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Kinetic modeling sheds light on the mode of action of recombinant factor VIIa on thrombin generation

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ABSTRACT

Introduction. The therapeutic potential of a hemostatic agent can be assessed by investigating its effects on the quantitative parameters of thrombin generation. For recombinant activated factor VII (rFVIIa) — a promising hemostasis-inducing biologic — experimental studies addressing its effects on thrombin generation yielded disparate results. To elucidate the inherent ability of rFVIIa to modulate thrombin production, it is necessary to identify rFVIIa-induced effects that are compatible with the available biochemical knowledge about thrombin generation mechanisms.

Materials and Methods. The existing body of knowledge about coagulation biochemistry can be rigorously represented by a computational model that incorporates the known reactions and parameter values constituting the biochemical network. We used a thoroughly validated numerical model to generate activated factor VII (FVIIa) titration curves in the cases of normal blood composition, hemophilia A and B blood, blood lacking factor VII, blood lacking tissue factor pathway inhibitor, and diluted blood. We utilized the generated curves to perform systematic fold-change analyses for five quantitative parameters characterizing thrombin accumulation.

Results. The largest fold changes induced by increasing FVIIa concentration were observed for clotting time, thrombin peak time, and maximum slope of the thrombin curve. By contrast, thrombin peak height was much less affected by FVIIa titrations, and the area under the thrombin curve stayed practically unchanged. Comparisons with experimental data demonstrated that the computationally derived patterns can be observed *in vitro*.

Conclusions. rFVIIa modulates thrombin generation primarily by accelerating the process, without significantly affecting the total amount of generated thrombin.

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Introduction

Activated factor VII, or factor VIIa (FVIIa), is a critical component of the human blood coagulation system [1,2]. Recombinant factor VIIa (rFVIIa) has been approved for use as a hemostatic agent for patients with hemophilia, congenital FVII deficiency, and Glanzmann's thrombocytopenia [3]. Following its initial approval, rFVIIa has become a prominent off-label drug to control hemorrhage in traumatic and surgical situations [4–6]. While available case studies and clinical trials indicate that rFVIIa can effectively decrease bleeding in trauma and surgery [7–11], existing evidence suggests the risk of serious thrombotic complications resulting from using rFVIIa in such settings [12,13]. The delicate biochemical balance between hemorrhage and thrombosis is essentially a quantitative phenomenon [1,14,15]. Therefore, the understanding of the effects of rFVIIa on the quantitative parameters of blood coagulation may significantly contribute to our ability to optimize clinical use of this biologic.

Factor VIIa is naturally present in the blood, and is an enzyme involved in the generation of thrombin [1,3]. Thrombin generation can be viewed as a process occurring in three phases: initiation, propagation, and termination [1,16,17]. During the initiation phase, the complex of FVIIa with tissue factor (TF) activates factors IX (FIX) and X (FX), and minute amounts of thrombin are generated by FXa (activated FX), leading to the activation of coagulation factors VIII (FVIII) and V (FV). During the propagation phase, the complex of FIXa and FVIIIa (activated FIX and FVIII, respectively) generates large amounts of FXa, which, in complex with FVa (activated FV), synthesizes the bulk of thrombin, giving the thrombin curve its characteristic one-peaked shape (illustrated by Fig. 1A). Thrombin is subsequently deactivated by natural anti-coagulants (anti-thrombin (AT), tissue factor pathway inhibitor (TFPI), and protein C) in the termination phase. These three phases are typically characterized *in vitro* by five quantitative parameters: clotting time, thrombin peak time, thrombin peak height, maximum slope of the thrombin curve (also known as maximum thrombin generation rate), and the area

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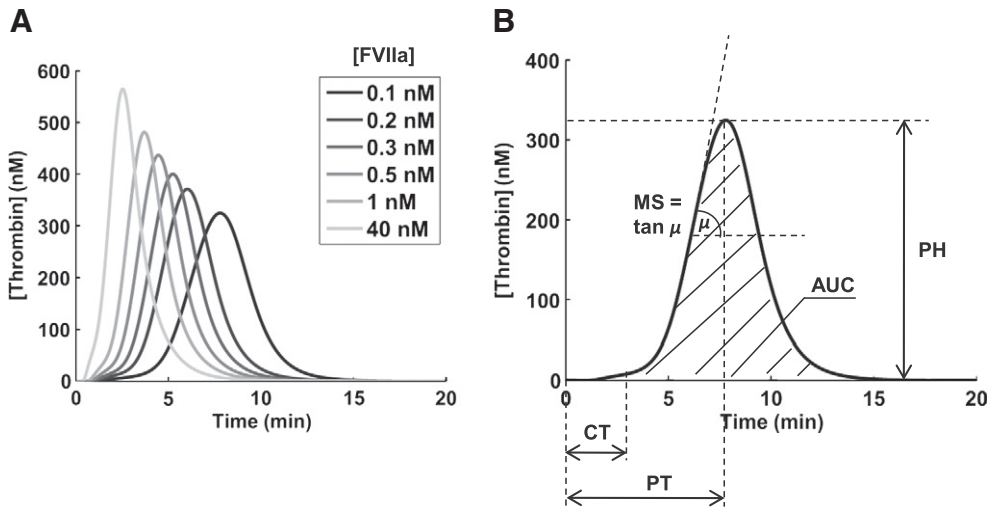

Fig. 1. Model-generated thrombin curves for normal blood composition. Thrombin generation was initiated at time 0 with 5 pM tissue factor. (A) Thrombin curves for different FVIIa concentrations. (B) Quantitative parameters of thrombin generation: the timing parameters (CT, PT, MS) and the amount parameters (PH, AUC). CT: clotting time (time to 10 nM thrombin); PT: thrombin peak time; MS: maximum slope of the thrombin curve; PH: thrombin peak height; AUC: area under the thrombin curve. The thrombin curve was generated for 0.1 nM FVIIa (mean FVIIa concentration for normal blood).

under the thrombin curve (AUC, sometimes referred to as endogenous thrombin potential) [18–22] (Fig. 1B). The first three parameters reflect the timing of thrombin accumulation, and can thus be termed the *timing* parameters. The remaining two parameters characterize the amount of generated thrombin, and can be termed the *amount* parameters. The ability of a biochemical agent to normalize some (or all) of the thrombin generation parameters can be used to assess its potential to correct blood clotting abnormalities.

