Computational analysis of intersubject variability and thrombin generation in dilutional coagulopathy

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BACKGROUND: Blood dilution is a frequent complication of massive transfusion during trauma and surgery. This article investigates the quantitative effects of blood plasma dilution on thrombin generation in the context of intersubject variability.

STUDY DESIGN AND METHODS: A thoroughly validated computational model was used to simulate thrombin generation curves for 472 healthy subjects in the Leiden Thrombophilia Study. Individual thrombin curves were calculated for undiluted blood and for different dilution scenarios. For every such curve, five standard quantitative parameters of thrombin generation were calculated and analyzed.

RESULTS: Thrombin generation parameters in diluted blood plasma displayed significant intersubject variability (with a coefficient of variation up to approx. 28%). Nevertheless, dilutional effects in the majority (or all) of the subjects in the study group were characterized by persistent patterns. In particular, the largest dilution-induced change typically occurred in the maximum slope (MS) of the thrombin curve, followed by a change in thrombin peak height (PH), whereas the smallest change often occurred in the area under the curve. The identified patterns demonstrated considerable robustness to variations in dilution scenario and tissue factor concentration.

CONCLUSION: Dilutional effects on thrombin generation in a human population can be predicted from trends identified for the "average" subject and then refined by performing an analysis of actual subjects in the study group. The MS and PH are dilution indicators that are both sensitive and reliable across a large subject group and could potentially be used as disease markers in the diagnosis of coagulopathic conditions.

Resuscitation efforts in trauma care, as well as various surgical procedures, often require the transfusion of fluids and blood products that do not contain (or contain reduced amounts of) the biochemical components of the blood coagulation system. This leads to the dilution of coagulation proteins and may thereby impair blood clotting, that is, induce

ABBREVIATIONS: AT = antithrombin; AUC = area under the thrombin curve; CT = clotting time (time to 10 nmol/L thrombin); FC(s) = fold change(s); LETS = Leiden Thrombophilia Study; MS = maximum slope of the thrombin curve; PH = thrombin peak height; PT = thrombin peak time; TAT = thrombin-antithrombin; TF = tissue factor; TFPI = tissue factor pathway inhibitor.

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dilutional coagulopathy.[1,2] Besides medical intervention, a decrease in the concentrations of clotting factors during hemorrhage can result from their consumption, as well as from the shift of fluids from the interstitial to the vascular space.[3] Systematic quantitative analysis of dilutional effects is complicated by the significant intersubject variability in coagulation factor levels.[4] This variability has a profound impact on the kinetics of blood coagulation,[4,5] resulting in potentially prothrombotic or prohemorrhagic phenotypes in apparently healthy subjects.[6,7] It is thus conceivable that the effects of dilution on blood clotting might be significantly different for different subjects. Alternatively, it can be hypothesized that dilutional effects follow major trends that hold for the vast majority of the human population and can be detected by analyzing the effects of dilution in a system with mean plasma concentrations of coagulation factors (i.e., the "average" subject). In this work, we investigated the possibility of the existence of such major trends for in vitro thrombin generation in blood plasma.

The generation of thrombin, due to its critical and diverse functions as a key coagulation enzyme, is central to blood clotting.[8,9] Thrombin formation is triggered by the protein tissue factor (TF), which activates the biochemical coagulation network when blood from a damaged vessel comes into contact with surrounding tissues.[10-13] Thrombin generation in vitro occurs in three phases: initiation, propagation, and termination[14,15] (or, alternatively, initiation, amplification, and propagation[10]), which are reflected by the peak-shaped thrombin generation curve (Fig. 1). Such curves are typically characterized by five quantitative parameters: clotting time (CT; which we define as the time to 10 nmol/L thrombin[16]), thrombin peak time (PT), maximum slope of the thrombin curve (MS), thrombin peak height (PH), and the area under the thrombin curve (AUC; Fig. 1A). While a number of recent studies have addressed the effects of dilution on these parameters,[17-20] it is still unknown which of the five parameters displays the highest sensitivity to blood plasma dilution. Furthermore, some of the studies of dilution-induced effects on thrombin generation parameters have reported disparate results (e.g., see De Smedt et al.,[17] Schols et al.,[19,20] Dunbar and Chandler[21]). The causes for disparities (which may be due to differences in experimental protocols[16,22,23] or be the result of intersubject variability[5]) are not immediately obvious, suggesting that new approaches, complementary to traditional experimentation, should be used to interpret the existing results and guide further experimental work.[16]

