Differential sialic acid content in adult and neonatal fibrinogen mediates differences in clot polymerization dynamics

Kimberly Nellenbach[1,2], Alexander Kyu[1], Nina Guzzetta[3], and Ashley C. Brown*[1,2]

[1]Joint Department of Biomedical Engineering, North Carolina State University and The University of North Carolina at Chapel Hill, Raleigh, NC 27695, USA;
[2]Comparative Medicine Institute, North Carolina State University, Raleigh, NC 27695, USA;
[3]Department of Anesthesiology, Emory University School of Medicine, Children's Healthcare of Atlanta, Atlanta, GA 30322, USA

*Corresponding Author: Ashley C. Brown, PhD
Address: Joint Department of Biomedical Engineering
North Carolina State University and University of North Carolina at Chapel-Hill
1001 William Moore Dr., Raleigh, NC, 27606
Phone: (919) 513-8231
Email: aecarso2@ncsu.edu

Text word count: 3991
Abstract word count: 250
Number of figures and tables: 6
Number of references: 27
Scientific category: Thrombosis and Hemostasis

Key Points:

- Increased sialic acid in neonatal fibrinogen influences fibrin knob-hole interactions during polymerization.
- Neonatal fibrin polymerization involves more B knob and less A knob mediated interactions compared to adults.

Abstract:

Neonates possess a molecular variant of fibrinogen, known as fetal fibrinogen, characterized by increased sialic acid, a greater negative charge, and decreased activity compared to adults. Despite these differences, adult fibrinogen is used for treatment of bleeding in neonates, with mixed efficacy. In order to determine safe and efficacious bleeding protocols for neonates, more information on neonatal fibrin clot formation and the influence of sialic acid on these processes is needed. Here, we examine the influence of sialic acid on neonatal fibrin polymerization. We hypothesized that the increased sialic acid content of neonatal fibrinogen promotes fibrin B:b knob hole interactions and consequently influences the structure and function of the neonatal fibrin matrix. We explored this hypothesis through analysis of structural properties and knob:hole polymerization dynamics of normal and desialylated neonatal fibrin networks and compare to those formed with adult fibrinogen. We then characterized normal neonatal fibrin knob:hole interactions by forming neonatal and adult clots with either thrombin or snake-venom thrombin like enzymes (SVTLEs) that preferentially cleave fibrinopeptide A or B. We determined that sialic acid content of neonatal fibrinogen is a key determinant of resulting clot properties. Experiments analyzing knob:hole dynamics indicated typical neonatal fibrin clots are formed with the release of more fibrinopeptide B and less fibrinopeptide A than adults. After the removal of sialic acid, fibrinopeptide release was roughly equivalent between adults and neonates indicating the influence of sialic acid on fibrin neonatal fibrin polymerization mechanisms. These results could inform future studies developing neonatal specific treatments of bleeding.

Introduction:

Recent studies have identified major differences in hemostasis between adults and neonates, including qualitative and quantitative differences in the coagulation protein fibrinogen[1–3]. Despite these differences, neonates with significant bleeding, including after procedures requiring cardiopulmonary bypass (CPB) or during extracorporeal membrane oxygenation (ECMO), are treated with adult specific treatment options, namely the transfusion of adult cryoprecipitate (fibrinogen component)[4–8]. Unfortunately the clinical effectiveness of such transfusions is often inconsistent and may result in deficient fibrin matrix insufficient to mitigate bleeding[9]. Our recent studies have identified significant structural and functional differences between neonatal and adult clots that might contribute to these outcomes. Structurally, neonatal clots appeared two dimensional with low degrees of fibrin cross-branching compared to the dense and heavily branched fibrin networks observed in adult clots. Functionally, neonatal clots are significantly softer than adult clots with faster degradation times[9]. Moreover, when neonatal and adult fibrinogen are mixed (to mimic transfusion of adult fibrinogen to neonates) the resultant fibrin networks maintain their distinct properties, are heterogeneous and are not seamlessly integrated[9]. Given these distinctions, a deeper understanding of the mechanistic differences between adult and neonatal fibrin polymerization is needed to improve outcomes related to neonatal bleeding.

The activation and polymerization of fibrinogen, a 340-kDa circulating glycoprotein, is essential for the formation of a stable blood clot and the cessation of bleeding. Post-injury, the proteolytic enzyme thrombin converts soluble fibrinogen to insoluble fibrin via cleavage of fibrinopeptides A and B, exposing fibrin knobs A and B. Fibrin protofibrils are then formed from the noncovalent binding of fibrin knobs to complementary fibrin holes a and b on adjacent proteins[10]. In adults, the driving force of polymerization occurs through fibrin A:a knob hole binding. A:a binding occur more rapidly than B:b binding and is required for the formation of typical fibrin clots in adults[11,12]. However, these mechanisms have not been explored in neonates. Additionally, recent evidence showed that neonates possess a molecular variant of fibrinogen, known as fetal fibrinogen[3,9]. Unlike in other proteins, such as hemoglobin, there is no evidence that neonates possess multiple forms of fibrinogen or any percentage of adult fibrinogen[13]. Initial studies characterizing fetal fibrinogen have identified an increased sialic acid content, greater negative charge, and increased clotting times compared to adult fibrinogen[1]. Of note, similarly to other post-translational modifications, sialic acid content in fibrinogen has been shown to influence fibrin clot properties. For example, an increased sialic acid content associated with liver disease has been shown to result in altered fibrin clot properties[14,15]. Additionally, studies on fibrinogen synthesized during trauma show a reduction in sialic acid content because of decreased galactose residues contributing to a faster rate of clot polymerization[16]. Furthermore, recent work from our group has found that the increased sialic acid in neonatal fibrin networks results in significantly greater fibroblast attachment compared to attachment on adult fibrin[17]. Although an increased sialic acid concentration has been identified in many neonatal cells and glycoproteins across various physiological systems due to its supportive functions in brain development, immune regulation, and gut maturation, its influence on fibrin polymerization mechanisms in neonates has not been thoroughly studied[18,19].

In this investigation, we examine the influence of sialic acid on neonatal fibrin polymerization. We hypothesized that the increased sialic acid content of neonatal fibrinogen would promote B:b knob hole interactions and consequently influence the structure and function of the neonatal fibrin matrix. We explore this hypothesis through analysis of structural properties and knob:hole polymerization dynamics of normal and desialylated neonatal fibrin networks and compare to those formed with adult fibrinogen.

Methods:

Isolation of fibrinogen:

After IRB approval and informed written parental consent, whole blood samples were collected from neonates (less than 30 days of age) undergoing elective cardiac surgery at the Children's Hospital of Atlanta (**Supplemental Table 1**). All samples were collected from an arterial line placed after the induction of anesthesia and prior to surgical incision and CPB. Preterm neonates, neonates with a known coagulopathy, or mothers with a known coagulopathy were excluded. At admission, liver function was assessed via blood levels of alkaline phosphatase, bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT). No signs of liver disease were observed. No fibrinogen interacting medications were administered during the perioperative period. Samples were centrifuged immediately 2x at 15,000 g for 15 mins to yield platelet poor plasma (PPP) and stored at -80°C until use. Pooled adult PPP was obtained from the New York Blood Center. Neonatal and adult human fibrinogen were isolated from plasma via ethanol precipitation[20–22]. SDS page was utilized to determine purity. We determined the isolation method primarily precipitated fibrinogen, however we also identified small amounts of FXIII, fibronectin, and von Willebrand factor in both samples. Purity was compared to a commercially available fibrinogen (Fib3, Fibrinogen with fibronectin, von Willebrand factor and factor XIII depleted, Enzyme Research Laboratories). Baseline FXIII concentration and plasmin activity levels were assessed and were similar between adult and neonatal samples (Abcam) (**Supplemental Figure 1**). Structural analysis of purified fibrin clots revealed similar clot structure to plasma clots and commercially purchased fibrinogen (**Supplemental Figure 2**). Additionally, ethanol purified fibrinogen clot structure was comparable to results seen in previous studies by our group utilizing Gly-Pro-Arg-Pro (GPRP) affinity beads to purify fibrinogen[9].

Investigation of sialic acid influence on fibrin properties:

Sialic acid was cleaved from neonatal and adult fibrinogen via neuraminidase digestion[23]. Fibrinogen solutions (5 mg/mL) were incubated with 0.025 U/mL Neuraminidase (Sigma Aldrich) for 4 hours at 35°C, then filtered with Pall Nanosep devices (100 kDa MWCO) and stored at -80°C until use. Sialic acid content was quantified via a Sialic Acid (NANA) Assay Kit (Abcam). Removal of sialic acid residues was confirmed by assaying the sialic acid concentration before and after neuraminidase digestion.

