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ORIGINAL ARTICLE

Mathematical modeling identifies clotting factor combinations that modify thrombin generation in normal and factor VIII-, IX-, or XI-deficient blood

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Abstract

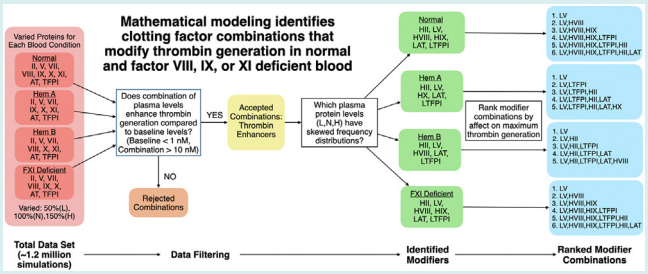
Background: In healthy individuals, plasma levels of clotting proteins naturally vary within a range of 50% to 150% of their mean values. We do not know how these variations modify thrombin generation.

Objectives: To assess the impact of protein level variations on simulated thrombin generation in normal and factor (F)VIII-, FIX-, or FXI-deficient blood.

Methods: We used a mathematical model of flow-mediated coagulation to simulate thrombin generation with all possible combinations of clotting protein variations within the normal range and for various tissue factor levels. We selected, analyzed, and ranked combinations that enhanced thrombin generation compared with baseline.

Results: Protein variations most strongly affected thrombin generation at intermediate tissue factor levels. Low tissue factor levels prevented coagulation initiation, while high tissue factor levels always triggered thrombin generation. At intermediate levels, we identified protein variations that substantially modified thrombin generation. Low-normal FV shortened lag times and increased thrombin generation, whereas high-normal FV lengthened lag times and reduced thrombin generation. With severe FVIII and FIX deficiencies, low-normal tissue factor pathway inhibitor α and antithrombin amplified the effect of low-normal FV. For moderate FVIII and FIX deficiencies, high-normal tissue factor pathway inhibitor α and antithrombin enhanced the impact of high-normal FV in reducing thrombin production. In normal and FXI-deficient blood, high-normal FVIII and FIX significantly boosted thrombin generation.

Conclusion: Our mathematical model predicted how variations in clotting protein levels, within the normal range, could contribute to the variability of bleeding phenotypes observed with clotting factor deficiencies. Our study generated experimentally testable hypotheses that could aid in developing new therapies toward normal hemostasis.



KEYWORDS
factor V, hemophilia, hemostasis, mathematical modeling, tissue factor

Essentials

- We do not know how normal variations in clotting factor levels affect thrombin generation.
- A mathematical model found thrombin generation modifiers in normal and factor-deficient blood.
- Factor V had the greatest single effect on thrombin generation for all blood types.
- Adding a second or third modifier influences thrombin, but more than 3 diminish the effects.

1 | INTRODUCTION

Plasma concentrations of clotting factors naturally vary between 50% and 150% of their mean (baseline) values in healthy individuals. This suggests the question: what are the effects of these variations on coagulation in response to vessel injury? In previous studies [1,2], we used a mathematical model of platelet deposition and coagulation under flow [3–7] to explore this question for normal (NRM) and hemophilia A (HA) blood with the factor (F)VIII level fixed at 1% of baseline. The model simulates the coagulation response after a small vascular injury. It tracks the reactions of the tissue factor (TF) pathway of coagulation in plasma, on the subendothelial (SE) surface, and on activated platelet surfaces. It also accounts for the transport of platelets and proteins to and from the vicinity of the injury and allows for the buildup of platelet aggregates and their effects on the coagulation reactions.

In our previous work, we used a model to assess the sensitivity of thrombin generation to plasma protein level variations (50%-150% of baseline) for high TF (15 fmol/cm^2) in NRM blood [1]. Our global sensitivity analysis showed that robust thrombin generation occurred for all combinations, which is in line with observations from other models [8–10]. The thrombin concentration at 20 minutes, the lag time to 1 nM thrombin, and the rate of thrombin generation were only slightly changed for varying protein combinations. One intriguing and initially surprising observation was that simulations with low NRM levels of FV had the shortest lag times compared with other proteins. We also observed that when plasma protein levels were varied individually in the NRM range, the greatest effects occurred at the extremes (50% and 150% of baseline) and that the effects varied monotonically between them. These results highlight the overall robustness of the coagulation system.

In a follow-up study [2], we used the same model and approach with severe HA [11]. We fixed FVIII to 1% of its baseline and carried out 10,000 simulations in which all other plasma protein concentrations were varied randomly between 50% and 150%. We did this for each of a range of TF exposures between 1 and 20 fmol/cm^2. For low TF exposure (<2-3 fmol/cm^2), none of the protein combinations prompted a thrombin burst, while at high TF exposure (>10 fmol/cm^2), all protein combinations led to a strong thrombin burst. To better detect sensitivity to plasma protein variations, we determined a critical TF density (5 fmol/cm^2) at which thrombin generation sharply transitioned between an attenuated (no burst) and amplified (burst) response. Simulations identified low NRM FV (50% of baseline) as the critical trigger for enhanced thrombin generation in HA blood, with thrombin generation being strongly augmented when prothrombin was also present at a high NRM level (150%). These observations were verified experimentally in thrombin generation and whole-blood microfluidic assays. Further explorations with the model suggested a potential biochemical mechanism: lowering FV reduces competition between FV and FVIII for FXa on activated platelets' surfaces, which increases activation of the low levels of FVIII present by FXa, leading to increased FVIIIa:FIXa formation, which thereby increases thrombin generation.

In these previous studies, the only roles of TF pathway inhibitor a (TFPIa) were to inhibit FXa in the plasma and TF:FVIIa on the SE, and the presence of TFPIa had little effect on the amount of thrombin produced. Motivated by recent studies [12–14] showing interactions between TFPIa and partially-activated forms of FV, we recently

extended our model to incorporate partially-activated FV and its interactions with TFPIa, along with expanded roles for TFPIa and antithrombin (AT) [15,16]. In that work, we introduced a new species, partially-activated FV (which we termed "FV-h"). We included its inhibition by TFPIa in the plasma and on activated platelet surfaces and allowed TFPIa to directly inhibit FXa bound to activated platelet surfaces. FV-h was produced by FXa acting on FV and could be released from platelets as they were activated. The role of AT was also extended to include its inhibition of thrombin, FIXa, FXa, and FXIa on activated platelet surfaces. With these changes, TFPIa and AT had more inhibitory influence than in the previous models, yet flow-mediated removal of enzymes was still the most effective inhibitor of thrombin generation within the reaction zone (RZ).

In this study, we revisited the impact of coagulation protein variations on thrombin generation using our recently extended model, which incorporated FV-h, new TFPIα interactions, and additional blood conditions. These conditions included NRM, HA, hemophilia B (HB), and FXI deficiency (FXID), each characterized by 1% of the deficient protein. For each of these blood conditions, we determined pairs of proteins and levels, which we term modifiers (eg, low NRM [LN] FV; high NRM [HN] FVIII), that could change a no thrombin burst situation to one where a strong burst occurs for some TF exposure. We determined which single modifier, which pair of modifiers, trio of modifiers, etc., were *most effective* at enhancing thrombin production. For all blood conditions, LN FV was the most effective single modifier, consistent with our previous work [1,2], but for the first time, the most effective pairs and trios were also identified. Large enhancements in thrombin production (beyond that elicited with LN FV) occurred with pairs and trios of modifiers. In HA, for example, LN FV, together with LN AT, was the most effective pair of modifiers, and LN levels of FV, AT, and TFPIa were the most effective trio. In addition, we performed similar analyses for NRM and 10% HA, 10% HB, and 10% FXID to determine which modifiers reduced thrombin generation to ineffective levels.

2 | METHODS

2.1 | Model overview

Here, we give a brief review of our previously developed mathematical model of flow-mediated coagulation [3–7,9,10]. More details about this model and its sensitivity to parameters can be found elsewhere [1]. The model simulates the coagulation reactions occurring in a small RZ above an injury where TF in the SE is exposed (Figure 1).