Here, we studied dilution by performing computational thrombin generation analyses on a data set obtained as a part of the Leiden Thrombophilia Study (LETS).[24] Computer modeling approaches that account for intersubject variability have been successfully applied to study thrombin generation in normal blood[4] and to analyze the factors contributing to different pathologic conditions and therapeutic interventions that could lead to thromboembolic events.[25-28] Our numeric modeling method relied on the use of the computational model of thrombin generation developed in K. Mann's laboratory.[14,29] This model has demonstrated adequate accuracy when benchmarked against experiments with synthetic blood plasma and natural blood systems and has been validated by the laboratories of K. Mann[15,30-32] and S. Diamond,[33,34] as well as by our own research group.[16]

We used the computational model to calculate the five thrombin generation parameters for every individual in the LETS control group in the case of normal (i.e., undiluted) blood plasma composition, as well as for different dilution scenarios. We found that the intersubject variability in thrombin generation parameters impacted by dilution can be significant. Despite this variability, we were able to detect robust patterns that characterize dilutional effects in the majority (or all) of the subjects and hold for several dilution scenarios and for different TF concentrations.

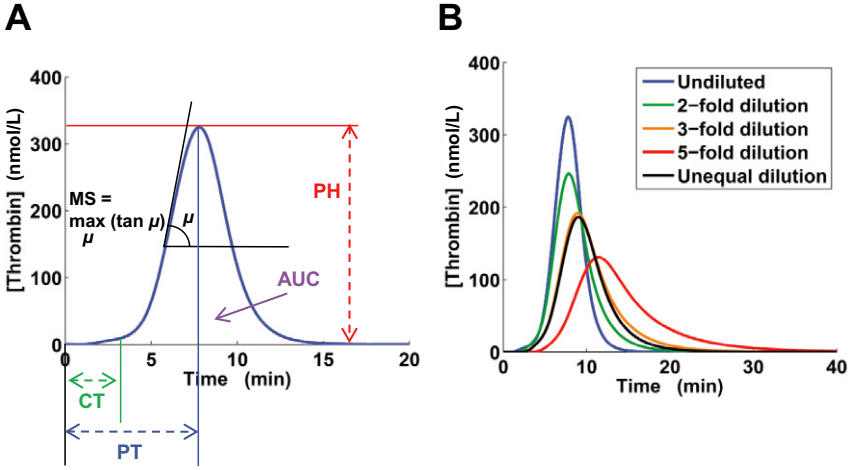

Fig. 1. Numeric simulation of thrombin generation for the average subject. Thrombin generation was initiated with TF at Time 0; coagulation factors were assumed to be at their mean plasma concentrations or corresponding diluted concentrations. (A) Quantitative parameters of thrombin generation: the timing parameters (CT, PT, MS) and the amount parameters (PH, AUC). The thrombin curve was generated for normal blood plasma composition. (B) Thrombin curves for different dilution scenarios.

MATERIALS AND METHODS

Study group

We performed computational analyses of an experimental data set obtained in the course of the previously published LETS.[24] The data set consisted of coagu-

lation factor level measurements for 472 healthy subjects from the LETS control group. In the LETS data set, we used the measurements for the following coagulation proteins: Factor (F)II, FV, FVII, FVIII, F IX, and FX, as well as the anticoagulants antithrombin (AT) and TF pathway inhibitor (TFPI). The results were normalized using average absolute concentration values as described by Brummel-Ziedins and colleagues.[4,35] The normalization yielded absolute concentrations for the coagulation factors in each individual, which were used to define the initial conditions in the computational model of thrombin generation (see below). The concentration of FVIIa in the model was chosen to equal 1% of the FVII concentration.[14,36]