The effects of rFVIIa on thrombin generation have been addressed in a number of independent studies [23–29], which provide evidence that rFVIIa can accelerate the onset of thrombin generation (i.e., reduce clotting time). However, for other thrombin generation parameters, consistent patterns have not been identified, and the reported experimental results lead to disparate conclusions. For example, experiments with platelet-poor human plasma have shown that rFVIIa does not affect thrombin peak time and height (Fig. 1 in Ref. [25]). Yet, a more recent investigation provides evidence that rFVIIa can noticeably impact both peak time and peak height in platelet-poor plasma with added phospholipids (Figs. 1 and 4 in Ref. [26]). In order to develop reliable clinical strategies for rFVIIa administration, it is necessary to identify persistent patterns in rFVIIa-induced effects *in vitro*, because such patterns might shed light on the *in vivo* mode of action of this therapeutic. The available but inconclusive experimental data indicate that new approaches, which are complementary to traditional experimentation, should be used to address this challenge.

Recent years have seen the rapid growth of quantitative knowledge about biochemical mechanisms underlying blood coagulation [30]. Here we propose to utilize this information to elucidate the characteristic mode of action of rFVIIa. To this end, we used the computational model developed in Kenneth Mann's laboratory [16,31], which is based on the existing body of knowledge about the biochemistry of thrombin generation [14,30]. This model has been benchmarked against experimental thrombin generation data in synthetic *in vitro* systems [17,20,22,32–35], and the modeling predictions are quantitatively [15,36] or semi-quantitatively [17,20,33–35] consistent with the *in vitro* measurements obtained for whole blood.

By performing systematic fold-change analyses for the parameters of thrombin generation, we established that the predominant mode of action of rFVIIa is acceleration of thrombin accumulation. Specifically, our results suggest that in blood with normal composition, hemophilia blood, blood lacking FVII, blood lacking TFPI (the major regulator of FVIIa activity), and diluted blood rFVIIa modulates primarily the timing parameters of the thrombin curve. By contrast, the amount parameters are much less affected (in some cases, practically unaffected) by pharmacological doses of rFVIIa. Comparisons with available experimental data demonstrate that the computationally derived patterns can indeed be observed *in vitro*. Moreover, such comparisons suggest that discrepancies in experimental data on modulation of thrombin generation by rFVIIa are due to particularities of experimental procedures rather than to intrinsic variability in the mode of action of this biologic. Finally, our results indicate that the mechanisms of action of rFVIIa assumed in the model may be sufficient to explain its ability to impact thrombin generation.

Materials and Methods

Model of thrombin generation

The Hockin–Mann computational model of thrombin generation is a system of nonlinear ordinary differential equations (ODEs) describing the mass-action kinetics of thrombin generation for an *in vitro* system that contains the main biochemical constituents of the coagulation system: pro-coagulant enzymes (FIIa, FVIIa, FIXa, FXa) and their respective zymogens (FII, FVII, FIX, FX), inactive and active cofactors (FV, FVa, FVIII, FVIIa), and the anti-coagulants TFPI and AT. In our work we used the updated version of the model [31] (see also Supplementary Material, Tables S1–S3), which is slightly different from the original model [16].

The model is based on the assumption that the biochemical system contains phospholipid surfaces at a saturating concentration [16]. While this is a strong assumption that might not always be satisfied for *in vitro* experimental studies, it is relevant for the modeling of blood clotting physiology. Indeed, *in vivo* blood clotting starts with the formation of a platelet plug, and the concentration of platelets in the plug (in the vicinity of a wound) is 100–200 times higher than the normal platelet count [37]. In this environment, the effective concentration of catalytic surfaces is comparatively high — a condition for which saturating concentration can be viewed as a reasonable first approximation. Because there is still controversy surrounding the TF-independent mechanism of FVIIa activation [38,39], our modeling approach takes into account only the generally accepted, TF-dependent FVIIa activation mechanism [1,2].

Computational procedures

The main output of the model was the concentration of active thrombin, which was calculated as a weighted sum — with weights 1.0 and 1.2, respectively — of the concentrations of thrombin and meizothrombin, thrombin's enzymatically active precursor. (The weights were chosen to reflect the relative activities of thrombin and meizothrombin towards a substrate used in experimental assays to determine thrombin activity [16]). In our description of the computational results, we used the term "thrombin" to designate active thrombin. We used the rate constants and initial concentrations of biochemical components for the model of blood with normal composition as given in the original model description [16,31]. These initial concentrations represent the mean values of coagulation proteins in human blood. In the computational experiments reflecting altered blood composition (e.g., hemophilic blood and diluted blood), the initial concentrations of the coagulation components were modified accordingly. We implemented the Hockin–Mann model using the SimBiology package from the software suite MATLAB 2010b (MathWorks, Natick, MA). To solve the ODEs constituting the model, we used the "sundials" solver available in SimBiology (absolute tolerance 1.0×10^{-18} M, relative tolerance 1.0×10^{-12}, maximal step size 0.05 s).

We selected FVIIa concentrations in the computational experiments to correspond to clinical doses of rFVIIa. Typical pharmacological rFVIIa concentrations reported in the literature vary, taking values such as 20 nM [28], 36 nM [20], and ≥25 nM [40]. To cover a broad range of possibilities, we considered FVIIa titrations with concentration changing from the "natural" value (0.1 nM for normal blood composition, or the corresponding diluted value) to 40 nM. In the computations modeling dilution, the dilution for all proteins (except TF) was effected by a 2-fold decrease in their original concentrations (the TF concentrations were not altered by dilution because TF is external to blood vessels). In all computational experiments, the TF concentration took the values 5, 10, 15, 20, and 25 pM, which were used in the work that introduced the Hockin–Mann model [16]. For every thrombin curve, we calculated clotting time, thrombin peak time, thrombin peak height, maximum slope of the thrombin curve, and the AUC. Clotting time was calculated as the first point in time when thrombin concentration equals or exceeds 10 nM [21,28,33]. Thrombin peak times were estimated as time points when the numerical derivative changed its sign from "+" to "-", and the numerical derivative was calculated directly from the trajectories by applying the standard 2nd order numerical differentiation formula. Maximum slope of the thrombin curve was calculated as the global maximum of the first derivative of the thrombin curve, and AUCs were calculated by numerical integration of thrombin curves over the chosen time interval by applying the trapezoidal rule.