The injury's severity is characterized by a prescribed density of exposed TF. Clotting proteins and platelets are transported into and out of the RZ by a combination of flow and diffusion, using a mass transfer coefficient whose value is a function of vessel and injury size, the flow's shear rate, and the species diffusivity. Importantly, the model distinguishes between proteins suspended in the plasma and proteins bound to the SE or to the surfaces of activated platelets. Protein concentrations in the RZ change due to enzymatic reactions, protein binding to or unbinding from the SE or the surfaces of activated platelets, and protein transport into and out of the zone. Similarly, platelet concentrations change as platelets adhere to the injured wall and become activated, and other platelets are transported into and out of the zone. The concentration of each species in the RZ plasma is tracked with an ordinary differential equation; this choice relies on the assumption that each species is uniformly distributed (well-mixed) within the RZ. An additional well-mixed endothelial zone is located adjacent to the RZ, in the direction perpendicular to the flow, and it is here that thrombin and surface-bound thrombomodulin form a complex that produces activated protein C. There are 3 forms of platelets in the model: unactivated platelets in the plasma and activated platelets in the growing thrombus, some of which are directly attached to the SE. Activation of platelets is achieved by contact with the SE, interaction with thrombin, or exposure to already activated platelets (this is an indirect way to model the release of agonists from platelet granules). Each activated platelet expresses specified types and numbers of binding sites to which coagulation proteins can selectively bind. Thus, activated platelets provide the membrane surface necessary for the formation of the platelet-bound FVIIIa:FIXa and FVa:FXa enzyme complexes. In this way, deposited platelets are procoagulant. They are also anticoagulant because their accumulation blocks access to and, therefore, the activity of extrinsic tenase (TF:FVIIa) on the SE surface.

2.2 | Data set generation

For each type of result presented below, we report on simulations carried out for 4 different blood conditions: NRM, FXID, HA, and HB, and for TF densities between 1 and 20 fmol/cm^2. We varied the plasma concentrations of the following 9 clotting proteins: clotting factors FII, FV, FVII, FXIII, FIX, FX, and FXI, and inhibitors TFPIa and AT.

For this study, we were interested in understanding which combinations of clotting proteins, if any, could either enhance thrombin generation or attenuate it for severe or moderate deficiencies, respectively. To this end, we performed simulations with many combinations of input protein concentrations and TF densities. When we look at the enhancement of thrombin production, the concentration of the protein deficient in each condition is set to 1% of its average healthy value; when we look at the attenuation of thrombin production for HA, HB, and FXID, the deficient protein's concentration is set to 10% of its average healthy value. When we refer to variations in protein levels, we mean that the concentrations of the nondeficient proteins are set to one of 50%, 100%, or 150% of their NRM healthy value, the range of variation reported in the literature [17,18]. When the plasma level of FV is changed, the total amount of FV and FV-h released by each platelet upon activation is changed in the same proportion. Below, we refer to "baseline" values, which means that for the NRM condition, all 9 protein concentrations are set to their

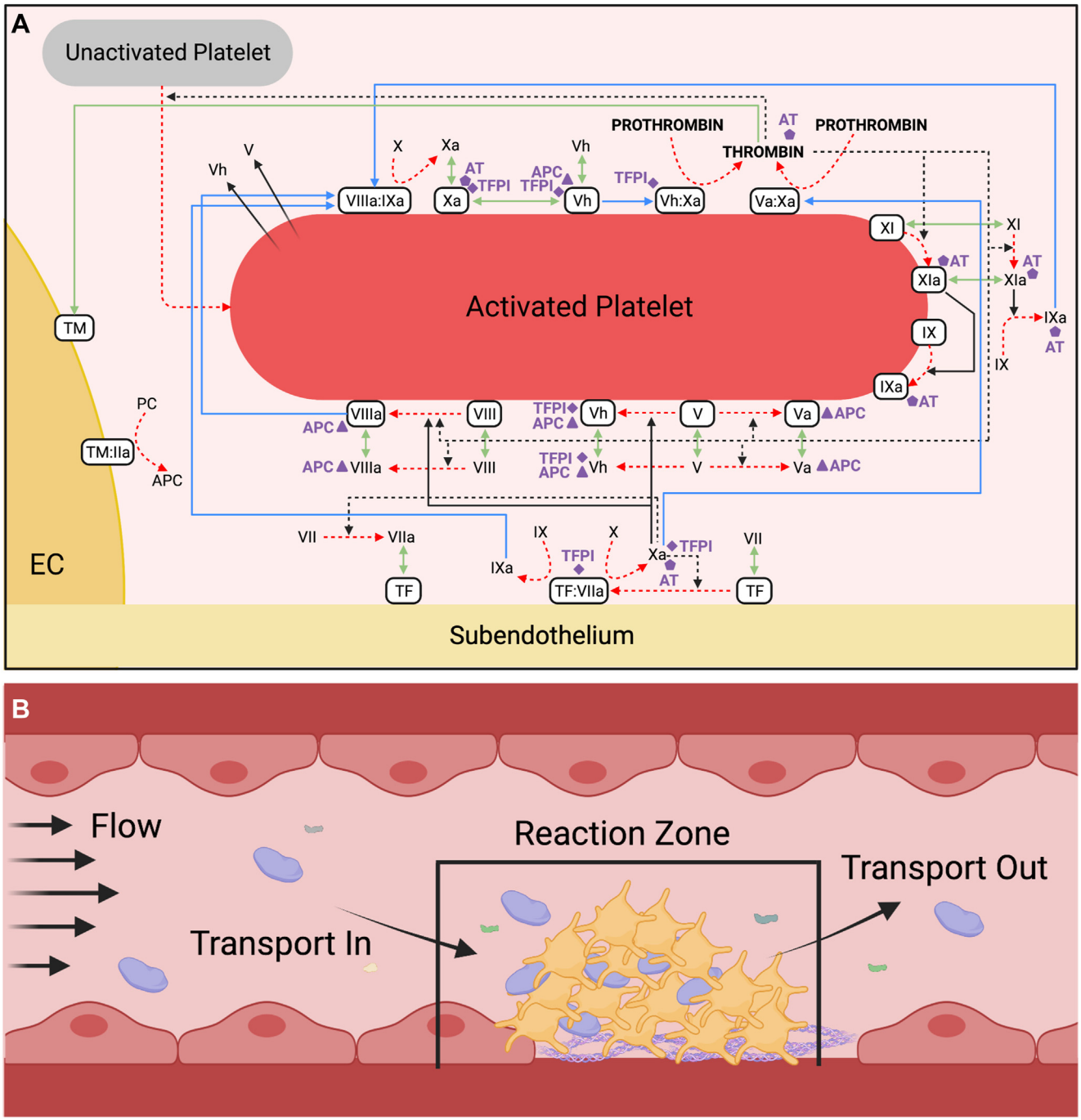


FIGURE 1 Schematic of coagulation reactions and reaction zone. Schematic of (A) coagulation reactions and the (B) reaction zone included in the model. (A) Dashed red arrows show cellular or chemical activation processes. Blue arrows show chemical transport in the fluid or on a surface. Green arrows depict binding and unbinding from cell surfaces. Rectangular boxes denote surface-bound species. Solid black lines show enzyme action in a forward direction, while dashed black lines show the feedback action of enzymes. Black lines with a fade indicate release from the platelet. Purple shapes show inhibitors. The lowercase letter "a" on any species means that it is in an "activated" form, eg, factor (F)X and FXa are clotting FX and activated clotting FX. (B) Schematic of vessel, reaction zone, and platelet and protein transport as incorporated in the model. APC, activated protein C; AT, antithrombin; TF, tissue factor; TFPI, tissue factor pathway inhibitor; EC, endothelial cell; FVh, partially activated FV; PC, protein C; TM, thrombomodulin.

average in healthy human blood, and for all the other blood conditions, protein concentrations other than that of the deficient one are set to these average concentrations.

Simulations for the NRM condition involved all combinations of the 9 plasma proteins and TF values ranging from 1 to 20 fmol/cm^2, which required a total of 393,660 simulations. For each of the HA, HB, and FXID

conditions, simulations involved combinations of all nondeficient proteins, while the deficient protein was fixed at either 1% or 10% of its NRM level. This led to 131,220 simulations for each deficient blood condition. The input conditions (initial plasma concentration and TF density) and simulated thrombin concentrations through time were recorded for each of the many simulations. These ~1.2 million simulations together make up the complete data set that we analyzed, as described in section 2.3.