Numeric model of thrombin generation and computational procedures

Thrombin generation was simulated using an updated version[29] of the computational model of thrombin generation developed in K. Mann's laboratory.[14] The model consists of a system of nonlinear ordinary differential equations describing thrombin generation in a limited system involving the key reactions known to generate thrombin in blood plasma containing phospholipid surfaces that support thrombin generation. The inputs of the model (i.e., the initial conditions for the differential equations) are constituted by the initial concentrations of the coagulation factors FII, FV, FVII, FVIIa, FVIII, F IX and FX, as well as the anticoagulants TFPI and AT and the thrombin generation inducer TF. The model output is the curve describing temporal changes in the concentration of active thrombin (Fig. 1; active thrombin, henceforth referred to as "thrombin," is a weighted sum of thrombin and meizothrombin concentrations[14,16]). Using the model, we generated such curves for different sets of initial conditions representing different subjects and different dilution scenarios. For each generated thrombin curve, we calculated the five thrombin generation parameters as described in Mitrophanov and Reifman.[16]

The numeric model of thrombin generation was implemented in the SimBiology toolbox of the MATLAB software suite (MathWorks, Natick, MA) as described in Mitrophanov and Reifman;[16] all computations were performed in MATLAB 2010b. In our simulations, we assumed that the concentration of phospholipid surfaces is not altered by dilution. This assumption reflects the experimental protocols used to study dilution in vitro.[17,19,20] It is also relevant for understanding dilutional coagulopathy in vivo, because it can be used to account for the process of compensatory platelet (PLT) resupply known to occur during hemorrhage.[37]

Thrombin generation simulations were run with the initial coagulation factor concentrations equal to the normal (i.e., undiluted) factor levels for each individual in the subject group and also for the case when the clotting factor levels have their average values for normal human plasma (the average subject).[14] Further, we generated thrombin curves with the normal factor levels decreased according to one of four dilution scenarios: two-, three-, and fivefold dilution, as well as unequal dilution. The x-fold dilution scenarios were equivalent to reducing the initial concentrations of all the coagulation proteins in the model by x-fold. The unequal dilution scenario[27] was based on the experimental data for an in vivo porcine model of dilutional coagulopathy, in which the degrees of dilution (dilution factors) were different for different coagulation factors.[38] Dilution factors for the unequal dilution scenario are shown in Table 1. In our model, [TF] (brackets designate concentration) is not affected by dilution. Unless otherwise stated, it had the default value of 5 pmol/L.[6] Because no data on the distribution of [TF] values in humans were available, the dependence of thrombin generation on [TF] was analyzed by performing computations with [TF] taking the values 2, 5, 10, 15, 20, and 25 pmol/L.[16] The effects of dilution on the thrombin generation parameters were characterized by two quantities, R-values, and fold changes (FCs). The R-value for a parameter is the ratio of the parameter value for diluted blood plasma to its value for undiluted blood plasma. FC = R if $R \geq 1$; otherwise FC = 1/R.

TABLE 1. Unequal dilution: individual dilution factors for the biochemical species with nonzero initial concentrations (except TF, which was not diluted)*[27]

Biochemical species	Dilution factor
FII (prothrombin)	0.38
FVII	0.36
FVIIa	0.36
FV	0.29
FVIII	0.27
F IX	0.33
FX	0.31
AT	0.41
TFPI	0.30

* Concentrations of coagulation proteins diluted according to the unequal dilution scenario were calculated by multiplying their normal (i.e., undiluted) concentrations by the corresponding dilution factors. These reduced concentrations were used as initial conditions for the differential equations constituting the computational model.[14,29]

Statistical analyses

Differences between thrombin curve parameter distributions were tested using the two-sided Wilcoxon sign-rank test. The degree of intersubject variability was estimated via coefficients of variation (CVs) for thrombin generation parameters as follows: CV = (standard deviation [SD])/(mean value). The propensity of dilutional effects at the subject level was characterized by estimating their probabilities, that is, by calculating the fractions of subjects for

whom the effect was detected. The standard error (SE) of the probability estimation was calculated as

$$SE = \sqrt{q(1-q)/N},$$

where q is the probability estimate and n = 472 is the subject group size.[39] SE = 0 when q equals 0 or 1, and in such cases the SE was omitted.