Selective cleavage of fibrinopeptides:

Neonatal and adult clots were formed with proteolytic enzymes: thrombin (Enzyme Research Labs), which cleaves both fibrinopeptides A and B, batroxobin (Prospec Bio), a snake venom thrombin-like enzyme (SVLTE) that preferentially cleaves fibrinopeptide A, and contortrixobin (MyBioSource), a SVLTE that preferentially cleaves fibrinopeptide B. Thrombin, batroxobin, or contortrixobin was added to purified fibrinogen (2.5 mg/mL) in sodium citrate to initiate clotting. A range of concentrations of thrombin (0.25-1.0 U/mL), batroxobin (0.5-1.0 U/mL) or controtrixobin (2.5-7.0 μg/mL) were utilized.

Structural characterization of fibrin matrices via confocal microscopy:

Confocal microscopy was utilized for imaging of fibrin clot structure[9,24–26]. 50 μl clots consisting of 2.5 mg/mL purified fibrinogen in 1M HEPES buffer (5 mM calcium, 7.4 pH) were formed with thrombin, batroxobin, or contortrixobin at the concentrations listed above. 10 μg/mL Alexa 488 labeled adult fibrinogen was added for visualization (Fisher Scientific). Our previous research determined that, at very low concentrations of adult fibrinogen, neonatal fibrin structure is not impacted[9]. Therefore, we expect effects on this group to be minimal. Clots were formed between a glass slide and coverslip and allowed to polymerize for two hours prior to imaging[27]. A Zeiss Laser Scanning Microscope (LSM 710, Zeiss Inc.) at 63x magnification was utilized for imaging and a minimum of three random 5.06 µm z-stacks were acquired per clot. ImageJ was used to create 3D projections from z-stacks. Fiber alignment was quantified through a Matlab algorithm previously developed by our group[9]. A minimum clot structure is required in order to run this code, therefore, if network formation was minimal, alignment data was not collected. Clot fiber density was determined from the ratio of black (fiber) over white (background) pixels in each image[28,29]. Additionally, clot structure in thrombin polymerized neonatal and adult clots was also assessed with cryogenic scanning electron microscopy. Here, 100 µl clots were formed with 0.5 U/mL thrombin and allowed to polymerize for 2h. Clots were rapidly frozen by plunging in subcooled liquid nitrogen and imaged at 1,000X. Three clots were imaged per group and three random images were taken per clot.

Analysis of enzyme-initiated polymerization dynamics:

Enzyme-initiated fibrin polymerization assays were used to evaluate clotting times and clot turbidity. 80 µl fibrin clots were formed in a 96-well plate with adult or neonatal fibrinogen (2.5 mg/mL), HEPES buffer, and the addition of thrombin, batroxobin, or contortrixobin. Turbidity curves were generated by reading the absorbance at 350 nm with a plate reader every 30 seconds for 3 hours. Analysis of turbidity curves included maximum absorbance and clotting time determined by the time needed to reach half the maximum absorbance value. Data was excluded if bubbles formed during polymerization, as they interfere with absorbances readings.

Analysis of clot degradation kinetics:

Clot degradation kinetics were determined for clots formed from purified fibrinogen in the presence of 0.25 U/mL thrombin, 0.5 U/mL batroxobin, or 2.5 ug/mL contortrixobin. 80 μl clots were formed in a 96-well plate as in absorbance-based assays and allowed to polymerize for 2 hours. An 80 μl overlay with 0.5 U/mL plasmin was then placed on top of clots to initiate degradation (Invitrogen). 5 μl aliquots of the clot liquor were taken at 0, 1, 2, 4, 6, 8, and 24 hours. Fibrinogen content of aliquots was determined via Nano-drop analysis. Degradation rates were determined from the time required to reach half max soluble protein content. Additional experiments were conducted using an overlay containing 10.8 mg/mL human plasminogen and 0.29 mg/mL tissue plasminogen activator (tPA). Aliquots were taken as described above and fibrinogen content was determined via Fibrinogen ELISA (Abcam).

Analysis of fibrinogen clottability:

Fibrinogen clottability was determined by a protein quantification assay which measures the fibrinogen content in the clot liquor (soluble portion of clot sample) that remains after polymerization. 50 μl clots were formed from purified fibrinogen at a concentration of 2.5 mg/ml in HEPES buffer and were polymerized with the initiation of 0.25 U/ml thrombin, 0.5 U/ml batroxobin, or 2.5 ug/mL contortrixobin. Before and after a 1-h polymerization, aliquots were taken and fibrinogen content was determined via ELISA. Percent of clottable fibrinogen was determined as: ([initial soluble protein – soluble protein in clot liquor] / initial soluble protein) x 100.

Analysis of mechanical clot properties:

Atomic Force Microscopy (AFM) was utilized to determine clot stiffness. Purified fibrin clots 50 μl in size were formed with thrombin, batroxobin, and contortrixobin at concentrations of 0.25 U/mL, 0.5 U/mL, and 2.5 μg/mL respectively and polymerized on a charged glass slide for 2 hours.

The AFM (Asylum MFP3D-Bio, Asylum Research) was operated in force contact mode with silicon nitride cantilevers with a particle diameter of 1.98 μm (Nanoandmore). 20 X 20 μm force maps were obtained on each clot and fit with a Hertz model to determine the elastic modulus. In order to reduce variation from potential edge of clot effects, force maps were conducted in the center of each clot. The average elastic modulus of an array of 256 contact points was reported for each force map. A minimum stiffness was required to conduct analysis, therefore data was excluded if force maps could not be taken.

Competitive binding of fibrin knobs:

A competitive binding assay was performed with synthetic fibrin knob peptides. Purified neonatal and adult fibrinogen solutions (2.5 mg/mL) were incubated with 1 mM A knob mimetic (GPRPFPAK), B knob mimetic (AHRPYAAK) or non-binding peptide (GPSPFPAK) (Genscript) for 20 minutes. Alexa 488 labeled adult fibrinogen was added for visualization and polymerization was then initiated with 0.5 U/mL thrombin. Confocal microscopy was utilized to examine resulting fibrin architecture at the conditions described above.

Quantitative release of fibrinopeptides:

To measure relative release of fibrinopeptides over time, 75 μl clots consisting of 1.0 mg/mL neonatal and adult fibrinogen were formed with 0.5 U/mL thrombin in 600 μl tubes. At 0, 5, 15, 30, 60, and 120 minute time points, the reaction was terminated by heating the solution at 97°C for 15 minutes[30]. The solution was kept on ice until the completion of the experiment. All samples were centrifuged at 15,000 g for 15 minutes at 4°C and the supernatant was removed and stored at -20°C until analysis. Quantitative analysis of fibrinopeptide concentration was conducted via ELISA (MyBioSource). Fibrinopeptide ELISA kits were reported to have high specificity with no observed cross reactivity between fibrinopeptide analogues. The neonatal to adult fold change of fibrinopeptide A or B concentration over time was reported.

Analysis of fibrinogen formulations in a post cardiopulmonary bypass model of coagulopathy:

Sialylation of adult fibrinogen was increased via sialyltransferase incubation[31]. Adult fibrinogen solutions (10 mg/mL) were incubated with 50 milliunits of alpha-2,3-Sialyltransferase and 5 μmol cytidine-5'-monophsospho-N-acetylneuraminic acid sodium for 24 hours (Sigma). The solution was filtered with Pall Nanosep devices and stored at -80°C until use. Sialic acid was removed with neuraminidase digestion as described above with an 8 hour incubation time. Neuraminidase digestion was also conducted on normal neonatal and adult fibrinogen for 8 hours. Sialic acid content was quantified as described above. Various formulations of fibrinogen were added to neonatal post-cardiopulmonary bypass (CPB) plasma samples in order to simulate treatment. Blood samples were collected from neonates prior to and after CPB and plasma was isolated as described above. 50 μl clots consisting of 90% platelet poor plasma were formed with and without the addition of 1.1 mg/mL adult fibrinogen, neonatal fibrinogen, and sialylated adult fibrinogen were formed using 0.5 U/mL thrombin. Previous studies from our group have determined that fibrinogen levels of post-CPB samples are an average of 2.1 mg/mL and baseline levels are 3.2 mg/mL[9]. Therefore, we supplemented fibrinogen additions up to baseline concentrations. Following 2 hours of polymerization, structure and degradation characteristics were analyzed as described above.

Analysis of porcine fibrinogen:

Sialic acid content was quantified for adult and neonatal porcine fibrinogen. Blood was collected from eight 1-yr-old female Yorkshire pigs and 8-week-old Yorkshire piglets before planned surgical procedures at North Carolina State University's School of Veterinary Medicine

(Raleigh, North Carolina). All samples were collected via jugular venous puncture after the induction of anesthesia and before surgical incision. Samples were centrifuged immediately for 15 mins at 15,000 g 2X to obtain platelet-poor plasma and stored at −80°C until use. Sialic acid was cleaved and quantified as described above.