2.3 | Data analyses

2.3.1 | Identifying modifiers of thrombin production

The overall data analysis of the ~1.2 million simulations is summarized in Figure 2. The effect of variations of plasma protein levels on thrombin generation depends on the density of TF exposed. At sufficiently high TF, all conditions with plasma proteins between 50% and 150% of NRM generate substantial thrombin, and for sufficiently low TF densities, little thrombin is made with any concentrations of the plasma proteins in this range. This is true for NRM blood and for each of the deficient conditions, but the meaning of "sufficiently high" and "sufficiently low" varied among the blood conditions. Even for HA and HB, sufficiently high TF densities led to enough FXa production by TF:FVIIa so that the deficit in platelet-bound FVIIIa:FIXa formation was overcome. For each condition, we sought to determine an intermediate range of TF levels in which varying the plasma protein levels could change thrombin production in a physiologically significant way, changing it from ineffective to effective (or *vice versa*) in its expected hemostatic action. To that end, we filtered the hundreds of thousands of simulation results generated as described above. The filtering was based on the thrombin concentration at 10 minutes for NRM blood and FXID blood and at 30 minutes for HA and HB blood.

We did this by looking at each TF level and each blood condition and checking for 2 things: (1) whether the baseline levels of plasma proteins result in a maximum thrombin concentration of less than 1 nM, and (2) whether some combination of protein levels lead to a maximum thrombin concentration of at least 10 nM. For each TF level for which both of these events occurred, we refer to the baseline protein levels as the "reference case," and we refer to each combination of protein levels that produced more than 10 nM thrombin as an "accepted sample." TF levels for which the pair of events did not occur were not considered further. We chose the bounds for the filtering because we judged maximum thrombin levels of less than 1 nM to be hemostatically ineffective and concentrations above 10 nM to be hemostatically effective because substantial platelet activation and fibrin formation can occur at such thrombin concentrations. We noted that modifying the bounds used for the filtering led to more or fewer accepted samples, and a second reason for the chosen bounds is that they produced a strong filter that resulted in a sufficiently large set of samples from which we could acquire meaningful statistics.

The procedure just described was used when considering thrombin enhancement. For thrombin generation attenuation, we used a similar but inverted filtering process. For each TF level, we looked for cases in which the baseline levels of plasma proteins led to the generation of more than 10 nM thrombin, and some other combination(s) of these protein levels produced less than 1 nM thrombin. When this occurred, the baseline levels were again referred to as

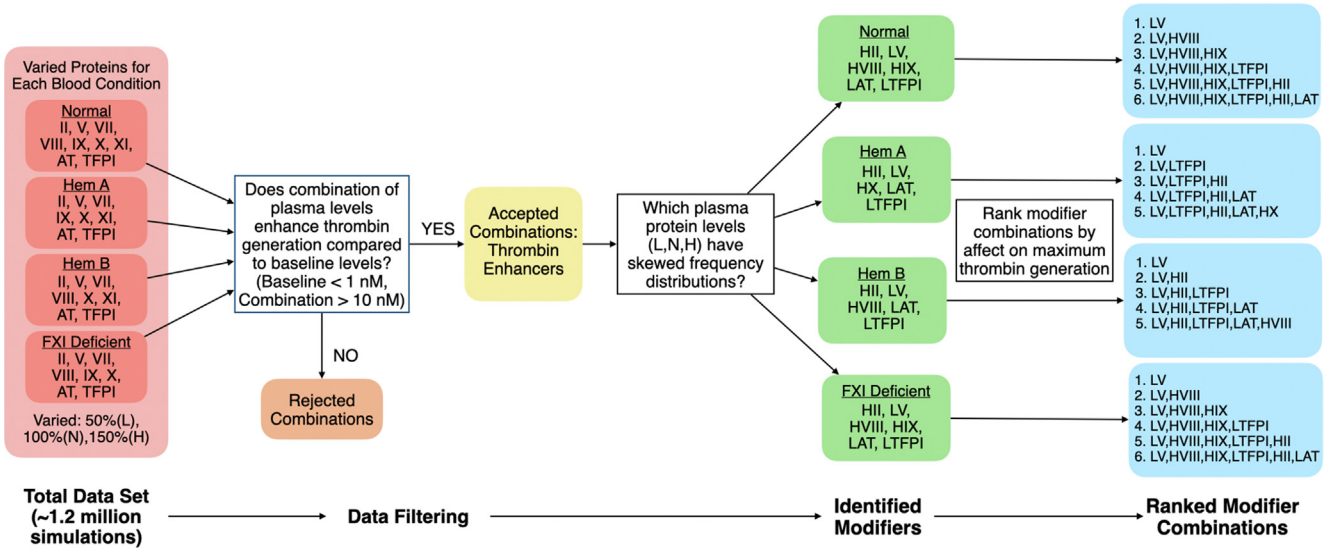


FIGURE 2 Schematic of methods workflow. For the blood conditions normal (N) and factor (F)VIII, FIX, and FXI deficient, the clotting proteins were varied to be 50%, 100%, and 150% of their baseline N levels (red boxes). Simulations were to determine if there were combinations of factor levels that enhanced thrombin generation compared with baseline levels (if no, orange; if yes, yellow). These thrombin-enhancing combinations were then filtered to identify the proteins and corresponding levels (together called modifiers, green). Finally, all combinations of modifiers, alone and together, were ranked in terms of maximum thrombin concentration. AT, antithrombin; H, high; Hem A, hemophilia A; Hem B, hemophilia B; L, low; TF, tissue factor; TFPI, tissue factor pathway inhibitor.

reference levels, and the protein combinations that led to greatly diminished thrombin production as accepted samples. We looked for thrombin enhancement with severe deficiencies in which the deficient protein was at 1% of its NRM level and for thrombin attenuation with moderate deficiencies in which the deficient protein was at 10% of its NRM level.

After the filtering process for enhancement (or attenuation), we counted the number of times within the entire set of accepted samples that each individual protein level occurred at the low NRM (50% of NRM), NRM, or high NRM (150% of NRM) value. We used the skew in those numbers as an indication of the potential importance of that protein. For example, if we had 100 accepted samples and for 1 protein, approximately 33% of the samples had low concentrations of that protein, 33% had NRM concentrations, and 33% had high concentrations, this would suggest that the level of that protein was not critical to the enhancement or attenuation. If instead we found that 50% of the samples contained low levels of that protein while 25% contained NRM levels and 25% high levels, this would suggest that a low level of that protein is potentially important to the enhancement or attenuation.

The results of the counting process for the entire set of accepted samples are summarized in the results section, shown as heatmaps for each blood condition in which the shades of blue or orange reflect the percentages of total counts for each protein and the black boxes outline proteins and levels that exceed 40%. We chose 40% as the cutoff since the probability of any of the 3 levels (50%, 100%, and 150%) occurring at more than 40% for a single protein by chance was <.005 (given our minimal sample size of n = 639) as computed by direct calculation.

2.3.2 | Ranking modifiers by thrombin produced

The filtering process identified the subsets of proteins and their levels (low, NRM, and high) that were most frequently associated with thrombin enhancement (or attenuation). The filtering process, however, did not rank these protein level changes by their effectiveness in thrombin enhancement (attenuation). To perform this ranking, we first established a reference TF level for each condition, namely the TF level, which, with baseline plasma protein concentrations, led to lag times of exactly 10 minutes (NRM and FXID) or 30 minutes (HA and HB). Using these TF levels allows for consistent ranking across blood conditions. For each blood condition and the corresponding reference TF level, we did simulations in which 1 or more of the previously identified protein levels were used along with baseline levels of all other proteins, and we recorded the thrombin at either 10 or 30 minutes. Then, for the group of simulations in which only 1 protein level was changed, we ordered the resulting thrombin from lowest to highest. We did the same for the group of simulations in which 2 protein levels were changed and for the group of simulations in which 3 protein levels were changed, etc.

3 | RESULTS

3.1 | Model verification and display of variability in thrombin generation at intermediate TF

Figure 3 shows thrombin concentration vs time for the 4 blood conditions and indicates the degree of sensitivity of thrombin generation to variations in TF levels and plasma protein levels within the NRM range. For high TF levels and all blood conditions, robust thrombin production (thrombin exceeding 10 nM) occurred for all protein levels considered, whereas, for low TF, the thrombin concentration did not reach 1 nM during the simulated 30 minutes for any of these protein levels. For the intermediate TF levels, some protein level combinations resulted in robust thrombin generation, while for others, the thrombin concentration did not reach 1 nM during the same simulation time. These results verified our model, showing that coagulation requires sufficient TF, and with excess TF, thrombin generation remains stable despite NRM protein level variations. However, these results also highlighted the fact that for intermediate TF, NRM protein level variations *can* lead to vastly different thrombin responses.