RESULTS

Thrombin curve generation

To choose a standard time interval for thrombin curve generation, we set the initial TF concentration to 2 or 5 pmol/L and calculated thrombin curves for coagulation factor concentrations at their average values for normal blood plasma,[14] as well as for the corresponding diluted concentrations for all considered dilution scenarios. In each of those cases, thrombin levels were sufficiently restored to zero after 40 minutes of thrombin generation. We then used this time interval to generate thrombin curves for the 472 subjects in the study group. Each of the generated curves had one local maximum (i.e., the thrombin peak), similarly to the curves shown in Fig 1.

Differential effects of dilution on thrombin generation in the average subject

We found that, in the average subject, dilutional effects can be characterized by general patterns that hold for different dilution scenarios. Indeed, for different dilution types, MS, PH, and AUC decreased, whereas CT and PT increased (Table 2; the only exception was a 2.50% decrease in CT for the twofold dilution). The largest FC occurred in MS, followed (in the order of decreasing FC) by PH, CT, PT, and AUC (Table 2). This pattern can be written as follows: FC(MS) > FC(PH) > FC(CT) > FC(PT) > FC(AUC). The only exception was the case of twofold dilution, in which the FC in the AUC (1.02-fold decrease) slightly exceeded the FC in PT (1.01-fold increase).

Intersubject variability of the thrombin generation parameters in the study group

All dilution scenarios caused significant changes in all of the five thrombin generation parameters ($p < 1.0 \times 10^{-6}$ for each thrombin generation parameter tested independently in the case of twofold dilution and $p < 1.0 \times 10^{-76}$ for all other dilution scenarios). The degree of variability in the thrombin generation parameters was substantial and depended on both the thrombin generation parameter and the dilution scenario (Fig. 2; Table 3). Table 3 shows the means and SDs of the thrombin generation parameters, which were used to compute the CVs. As expected, due to the nonlinearity of the equations constituting the computational model, the subject-group averages for the thrombin parameters (Table 3) were slightly different from the corresponding values for the average subject (Table 2).

For each dilution type (including undiluted blood plasma), the thrombin generation parameters listed in the order of decreasing CV were as follows: MS (CV, 26.48%-29.28%), AUC (CV, 19.67%-20.35%), PH (CV, 17.66%-21.40%), CT (CV, 12.05%-16.64%), and PT (CV, 8.77%-10.20%). The only exception was detected for undiluted blood plasma: the CV for PH (21.40%) exceeded that for AUC (20.21%) by a small margin. For all thrombin generation parameters except AUC, the CVs slightly decreased with dilution. For the AUC, the largest CV (20.35%) was detected for twofold dilution, and the three largest CVs (for two- and threefold dilutions and for undiluted blood plasma) differed by less than 0.7%. These results indicate that intersubject variability causes significant deviations of the thrombin generation parameters from their mean values for all dilution types. It can thus be expected that some of the thrombin generation patterns identified for the average subject may not hold for all subjects in the study group.

Subject-level characterization of dilutional effects in the study group

Threefold dilution caused a decrease in CT for 3.18% (SE, 0.81%) of the subjects (Fig. 3B); for unequal dilution, CT decreased in 4.24% (SE, 0.93%) of the subjects (Fig. 3D). This is in contrast to the behavior of CT for the average subject, which increased with dilution (Table 2). Considerable heterogeneity was detected for CT and PT in the case of twofold dilution, where CT increased in 40.04% (SE 2.26%) of the subjects and PT increased in 60.38% (SE 2.25%) of the subjects (Fig. 3A). Yet, for all other thrombin parameter/dilution scenario combinations, the direction

TABLE 2. Thrombin generation parameter values for the average subject (the subject whose undiluted clotting factor levels were equal to the corresponding average values for human plasma[14])

Dilution scenario	CT (min)	PT (min)	MS (nmol/L/min)	PH (nmol/L)	AUC (nmol/L × min)
Undiluted blood	3.28	7.80	124.48	324.75	1306.55
Twofold dilution	3.20	7.87	89.44	246.15	1284.41
Threefold dilution	3.89	8.92	60.41	191.69	1276.60
Fivefold dilution	5.51	11.43	31.4 7	130.86	1264.67
Unequal dilution	3.87	9.03	56.70	186.29	1163.79

