF in a ratio of 1:10 to recalcified plasma deficient in (A, H) factor (F)XII, (B, I) FXI, (C, J) FIX, (D, K) prekallikrein (PK), or (E, L) pretreated with 50 μg/mL of corn trypsin inhibitor (CTI). (F, M) FXIIa generation assay of plasma was performed in the presence of human or rhesus AF. Ellagic acid served as a positive control. (G, N) FXIIa generation assay of human or rhesus AF was performed with or without ellagic acid. FXII and ellagic acid served as a positive control. Data are mean ± SD (n = 3). The Mann–Whitney U-test was used for statistical comparisons. Veh, vehicle.

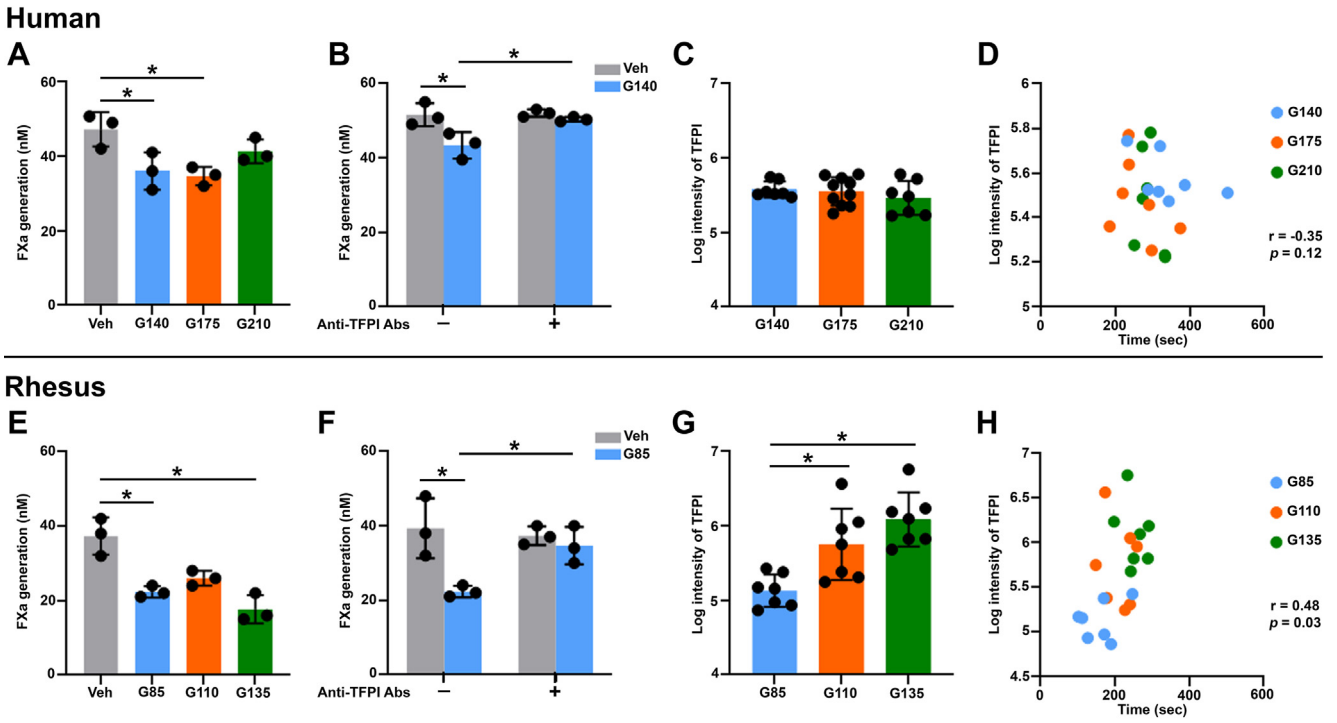


FIGURE 5 Factor (F)Xa generation and tissue factor pathway inhibitor (TFPI) in amniotic fluid (AF). (A, E) FXa generation after initiation with tissue factor was assessed in the presence of pooled human or rhesus AF at various gestational time points. (B, F) Anti-TFPI K1 and K2 antibodies (10 μg/mL) were added to the assay in the presence of human or rhesus AF. (C, G) Logarithmically normalized expression of TFPI was determined by human or rhesus AF proteomics. (D, H) The correlation between logarithmically normalized expression of TFPI in proteomics and recalcified clotting time of plasma with the addition of human or rhesus AF. Data are mean ± SD (n = 3 in FXa generation assay and n = 7 in proteomics). The Mann–Whitney U-test was used for statistical comparisons. G, gestational day. Veh, vehicle.