3.2 | Model identified thrombin enhancers/attenuators in severe/moderate factor deficiencies

The heatmaps shown in Figure 4 are based on the "accepted" simulations for each blood condition. As defined in the Methods section, the specific combination of TF and protein levels in these simulations (1) generated at least 10 nM thrombin, while (2) less than 1 nM thrombin was generated for the same TF level with baseline protein levels. These accepted combinations were "thrombin enhancers" because they enhanced thrombin from an ineffective level to an effective level. The heatmaps show the frequency with which a particular protein and its level (low, NRM, or high) occurred among the thrombin enhancers. For example, Figure 4A shows that for NRM blood, 76% of the thrombin enhancers had HN FVIII, 24% of them had NRM FVIII, and none of them had LN FVIII.

Figure 4A, B for NRM and FXID blood highlight the 6 proteins and protein levels found most frequently among the thrombin enhancers: LN levels of FV, TFPIa, and AT, and HN levels of FVIII, FIX, and prothrombin. Each of LN FV, HN FVIII, and HN FIX were found in more than 70% of the thrombin enhancers for NRM blood and in more than 65% of those for FXID blood. Figure 4C, D show analogous results for HA and HB. Four proteins and protein levels were frequently found among the thrombin enhancers for both HA and HB: LN levels of FV, TFPIa, and AT, and an HN level of prothrombin. In addition, a HN FX concentration for HA and a HN FVIII concentration for HB were seen in about half of the respective sets of simulations.

We also looked for protein and protein level combinations that *reduced* thrombin with milder deficiencies, ie, deficient protein levels

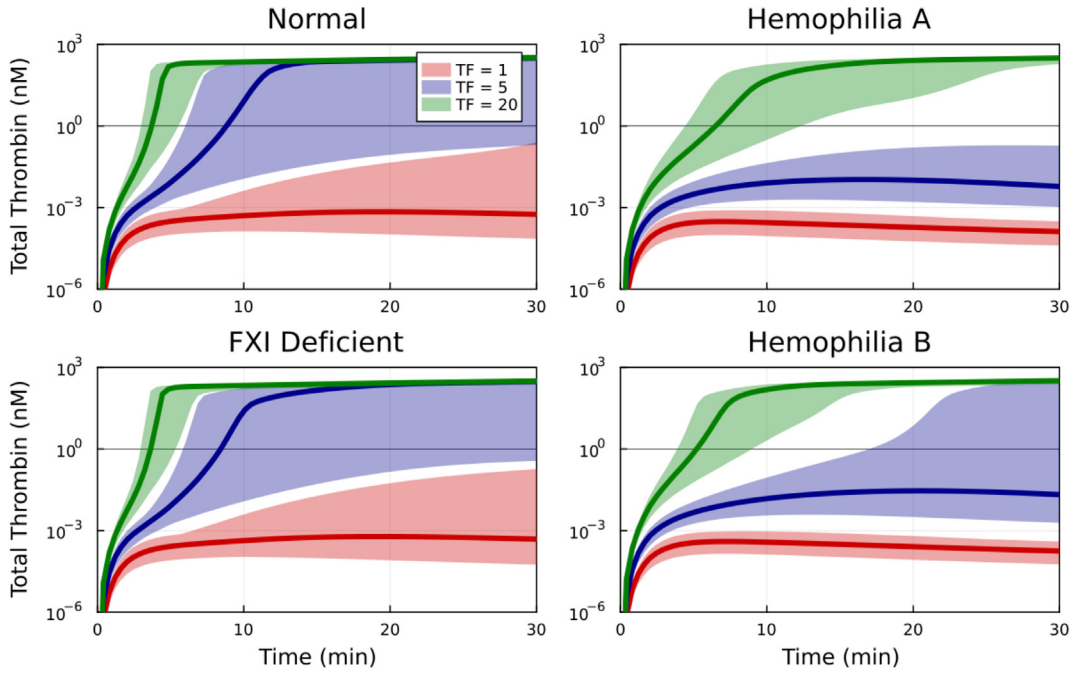


FIGURE 3 Range of simulated thrombin generation for variations in plasma protein concentrations at different tissue factor (TF) exposures. Thrombin concentration vs time for baseline protein levels in normal blood and severe (1%) protein deficiencies for low, medium, and high TF levels (red, blue, and green solid curves are for 1, 5, and 20 fmol/cm^2, respectively). The shaded region surrounding each solid curve shows the entire range of thrombin concentrations achieved by the model at the corresponding TF levels when sampling all of the nondeficient proteins at 50%, 100%, or 150% of their baseline values.

were set to 10% of their baseline value rather than 1%. For these experiments, simulations were "accepted" if they (1) achieved less than 1 nM thrombin for their combination of protein levels and TF, while (2) a corresponding simulation with baseline plasma protein levels and the same TF achieves more than 10 nM thrombin. These were "thrombin attenuators," and their heatmaps are shown in Figure 5.

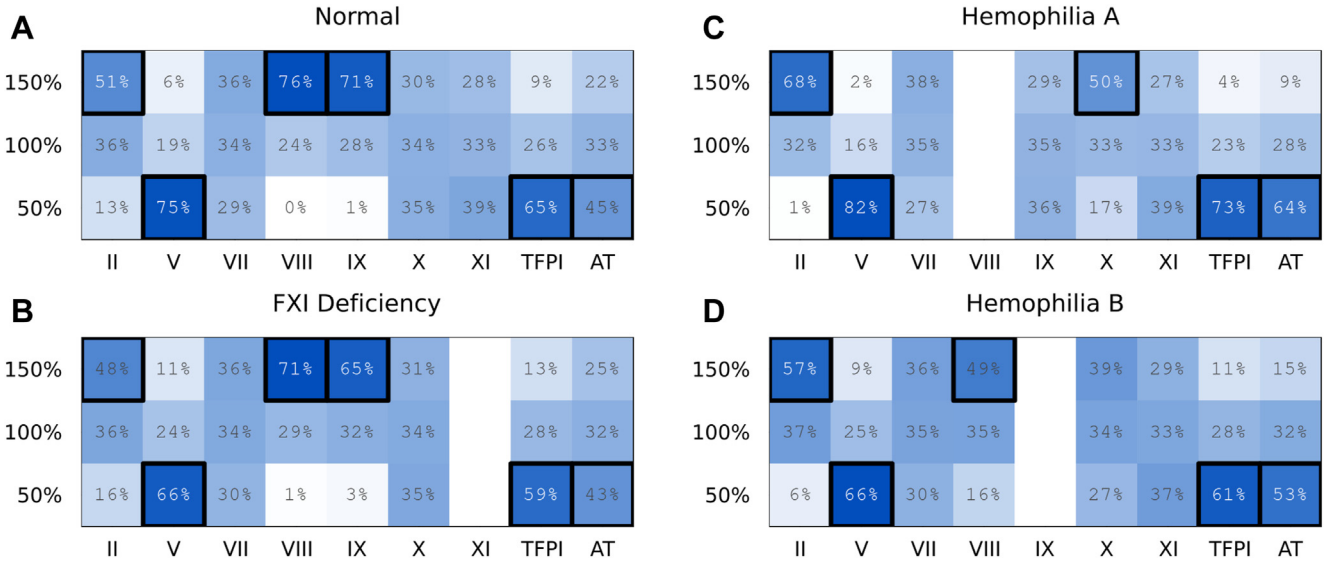


FIGURE 4 Heatmap of retained enhanced samples. With deficient proteins at 1% of baseline, initial plasma protein concentrations were exhaustively sampled at 50%, 100%, or 150% of their normal values. Samples that generated greater than 10 nM of total thrombin when the corresponding reference simulation produced less than 1 nM were categorized as *thrombin enhancing*. The enhancing samples were grouped by their initial plasma protein levels and summarized in the heatmaps. The numbers in each box indicate the percentage of the enhancing samples that utilized the particular factor level (50%, 100%, or 150% of normal). Black outline boxes indicate strongly skewed protein concentrations that warranted further analysis. Tissue factor levels for the selected samples were 3 to 4 fmol/cm^2 for both normal and factor (F)XI-deficient, 7 to 8 fmol/cm^2 for hemophilia A, and 5 to 6 fmol/cm^2 for hemophilia B blood. AT, antithrombin; TFPI, tissue factor pathway inhibitor.