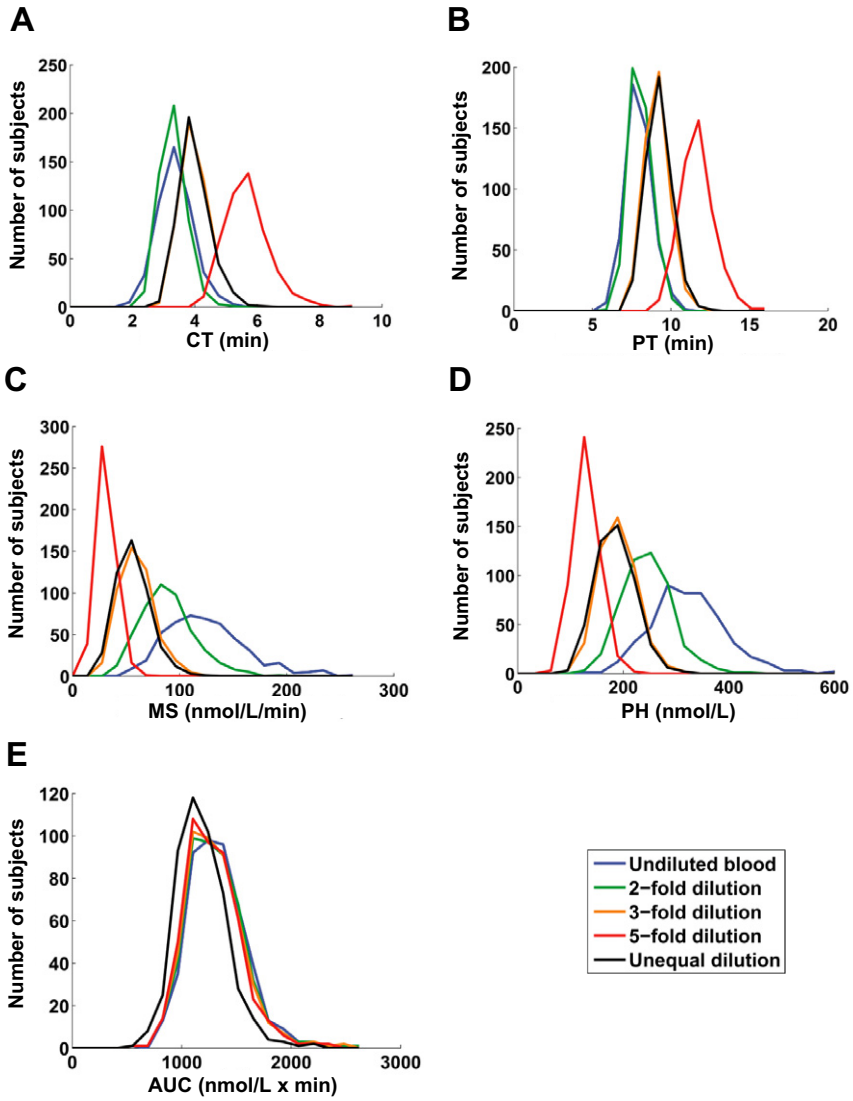

Fig. 2. Thrombin generation parameter distributions in the study group for different blood dilution scenarios. Plots were generated using the MATLAB function HIST with 20 bins.

of dilution-induced change for all or almost all (≥99.58% [SE ≤ 0.30%]) subjects coincided with those for the average subject (Fig. 3).