3.10 | AF triggers platelet activation through thrombin generation in plasma

To assess the effect of AF-mediated thrombin generation on platelet activation, we used flow cytometry to analyze platelet α-granule secretion and integrin $\alpha_{IIb}\beta_3$ activation in plasma settings. Platelets were incubated in recalcified fibrinogen-depleted plasma for 10 minutes, followed by staining with antigen presenting cell CD62P and FITC PAC-1. The surface levels of platelet P-selectin and PAC1 on platelets significantly increased in the presence of human and rhesus AF (Figure 7A, E). In separate experiments, the addition of FXa inhibitor rivaroxaban, thrombin inhibitor hirudin, or phospholipid inhibitor annexin V suppressed the effect of AF on platelet activation, indicating that the effect of AF on platelet activation resulted from thrombin generation (Figure 7B, F). We next examined the direct effect of AF on platelets. There was no significant change in α-granule secretion and integrin $\alpha_{IIb}\beta_3$ activation in resting or agonist-stimulated platelets in the presence of either human or rhesus AF (Figure 7C, D, G, H).

4 | DISCUSSION

To our knowledge, this study is the first comprehensive evaluation regarding the effects of AF on coagulation throughout gestation, integrating proteomic and lipidomic data with *in vitro* studies and utilizing both human and nonhuman primate samples. We found that FXa generation was moderately reduced by AF, which we hypothesize is due to the presence of TFPI in AF. Yet, the abundance of phospholipids in AF was found to promote thrombin generation, which overwhelmed the anticoagulant effects of TFPI in promoting global thrombin generation and subsequent platelet activation. This integrative approach offers a detailed understanding of the potential role of AF in both normal and pathological hemostatic processes. This study also offers insights into when, in the rare scenario, AF comes into contact with maternal blood – a recognized mechanism for AF embolism, an uncommon but nonetheless obstetric emergency.

AF is initially derived from maternal plasma [19]. Given that embryonic skin contains only simple epithelium, AF could diffuse through the nonkeratinizing skin under hydrostatic and osmotic forces in early gestation [20]. The composition of AF at this early stage is reasonably comparable to maternal plasma [21]. By the second half of pregnancy, full keratinization of the fetal skin no longer allows fluid to transfer as easily as before [22,23]. As gestation progresses, fetal urination and lung secretion prevail and significantly contribute to the composition of AF [19]. Yet, AF does not mix in the fetal circulation under physiological conditions. Concurrently, as fetal lung tissue begins developing and producing phospholipids, one of the phospholipids that possesses procoagulability, phosphatidylserine, is synthesized by fetal lung cells as well as from cellular turnover and apoptosis during fetal development [24,25]. These observations may explain why we observed higher concentrations