Results

Generation of thrombin curves

For accurate estimation of thrombin generation parameters, the thrombin curves selected for analysis should span the time interval from 0 — when clotting is initiated by adding TF — to the time when thrombin generation can be considered complete. By this time, the thrombin peak has already occurred and the concentration of thrombin is almost zero due to binding with AT [16,19] (Fig. 1A). This stage is reached in less than 20 min in *in vitro* experiments with blood from healthy individuals, when clotting is initiated with 5 pM TF [33].

For normal blood composition, with the concentrations of FVIIa and TF varying in the specified ranges (see Methods), we computed thrombin curves over a 20 min (1200 s) time interval. Because the AUC value was determined by integrating the thrombin curve over the chosen time interval, it was important to verify that the interval was large enough to capture the three phases of thrombin generation (i.e., that the thrombin level at the end of the 1200 s time interval was sufficiently close to 0). To test that, we compared the AUC values computed for the 1200 s interval with those for the 2000 s interval (here termed the reference interval). The maximum relative error in the AUC calculations was 0.002%. We concluded that the thrombin curves for normal blood composition were sufficiently close to 0 at 1200 s for all considered values of TF and FVIIa concentrations. The same strategy was used to select time intervals in subsequent computational experiments that reflected pathological blood composition. The corresponding time intervals, reference intervals, and AUC errors are given in Table 1. In all our computational experiments, thrombin generation curves had one local maximum (thrombin peak), as illustrated by Fig. 1A.

FVIIa titration curves are characterized by monotonicity and convergence to plateau

For normal blood composition, we generated thrombin curves and computed the five quantitative parameters — clotting time, thrombin peak time, thrombin peak height, maximum slope, and the AUC (Fig. 2). Our computations showed that the parameters were monotonic functions of [FVIIa] (brackets designate concentration) and approached a plateau as the level of FVIIa increased. As [FVIIa] increased, clotting time and thrombin peak time decreased, while maximum slope and thrombin peak height increased, which is consistent with the pro-coagulant function of FVIIa. The effect of changes in FVIIa concentration on the AUC (a slight decrease) was negligible, and the above patterns held for all considered TF concentrations. The effects of FVIIa titration were significant for [FVIIa] between 0.1 nM (mean value for normal blood) and 5 nM; above 5 nM, the FVIIa concentration had little influence on the thrombin generation parameters. These results are consistent with whole blood experiments, in which clotting times were determined (by using a Hemochron instrument) for rFVIIa titrations with [rFVIIa]≥10 nM in the presence of 10 pM TF (Fig. 2 in Ref. [38]). Moreover, experiments with platelet poor plasma showed that thrombin generation curves obtained in the presence of 10 nM rFVIIa and 100 nM rFVIIa were almost identical (Fig. 3a in Ref. [27]).

Notably, the decreasing FVIIa titration curves for clotting time, maximum slope and thrombin peak height for normal blood composition (Fig. 2A,C,D) possess the properties of monotonicity and convergence to plateau similar to those observed for rFVIIa titration curves measured experimentally in synthetic plasma lacking FVII [20]. Further, experiments with platelet poor plasma from patients with hemophilia A demonstrated that addition of rFVIIa had little effect on the thrombin generation parameters when [rFVIIa] varied between 10 nM and 200 nM, and thrombin generation was initiated with 5 pM TF (Fig. 4 in Ref. [41]). These results suggest that monotonic curves approaching plateau (Fig. 2) characterize not only normal blood composition, but also various blood pathologies. Indeed, in our computational experiments for altered blood (described in the subsequent sections of this report), FVIIa titration curves for the five thrombin curve parameters had monotonicity and convergence properties similar to the FVIIa titration curves for normal blood

Table 1
Time intervals for thrombin curve generation (see Results for details).

	Time interval, s	Reference interval, s	Relative AUC error, %
Normal blood	(0, 1200)	(0, 2000)	2.0×10^{-3}
No FVIII	(0, 4000)	(0, 5000)	7.6×10^{-3}
No FIX	(0, 4000)	(0, 5000)	1.5×10^{-2}
No FVII	(0, 1200)	(0, 2000)	1.7×10^{-4}
No TFPI	(0, 1200)	(0, 2000)	7.0×10^{-5}
Diluted blood	(0, 2000)	(0, 3000)	1.0×10^{-3}