Due to considerable intersubject variability (Figs. 2 and 3 and Table 3), the magnitude of change for different thrombin generation parameters deviated from what could be expected based on the average subject analysis. In certain cases, the probability of deviations from the average subject behavior was comparatively small: for example, for twofold dilution, FC(PH) > FC(MS) in 2.33% (SE 0.69%) of the subjects, whereas for the average subject FC(PH) < FC(MS) (Table 2). However, in other cases such deviations were detected in a large fraction of the subject group. Indeed, for twofold dilution, FC(PT) > FC(CT) in 23.94% (SE 1.96%) of the subjects, although for the average subject FC(PT) < FC(CT). For threefold dilution, the same effect was detected for 23.52% (SE, 1.95%) of the subjects and for unequal dilution it was detected for 33.69% (SE, 2.18%) of the subjects. Thus, the probability of deviating from a pattern detected for the average subject can strongly depend on the pattern in question. At the same time, such probabilities also depend on the dilution scenario. Indeed, the pattern FC(MS) > FC(PH) > FC(CT) > FC(PT) > FC(AUC) was detected in 41.31% (SE, 2.27%), 75.42% (SE, 1.98%), and 99.79% (SE, 0.21%) of the subjects for two-, three-, and fivefold dilution, respectively, and in 55.30% (SE, 2.29%) of the subjects for unequal dilution.

The pattern FC(MS) > FC(PH) > FC(*X*), where *X* represents CT, PT, or AUC held for 97.46% (SE, 0.72%) of the subjects in the case of twofold dilution and for all subjects for other dilution scenarios. AUC was the parameter with the smallest dilution-induced FC in 55.72% (SE, 2.29%), 94.07% (SE, 1.09%), and 100% of the subjects for two-, three-, and fivefold dilution, respectively, and in 65.47% (SE, 2.19%) of the subjects for unequal dilution. In all subjects, the parameter with the smallest FC was either AUC, CT, or PT.

Dilution-induced abnormal states in the study group

According to an operational definition of traumatic coagulopathy, a trauma patient is considered coagulopathic if his or her prothrombin time is more than 1.5-fold larger than the typical normal value for this parameter.[37,40,41] For this threshold, we determined the fractions of subjects for which the FC in a thrombin generation parameter takes on above-threshold (i.e., abnormal) values. These fractions were the highest for MS and PH. Indeed, for threefold, fivefold, and unequal dilutions, the fractions of subjects with abnormal PH were 98.73 (SE, 0.52), 100, and 100%, respectively, and all of the subjects had abnormal MS values. For fivefold dilution, 84.96% (SE, 1.65%) of the subjects had abnormal CT and 37.71% (SE, 2.23%) of the subjects had abnormal PT. For other dilution scenarios, PT was within the normal range for all subjects. CT was abnormal in 2.33 (SE, 0.69) and 2.12% (SE, 0.66%) of the subjects for threefold and unequal dilution, respectively. For twofold dilution, 14.41% (SE 1.62%) of the sub-

TABLE 3. Thrombin generation parameters in the LETS control group*

Dilution scenario	CT (min)	PT (min)	MS (nmol/L/min)	PH (nmol/L)	AUC (nmol/L × min)
Undiluted blood	3.37 (0.56)	7.96 (0.81)	124.92 (36.58)	324.74 (69.51)	1332.86 (269.42)
Twofold dilution	3.30 (0.43)	8.04 (0.71)	88.97 (24.55)	244.87 (47.18)	1310.60 (266.70)
Threefold dilution	4.00 (0.48)	9.11 (0.80)	59.96 (16.27)	190.45 (35.11)	1302.36 (264.90)
Fivefold dilution	5.67 (0.68)	11.68 (1.07)	31.25 (8.27)	130.04 (22.96)	1286.53 (255.15)
Unequal dilution	3.98 (0.49)	9.23 (0.81)	56.35 (15.91)	185.17 (35.90)	1185.29 (233.12)

* The values of thrombin generation parameters were computed for each subject individually and then were used to calculate population-wide characteristics, such as population means. Data are expressed as mean (SD).