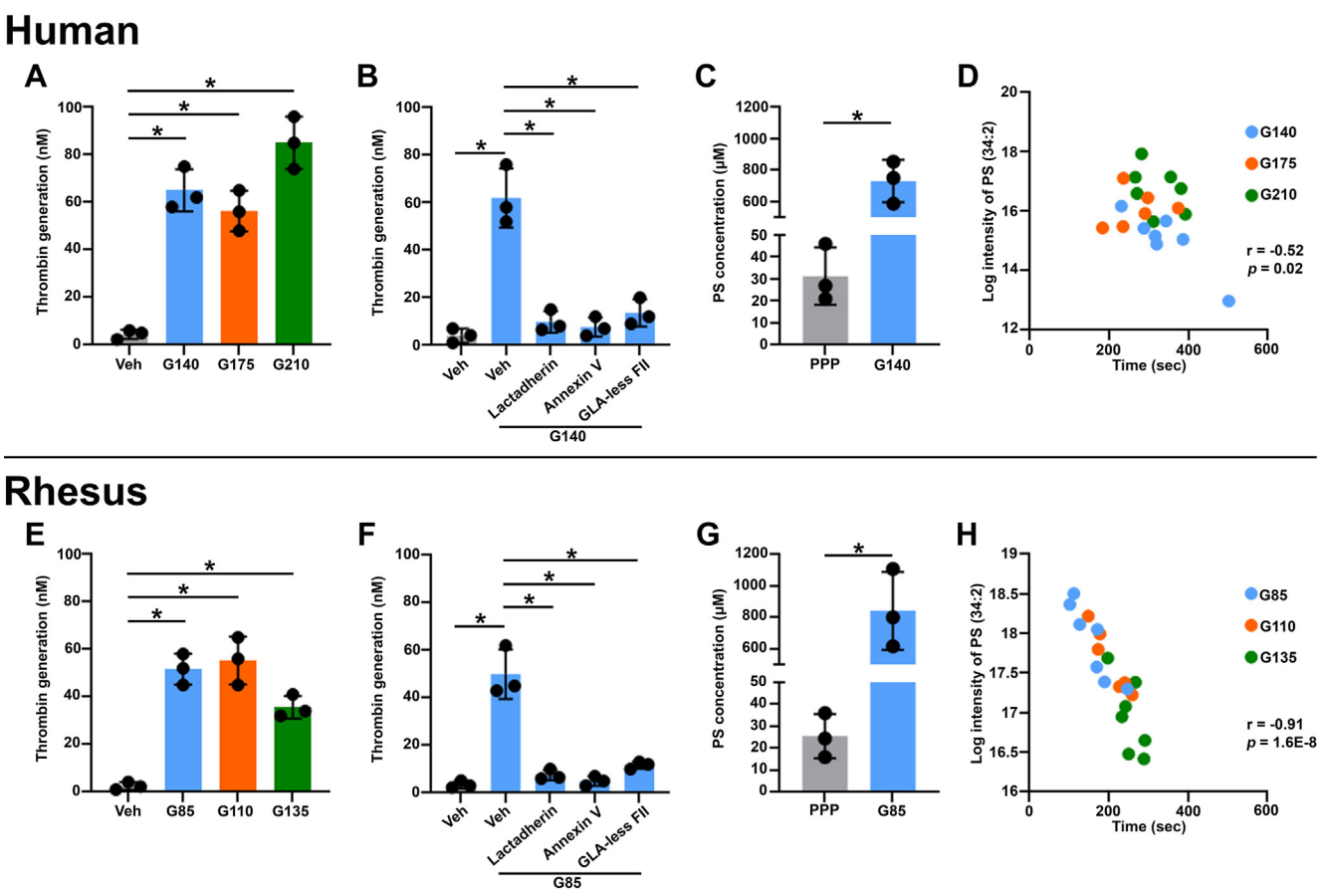


FIGURE 6 Thrombin generation and phosphatidylserine (PS) in amniotic fluid (AF). (A, E) Thrombin generation assay was evaluated in the presence of pooled human or rhesus AF at various gestational time points. (B, F) Lactadherin (30 nM) or annexin V (10 nM) was added. In selected experiments, γ-carboxyglutamic acid (GLA)-domainless prothrombin was used. (C, G) The phosphatidylserine level was quantified as L-serine generation following enzymatic cleavage of phosphatidylserine in lipid extracts of human or rhesus AF. (D, H) The correlation between logarithmically normalized expression of phosphatidylserine (34:2) in lipidomics and recalcified clotting time of plasma with the addition of human or rhesus AF. Data are mean ± SD (n = 3 in thrombin generation assay and n = 7 in lipidomics). The Mann–Whitney U-test was used for statistical comparisons. G, gestational day. PPP, platelet poor plasma; Veh, vehicle.

of phospholipids, and in particular phosphatidylserine, in AF than in plasma. Given our observation that the procoagulant properties of AF are dynamic, with AF being the most procoagulant in the first trimester, it is possible that this effect supports fetal hemostasis and barrier function in the earliest period of gestation and may be necessary to achieve successful growth and development.