Statistical analysis:

Statistical analysis was performed using GraphPad Prism Software. Data was analyzed via a two-way Analysis of Variance (ANOVA) with a Tukey's post hoc test using a 95% confidence interval for most measurements. For analysis of sialic acid concentration, fiber density with and without sialic acid, competition assays, and clottability, degradation, and clot stiffness in the presence of SVTLE's, an unpaired, two tailed Student's t-test was conducted. For analysis of cumulative fibrinopeptides, a one-way ANOVA was utilized. Statistical significance was achieved for $P<0.05$. Data is defined as interval and is presented as average +/- SD.

Results:

Influence of sialic acid on fibrin polymerization:

The influence of sialic acid on neonatal fibrin polymerization was explored with various assays (**Figure 1**). First, sialic acid concentration was quantified; neonatal fibrinogen was shown to have significantly greater concentration than adults (Adults: 3.07 +/- 0.09 μg/mL, Neonates: 5.88 +/- 1.08 μg/mL, P= 0.011; **Figure 1A)**. Sialic acid concentration of the fibrinogen solutions prior to neuraminidase cleaving was always undetectable, as our assay does not detect bound residues. After removal of sialic acid via neuraminidase digestion, clot structure was assessed via confocal microscopy (**Figure 1B)**. When sialic acid was removed and clots were formed with thrombin, neonatal and adult fibrin clots appeared similar in structure with no significant differences in fiber density (Adults: 0.71 +/- 0.57 black/white pixels, Neonates: 0.75 +/- 0.66 black/white pixels, P=0.96). When desialylated fibrinogen was polymerized with batroxobin (fpA cleavage), clot structure was porous and similar between adults and neonates with no significant differences in fiber density (**Supplemental Figure 3**; Adults: 0.97 +/- 0.56 black/white pixels, Neonates: 0.69 +/- 0.18 black/white pixels, P=0.36). However, when polymerization of desialylated fibrinogen was initiated with contortrixobin (FpB cleavage), fibrin clots had low degrees of branching and neonatal networks had significantly higher fiber density (**Supplemental Figure 3**; Adults: 0.23 +/- 0.09 black/white pixels, Neonates: 0.60 +/- 0.28 black/white pixels, P=0.046). When polymerized with thrombin, neonatal and adult desialylated fibrinogen had similar polymerization behavior (**Figure 1C)**. No significant differences were observed in max turbidity or (**Figure 1D,** Max turbidity; Adults: 0.4 +/- 0.2 abs350, Neonates: 0.32 +/- 0.09 abs350, P=0.99), clottibility (**Figure 1E**, Adults: 94.07 +/- 0.25 %, Neonates: 93.37 +/- 0.89, P=0.42). When fibrinolysis was initiated on normal and adult desialylated fibrinogen, degradation rates were similar between groups (**Figure 1F,** Adults: 5.9 +/- 0.53 hours, Neonates: 5.48 +/- 0.51 hours).

Structural characterization of fibrin matrices:

Structure of clots formed from neonatal and adult fibrinogen formed with venom enzymes was evaluated via confocal microscopy (**Figure 2**). In clots formed with thrombin, adult fibrin networks were dense and highly branched compared to the thin networks with low degrees of branching seen with neonatal fibrin. Significantly higher fiber densities were observed with mid to high concentrations of thrombin. Increased fibrin alignment was observed in neonatal clots, but did not reach statistical significance. Adult clots formed with batroxobin (FpA cleavage) were also highly branched and appeared similar in fibrin structure to clots formed with thrombin. Neonatal clots formed with batroxobin were highly porous with little network structure visible. At higher batroxobin concentrations, fiber density was significantly higher in adults compared to neonatal clots (batroxobin fiber density: 1.0 U/mL: Adults: 0.79 +/- 0.49 black/white pixels, Neonates: 0.12 +/- 0.11 black/white pixels P=0.002); all concentrations yielded low alignment values that were comparable across age groups. When clotting was initiated with contortrixobin (FpB cleavage), adult clots were very porous and lacked 3D structure. At high concentrations of contortrixobin, neonatal clots had significantly greater fiber density than adults (contortrixobin fiber density 7.0 ug/mL: Adults: 0.11 +/- 0.08 black/white pixels, Neonates: 0.51 +/- 0.32 black/white pixels, P=0.013; no significant differences in fibrin alignment were observed across age groups. In our analysis using cryogenic scanning electron microscopy in thrombin polymerized clots we observed significantly greater fiber densities in the adult groups compared to neonatal clots (**Supplemental Figure 4,** Adults: 0.61 +/- 0.06 black/white pixels, Neonates: 0.31 +/- 0.08 black/white pixels, P=0.007.

Analysis of enzyme initiated polymerization dynamics:

Polymerization time and maximum fibrin clot turbidity in the presence of proteolytic enzymes were determined by monitoring changes in absorbance during clot formation (**Figure 3, Supplemental Figure 5**). In the presence of thrombin, adult clots reached significantly greater maximum turbidities than neonatal clots and had comparable clotting times (thrombin max turbidity 1.0 U/mL: Adults: 0.82 +/- 0.35 OD, Neonates: 0.22 +/- 0.09 OD, P=0.0001, Time to half max turbidity, 1.0 U/mL: Adults: 11.0 +/- 4.81 s, Neonates: 9.83 +/- 5.62 s). In batroxobin (FpA cleavage) initiated polymerization, adult clots had faster rates of clot formation and reached significantly greater maximum turbidities compared to neonates (batroxobin max turbidity 1.0 U/mL: Adults: 0.86 +/- 0.14 OD, Neonates: 0.39 +/- 0.29 OD P=0.018, Time to half max turbidity 1.0 U/mL: Adults: 19.3 +/- 8.18 s, Neonates: 20.5 +/- 1.68 s). Finally, when polymerization was initiated with contortrixobin (FpB cleavage), significantly greater clotting times and maximum turbidites were reached in neonatal fibrin clots compared to adult clots (contortrixobin max turbidity 7.0 ug/mL: Adults: 0.07 +/- 0.06 OD, Neonates: 0.35 +/- 0.19 OD P=0.0004, Time to half max turbidity 7.0 ug/mL: Adults: .63 +/- .25 s, Neonates: 30.0 +/- 1.730 s).

Analysis of clot degradation kinetics:

Fibrin clot degradation was initiated via plasmin and assessed by determining changes in soluble protein concentration over time (**Figure 4C**). Degradation rates were established by determining the time required to reach maximum soluble protein content; a greater time required translates to a slower rate of fibrin clot degradation. In the presence of thrombin and batroxobin (FpA cleavage), adult fibrin clots had significantly greater time to half degradation rate than neonatal clots (thrombin: Adults: 6.06 +/- 3.07 hours, Neonates: 1.81 +/- 1.75 hours, P=0.015; batroxobin: Adults: 4.3 +/- 2.82 hours, Neonates: 1.36 +/- 1.53 hours, P=0.048). When clots were formed with Contotrixobin (FpB cleavage), neonatal clots exhibited slightly greater time to half degradation than adults, although statistical significance was not reached (Adults: 1.53 +/- 2.13 hours, Neonates: 2.27 +/- 1.79 hours, P=0.59). Degradation rates were also analyzed using a plasminogen and tPA overlay (**Supplemental Figure 6).** When clotting was initiated with thrombin and batroxobin, adult fibrin clots had significantly greater time to half degradation compared to neonatal clots (thrombin; Adults: 12.88 +/- 1.99 hours, Neonates: 7.98 +/- 1.01 hours, P=0.018, batroxobin; Adults: 10.89 +/- 2.9 hours, Neonates: 5.01+/- 1.9 hours, P=0.043. When clots were formed with contortrixobin, neonatal samples had longer degradation times, although not statistically significant (Adults: 1.9 +/- 1.44 hours, Neonates: 5.56 +/- 2.55 hours, P=0.096). Due to the similar results between degradation analyses, it is likely that differences observed are due to fibrinolytic discrepancies rather than differences in plasmin generation.

Analysis of fibrinogen clottability and clot mechanical properties:

To determine the stability of neonatal and adult fibrin clots formed with various fibrinopeptide releasing enzymes, we assed fibrinogen clottability and stiffness in the presence of mid-range concentrations of thrombin, batroxobin, and contortrixobin (**Figure 4A**). When clots were formed with 0.25 U/mL thrombin and 0.5 U/mL batroxobin (FpA cleavage), adult clots had significantly greater fibrinogen clottability measurements than neonatal clots (thrombin: Adults: 93.65 +/- 8.26 % clottibility, Neonates: 53.8 +/- 31.21 % clottibility, P=0.048; batroxobin: Adults: 90.14 +/- 12.22 % clottibility, Neonates: 16.8 +/- 11 % clottibility, P=0.0001.) When clots were formed with 2.5 μg/mL contortrixobin (FpB cleavage), neonatal clottibility was significantly higher than adult samples (Adults: 18.29 +/- 9.75, Neonates: 84.62 +/- 19.42, P=0.0009). AFM was utilized to determine clot elastic moduli (**Figure 4B**). Adult clots were significantly stiffer than neonatal clots formed from thrombin or batroxobin (thrombin: Adults: 2.41 +/- 0.33 kPa, Neonates: 1.36 +/- 0.16 kPa,P<0.0001; batroxobin: Adults: 0.42 +/- 0.09, kPa Neonates: 0.12 +/- 0.09 kPa, P=0.062). Conversely, in the presence of contortrixobin, neonatal clots had significantly greater stiffness values than adults (Adults: 0.25 +/- 0.34 kPa, Neonates: 0.81 +/- 0.52 kPa, P=0.39).