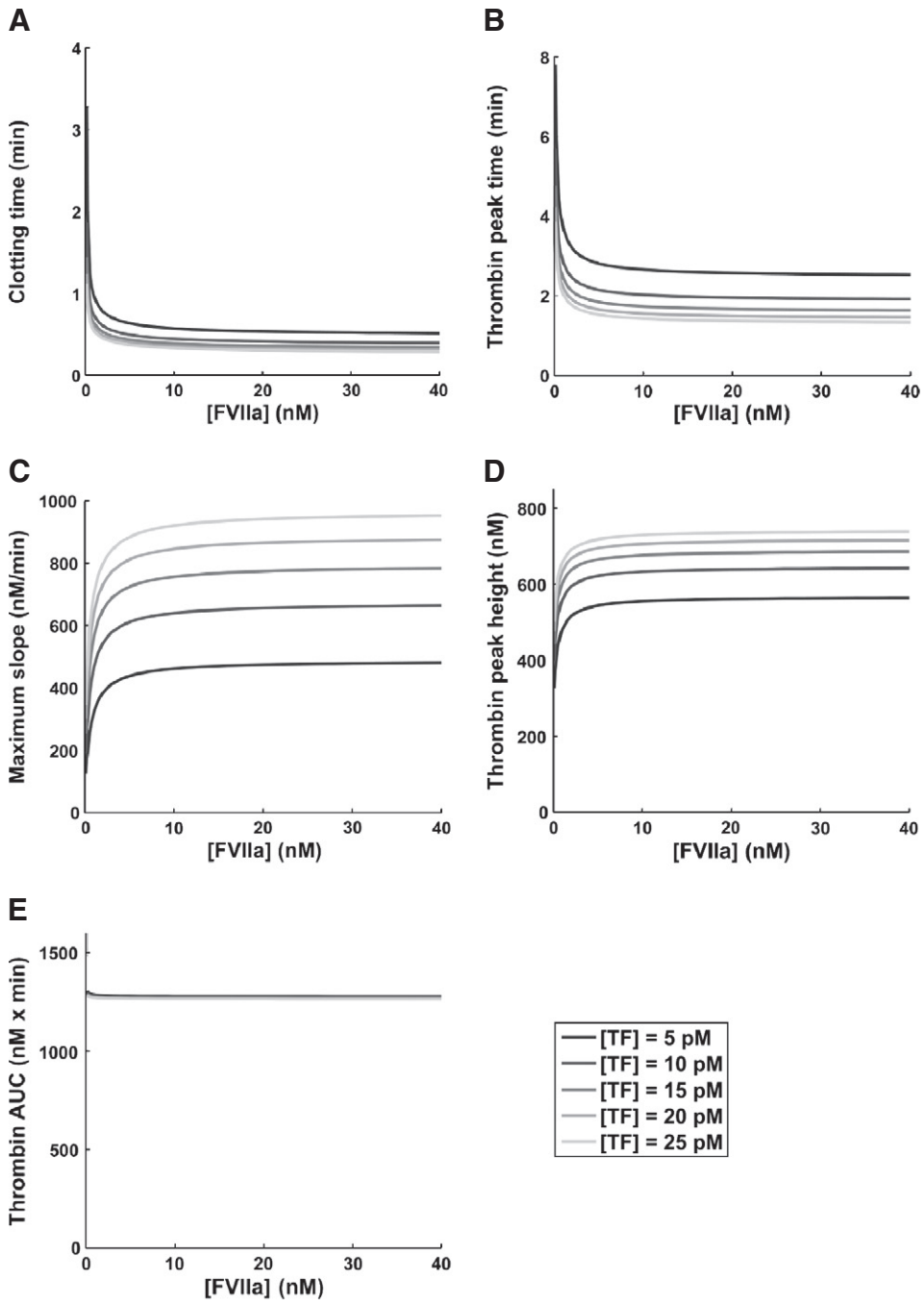

Fig. 2. Model-generated FVIIa titration curves for the quantitative parameters of thrombin generation in the case of normal blood composition. TF = tissue factor.

composition. The monotonicity properties of the titration curves allowed us to devise a strategy to compare FVIIa-induced effects for different parameters of thrombin generation, as described below.

Acceleration of thrombin accumulation is the primary mode of action of FVIIa in blood with normal composition

To elucidate the predominant mode of action of FVIIa, we compared the fold changes in the quantitative parameters of thrombin curves induced by changes in [FVIIa]. To this end, we normalized the titration curves (Fig. 2) by the values corresponding to the minimal FVIIa concentration. To evaluate the magnitude of induced effects, we considered the reciprocal values for the parameters that decreased upon addition of FVIIa, i.e., clotting time, thrombin peak time, and the AUC. This allowed us to compare the magnitude of fold changes in all the thrombin curve parameters, both the ones that increased and the ones that decreased in FVIIa titration experiments.

Fig. 3A illustrates these comparisons, where the largest changes were observed in the values of clotting time. This result is consistent with the biochemical role of FVIIa, which is directly involved in the initiation phase of thrombin generation. We focused on comparisons of fold changes in thrombin generation parameters for [FVIIa] varying within the therapeutic range (i.e., [FVIIa] ≥ 20 nM). For every [FVIIa] and [TF], fold changes in clotting time were larger than the corresponding fold changes in thrombin peak time and maximum slope of the thrombin curve by at least 0.84 and 0.91, respectively. The

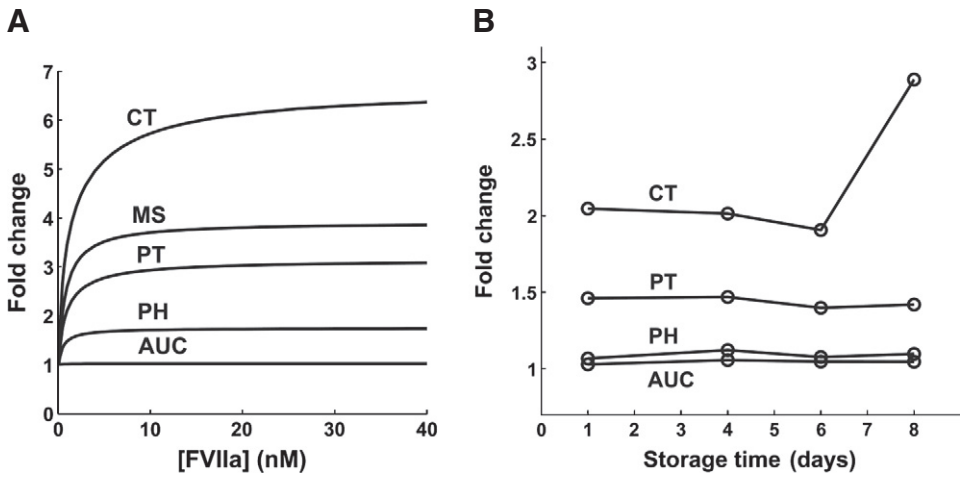

Fig. 3. FVIIa-induced fold changes in the quantitative parameters of thrombin generation for the case of normal blood composition. (A) Computational results obtained using the Hockin–Mann model (see Methods); shown are the curves corresponding to [TF] = 5 pM (TF = tissue factor). CT: clotting time; PT: thrombin peak time; MS: maximum slope of the thrombin curve; PH: thrombin peak height; AUC: area under the thrombin curve. (B) Fold change in the value of thrombin generation parameters upon addition of rFVIIa to stored blood platelet concentrates. Raw data were taken from Ref. [28]; fold changes (circles) were calculated with respect to control values (no added rFVIIa) of thrombin generation parameters. For CT and PT, fold change was calculated as the value measured in the presence of rFVIIa divided by the control value (no rFVIIa); for PH and AUC, fold change was calculated as the control value divided by the value determined in the presence of rFVIIa.

fold changes in thrombin peak time and maximum slope varied within substantially overlapping intervals (Table 2). For [TF] = 25 pM, fold changes in peak time exceeded those in maximum slope, whereas for all other considered TF concentrations, the opposite was true. Each of those two parameters changed at least 2.7-fold, while thrombin peak height changed at most 1.74-fold, and the AUCs remained practically unchanged (Table 2). Taken together, these data suggest that rFVIIa, when added to normal blood, acts primarily by accelerating thrombin accumulation. Notably, both the initiation phase (characterized by clotting time) and the propagation phase (characterized by maximum slope of the thrombin curve) were accelerated, but the initiation phase was affected the most.