The amnion, a single layer of epithelium lining the amniotic sac, functions as a crucial barrier that separates AF from the vascularized chorion [26]. The intricate interactions between AF and the amniotic epithelium likely contribute to a healthy intrauterine environment for optimal fetal growth and development during pregnancy, which includes regulation of AF composition, immune modulation, as well as secretion of growth factors and cytokines [27]. Recent work revealed that TF-FVIIa within the AF is capable of activating protease-activated receptor 2 on the fetal epithelium, likely contributing to fetal homeostasis during the early phases of development [7]. Here, we identified a key regulator of TF-FVIIa interaction, TFPI, as a critical regulator of AF's procoagulant activity. TFPI plays a key role in inhibiting coagulation by blocking the extrinsic pathway-mediated generation of FXa [28,29]. We observed that AF-derived TFPI was able to suppress FXa generation. When an anti-TFPI antibody was introduced, this inhibition was reversed, restoring FXa generation and enhancing AF-mediated coagulation *in vitro*. These findings suggest that AF's procoagulant properties are, at least in part, modulated by TFPI. This observation underscores AF's dual role in regulating hemostasis, as it contains both procoagulant and anticoagulant factors, reflecting the tightly regulated mechanisms that control blood coagulation. This balance may be particularly crucial during pregnancy, where a finely tuned hemostatic environment is essential to prevent both hemorrhage and thrombosis. Along these lines, one such study demonstrated that depletion of TFPI in a rabbit model increased susceptibility for the development of disseminated intravascular coagulopathy after injection of TF or endotoxin, a condition similar to the aftermath of AF embolism [30]. Consequently, understanding the role of TFPI and other hemostatic regulators in modulating AF's coagulation effects may provide valuable insights into the mechanisms underlying pregnancy-related hemostasis.