Competitive binding of fibrin knobs:

To explore differences in competitive binding of fibrin knobs between neonatal and adult polymerization patterns, clots were formed in the presence of excess fibrin knob A, knob B, and nonbinding peptide mimetics prior to polymerization with thrombin (**Figure 5)**. When fibrinogen was incubated with a nonbinding peptide control, clot structure appeared looser most likely due to non-specific interactions from the peptide. However, structural trends were similar to native controls and the presence of the peptide did not appear to significantly alter polymerization (Fiber density; Adults: 1.18 +/ 0.38 black/white pixels, Neonates: 0.4 +/- 0.13 black/white pixels, P=0.007). However, when clots were formed in the presence of excess fibrin knob A, adult fibrin clot structure was significantly altered; clot architecture was heterogenous and lacked normal matrix formation. Under the same conditions, neonatal fibrin clots had significantly greater fiber density than adult clots and formed relatively complete fibrin networks (Adults: 0.16 +/- 0.04 black/white pixels, Neonates: 0.43 +/- 0.05 black/white pixels, P=0.0001). In the presence of excess fibrin knob B, the reverse was found. Adult fibrin clots had significantly greater fiber density than neonatal clots and formed a network similar to controls (Adults: 0.69 +/- 0.27 black/white pixels, Neonates: 0.2 +/- 0.13 black/white pixels, P=0.017).

Quantitative release of fibrinopeptides:

The release of fibrinopeptide A and B over a two hour time period was quantified via ELISAs and reported as the neonatal to adult fold change (**Figure 6A).** The ratio of neonatal to adult fibrinopeptide A concentration was less than 1 throughout the duration of the two hour clotting time and greater than 1 for fibrinopeptide B. At 60 and 120 mins, we observed statistically significant differences in fold changes between fibrinopeptide A and B release (60 minutes: FPA: 0.21 +/- 0.08 Neonate: Adult fold change, FPB: 1.33 +/- 0.07 Neonate: Adult fold change P=0.0025; 120 minutes: FPA: 0.16 +/- 0.13 Neonate: Adult fold change, FPB: 1.73 +/- 0.63 Neonate: Adult fold change, P=0<0.0001). We also observed significant differences in fold changes of cumulative release of fibrinopeptides (**Figure 6B,** FPA: 0.37 +/- 0.63 Neonate: Adult fold change, FPB: 1.25 +/- 0.15 Neonate: Adult fold change, P=0.003). When sialic acid was removed, fibrinopeptide release was similar between adults and neonates (**Figure 6 C-D**). The neonate:adult fold change in fibrinopeptide release of desiaylated fibrinogen was significantly greater at 60 and 120 mins, as well as cumulatively, compared normal fibrinogen. (Desialyated fibrinogen, Cumulative release; FPA: 0.97 +/- 0.7 Neonate: Adult fold change, FPB: 1.062 +/- 0.06 Neonate: Adult fold change, P=0.59).

Analysis of fibrinogen formulations in a post-CPB model of coagulopathy:

Sialic acid concentration of adult fibrinogen incubated with CMP-sia and sialyltransferase was determined after 8 hour neuraminidase digestion (**Supplemental Figure 7A**). 24 hour enzyme incubation proved to be sufficient at increasing sialylation of adult fibrinogen to levels seen in neonatal samples (Adult: 3.492 +/- 1.043 μg/mL, Neonate: 6.36 +/- 0.91, Sialylated Adult: 5.288 +/- 0.53, Adult vs. Neonate: P=0.003, Adult vs. Sialylated Adult: P=0.037, Neonate vs. Sialylated Adult:P=0.23). Clots were formed with sialylated adult fibrinogen to determine fiber structure (**Supplemental Figure 7B**). Sialylated adult clots had fiber density and alignment values that were between averages from adult and neonatal fibrin clots (Fiber Density: Adult: 0.71 +/- 0.25 black/white pixels, Neonate: 0.30 +/- 0.21 black/white pixels, Sialylated Adult: 0.45 +/- 0.09 black/white pixels, Alignment: Adults: 1.07 +/- 0.01 AI, Neonate: 1.21 +/- 0.18 AI, Sialylated Adult: 1.17 +/- 0.09 AI). To simulate treatment of bleeding, various fibrinogen formulations were added to post-CPB neonatal plasma and clot structure was analyzed (**Supplemental Figure 7C**). The addition of adult fibrinogen to CPB samples resulted in heterogeneous clots with low alignment and areas of dense fibers and others with high porosity while the addition of neonatal fibrinogen resulted in more homogenous clots with alignment values more similar to baseline compared to adult (Fiber Density: Baseline: 0.29 +/- 0.06 black/white pixels, CPB: 0.40 +/- 0.01 black/white pixels, CPB + Adult Fibrinogen: 0.37 +/- 0.05 black/white pixels, CPB + Neonatal Fibrinogen 0.56 +/- 0.05 black/white pixels, CPB + Sialylated Adult Fibrinogen: 0.36 +/- 0.01 black/white pixels, Alignment: Baseline: 1.13 +/- 0.03 AI, CPB: 1.23 +/- 0.09 AI, CPB + Adult Fibrinogen: 1.05 +/- 0.02 AI, CPB + Neonatal Fibrinogen: 1.09 +/- 0.01 AI, CPB + Sialylated Adult Fibrinogen: 1.15 +/- 0.06 AI). Degradation rates were also analyzed in these samples (**Supplemental Figure 7D**). We determined CPB samples had the fastest rate of fibrinolysis and that the addition of adult, neonatal, and sialylated adult fibrinogen slowed this rate**.** Additionally, CPB clots in the presence of neonatal fibrinogen had the most similar values to baseline neonatal degradation rates (Baseline: 1.68 +/- 0.42 hours. CPB: 0.73 +/- 0.26 hours, CPB + Adult Fibrinogen: 2.6 hours, CPB + Neonatal Fibrinogen: 1.43 +/- 0.62 hours, CPB + Sialylated Adult Fibrinogen: 2.25 +/- 0.15 hours).

Analysis of porcine fibrinogen:

The sialic acid content of adult and neonatal fibrinogen isolated from plasma collected from Yorkshire pigs was determined after neuraminidase digestion **(Supplemental Figure 8**). Neonatal porcine fibrinogen had significantly higher sialic acid content than adult porcine fibrinogen (Adults: 3.09 +/- 0.09 μg/mL, Neonates: 6.32 +/- 1.23 μg/mL, P<0.002).

Discussion:

Here we characterize the role of sialic acid on neonatal fibrin polymerization dynamics by performing structural and functional assays on normal and desialylated fibrinogen, comparing properties of clots formed with selective fibrinopeptide cleavage, and directly quantifying the release of fibrinopeptides in both the presence and absence of sialic acids. Our results show a significantly increased sialic acid concentration in neonatal fibrinogen compared to adult fibrinogen, confirming findings of previous studies. We show for the first time that this modification influences the structural and functional properties of neonatal clots. Through a variety of structural and functional assays, we determined that the sialic acid content in neonatal fibrinogen plays a key role in determining clot properties. Assays using SVTLE's revealed that neonatal fibrin formation involves more B knob mediated interactions than adult fibrin polymerization. We also found a greater quantitative release of fibrinopeptide B, and less fibrinopeptide A, when forming neonatal fibrin networks compared to adults. When sialic acid was removed, no significant differences were observed in fibrinopeptide release indicating that the increased sialic acid content in neonatal fibrinogen significantly influences polymerization mechanisms.

Previous studies have shown that post-translational modifications of fibrinogen, including sialic acid, impact fibrin polymerization patterns and resultant clot structure. In the dysfibrinogenemia associated with liver disease, an increased sialic acid content is associated with altered fibrin polymerization times and clot structure[14,32,33]. Fibrinogen isolated from patients with cirrhosis is hypersialylated and exhibits a decreased rate of polymerization. Despite the delay in clot formation, the clot that was ultimately formed was less permeable compared to those generated from plasma from healthy individuals suggesting a structurally more thrombogenic clots. The explanation for this paradox is unknown though one potential hypothesis is that hypersialylation may cause a decrease in permeability by electrostatic changes within the clot. Nevertheless, desialylation of fibrinogen with neuraminidase corrected prolonged polymerization times thus validating the inhibitory effect of sialic acid on polymerization. Our results confirm a greater sialiac acid content in neonatal fibrinogen compared to adult fibrinogen, and that neonatal fibrin clot structural and mechanical properties are significantly different than that of adults. Conversely, desiaylated adult and neonatal fibrinogen show roughly equivalent structure, polymerization kinetics, and clottability results. Our data indicate that sialic acid content is likely contributing to the mechanistic differences identified between neonatal and adult fibrin network formation.