Our computational results are consistent with recently published experimental studies of TF-initiated blood clotting in stored platelet concentrates [28]. The concentration of platelets in the platelet concentrates was adjusted to 200×10^9 platelets/L, which corresponds to normal platelet concentration. For each thrombin generation parameter, two values were obtained: one for control (system with added buffer) and one for the system with 25 nM added rFVIIa. We estimated the effects of rFVIIa in those experiments by dividing the value obtained with added rFVIIa by the corresponding control values (in the case of clotting times and thrombin peak times, we calculated the reciprocals) (Fig. 3B). As can be seen, there are quantitative differences between fold changes predicted by our model (Fig. 3A) and the experimentally observed fold changes (Fig. 3B). These discrepancies could be expected, given the differences between the specific experimental setup used to obtain the data for stored platelet concentrates [28] and our computational methodology. Therefore, here we are interested in a semi-quantitative comparison. Fig. 3B shows that the largest fold change — a decrease — was observed for clotting times, and the second-largest fold change — also a decrease — was observed for thrombin peak times. A slight increase characterized thrombin peak heights, and the AUCs were practically unchanged. (Experimental data for maximum slope of the thrombin curves were not available.) These results suggest that even when the concentration of phospholipids is not saturating, the mode of action of rFVIIa in blood with normal composition may follow the general pattern captured by the computational model.

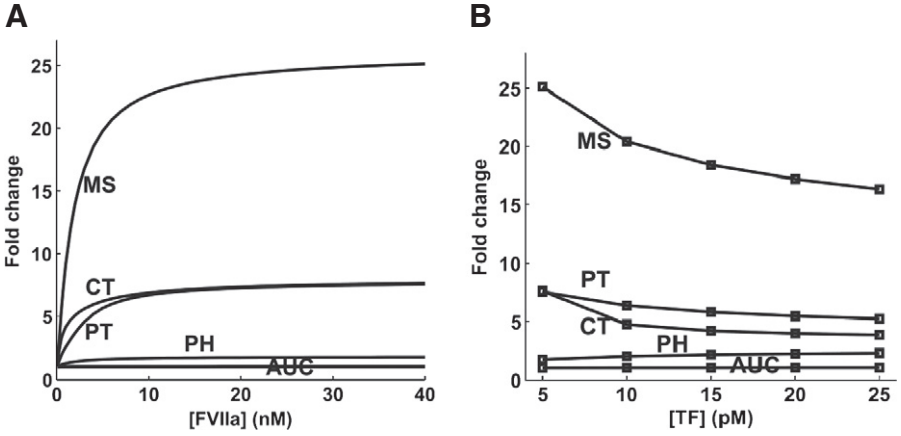

Fig. 4. FVIIa-induced fold changes in the quantitative parameters of thrombin generation in the case when blood contained no FVIII. CT: clotting time; PT: thrombin peak time; MS: maximum slope of the thrombin curve; PH: thrombin peak height; AUC: area under the thrombin curve. (A) [TF] = 5 pM (TF = tissue factor); varying [FVIIa]. (B) [FVIIa] = 40 nM (maximal of the considered values); the squares designate the computed values for varying [TF].

Table 2
Fold change ranges for the quantitative parameters of thrombin generation. The ranges were calculated with [TF] taking the values 5, 10, 15, 20, 25 pM, and [FVIIa] ≥ 20 nM.

	CT	PT	MS	PH	AUC
Normal blood	(3.65, 6.37)	(2.80, 3.08)	(2.73, 3.86)	(1.47, 1.74)	(1.01, 1.02)
No FVIII	(3.65, 7.65)	(5.11, 7.56)	(15.77, 25.12)	(1.71, 2.30)	(1.02, 1.04)
No FIX	(3.69, 7.93)	(5.20, 7.93)	(16.15, 25.79)	(1.72, 2.30)	(1.02, 1.04)
No FVII	(3.20, 3.55)	(2.02, 2.33)	(1.86, 1.90)	(1.28, 1.28)	(1.01, 1.01)
No TFPI	(3.01, 3.28)	(2.39, 2.57)	(2.19, 2.34)	(1.29, 1.37)	(1.01, 1.01)
Diluted blood	(3.57, 4.45)	(2.78, 2.88)	(2.49, 2.86)	(1.32, 1.49)	(1.01, 1.01)

CT = clotting time; PT = thrombin peak time; MS = maximum slope of the thrombin curve; PH = thrombin peak height; AUC = area under the thrombin curve.

FVIIa modulates predominantly maximum slope of the thrombin curve in hemophilia blood

Because the bulk of thrombin is generated by the concerted action of FVIIIa and FIXa (the *intrinsic tenase* complex), the propagation phase of thrombin generation is shaped primarily by these two proteins [1,30]. While FVIIa directly participates in the activation of FIX, its role in the formation of FVIIIa is indirect. We investigated the activity of FVIIa in the coagulation systems lacking either of these two proteins. Such systems represent the well-known and widely studied blood pathologies hemophilia A (decreased level of FVIII) and hemophilia B (decreased level of FIX) [38].

To elucidate the effects of FVIIa in blood lacking FVIII, we set the initial concentration of FVIII in the computational model to 0, which resulted in the absence of FVIIIa in the system. Similarly to the case of normal blood composition, for [FVIIa] ≥ 20 nM the three timing parameters — clotting time, thrombin peak time, and maximum slope — were affected the most, and the AUC stayed practically unchanged (Fig. 4, Table 2). However, in contrast to the normal blood composition case, the parameter that changed the most (>15-fold) was maximum slope, with changes in clotting time and thrombin peak time being noticeably smaller. Furthermore, for [TF] > 5 pM fold changes in peak time exceeded those in clotting time by at least 1.38; however, for [TF] = 5 pM the fold changes in those two parameters were practically the same (Fig. 4). For every [FVIIa] ≥ 20 nM and every [TF], fold changes in clotting time exceeded the corresponding fold changes in peak height by at least 1.39. Fold changes in thrombin peak height (from ~1.71-fold to ~2.30-fold) were larger than those in the case of normal blood composition, which suggests that rFVIIa can noticeably impact thrombin peak height in hemophilia A blood. Experimental support for this theoretical conclusion can be found in the literature (e.g., Fig. 4 in Ref. [26]).