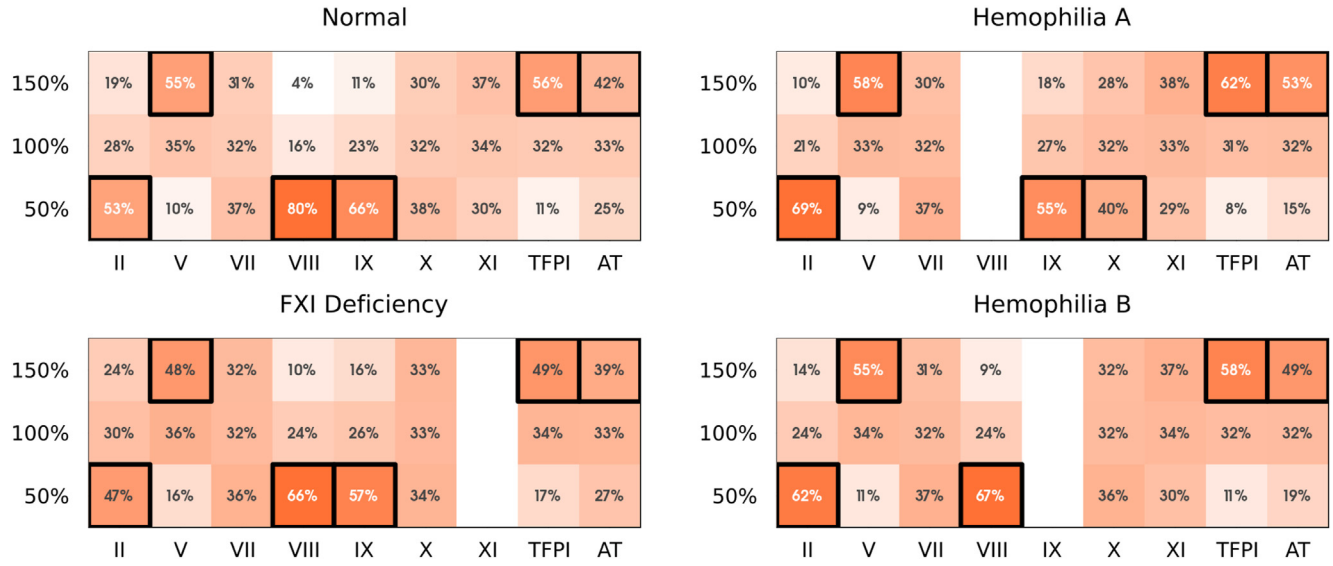


FIGURE 5 Heatmap of retained attenuated samples. With deficient proteins at 10% of baseline, initial plasma protein concentrations were exhaustively sampled at 50%, 100%, or 150% of their normal values. Samples that generated *less* than 1 nM of total thrombin when the corresponding reference simulation produced greater than 10 nM were categorized as *thrombin attenuating*. The attenuating samples were grouped by their initial plasma protein levels and summarized in the heatmaps. The numbers in each box indicate the percentage of the enhancing samples that utilized the particular factor level (50%, 100%, or 150% of normal). Black outline boxes indicate strongly skewed proteins taken from the attenuating samples above. Tissue factor levels for the selected samples were 6 to 9, 5 to 9, 6 to 9, and 5 to 7 fmol/cm^2 for normal, factor (F) XI-deficient, hemophilia A, and hemophilia B blood, respectively. AT, antithrombin; TFPI, tissue factor pathway inhibitor.

In Figure 5A, B for NRM and FXID blood, the proteins and levels most frequently identified as thrombin attenuators were low-normal levels of FVIII, FIX, and prothrombin, and high-normal levels of FV, TFPIα, and AT. These are the same proteins identified in Figure 4A, B but with their levels changing in the opposite direction, eg, LN instead of HN. LN FVIII and LN FIX were most frequently present.

Figure 5C, D show results for HA and HB, where the same 5 proteins identified in the enhancement case (Figure 4C, D) were frequently observed in the accepted simulations, but with their levels shifted in the opposite direction. Interestingly, for HA, an additional protein and level (LN FIX) was found to attenuate thrombin generation; HN FIX did not enhance thrombin generation in HA.

3.3 | Low NRM FV is the most effective single modifier of thrombin enhancement; modifier combinations show nesting behavior with diminishing returns

We identified 5 or 6 proteins and corresponding plasma levels for each blood condition that most strongly *modified* thrombin. Next, we ranked the *modifiers* (protein and level, eg, LN FV) within groups of a single modifier, duos, trios, etc., by the maximum thrombin they produced over the specified time simulated. For instance, among all possible pairs of modifiers with HA, the combination of HN prothrombin and HN FX resulted in the lowest thrombin concentration after 10 minutes (∼10 nM), while LN FV and LN TFPI yielded the highest thrombin concentration after 10 minutes (∼190 nM). To perform these rankings, we set the TF level for each blood condition so that the lag time was exactly 10 minutes (NRM and FXID) or 30 minutes (HA and HB). This way, the resulting thrombin concentrations could be compared easily with the 1 nM baseline.

The highest-ranked (most effective) modifiers for all blood conditions are listed in the Table, and the comprehensive rankings for all modifiers and blood conditions are shown in Figure 6. For *all* blood conditions, LN FV was the highest-ranked single modifier of thrombin production. The most effective duo of modifiers differed across blood conditions, but an interesting nesting behavior was observed: each time the number of modifiers in the combination increased, all of the prior modifiers were retained, and a new one was added. For instance, again, in HA, we found that LN FV was the most effective single modifier; LN FV and LN TFPIa were the most effective duo; LN FV, LN TFPIa, and HN prothrombin was the most effective trio; LN FV, LN TFPIa, HN prothrombin, and LN AT was the most effective quartet; and LN FV, LN TFPIa, HN prothrombin, LN AT, and HN FX was the most effective quintet.

The highest-ranked modifiers and their nesting behaviors were the same for NRM and FXID. LN FV alone increased thrombin at 10 minutes by about 10-fold; LN FV with HN FVIII increased it by another factor of 5 to 9; and with HN FIX, thrombin increased further by about a factor of 2. For HA, LN FV alone increased thrombin at 10 minutes by about 20-fold; LN FV with LN TFPIa increased it by

TABLE Most effective modifier combinations to enhance thrombin generation for each blood condition.

Normal (TF = 4.33)			Hemophilia A (TF = 8.74)		
Modifiers	**Max IIa (nM)**	**Lag time (min)**	**Modifiers**	**Max IIa (nM)**	**Lag time (min)**
⇣V ⇡VIII ⇡IX ⇣TFPI ⇡II ⇣AT	204.1	6.6	⇣V ⇣TFPI ⇡II ⇣AT ⇡X	317.8	10.8
⇣V ⇡VIII ⇡IX ⇣TFPI ⇡II	189.9	6.8	⇣V ⇣TFPI ⇡II ⇣AT	309.2	11.3
⇣V ⇡VIII ⇡IX ⇣TFPI	166.0	6.9	⇣V ⇣TFPI ⇡II	274.9	12.3
⇣V ⇡VIII ⇡IX	129.4	7.5	⇣V ⇣TFPI	190.0	13.6
⇣V ⇡VIII	64.0	8.0	⇣V	18.1	17.4
⇣V	9.2	8.8	Reference	1.0	30
Reference	1.0	10			
FXI deficiency (TF = 4.07)			**Hemophilia B (TF = 6.37)**		
Modifiers	**Max IIa (nM)**	**Lag time (min)**	**Modifiers**	**Max IIa (nM)**	**Lag time (min)**
⇣V ⇡VIII ⇡IX ⇣TFPI ⇡II ⇣AT	131.3	6.6	⇣V ⇡II ⇣TFPI ⇣AT ⇡VIII	332.3	12.2
⇣V ⇡VIII ⇡IX ⇣TFPI ⇡II	119.4	6.7	⇣V ⇡II ⇣TFPI ⇣AT	327.3	12.9
⇣V ⇡VIII ⇡IX ⇣TFPI	106.9	6.9	⇣V ⇡II ⇣TFPI	316.6	14.2
⇣V ⇡VIII ⇡IX	83.7	7.5	⇣V ⇡II	299.4	17.7
⇣V ⇡VIII	49.0	8.0	⇣V	235.8	20.4
⇣V	11.3	8.8	Reference	1.0	30
Reference	1.0	10			

⇡, high-normal levels; ⇣, low-normal levels; AT, antithrombin; II, prothrombin; V, factor V; VIII, factor VIII; IX, factor IX; X, factor X; XI, factor XI, TF, tissue factor; TFPI, tissue factor pathway inhibitor.

another factor of 10; and with HN prothrombin, thrombin increased further by about a factor of 1.5. Adding more modifiers beyond the trios had diminishing effects on thrombin at 10 minutes for NRM and FXID and at 30 minutes for HA. For HB, LN FV alone increased thrombin at 30 minutes from 1 nM to greater than 200 nM. Additional modifiers beyond LN FV had diminishing effects on thrombin at 30 minutes for HB. Tests in which TF was changed by ±5% did not change the rankings.