Our initial studies provided an in-depth analysis of adult and neonatal fibrin network properties when formed with fibrinopeptide cleaving enzymes specific for either fibrinopeptide A or B. Our results revealed significantly different characteristics between adult and neonatal fibrin matrices at equivalent enzyme concentrations. When initiating cleavage with batroxobin (FpA cleavage), adult clots were similar in fiber density to naturally occurring adult clots formed with thrombin. They also displayed similar clotting kinetics. Neonatal clots formed with batroxobin were highly porous and lacked substantial structure. On the other hand, with low doses of contortrixobin (FpB cleavage), neonatal clots were similar in structure to thrombin cleaved clots while adult clots showed a lack of network formation. Other clot properties, including clottability, turbidity, clot stiffness, and clot degradation, also reflected similar trends. We next examined fibrinopeptide release during adult and neonatal fibrin polymerization. Neonatal clots release more fibrinopeptide B and less fibrinopeptide A than adults, and was statistically significant after 60 minutes of clotting. Lastly, we investigated the role of sialic acid concentration on fibrin knob:hole dynamics. We removed sialic acid from both adult and neonatal fibrinogen, and, again, measured fibrinopeptide release. In the absence of sialic acid, fibrinopeptide A and B release was roughly equivalent between adults and neonates, indicating that this modification does contribute to differences polymerization mechanisms in neonates.

We also performed preliminary studies on how the modulation of sialic acid content in adult fibrinogen can affect network properties. Sialylation of adult fibrinogen appeared to alter resulting fibrin clot structural characteristics; however, it should be noted that the location of the newly added residues is unknown. As this can impact polymerization dynamics and resulting clot properties, future studies exploring this should be conducted. In simulating treatment to post-CPB neonatal bleeding, we found that the addition of adult, neonatal, and sialylated adult fibrinogen appeared to enhance clot structure and function. However, as seen with previous studies, the addition of neonatal fibrinogen resulted in properties most similar to baseline values. The addition of sialylated adult fibrinogen appeared to enhance post-CPB matrix properties in a manner distinct from normal adult fibrinogen. However, due to the low sample size owing to logistical constraints of procuring neonatal samples, future studies are necessary to explore these findings. Additionally, *in vivo* animal studies should explore how modulation of sialic acid in adult fibrinogen can affect bleeding and thrombosis outcomes.

In this study, fibrinogen was isolated from neonates undergoing elective cardiac surgery. Patients with a coagulopathy, mother with a coagulopathy, or those on hemostatic altering medications were excluded from this study. Despite this exclusionary criteria, it's possible that the structural cardiac defects could result in high shear stress which has been associated with coagulopathies such as acquired von Willebrand disease and platelet irregularities[34]. Ideally fibrinogen purification would have been from samples collected from healthy neonates, however; due to the logical constraints on obtaining blood from healthy neonates, we utilized available specimens. However, recent studies from our group using healthy adult and neonatal porcine samples identified similar age-dependent differences in fibrinogen as those observed between the human samples analyzed in these studies[29]. Moreover, we determined adult porcine fibrinogen has significantly lower sialic acid content than neonatal porcine fibrinogen (**Supplemental Figure 7)**, which is similar to what we observed in human patients. These similar findings indicate that our results obtained from neonates undergoing elective cardiac surgery are likely more widely applicable to healthy human neonates as well.

Here, we focus solely on sialic acid as previous studies have identified the increased concentration in neonatal fibrinogen but have not analyzed its potential effects on fibrin network formation in neonates. However, due to the complexities of polymerization and post-translational modifications, there are likely other contributing factors. For example, there are many fibrinogen interacting coagulation factors such as factors II,VII, IX, and plasminogen that that have been identified as being decreased in neonates relative to adults that may play a role[35,36]. Furthermore, the action of FXIII crosslinking, which has been shown to impact fibrin clot structure and elastic moduli, was not explored in this study[37,38]. Similar FXIII concentrations were identified between adult and neonatal samples, therefore we expect similar effects between groups in our thrombin polymerized clots. However, future studies controlling for the potential effects of neonatal vs adult FXIII action should be investigated. Additional differences in post-translational modifications have been identified in neonatal fibrinogen, such as an increased phosphorus content, although phosphorus has not been shown to impact fibrin polymerization[39]. Future studies utilizing tandem mass spectroscopy to identify type and location of other post-translational modifications that may reveal more mechanistic differences in polymerization should be explored. It should be noted that there is precedent from other studies exploring the effects of abnormal sialic content on fibrin polymerization and network characteristics[14,33]. The results from our study indicate that the increased sialic acid concentration found in neonatal fibrinogen may contribute to altered fibrinopeptide release and subsequent clot matrix properties though the underlying mechanisms remain unknown. Future studies researching the potential of affinity, steric hindrance, and increased negative charge are needed to elucidate the underlying process. Additionally, further investigation on our hypothesis using genetically modified fibrinogen with dysfunctional and A and B knob sites could support our findings. Furthermore, previous studies have identified sialic acid residues to act as low affinity calcium binding sites that aid in fibrin network formation[40]. Future experiments studying the role of calcium binding on these processes in neonatal fibrinogen should be explored. Furthermore, we assessed only complete cleavage of sialic acid residues, and not how partial removal would impact polymerization dynamics. Additionally, the underlying purpose for these age-dependent changes is unclear, although many have posited that developmental differences in hemostatic proteins provides antithrombotic and hemorrhagic protection[41].

In conclusion, our data suggest that molecular differences in fibrin polymerization between adult and neonatal fibrinogen determine vastly differing structural and mechanistic clot properties. In neonates, fibrin polymerization appears to be more strongly influenced by B knob interactions while, in adults, it is dominated by A knob interactions. The increased sialic acid content of neonatal fibrinogen could drive the increased B knob interactions by promoting more release of fibrinopeptide B. The findings provide a plausible explanation for our previous observation that adult and neonatal fibrinogen, when mixed, do not seamlessly integrate with each other[9]. For many reasons, neonates receive a substantial amount of blood products during cardiac surgeries and ECMO support and experience a significant risk of thrombosis. It may prove advantageous to research neonatal specific treatment options as well as therapeutics that target the fibrin A knob for treatment of bleeding and fibrin B knob for treatment of thrombosis.

Acknowledgements:

CDMRP W81XWH-15-1-0485 from the U.S. Department of Defense (Washington, D.C.), DMR-1847488 from the National Science Foundation (Alexandria, Virgina), and NIH NHLBI 1R01HL146701, R01HL130918-01A1 from the National Institutes of Health (Bethesda, Maryland).

Authorship:

Contribution: K.N performed experiments, analyzed results, and wrote paper. A.K assisted in quantification of structural results and clot degradation experiments and data analysis. N.G assisted in research design and paper writing. ACB designed and supervised the study, data analysis, and paper writing.

Conflict-of-interest disclosure: The authors have nothing to disclose.

References:

1. Revel-Vilk S. The conundrum of neonatal coagulopathy. *Hematology*. 2012;2012(1):450–454.
2. Haidl H, Zöhrer E, Pohl S, et al. New insights into neonatal coagulation: normal clot formation despite lower intra-clot thrombin levels. *Pediatric Research*. 2019;86(6):719–724.
3. Ignjatovic V, Ilhan A, Monagle P. Evidence for age-related differences in human fibrinogen. *Blood Coagul. Fibrinolysis*. 2011;22(2):110–117.
4. Guzzetta NA, Allen NN, Wilson EC, et al. Excessive postoperative bleeding and outcomes in neonates undergoing cardiopulmonary bypass. *Anesth. Analg.* 2015;120(2):405–410.
5. Wolf MJ, Maher KO, Kanter KR, et al. Early postoperative bleeding is independently associated with increased surgical mortality in infants after cardiopulmonary bypass. *The Journal of Thoracic and Cardiovascular Surgery*. 2014;148(2):631-636.e1.
6. Dönmez A, Yurdakök O. Cardiopulmonary Bypass in Infants. *Journal of Cardiothoracic and Vascular Anesthesia*. 2014;28(3):778–788.
7. Cashen K, Meert K, Dalton H. Anticoagulation in Neonatal ECMO: An Enigma Despite a Lot of Effort! *Front Pediatr*. 2019;7:.
8. Van Ommen CH, Neunert CE, Chitlur MB. Neonatal ECMO. *Front Med (Lausanne)*. 2018;5:.
9. Brown AC, Hannan R, Timmins LH, et al. Fibrin network changes in neonates after cardiopulmonary bypass. *Anesthesiology*. 2016;124(5):1021–1031.
10. Weisel JW, Litvinov RI. Mechanisms of fibrin polymerization and clinical implications. *Blood*. 2013;121(10):1712–1719.
11. Weisel JW, Litvinov RI. Fibrin Formation, Structure and Properties. *Subcell Biochem*. 2017;82:405–456.
12. Litvinov RI, Gorkun OV, Owen SF, Shuman H, Weisel JW. Polymerization of fibrin: specificity, strength, and stability of knob-hole interactions studied at the single-molecule level. *Blood*. 2005;106(9):2944–2951.
13. Regulation of human fetal hemoglobin: new players, new complexities | Blood | American Society of Hematology.
14. Martinez J, MacDonald KA, Palascak JE. The role of sialic acid in the dysfibrinogenemia associated with liver disease: distribution of sialic acid on the constituent chains. *Blood*. 1983;61(6):1196–1202.
15. Lisman T, Ariëns RAS. Alterations in Fibrin Structure in Patients with Liver Diseases. *Semin Thromb Hemost*. 2016;42(4):389–396.
16. Brennan SO. Variation of fibrinogen oligosaccharide structure in the acute phase response: Possible haemorrhagic implications. *BBA Clin*. 2015;3:221–226.
17. Neonatal Fibrin Scaffolds Promote Enhanced Cell Adhesion, Migration, and Wound Healing In Vivo Compared to Adult Fibrin Scaffolds | SpringerLink.
18. Edelberg JM, Enghild JJ, Pizzo SV, Gonzalez-Gronow M. Neonatal plasminogen displays altered cell surface binding and activation kinetics. Correlation with increased glycosylation of the protein. *J Clin Invest*. 1990;86(1):107–112.
19. Komlos L, Levinsky H, Goldman J, et al. Sialic acid content and sialyltransferase activity in lymphocytes from neonates. *Biol. Neonate*. 1988;54(5):270–274.
20. Qiu LL, Levinson SS, Keeling KL, Elin RJ. Convenient and effective method for removing fibrinogen from serum specimens before protein electrophoresis. *Clin. Chem.* 2003;49(6 Pt 1):868–872.
21. Dietrich M, Heselhaus J, Wozniak J, et al. Fibrin-Based Tissue Engineering: Comparison of Different Methods of Autologous Fibrinogen Isolation. *Tissue Engineering Part C: Methods*. 2012;19(3):216–226.

22. Aper T. Fibrinogen Preparations for Tissue Engineering Approaches. *J Bioengineer & Biomedical Sci*. 2012;02(03):
23. Diaz-Mauriño T, Castro C, Albert A. Desialylation of fibrinogen with neuraminidase. Kinetic and clotting studies. *Thromb. Res.* 1982;27(4):397–403.
24. Valladolid C, Martinez-Vargas M, Sekhar N, et al. Modulating the rate of fibrin formation and clot structure attenuates microvascular thrombosis in systemic inflammation. *Blood Advances*. 2020;4(7):1340–1349.
25. Collet J-P, Moen JL, Veklich YI, et al. The αC domains of fibrinogen affect the structure of the fibrin clot, its physical properties, and its susceptibility to fibrinolysis. *Blood*. 2005;106(12):3824–3830.
26. Baker SR, Zabczyk M, Macrae FL, et al. Recurrent venous thromboembolism patients form clots with lower elastic modulus than those formed by patients with non- recurrent disease. *J Thromb Haemost*. 2019;17(4):618–626.
27. Sproul EP, Hannan R, Brown AC. Characterization of fibrin-based constructs for tissue engineering. *Biomaterials for Tissue Engineering: Methods and Protocols, Methods in Molecular Biology*. 2018;85–99.
28. Nellenbach K, Guzzetta NA, Brown AC. Analysis of the structural and mechanical effects of procoagulant agents on neonatal fibrin networks following cardiopulmonary bypass. *J. Thromb. Haemost.* 2018;16(11):2159–2167.
29. Nellenbach KA, Nandi S, Kyu A, et al. Comparison of Neonatal and Adult Fibrin Clot Properties between Porcine and Human Plasma. *Anesthes*. 2020;
30. Lord ST, Strickland E, Jayjock E. Strategy for recombinant multichain protein synthesis: fibrinogen B beta-chain variants as thrombin substrates. *Biochemistry*. 1996;35(7):2342–2348.
31. Raju TS, Briggs JB, Chamow SM, Winkler ME, Jones AJ. Glycoengineering of therapeutic glycoproteins: in vitro galactosylation and sialylation of glycoproteins with terminal N-acetylglucosamine and galactose residues. *Biochemistry*. 2001;40(30):8868–8876.
32. Gralnick HR, Givelber H, Abrams E. Dysfibrinogenemia Associated with Hepatoma. *New England Journal of Medicine*. 1978;299(5):221–226.
33. Varki A. Sialic acids in human health and disease. *Trends Mol Med*. 2008;14(8):351–360.
34. Loeffelbein F, Funk D, Nakamura L, et al. Shear-stress induced acquired von Willebrand syndrome in children with congenital heart disease. *Interactive CardioVascular and Thoracic Surgery*. 2014;19(6):926–932.
35. Saracco P, Rivers RPA. Pathophysiology of Coagulation and Deficiencies of Coagulation Factors in Newborn. *Neonatology: A Practical Approach to Neonatal Diseases*. 2018;1431–1453.
36. Pichler E, Pichler L. The neonatal coagulation system and the vitamin K deficiency bleeding - a mini review. *Wien Med Wochenschr*. 2008;158(13–14):385–395.
37. Duval C, Allan P, Connell SDA, et al. Roles of fibrin α- and γ-chain specific cross-linking by FXIIIa in fibrin structure and function. *Thrombosis and Haemostasis*. 2014;111(5):842–850.
38. Kurniawan NA, Grimbergen J, Koopman J, Koenderink GH. Factor XIII stiffens fibrin clots by causing fiber compaction. *J Thromb Haemost*. 2014;12(10):1687–1696.
39. Witt I, Hasler K. Influence of organically bound phosphorus in foetal and adult fibrinogen on the kinetics of the interaction between thrombin and fibrinogen. *Biochim Biophys Acta*. 1972;271(2):357–362.

40. Dang CV, Shin CK, Bell WR, Nagaswami C, Weisel JW. Fibrinogen sialic acid residues are low affinity calcium-binding sites that influence fibrin assembly. *J Biol Chem*. 1989;264(25):15104–15108.
41. Ignjatovic V, Mertyn E, Monagle P. The coagulation system in children: developmental and pathophysiological considerations. *Semin Thromb Hemost*. 2011;37(7):723–729.

Figures:

Figure 1

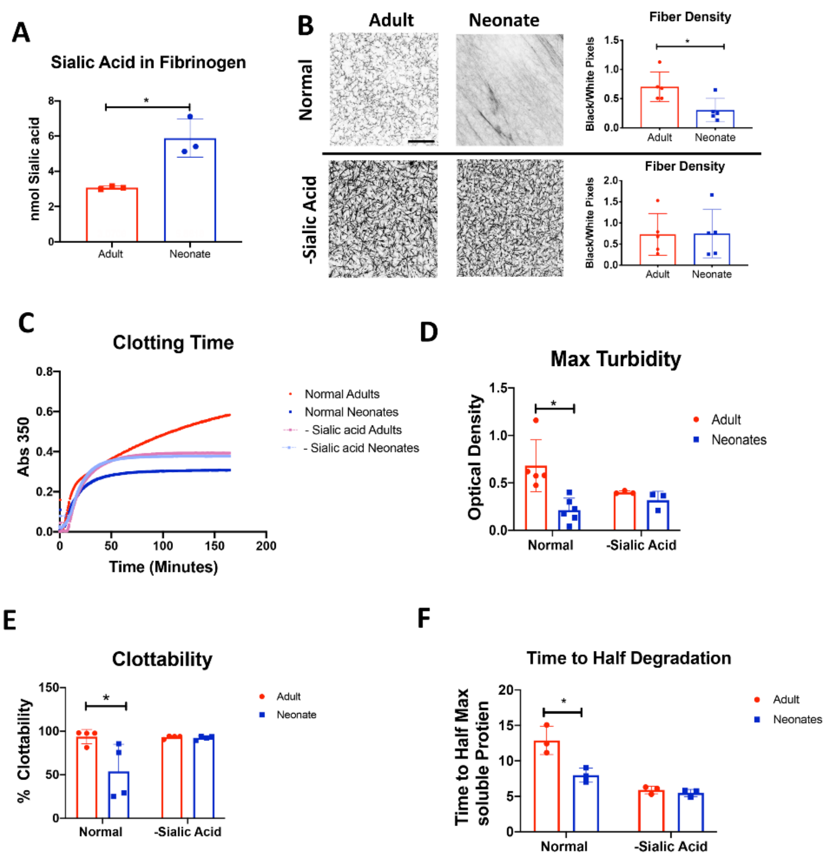

Figure 1. Influence of sialic acid on neonatal fibrin network properties. A) Sialic acid concentration of neonatal fibrinogen is significantly greater than adult fibrinogen. N=3 triplicate experiments. **B)** Representative images of confocal microscopy analysis of clots taken at 63x. Prior to removal of sialic acid, fiber density was statistically significant between adults and neonates. After removal, resulting clot structures were roughly equivalent in fiber density. N=5 clots/group. Scale: 10 µm. **C)** Polymerization curves were analyzed via turbidity measurements on a plate reader. **D)** Max turbidity values were significantly lower in neonates compared to adults prior to removal of sialic acid and similar after. N=5 **E)** Normal neonatal fibrinogen had significantly lower clottability than adult fibrinogen and after digestion of sialic acid clottability was similar. N=4. **F)** Fibrinolysis was initiated with plasminogen and tPA. Normal neonatal fibrinogen had statistically faster degradation rates than adult fibrinogen. After removal of sialic acid, degradation rates were similar. N=3 Average +/- standard deviation is shown. P*<0.05.