The significant difference between the fold change analysis results for normal blood composition (Fig. 3A) and hemophilia A blood (Fig. 4A) likely arises from the special functional role of the intrinsic tenase complex (of which FVIIIa is a part) in the thrombin generation network. Indeed, the intrinsic tenase complex is the key element of a positive feedback loop that is necessary for amplifying thrombin generation after the initial amount of thrombin is formed [1,30]. It is generally known that positive feedback strongly impacts many qualitative and quantitative dynamic properties of biological systems [42,43]. Therefore, the disruption of a critical positive feedback loop in the thrombin generation network can be expected to cause a drastic difference in fold change analysis results. This difference was demonstrated in our computational experiments with blood lacking FVIII, because setting the initial FVIII concentration to 0 prevents the intrinsic tenase complex formation.

If the differences in the fold change analysis results were indeed due primarily to the disruption of the positive feedback loop, then elimination of another component of the intrinsic tenase — factor IXa — would produce similar results. To test this hypothesis, we performed thrombin curve analysis in the system containing FVIII but lacking FIX by setting the initial FIX concentration in the model to 0, thereby preventing FIXa from being generated. As expected, the results of this analysis were very similar to the case of no FVIII (Table 2). Furthermore, these results were consistent with the experimental data obtained by Butenas et al. for an *in vitro* system containing phospholipids and coagulation proteins, with protein composition analogous to that in our computational experiments [37]. In those experiments, coagulation was initiated with 5 pM TF in a mixture of coagulants and anti-coagulants lacking FIX, and the concentration of platelets was 5-fold higher than the normal platelet concentration. (The latter condition is beneficial for our comparison, because the Hockin–Mann model reflects the case of high phospholipid surface concentrations [16].) As a result of increasing the concentration of rFVIIa from 0.1 nM to 10 nM, the duration of the initiation phase (which roughly corresponds to clotting time) decreased ~8-fold and the thrombin peak height increased by less than 6.8-fold, whereas maximum slope of the thrombin curve increased by >11-fold (see Fig. 1A in Ref. [37]).

In summary, our analysis supported the notion that FVIIa acts on hemophilia A and B blood predominantly by accelerating thrombin accumulation. However, by contrast with the case of normal blood composition, the thrombin generation phase that was affected the most was the propagation phase, the difference being due to the disruption of a critical positive feedback loop in the system. Our results suggest that FIXa contributes to the action of FVIIa in a similar way to FVIIIa, which is consistent with the biochemical roles of FVIIIa and FIXa as the components of the intrinsic tenase complex. Yet, there are quantitative differences in the FVIIa-induced fold changes between the systems lacking FVIII and FIX, which might result from the fact that TF:FVIIa directly activates FIX, but not FVIII [1].

FVIIa accelerates thrombin accumulation in diluted blood, blood lacking FVII, and blood lacking TFPI

Dilutional coagulopathy is one of the critical complications of trauma that is associated with heavy bleeding. This type of coagulopathy results from resuscitation efforts aimed to maintain normal blood volume and pressure when the volume of circulating blood is rapidly decreasing due to severe hemorrhage [44–46]. We modeled blood dilution by decreasing the normal concentration of the clotting factors in the Hockin–Mann model by 2-fold (see Methods). This degree of dilution was chosen because in clinical practice a decrease in coagulant levels by 50% is a typical threshold that triggers fresh frozen plasma transfusion aimed at normalizing coagulant levels [47]. While dilution decreases platelet concentration, this degree of dilution should not be prohibitive for platelet plug formation in which the local concentration of platelets increases by 2 orders of magnitude [37]. Therefore, the saturating phospholipid level assumption underlying the Hockin–Mann model [15] does not appear to be severely violated during clot formation under the 2-fold dilution conditions.

To investigate the effects of FVIIa in diluted blood, we performed computational FVIIa titration analysis for the mathematical model of coagulation in diluted blood (see Methods). We found that, in contrast to the case of normal blood composition, for [FVIIa] ≥ 20 nM and all considered TF concentrations fold changes in thrombin peak time exceeded those for maximum slope. For [TF] = 5 pM and [TF] = 25 pM, the fold changes for those two parameters were practically the same (Fig. 5A). Similarly to the case of normal blood composition, in diluted blood clotting time was characterized by the largest fold changes

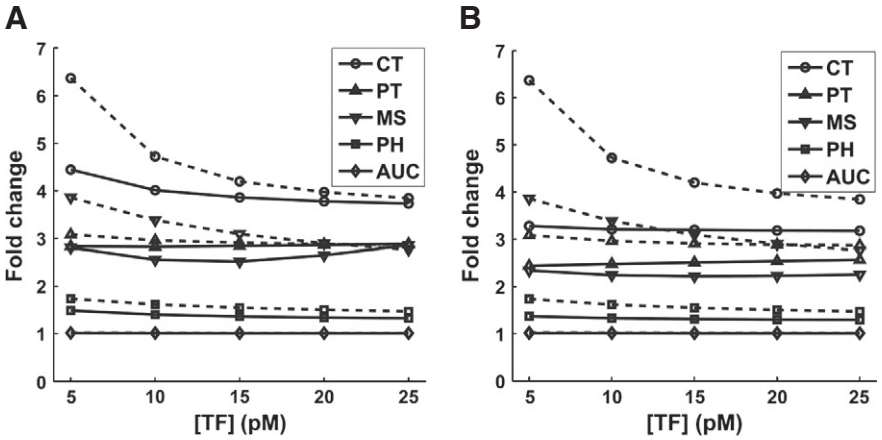

Fig. 5. FVIIa-induced fold changes in the quantitative parameters of thrombin generation for [FVIIa] = 40 nM. The markers designate the computed values for varying [TF] (TF = tissue factor). (A) Solid lines: diluted blood; dashed lines: normal blood composition. (B) Solid lines: no TFPI; dashed lines: normal blood composition. CT: clotting time; PT: thrombin peak time; MS: maximum slope of the thrombin curve; PH: thrombin peak height; AUC: area under the thrombin curve.