Thrombin generation time courses that resulted from the highest-ranking modifiers are shown in Figure 7. Overall, as the number of modifiers increased, the lag times shortened, and the thrombin concentrations increased. The precise lag times are listed in the Table. The shortening of lagtimes followed a similar pattern as did the increases in max thrombin; namely, the largest changes from baseline occurred with the addition of the first 3 modifiers. For each blood condition and corresponding highest-ranking modifiers, the same trends were found using 0.5%, 1%, 5%, and 10% FVIII, FIX, and FXI (Supplementary Figures S1–S3).

The larger impact of LN FV on thrombin at 30 minutes in HB compared with HA was at first a bit puzzling. However, the lag times in these cases were affected by comparable amounts (decreases of 10-12 minutes; see the Table). Thus, the differences in thrombin at 30 minutes were largely a result of the rate of thrombin generation that occurred *after* thrombin reached 1 nM; in Figure 7, the slopes of the thrombin curves that occurred just after the lag times were much steeper in HB than they were in HA. This is consistent with previous results from our model showing a difference in the rates of thrombin generation between HA and HB [3]. Additionally, this is consistent with the idea that HB is less clinically severe than HA.

Lastly, for each set of modifiers and each blood condition, we varied the TF level between 1 and 12 fmol/cm^2 and recorded the thrombin concentration at 10 (NRM and FXID) or 30 (HA and HB) minutes. As seen in Figure 8, the resulting curves show a threshold-like dependence of the thrombin concentration achieved at these specified times on the level of TF. At low TF, there was only sub-nanomolar thrombin at the specified times. For relatively small increases in TF, there were substantial increases in thrombin levels. However, at the highest TF levels, where thrombin was already in the hundreds of nanomolar range, further increases in TF resulted in only slight increases in thrombin. For each blood condition, as the number of modifiers was increased, the TF dependence curves shifted to the left, showing that less TF was required to elicit the same amount of thrombin by the specified times. These data suggest that modifiers change the sensitivity of the coagulation system to TF.

4 | DISCUSSION

In the current study, we used an enhanced version [15] of our mathematical model to explore how natural variations of coagulation factor and plasma inhibitor levels affect thrombin production in NRM blood and in severe (1%) and moderate (10%) HA, HB, and

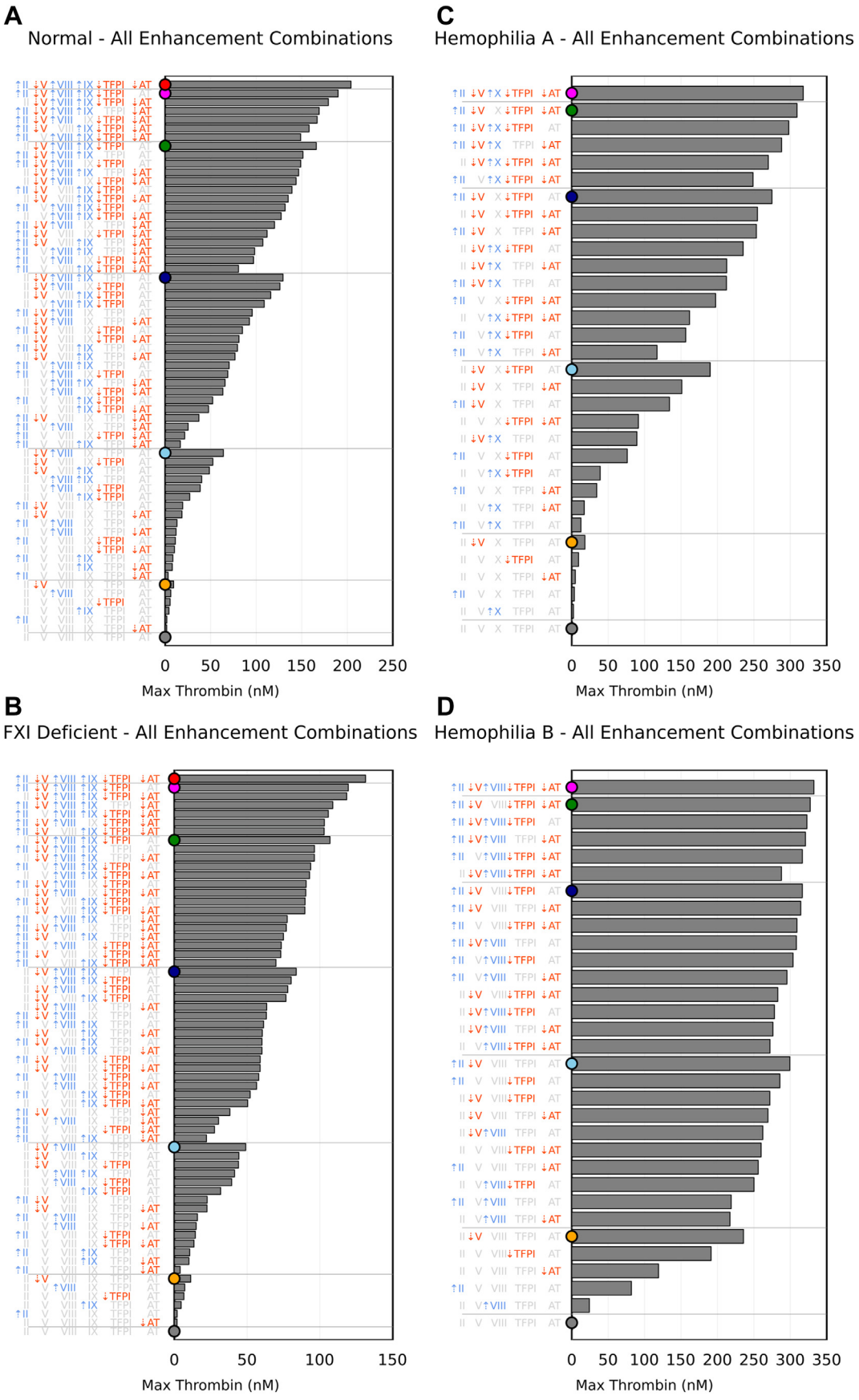


FIGURE 6 Maximum thrombin generated with the modified combinations identified through the filtering process for each blood condition. Initial plasma protein levels strongly associated with thrombin enhancement were identified from an exhaustive sampling procedure for (A)