Figure 2

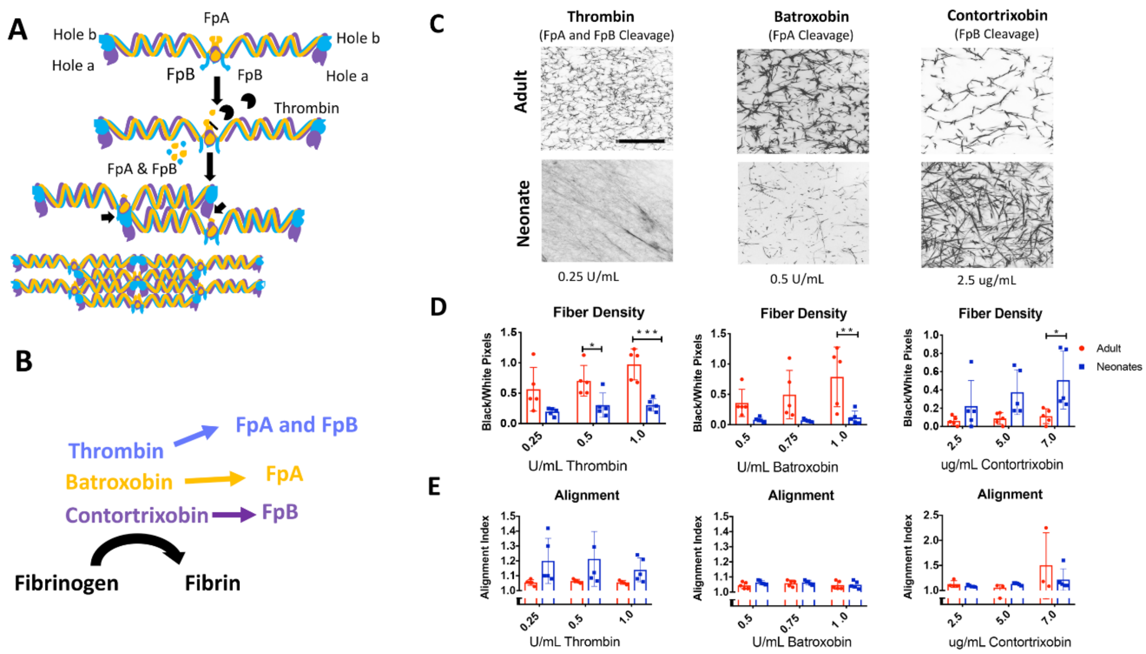

Figure 2. Selective cleave of fibrinopeptides. A) Fibrin polymerization occurs through the noncovalent binding of exposed fibrin knobs A and B to fibrin holes a and b on adjacent proteins. These mechanisms have not been explored in neonates. **B)** Snake venom thrombin-like enzymes were utilized to selectively cleave fibrinopeptides and activate fibrin. **C)** Representative images from confocal microscopy at 63x magnification of adult and neonatal fibrin clots at equal enzyme concentrations reveals differences in **D)** fiber density and **E)** fiber alignment. Scale: 10 µm. Fiber density N=5/group, fiber alignment N=4-5/group. Average +/- standard deviation is shown. P*<0.05, P***<0.001.

Figure 3

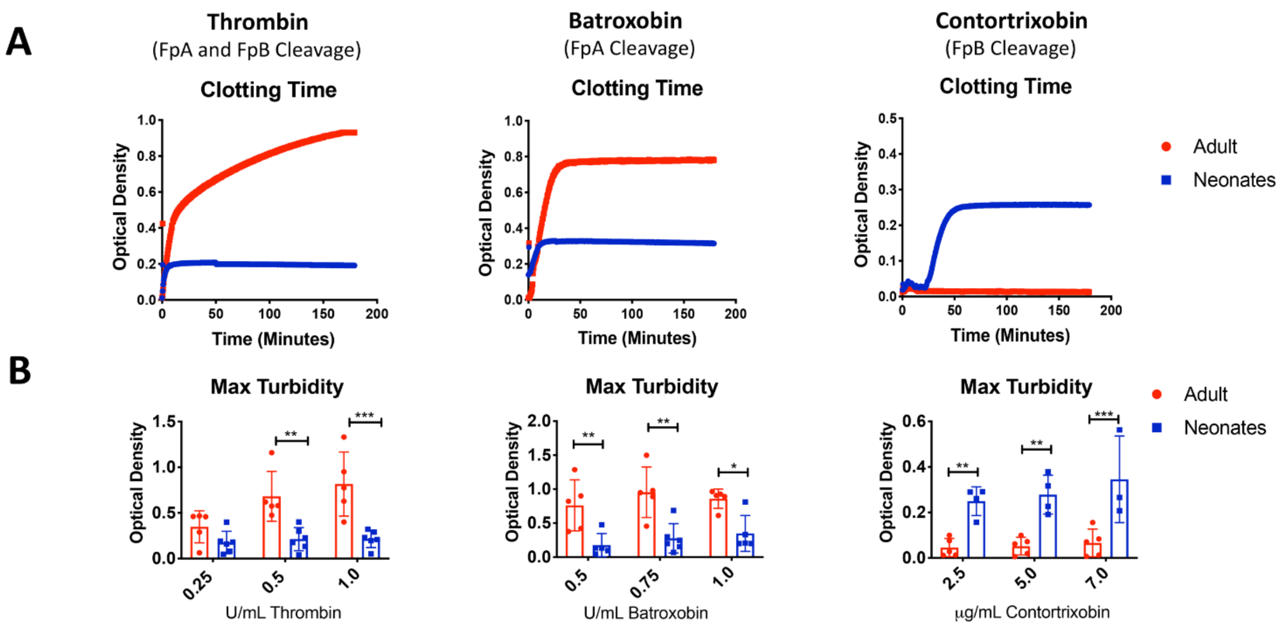

Figure 3. Clotting time. A) Representative clotting time curves from absorbance based turbidity assays using neonatal and adult fibrinogen at equal enzyme concentrations. **B)** Maximum turbidity data gathered from clotting curves reveals significant differences between adult and neonatal fibrin clots. Average maximum turbidity +/- standard deviation is shown. N=3-6/group. P*<0.05, P**<0.01, P***<0.001.

Figure 4:

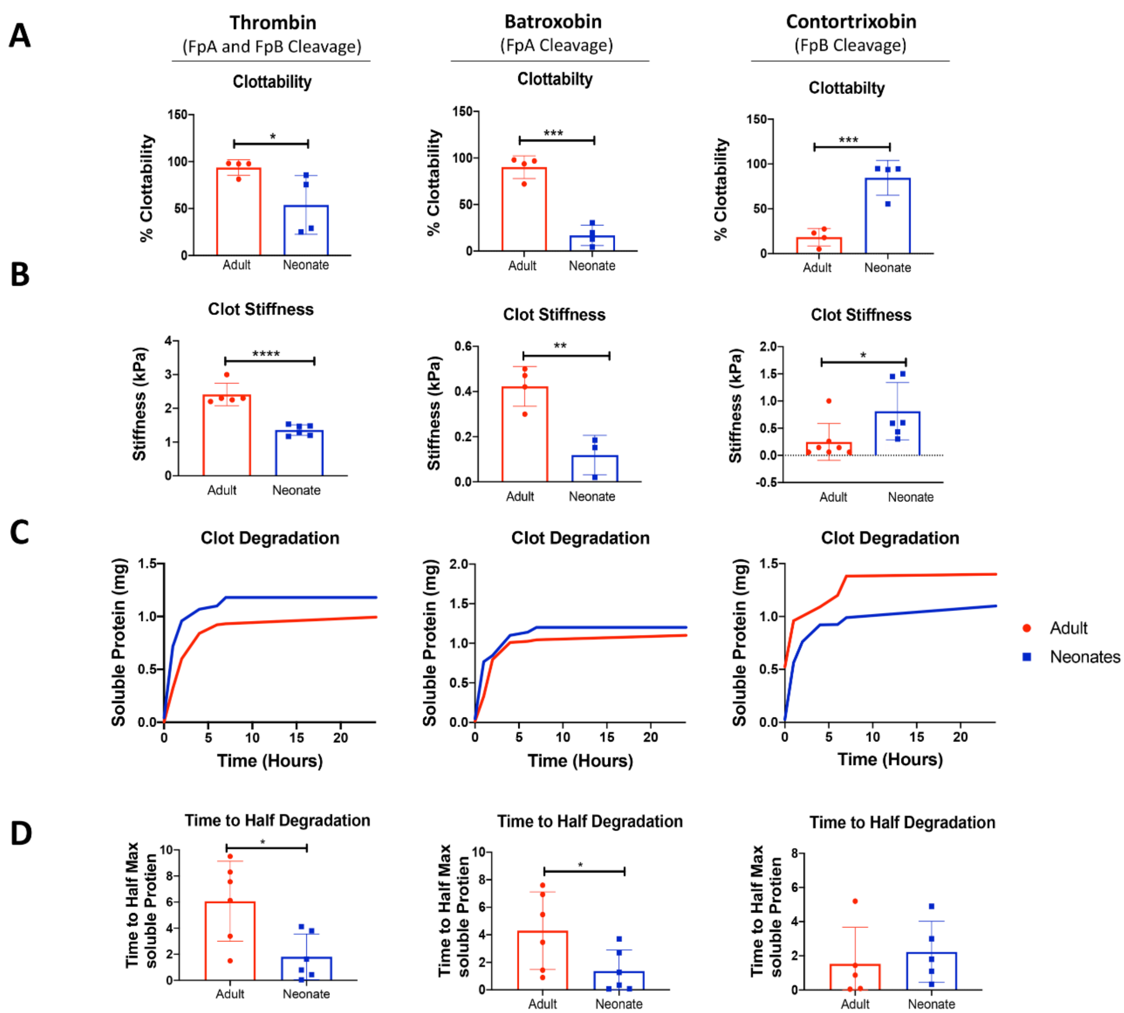

Figure 4. Functional analysis of clots formed with selective cleavage of fibrinopeptides. A) Clottability of neonatal and adult fibrinogen with mid-range concentrations of fibrinopeptide cleaving enzymes. N=4/group. **B)** Atomic force microscopy was utilized to determine stiffness for clots formed with neonatal and adult fibrinogen with mid-range concentrations of fibrinopeptides. N=3-7/group. **C)** Representative degradation curves and **D)** degradation rates for clots formed with venom enzymes were determined from the time taken to reach half- maximum soluble protein. N=5-6/group. Average values +/- standard deviation is reported. P*<0.05, P**<0.01, P***<0.001, P****<0.0001.

Figure 5:

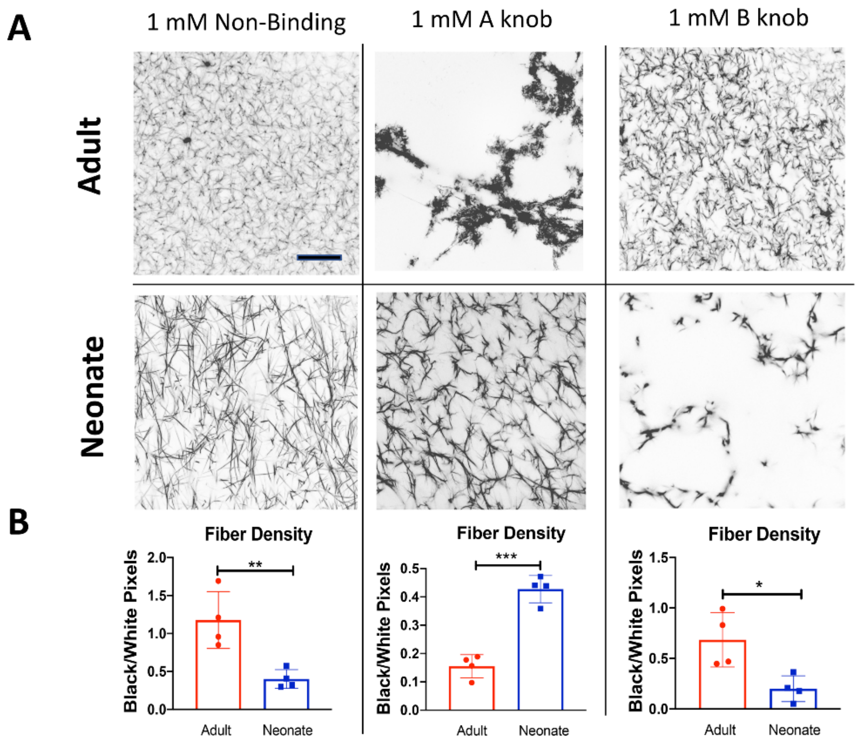

Figure 5. Competition assay to reveal polymerization mechanism. High concentrations of fibrin knob A and B mimetic peptides were added to neonatal and adult fibrinogen solutions prior to initiation of clotting with thrombin. **A)** Representative images are shown from confocal microscopy imaging of clots at 63x. **B)** Fiber density analysis reveals significant differences between adult and neonatal clots after equivalent peptide incubation times. N=4/group. Scale: 10 µm. Mean fiber density +/- standard deviation is reported. P*<0.05, P**<0.01, P***<0.001

Figure 6:

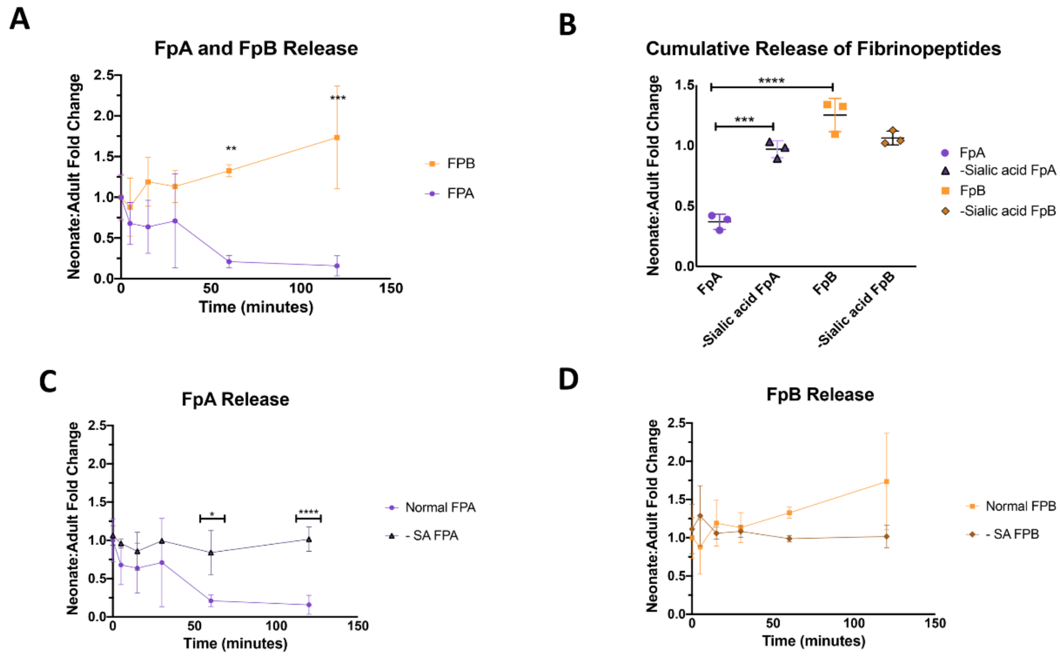

Figure 6. Quantitative release of fibrinopeptides. Clotting was initiated with addition of thrombin to normal neonatal and adult fibrinogen. **A)** Fibrinopeptide A and B concentration was determined for each time point over 120 minutes and reported as the neonatal to adult fold change. Mean fold change +/- standard deviation is shown. **B)** Cumulative release of fibrinopeptides was determined for normal and desialylated fibrinogen during clotting and reported as the neonatal to adult fold change for each fibrinopeptide. **C)** Fibrinopeptide A release over time in desialylated fibrinogen is compared to normal fibrinogen. The neonatal:adult fold change is reported. **D)** Fibrinopeptide B release over time in desialylated fibrinogen is compared to normal fibrinogen. The neonatal:adult fold change is reported. N=3 triplicate experiments/group. Mean +/- standard deviation is shown. P*<0.05, P**<0.01, P***<0.001, P****<0.0001.