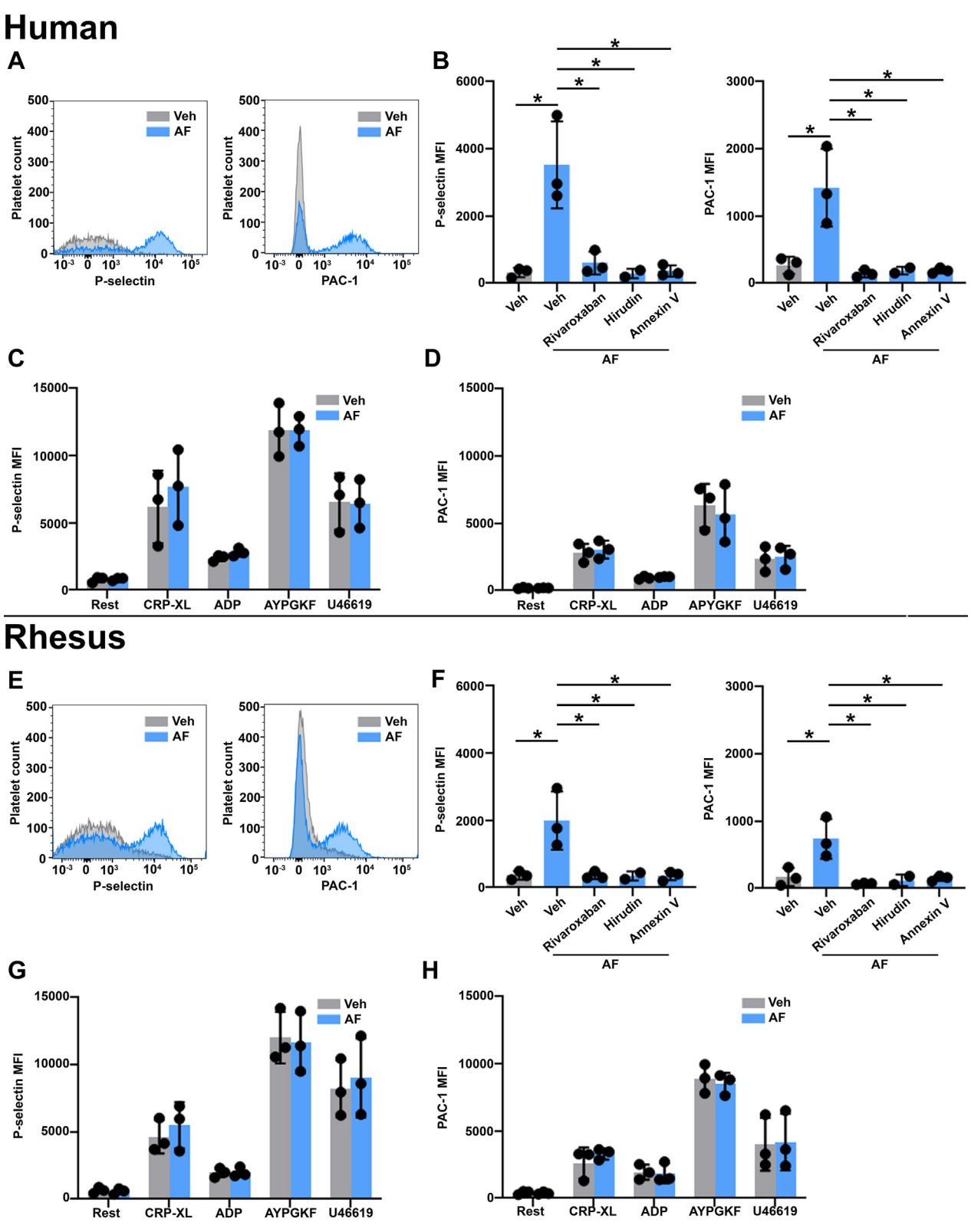


FIGURE 7 Platelet activation and amniotic fluid (AF). Platelet activation was assessed by flow cytometry analysis of platelet degranulation and integrin activation. Washed platelets (2×10^7/mL) in recalcified (20 mM of $CaCl_2$) fibrinogen-depleted plasma were treated with human AF, rhesus AF, or vehicle (Veh; modified HEPES/Tyrode buffer), staining with (A, E) APC CD62P (P-selectin) and FITC PAC-1 (PAC-1) to monitor platelet α-granule secretion and integrin activation. (B, F) In selected experiments, rivaroxaban, hirudin, or annexin V was added. Alternatively, washed platelets (2×10^7/mL) were pretreated with human AF, rhesus AF, or Veh, followed by cross-linked collagen-related peptide (CRP-XL) (10 μg/mL), adnosine diphosphate (ADP) (30 μM), AYPGKF (250 μM), or U46619 (2 μM) stimulation and staining with (C, G) P-selectin and (D, H) PAC-1. Data are mean ± SD (n = 3). The Mann–Whitney U-test was used for statistical comparisons. *$p < .05$. MFI, mean fluoresence intensity; Veh, vehicle.

This work also identified the pivotal role of phosphatidylserine, a phospholipid present in high concentrations in AF, in driving its procoagulant properties [18,31]. The presence of phosphatidylserine is inversely correlated with clotting time, emphasizing its role in enhancing thrombin generation and accelerating clot formation upon exposure to blood. This phospholipid-dependent mechanism may play a critical role in maintaining hemostatic balance during pregnancy, particularly in early gestation when AF's procoagulant effects peak. While this observation is speculative, it generates intriguing hypotheses and invites comparisons to the mechanisms of obstetric antiphospholipid syndrome (APS). Obstetric APS is characterized by prothrombotic antibodies targeting phospholipids and phospholipid-binding proteins, leading to severe complications such as recurrent miscarriage, preeclampsia, and placental insufficiency, as well as inhibiting phospholipid-dependent thrombin generation [32]. Although the mechanisms driving pregnancy loss in obstetric APS have been theorized to be prothrombotic, our findings suggest that phospholipid-dependent clotting may be a normal physiological process in a healthy pregnancy, supporting hemostasis at the maternal-fetal interface and preparing the body for the heightened hemostatic demands of delivery. In obstetric APS, disruption of this phospholipid-driven coagulation may contribute to the increased risk of pregnancy complications. Further studies are needed to better define the potential role of AF in this obstetric pathology. Understanding how antiphospholipid antibodies interfere with normal phospholipid function in AF could offer valuable insights into the pathophysiology of obstetric APS and inform the development of targeted therapies.

An increasing trend in TFPI expression across gestation was observed, which was associated with longer clotting times and was more pronounced and consistent in rhesus AF compared with human AF. While biological differences between species may account for this, the observation could also be attributed to lower interindividual variability in rhesus AF in the context of a longitudinal vs cross-sectional sampling design. In support of this overall trend, we also observed a strong and inverse correlation between the expression of phosphatidylserine (34:2) and clotting time in rhesus AF over gestation.

Platelet activation has been considered a significant factor in the development of AF embolism, as it manifests several thromboembolic characteristics [33,34]. Therefore, we examined whether AF activates platelets, which may also contribute to maternal thrombosis when AF encounters maternal blood. We demonstrated that although AF itself does not directly affect platelet activation, AF activates platelets through thrombin generated in the plasma.