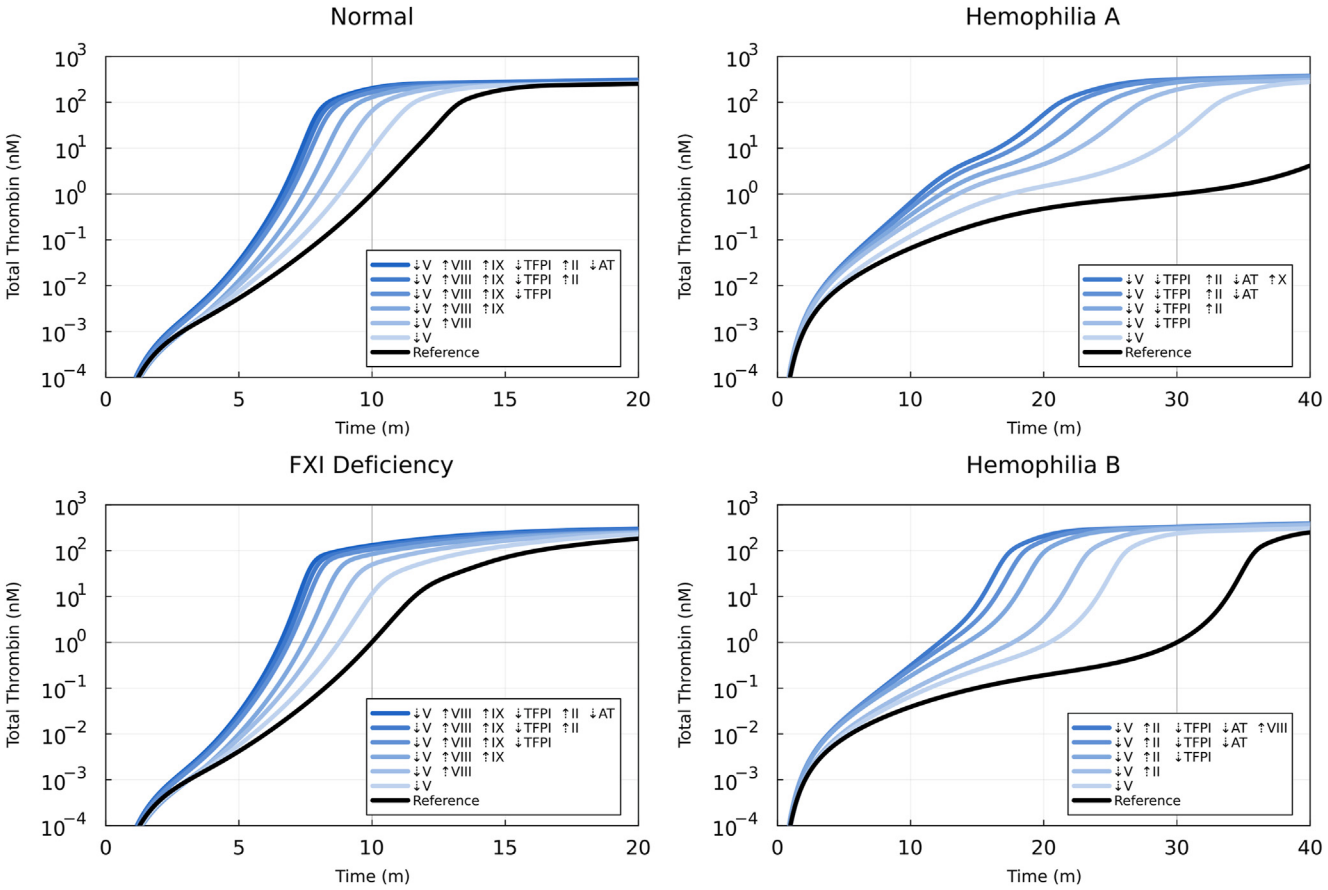


FIGURE 7 Thrombin curves for most effective modifier combinations. Thrombin generation through time using tissue factor levels 4.33 fmol/cm^2 for normal blood, 4.07 fmol/cm^2 for factor (F)XI-deficient blood, 8.74 fmol/cm^2 for hemophilia A blood, and 6.37 fmol/cm^2 for hemophilia B blood. AT, antithrombin; TFPI, tissue factor pathway inhibitor.

FXID. Each plasma protein level was considered at 50%, 100%, and 150% of its average level, and the sensitivity of thrombin production metrics, including lag time and thrombin concentrations at specified times, was elucidated when varying the protein levels simultaneously. For each blood condition, we found that (1) for sufficiently low TF levels, no protein level combination led to robust thrombin generation; (2) for sufficiently high TF levels, all the protein level combinations led to robust thrombin generation; and (3) for an intermediate range of TF values, different protein level combinations could lead to negligible or substantial thrombin generation. We focused our study on the intermediate range because it allowed us to identify plasma protein level combinations that caused a switch between weak and strong thrombin responses. We believe that identifying these combinations and corresponding TF ranges could lead to a better understanding of the different bleeding phenotypes seen with HA, HB, and FXID.

For severe HA, HB, and FXID, we identified modifiers that, at certain TF levels, enhanced thrombin generation from a negligible level (subnanomolar) to a physiologically effective one (greater than 10 nM). These combinations could reasonably be expected to improve hemostasis in vascular beds with those TF levels present. For moderate HA, HB, and FXID, we found modifiers that reduced thrombin from effective to ineffective levels; these modifiers, therefore, could be associated with more frequent or severe bleeding.

In all blood conditions examined, we found that LN FV in the plasma, along with a 50% reduction in platelet FV, was consistently associated with notable increases in thrombin, compared with baseline, for the respective sensitive ranges of TF values. The result for HA was consistent with our previous results [2] but now reflects the greater importance of lowering TFPIa and AT than before; the results for the other blood conditions are all new. At least part of the explanation for LN FV being the most impactful modifier for *all* blood conditions is reduced substrate competition for activation by FXa. Early in the clotting process, FXa made by TF:FVIIa can bind to activated platelets, and there activate FVIII to FVIIIa and FV to FV-h. LN FV allows more of the platelet-bound FXa to interact with FVIII

normal, (B) factor (F)XI-deficient, (C) hemophilia A, and (D) hemophilia B blood. These simulations were further examined and organized by the number of initial plasma protein levels that were modified. Horizontal bars indicate the maximum thrombin achieved in 10 minutes for the identified sample with normal or FXI-deficient blood or in 30 minutes with hemophilia A or B blood. Large circles on the bar indicate the largest thrombin-producing sample for the number of modified plasma levels. AT, antithrombin; TFPI, tissue factor pathway inhibitor.

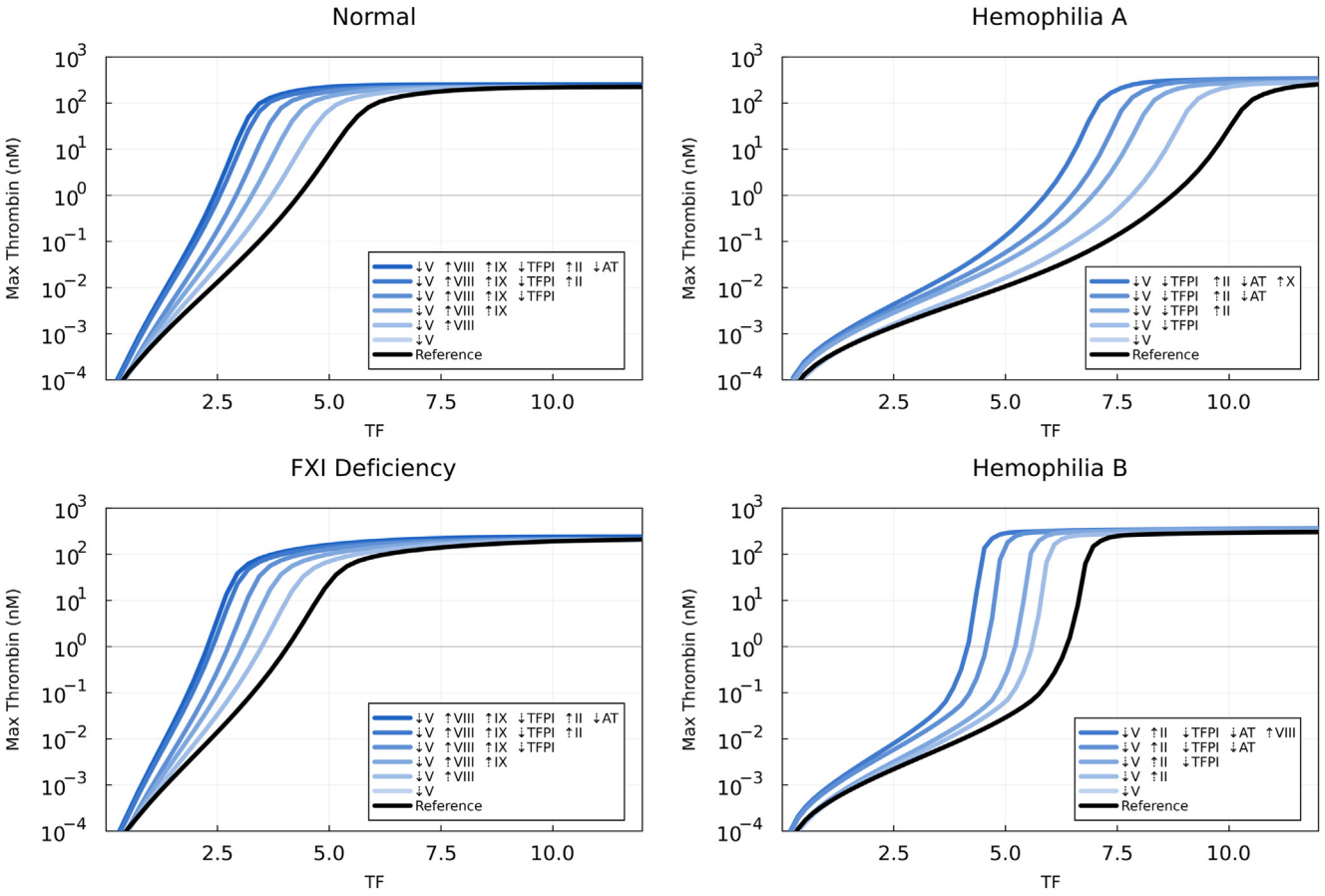


FIGURE 8 Tissue factor threshold curves for the most effective modifier. Curves show the maximum thrombin over a 10-minute (normal and factor [F]XI deficient) or 30-minute (hemophilia A and B) simulation as a function of tissue factor density (fmol/cm^2). AT, antithrombin; TFPI, tissue factor pathway inhibitor.