(Table 2), and thrombin peak height changed considerably less than any of the three timing parameters. Indeed, for every [TF] and [FVIIa] ≥ 20 nM, fold changes in maximum slope exceeded those in thrombin peak height by at least 1.13. The AUC was practically unchanged by FVIIa action (Fig. 5A, Table 2). We thus conclude that FVIIa acts on diluted blood primarily by accelerating the initiation and propagation phases of thrombin accumulation. This conclusion is consistent with experimental data on rFVIIa-induced decrease in clotting time for *in vitro* [48–50] and *in vivo* [24] hemodilution.

FVII and TFPI are the primary regulators of the activity of FVIIa. FVII is the inactive precursor of FVIIa, which competes with FVIIa for binding with TF [51]. TFPI is an anti-coagulant, which acts through the formation of complexes with TF and FXa [16,52] (see also Supplementary Material, Table S1). TFPI inhibits the activity of TF and the formation of FXa (Supplementary Material, Fig. S1). We performed fold change analysis for the cases when initial concentrations of TFPI or FVII were equal to 0, with other initial concentrations corresponding to normal blood composition. In both of these cases, the FVIIa-induced fold changes in thrombin generation parameters (for [FVIIa] ≥ 20 nM) varied within non-intersecting parameter-specific intervals (Table 2). The pattern of fold changes was as follows: clotting time > thrombin peak time > maximum slope > thrombin peak height > the AUC (practically no change) (Fig. 5B). This pattern for clotting time, maximum slope, and thrombin peak height has been confirmed by *in vitro* experiments with synthetic plasma lacking FVII [20] (thrombin peak time and the AUC were not considered in that work). For [FVIIa] ≥ 20 nM and all considered [TF] values, fold changes in clotting time and maximum slope of the thrombin curve in the case of no FVII decreased (in comparison with the case of normal blood composition) by at least 0.45 and 0.83, respectively. In the case of no TFPI, the respective numbers were 0.64 and 0.51. Taken together, our results indicate that even in the absence TFPI or FVII, rFVIIa has the ability to speed up both initiation and propagation phases of thrombin accumulation. However, in the presence of TFPI and FVII the acceleration effect of FVIIa on thrombin generation is significantly more pronounced (Fig. 5B).

Discussion

Native factor VIIa and recombinant factor VIIa — a promising hemostatic agent — modulate the enzymatic network responsible for thrombin generation. This is the first study to apply computational fold-change analyses to elucidate the effects of a therapeutic agent on the dynamics of thrombin accumulation. By performing computational experiments with the well-established Hockin–Mann model of thrombin generation [16,31], we have demonstrated that the primary mode of action of FVIIa is acceleration of thrombin accumulation. This general pattern is manifested through considerable changes in clotting time, thrombin peak time, and maximum slope of the thrombin curve. By contrast, the parameters that do not characterize thrombin accumulation rate — thrombin peak height and the area under the thrombin curve (termed amount parameters) — are much less affected by FVIIa; in all our computations, the AUC remained practically unchanged. This mode of action of FVIIa has been verified in numerical experiments addressing thrombin generation in blood with normal composition (Fig. 3), hemophilia A and B blood (Fig. 4), diluted blood (Fig. 5A), blood lacking TFPI (Fig. 5B), and blood lacking FVII (Table 2). Remarkably, our results for hemophilia blood show that thrombin peak time and maximum slope, which characterize the *propagation* phase of thrombin generation, can be affected by FVIIa more than clotting time, which characterizes the duration of the *initiation* phase (Fig. 4). This suggests that rFVIIa can strongly impact *both* the initiation and propagation phases. Our findings indicate that the quantitative effects of FVIIa depend on blood composition, and are modulated by both pro- and anti-coagulants, but the ability of FVIIa to speed up thrombin accumulation demonstrates significant robustness. Taken together, our results suggest that acceleration of thrombin accumulation might be the foundation of the therapeutic potential of rFVIIa for a broad range of pathologic conditions.

Our results indicate that pharmacological doses of rFVIIa can reduce clotting time in blood with normal or pathological composition at least 3-fold (Table 2). For all considered blood compositions except hemophilia blood, clotting time was the parameter with the largest FVIIa-induced fold changes. The ability of rFVIIa to reduce clotting time was confirmed by TF-induced thrombin generation *in vitro* experiments in blood with normal composition [23,28,38], hemophilia blood [26,29,38], diluted blood [48,50], and blood lacking FVII [20]. These computational and experimental results allow us to conclude that the ability of rFVIIa to reduce clotting time is an inherent property of the biochemical mechanisms governing the activity of rFVIIa, and this property is correctly reproduced by the computational model.

For other parameters of thrombin generation, we elucidated general patterns of FVIIa activity that would hardly be possible to infer from the available experimental data alone due to a lack of agreement between the reported experimental results [23–29]. Indeed, our computations predicted that pharmacological doses of rFVIIa decrease thrombin peak time by at least 2-fold (Table 2). While some of the published experimental papers supported the ability of rFVIIa to reduce thrombin peak time [26,28], other articles reported

no significant rFVIIa-induced effects on this parameter [25,29]. Our computations suggest that rFVIIa cannot induce any significant changes in the AUC, with fold changes never exceeding 1.04 (Table 2), which is in agreement with the work of Svendsen et al. on stored platelet concentrates [28] (Fig. 3B). Yet, Altman et al. showed that rFVIIa can induce up to 2-fold increases in the AUC measured in platelet-rich plasma [23]. While such disparities may be due largely to dissimilarities in experimental procedures and conditions (which is not uncommon in hematology research [19,38,39]), one could hypothesize that the lack of robustness in the modulation of thrombin generation is an inherent property of the molecular mechanism of action of rFVIIa. Indeed, the capacity of rFVIIa to preferentially affect some of the thrombin generation parameters, but not the others, might display a strong and complicated dependence on the concentrations of rFVIIa and TF, as well as other coagulation components. Our results, however, do not provide support for this hypothesis, and instead suggest that the mode of action of rFVIIa is characterized by general patterns compatible with the existing knowledge about the biochemistry of thrombin generation.