In conclusion, AF demonstrates a significant procoagulant effect, accelerating clotting and fibrin production, which is dynamic through gestation and largely dependent on phospholipids, as indicated by the counteraction of this effect through phospholipid inhibition. Importantly, the consistent correlation between clotting time, expression of coagulation proteins, and phosphatidylserine levels observed in both rhesus and human AF further underscores the translational strength of the nonhuman primate model. This supports its use in future investigations into the procoagulant properties of AF and their implications in pregnancy-related hemostasis and pathologies, an understudied but biologically significant topic.

4.1 | Strengths and limitations

Our study had several strengths, particularly in leveraging unique AF samples from both a translational rhesus model and human pregnancies to address critical knowledge gaps in understanding the procoagulant properties of AF. Rhesus macaques and humans share similar physiological, genetic, reproductive, and developmental traits, making the rhesus model ideal for pregnancy studies and longitudinal AF sampling, which would be neither feasible nor ethical in humans [12]. Furthermore, all rhesus samples were collected by a single board-certified maternal-fetal medicine specialist (J.O.L.) under ultrasound guidance, ensuring rigor and reproducibility in the collection process [12]. The rhesus subjects were also controlled for age, size, and housing conditions, minimizing the confounding variables often present in human cross-sectional study designs. Additionally, our ability to deeply phenotype the prothrombotic contents of AF and corroborate these observations with direct mechanistic *in vitro* experiments further strengthens the findings of our study.

There are some limitations in this study. First of all, the limited size of the human plasma pool (n = 4) may introduce potential bias in the results. Second, our study was not able to specify the subtypes of TFPI present in AF. When referring to TFPI, we generally mean TFPI-1. However, TFPI-2, a placenta-derived protein that is structurally homologous to TFPI-1, exhibits distinct biological functions [35]. Unlike TFPI-1, TFPI-2 may possess procoagulant characteristics and has been associated with an elevated risk of venous thromboembolism [36]. Since TFPI-2 levels in AF have not yet been established, further studies are needed to comprehensively understand the roles of different TFPI subtypes in the amniotic environment. Additionally, the relatively high concentrations of annexin V and lactadherin used in our study could block the prothrombinase-activating effects not only of phospholipids but also of other procoagulant anionic molecules such as skeletal muscle myosin [37], potentially masking their contribution to prothrombin activation. Lastly, although previous studies found no *in vitro* evidence for an effect of AF on fibrinolysis [4,8,38,39], we did not specifically assess the potential impact of AF on fibrinolysis in this current study. Therefore, more research is required to better characterize the full spectrum of procoagulant properties in AF.

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AUTHOR CONTRIBUTIONS

C.J.Y., J.J.S., C.P., C.S.C., A.P.R., O.J.T.M., B.P.S., and J.O.L. contributed to the conception and design of the study. C.J.Y., L.E.S.-K., P.A.W., K.D.Z., A.C., H.H.V., and J.O.L. acquired the data. C.P., A.P.R., O.J.T.M., B.P.S., and J.O.L. supervised the work. C.J.Y., L.E.S.-K., J.J.S., A.E.B., C.P., P.A.W., A.P.R., K.D.Z., K.K., C.S.C., L.M.B., O.J.T.M., B.P.S., and J.O.L. performed the data analysis and interpretation. C.J.Y., L.E.S.-K., J.J.S., A.C., P.A.W., A.P.R., K.D.Z., B.P.S., and J.O.L. assisted in drafting the article. C.J.Y., L.E.S.-K., J.J.S., A.C., P.A.W., A.P.R., K.D.Z., B.P.S., J.O.L., A.E.B., O.L.H., C.S.C., L.M.B., H.H.V., and O.J.T.M. assisted in revising the manuscript critically for important intellectual content. All authors had full access to all the data in the study and read and approved the final version of the manuscript to be submitted. The corresponding author had final responsibility for the decision to submit for publication.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [40] with the dataset identifier PXD043519.