because there is less FV to compete with, resulting in more FVIIIa production during the critical early steps of coagulation. In the model, the rate of formation of the tenase (FVIIIa:FIXa) complex is proportional to both the concentration of platelet-bound FVIIIa and the concentration of platelet-bound FIXa. The activation of FVIII on platelets, even with low FVIII in HA, is increased when FV's concentration is low, and this leads to an increased rate of tenase formation.

In NRM and FXID with NRM levels of FVIII and FIX, a reduction of FV is favorable because FVIIIa activation is enhanced, leading to an increase in tenase production. In HA, reducing the competition is even more important because FVIII is in short supply. In HB, lower FV does not increase the FIXa concentration, but an increase in the concentration of platelet-bound FVIIIa can at least partially compensate for the low platelet-bound FIXa concentration in the expression for the rate of tenase formation.

When quantifying the effect of the modifiers on thrombin generation, we noticed several patterns that we believe to be important. One was the nesting of modifiers; for example, the most effective 3 modifiers included the most effective 2 modifiers, which in turn contained the single most important modifier. This suggests that if modifying a single plasma protein level generated insufficient improvement, adding a second modification on top of the first one could be an effective strategy. This notion was further supported by the observation that thrombin generation was further augmented greatly with a second modification and also with a third. For example, in NRM blood, a low-normal FV level alone elicited a 9-fold increase, adding high-normal FVIII led to an additional 7-fold increase, and the combination of low-normal FV, high-normal FVIII, and high-normal FIX resulted in a further 2-fold increase. Further enlargement of the set of modifiers beyond 3 had a diminishing impact. In summary, our results suggest that lowering FV and modifying just 1 or 2 other protein levels, depending on the deficiency, could be a useful strategy for designing effective prohemostatic therapies.

Bleeding in hemophilia is generally associated with muscle bleeds, particularly joint bleeds. A survey of TF expression in various tissues, as measured by immunohistochemistry, shows that deep skeletal muscle and joints had low to undetectable levels of TF [19]. We know of no other studies of tissue samples that have measured TF levels. By contrast, in persons with hemophilia who have experienced trauma, up to 8% show intracranial hemorrhage despite the brain having very high levels of TF [20]. So, it is not easy to directly correlate TF levels with bleeding. However, it is known that robust thrombin generation has a threshold-like dependence on TF [3,21–23]. One way of thinking about our results is that each individual has a unique TF threshold that

depends on their plasma levels of clotting proteins. As illustrated in Figure 5, deficiencies in FVIII and FIX required higher TF values than those with NRM or FXID blood to generate comparable amounts of thrombin. These results suggest there is a "critical" TF level for each individual that is increased with protein deficiencies but can be decreased with modified plasma levels of clotting proteins. A major outcome of this study is that we determined the specific combination of modifiers that lowered the critical TF values for NRM blood, HA, HB, and FXID (see Figure 8). We speculate that lowered TF thresholds enable substantial and physiologically effective thrombin generation in locations with low TF (eg, joints) where, without these modifications, it would not occur.

There are consequences of our modeling assumptions and limitations of the model. The mathematical model of the extrinsic pathway of coagulation and platelet deposition under flow used in this study was originally inspired by the cell-based model of hemostasis [24] in that it incorporates the assumption that critical reactions of the coagulation system occur on the surfaces of activated platelets. A consequence of this view and of the fact that each platelet's surface can host a limited number of relevant coagulation enzymes is that the rates and extents to which these reactions can occur are regulated by the rate and extent of accumulation of activated platelets on the injury.

In the mathematical model, platelet accumulation is not specified; it occurs at a rate and to an extent that is an outcome of the model's dynamics, and it depends in large part on the platelet count, the near-wall blood flow velocity, the prescribed rates at which a platelet binds to the SE and becomes activated, and the rates at which a platelet is activated by soluble agonists in the surrounding plasma. While the model does not treat in detail platelet responses to external stimuli, the molecular machinery of platelet adhesion and cohesion, or the dynamics of intraplatelet signaling pathways, we were able to obtain insights about how thrombin generation would be affected by variations in those processes by doing simulations in which the values of parameters in the model (eg, the platelets' rates of adhesion to injury or activation by thrombin) were varied [3–5]. The model predicts, for example, only a mild reduction of thrombin production with a 90% reduction of platelet count and a severe reduction of thrombin production with a 99% reduction of platelet count [3], corresponding to clinical observations of moderate and severe thrombocytopenia. These predictions arise as a direct consequence of the different rates of platelet accumulation that occur with the model for different platelet counts.

The mathematical model also incorporates the strong impact of flow-mediated delivery and removal of species on those species' concentrations near the injury and, therefore, on the speed at which reactions involving those species occur, but it ignores other aspects of hemodynamics. Because the model does not incorporate spatial variations in concentrations within the thrombus or the physical feedback of a large growing thrombus on the blood flow, the model is limited to small injuries. When it is applied to small injuries, the model makes predictions that agree with a much more complex model, which includes spatial heterogeneity and detailed fluid dynamics [25,26] along with the coagulation reactions considered here.

The current model does not include fibrinogen, production of fibrin by thrombin, or fibrin polymerization. Therefore, it does not account for the dynamic effects of fibrin-mediated sequestration of thrombin [27–29]. In simulations with a closely related model, we included a simple treatment of fibrin accumulation and thrombin sequestration [7] and found that the extent of thrombin removal by sequestration was substantially less than the extent of thrombin removal by flow. Because the flow velocities considered here are higher than in that study [7], we expect that flow-mediated thrombin removal would greatly exceed fibrin-mediated thrombin sequestration.

The current mathematical model and analysis are focused on thrombin generation, which alone is insufficient to predict clinical bleeding. Speaking broadly, thrombin generation capacity shows a general correlation with clinical bleeding. But, up to 15% of patients have a bleeding risk that is discordant with their thrombin generation levels [30]. Bleeding risk is a clinical assessment that is somewhat difficult to define in part because it is a subjective assessment from patients and physicians (although tools like the International Society on Thrombosis and Haemostasis Bleeding Assessment Tool [31] can aid in assigning bleeding risk). In persons with HA and HB, a large part of the assessment can be based on bleeding into joints, with the development of a target joint being most commonly seen in severe hemophilia. Looking at patients with a phenotype discordant with their factor levels, there is a suggestion that thrombophilic mutations, particularly in the fibrinolytic system, rather than levels of other coagulation factors, modulate disease severity [32]. Other studies have suggested that disease severity, as measured by joint bleeding, may be related to thrombomodulin levels [33]. Broadly speaking, data suggest that dysregulation of fibrinolysis, particularly with regards to the activation of thrombin-activatable fibrinolysis inhibitor, contributes to joint damage in various, not yet fully understood ways [34,35].

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AUTHOR CONTRIBUTIONS

M.T.S. contributed to conceptualization, methodology, software, validation, writing – original draft, writing – review and editing, and visualization. K.B.N. contributed to writing – review and editing, visualization, and funding acquisition. D.M.M. contributed to writing – review and editing, visualization, and funding acquisition. S.S.S., K.L., and A.L.F. contributed to conceptualization, methodology, writing – original draft, writing – review and editing, visualization, and funding acquisition.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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