Our results demonstrate that the coagulation potential of blood is essentially saturated at [FVIIa] = 15 nM (e.g., Fig. 2, Fig. 3A, Fig. 4A). However, clinical evidence suggests that in hemophilia patients, dose-dependent improvements in rFVIIa-induced hemostasis might be possible beyond the 90 μg/kg dose ([rFVIIa] ≈ 36 nM) — the "standard" dose for this therapeutic [53,54]. This apparent discrepancy might be accounted for by the *in vivo* factors affecting the action of rFVIIa (such as the decay of rFVIIa in the body, the details of tissue factor presentation, and clotting factor dilution or resupply caused by blood flow). This conclusion is supported by *in vitro* experiments carried out by Butenas et al., who demonstrated rFVIIa saturation in whole-blood models of hemophilia [38]. However, the *in vitro* studies by Sørensen et al. [55] and Allen et al. [56] do not show such saturation for rFVIIa. To indicate potential causes for these disparities, we note that the TF concentration used by Sørensen et al. [55] and the source of TF (LPS-activated monocytes in cell culture) used by Allen et al. [56] were significantly different from those used by Butenas et al. [38]. The existing disparate experimental evidence, as well as the general complexity of the blood coagulation system, warrant further investigations of saturation effects for rFVIIa activity, which might shed light on the optimal dosing strategies for this therapeutic. It is of note, however, that the model predicted saturation behavior for FVIIa-induced effects in dilutional coagulopathy, which was similar to the behavior shown in Fig. 2. This provides further support to the emerging view that, in the case of traumatic coagulopathy, the use of rFVIIa doses exceeding 100 μg/kg may be unjustified [57], and in many cases low rFVIIa doses could in fact be sufficient [58].

Anomalous values of quantitative parameters of *in vitro* thrombin generation can be correlated with hemorrhagic or thrombotic tendencies *in vivo* [18,22,32]. Therefore, thrombin generation curves can potentially be used both as a diagnostic tool for bleeding abnormalities and as a tool to guide therapeutic intervention aimed at normalizing thrombin generation parameters [59]. In an *in vivo* porcine model of dilutional coagulopathy, hemodilution did not impact the AUC, but increased prothrombin time and decreased thrombin peak height [60]. This result suggests that clotting time and peak height, but not the AUC, are more predictive of therapeutic efficacy during *in vivo* clotting in dilutional coagulopathy. Of note, the fold change in peak height (~ 1.9-fold) observed in the porcine model of dilutional coagulopathy was larger than the corresponding fold change in prothrombin time (~ 1.3-fold) [60]. Because rFVIIa affects clotting time considerably more than thrombin peak height in diluted blood (Table 2; Fig. 5A), a dose of rFVIIa that normalizes peak height might cause the corresponding clotting rate to exceed its normal level, resulting in increased clotting potential and leading to thromboembolic complications. By contrast, in hemophilia all (or most) of the thrombin generation parameters can be altered considerably [38,61,62], which might contribute to the comparative rarity of thromboembolic complications during the rFVIIa use in hemophilia. The example of dilutional coagulopathy suggests that the ultimate goal of hemostatic therapy should be to induce a *balanced* improvement in several (all) thrombin generation parameters, so as to avoid significant post-therapeutic distortions in the thrombin generation curve. One strategy potentially leading to a more balanced improvement in thrombin generation is to combine the administration of rFVIIa with other therapeutics. Indeed, a combination of rFVIIa and prothrombin has been demonstrated to have higher efficacy than rFVIIa alone in a cell-based *in vitro* model of hemophilia [56]. Furthermore, in an *in vitro* model of severe dilutional coagulopathy, increasing the level of fibrinogen can enhance the efficacy of rFVIIa [49].

The limitations of this study stem from its focus on blood coagulation *in vitro*, as well as from inevitable simplifications necessary to represent complex biological systems by mechanistic, computational models. First, the current version of the model does not reflect platelet activation dynamics, assuming instead that catalytically active phospholipid surfaces are present at the moment of clotting initiation [16,31]. This model assumption could possibly lead to over-estimation of the rate of thrombin generation [63]. However, our fold change analysis focused on ratios, rather than absolute values, of the thrombin generation parameters, which are likely less sensitive to the details of platelet activation dynamics. Second, the model does not reflect the specifics of clotting factor interactions with platelets. Therefore, the platelet-dependent, TF-independent rFVIIa effects (which might become more significant when platelet concentration is increased [64], e.g., in the platelet plug) could not be investigated directly. However, it has been shown that a 5-fold increase in platelet concentration does not alleviate the need for TF in clotting activation in hemophilia blood [37]. Further studies are needed to resolve the existing controversy [38,39] about the TF-independent mechanism of rFVIIa activity. Finally, we do not consider the effects of blood flow on rFVIIa activity, which can be the subject of a separate investigation. Despite these potential limitations, computational modeling of *in vitro* blood clotting can provide insights into the complex *in vivo* dynamics of clot formation. This has been demonstrated in a combined computational/experimental study of serine protease inhibitors [65], and in a study that compared computationally derived points of fragility of the coagulation system with existing therapeutic strategies [66]. Thus, the results of our fold change analyses can be regarded as rational (and realistic) hypotheses regarding the *in vivo* mode of action of rFVIIa.

It is becoming increasingly clear that the results of *in vitro* thrombin generation experiments can be considerably affected by the choice of experimental protocol [48,67–69]. This motivated us to seek alternative approaches to study quantitative effects that could be masked by the "noisy" character of the available experimental data. In this work, we used a computational model to elucidate robust patterns that characterize the effects of rFVIIa on thrombin generation. While our results can be used to interpret the existing experimental datasets, the current scarcity of quantitative data precludes a comprehensive and direct comparison of our fold-change analysis results (Figs. 2–5, Table 2) with relevant *in vitro* measurements. It is our hope that this work has demonstrated the need to obtain high-fidelity measurements of rFVIIa-stimulated thrombin generation for *in vitro* systems in which *1*) non-physiological experimental biases are minimized; *2*) TF concentration is quantitatively controlled; *3*) the clotting factor composition is known; and *4*) the concentration of phospholipid surfaces (preferably platelets) is sufficiently high to reflect the conditions existing in a platelet plug.

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Conflict of interest statement

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.thromres.2011.05.013.

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