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REFERENCES

[1] Weiner AE, Reid DE, Roby CC. The hemostatic activity of amniotic fluid. *Science*. 1949;110:190–1.
[2] Gupta R, Cabacungan ET. Neonatal birth trauma: analysis of yearly trends, risk factors, and outcomes. *J Pediatr*. 2021;238:174–80.e173.
[3] Yang RL, Lang MZ, Li H, Qiao XM. Immune storm and coagulation storm in the pathogenesis of amniotic fluid embolism. *Eur Rev Med Pharmacol Sci*. 2021;25:1796–803.
[4] Oda T, Tamura N, Shen Y, Kohmura-Kobayashi Y, Furuta-Isomura N, Yaguchi C, et al. Amniotic fluid as a potent activator of blood coagulation and platelet aggregation: study with rotational thromboelastometry. *Thromb Res*. 2018;172:142–9.
[5] Sarig G, Klil-Drori AJ, Chap-Marshak D, Brenner B, Drugan A. Activation of coagulation in amniotic fluid during normal human pregnancy. *Thromb Res*. 2011;128:490–5.
[6] Lockwood CJ, Bach R, Guha A, Zhou XD, Miller WA, Nemerson Y. Amniotic fluid contains tissue factor, a potent initiator of coagulation. *Am J Obstet Gynecol*. 1991;165:1335–41.
[7] Hu Y, Scharrer A, Hau C, Ay C, Berckmans RJ, Ruf W, et al. Coagulation signaling from amniotic fluid to fetal skin. *Blood Adv*. 2022;6:5538–41.
[8] Hell L, Wisgrill L, Ay C, Spittler A, Schwameis M, Jilma B, et al. Procoagulant extracellular vesicles in amniotic fluid. *Transl Res*. 2017;184:12–20.e1.
[9] Benson MD, Cheema N, Kaufman MW, Goldschmidt RA, Beaumont JL. Uterine intravascular fetal material and coagulopathy at peripartum hysterectomy. *Gynecol Obstet Invest*. 2012;73:158–61.
[10] Marik PE, Plante LA. Venous thromboembolic disease and pregnancy. *N Engl J Med*. 2008;359:2025–33.
[11] Devis P, Knuttinen MG. Deep venous thrombosis in pregnancy: incidence, pathogenesis and endovascular management. *Cardiovasc Diagn Ther*. 2017;7:S309–19.
[12] Shorey-Kendrick LE, Crosland BA, Spindel ER, McEvoy CT, Wilmarth PA, Reddy AP, et al. The amniotic fluid proteome changes across gestation in humans and rhesus macaques. *Sci Rep*. 2023;13:17039. https://doi.org/10.1038/s41598-023-44125-3
[13] Bhatti G, Romero R, Gomez-Lopez N, Chaiworapongsa T, Jung E, Gotsch F, et al. The amniotic fluid proteome changes with gestational age in normal pregnancy: a cross-sectional study. *Sci Rep*. 2022;12:601. https://doi.org/10.1038/s41598-021-04050-9
[14] Bhatti G, Romero R, Gomez-Lopez N, Chaiworapongsa T, Than NG, Theis KR, et al. The amniotic fluid proteome changes with term labor and informs biomarker discovery in maternal plasma. *Sci Rep*. 2023;13:3136. https://doi.org/10.1038/s41598-023-28157-3
[15] Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol*. 2010;8:e1000412. https://doi.org/10.1371/journal.pbio.1000412
[16] Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005;102:15545–50.
[17] Uszyński M, Zekanowska E, Uszyński W, Kuczyński J. Tissue factor (TF) and tissue factor pathway inhibitor (TFPI) in amniotic fluid and blood plasma: implications for the mechanism of amniotic fluid embolism. *Eur J Obstet Gynecol Reprod Biol*. 2001;95:163–6.
[18] Zhou J, Liu S, Ma M, Hou J, Yu H, Lu C, et al. Procoagulant activity and phosphatidylserine of amniotic fluid cells. *Thromb Haemost*. 2009;101:845–51.
[19] Cunningham FG, Leveno KJ, Bloom SL, Dashe JS, Hoffman BL, Casey BM, et al. *Williams obstetrics*. 25th ed. New York, NY: McGraw-Hill Education; 2018:83–7.
[20] Beall MH, van den Wijngaard JP, van Gemert MJ, Ross MG. Amniotic fluid water dynamics. *Placenta*. 2007;28:816–23.
[21] Brace RA. Physiology of amniotic fluid volume regulation. *Clin Obstet Gynecol*. 1997;40:280–9.
[22] Sulibarska J, Kocyłowski R, Komorowicz I, Grzesiak M, Bogdański P, Barałkiewicz D. Concentrations of mineral in amniotic fluid and their relations to selected maternal and fetal parameters. *Biol Trace Elem Res*. 2016;172:37–45.
[23] Dale BA, Holbrook KA, Kimball JR, Hoff M, Sun TT. Expression of epidermal keratins and filaggrin during human fetal skin development. *J Cell Biol*. 1985;101:1257–69.

[24] Schlegel RA, Williamson P. Phosphatidylserine, a death knell. *Cell Death Differ*. 2001;8:551–63.

[25] Gluck L, Kulovich MV, Borer Jr RC, Brenner PH, Anderson GG, Spellacy WN. Diagnosis of the respiratory distress syndrome by amniocentesis. *Am J Obstet Gynecol*. 1971;109:440–5.

[26] Miki T, Lehmann T, Cai H, Stolz DB, Strom SC. Stem cell characteristics of amniotic epithelial cells. *Stem Cells*. 2005;23:1549–59.

[27] Underwood MA, Gilbert WM, Sherman MP. Amniotic fluid: not just fetal urine anymore. *J Perinatol*. 2005;25:341–8.

[28] Baugh RJ, Broze GJ Jr, Krishnaswamy S. Regulation of extrinsic pathway factor Xa formation by tissue factor pathway inhibitor. *J Biol Chem*. 1998;273:4378–86.

[29] Puy C, Tucker EI, Matafonov A, Cheng Q, Zientek KD, Gailani D, et al. Activated factor XI increases the procoagulant activity of the extrinsic pathway by inactivating tissue factor pathway inhibitor. *Blood*. 2015;125:1488–96.

[30] Warr TA, Rao LV, Rapaport SI. Disseminated intravascular coagulation in rabbits induced by administration of endotoxin or tissue factor: effect of anti-tissue factor antibodies and measurement of plasma extrinsic pathway inhibitor activity. *Blood*. 1990;75:1481–9.

[31] Bishop AJ, Israels LG, Chernick V, Israels ED. Placental transfer of intravascular coagulation between mother and fetus. *Pediatr Res*. 1971;5:113–25.

[32] Knight JS, Branch DW, Ortel TL. Antiphospholipid syndrome: advances in diagnosis, pathogenesis, and management. *BMJ*. 2023;380:e069717. https://doi.org/10.1136/bmj-2021-069717

[33] Furukawa S, Urabe H, Nagai Y, Sameshima H, Ikenoue T, Sato Y. A rare case of amniotic fluid embolism with massive platelet aggregations in pulmonary capillaries. *J Obstet Gynaecol Res*. 2010;36:397–400.

[34] Salem HH, Walters WA, Perkin JL, Handley CJ, Firkin BG. Aggregation of human platelets by amniotic fluid. *Br J Obstet Gynaecol*. 1982;89:733–7.

[35] Kobayashi H, Matsubara S, Yoshimoto C, Shigetomi H, Imanaka S. Tissue factor pathway inhibitor 2: current understanding, challenges, and future perspectives. *J Obstet Gynaecol Res*. 2023;49:2575–83.

[36] Miyagi E, Arakawa N, Sakamaki K, Yokota NR, Yamanaka T, Yamada Y, et al. Validation of tissue factor pathway inhibitor 2 as a specific biomarker for preoperative prediction of clear cell carcinoma of the ovary. *Int J Clin Oncol*. 2021;26:1336–44.

[37] Morla S, Deguchi H, Fernández JA, Ruf W, Brekken RA, Griffin JH. Procoagulant activities of skeletal muscle and cardiac myosins require both myosin protein and myosin-associated anionic phospholipids. *Blood*. 2021;137:1839–42.

[38] Liu EH, Shailaja S, Koh SC, Lee TL. An assessment of the effects on coagulation of midtrimester and final-trimester amniotic fluid on whole blood by thrombelastograph analysis. *Anesth Analg*. 2000;90:333–6.

[39] Harnett MJ, Hepner DL, Datta S, Kodali BS. Effect of amniotic fluid on coagulation and platelet function in pregnancy: an evaluation using thromboelastography. *Anaesthesia*. 2005;60:1068–72.

[40] Perez-Riverol Y, Bai J, Bandla C, García-Seisdedos D, Hewapathirana S, Kamatchinathan S, et al. The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Res*. 2022;50:D543–52.

SUPPLEMENTARY MATERIAL

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