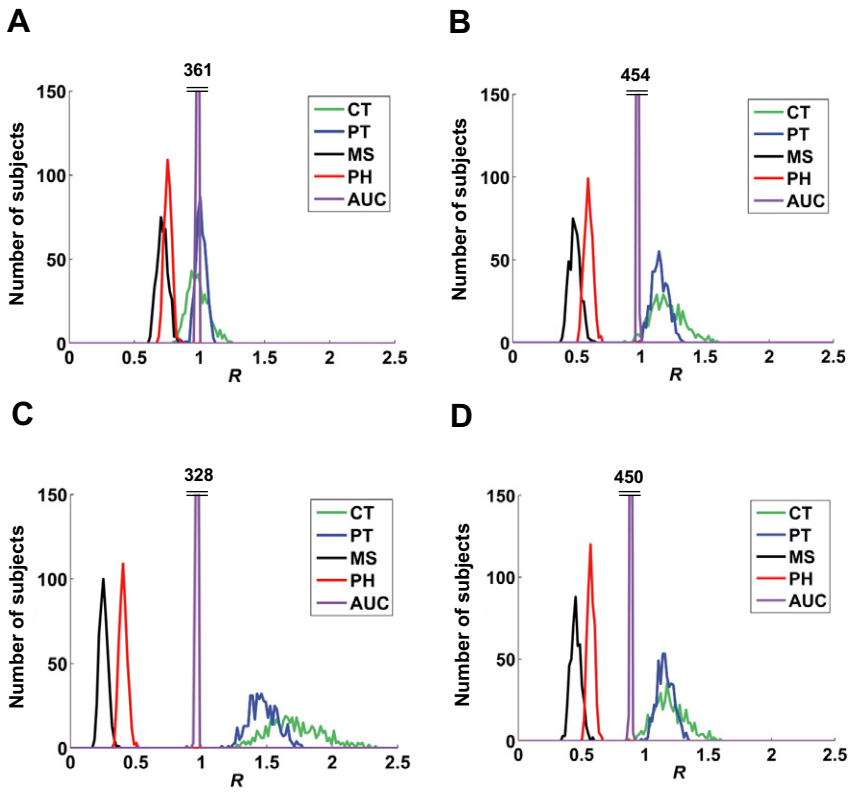

Fig. 3. R-value distributions for the five thrombin generation parameters and the four dilution scenarios calculated for the study group. For each thrombin generation parameter and each subject, R = (parameter value in diluted blood plasma)/(parameter value in undiluted blood plasma). Plots were generated using the MATLAB function HIST with 150 bins. (A) Twofold dilution; (B) threefold dilution; (C) fivefold dilution; (D) unequal dilution. The numbers above double-dash signs indicate the heights of the AUC distribution peaks.

jects had abnormal MS values, and the values for the other parameters were within the normal range for all subjects. For all dilution scenarios, none of the subjects had abnormal AUC values. Based on these results, we conclude that, for twofold dilution, the vast majority of the subjects retained normal thrombin generation parameters. For other dilution scenarios, thrombin generation was abnormal in all subjects, which could be attributed to the abnormal values in MS and PH.

Robustness of blood dilution effects under TF level variations

TF concentration in vivo might vary depending on the subject, blood vessel, and trauma type.[42] Thus, we wished to investigate the robustness of the results obtained for the default TF concentration value (i.e., [TF] = 5 pmol/L) to changes in [TF]. In the average subject, the pattern FC(MS) > FC(PH) > FC(CT) > FC(PT) > FC(AUC) was found to hold for other TF concentration values, with some exceptions occurring only at [TF] = 2 pmol/L and [TF] = 5 pmol/L (Fig. 4), suggesting that the occurrence of a pattern can depend on TF concentration. Yet, the pattern FC(MS) > FC(PH) > FC(*X*), where *X* is CT, PT, or AUC, was found to hold for the average subject under all considered conditions (Fig. 4). This finding demonstrates that some patterns can be persistent enough to hold over a wide range of TF concentrations.

For all considered TF concentration values and all dilution scenarios, dilution caused significant changes in all thrombin generation parameters ($p < 1.0 \times 10^{-7}$ for each thrombin generation parameter tested independently in the case [TF] = 2 pmol/L and $p < 1.0 \times 10^{-77}$ for [TF] > 5 pmol/L). The pattern FC(MS) > FC(PH) > FC(*X*), where *X* is CT, PT, or AUC, held for the majority of the study group for all dilution scenarios and all considered TF concentration values (Table 4). The frequency of occurrence of the pattern did depend on TF concentration, but for the majority of dilution scenario/TF concentration combinations, the pattern occurred in more than 95% (SE, <1%) of the subjects. This finding suggests that MS was indeed the most sensitive, and PH was the second most sensitive, thrombin generation parameter for most subjects under a wide variety of conditions. Notably, MS and PH demonstrated a coherent response to dilution:

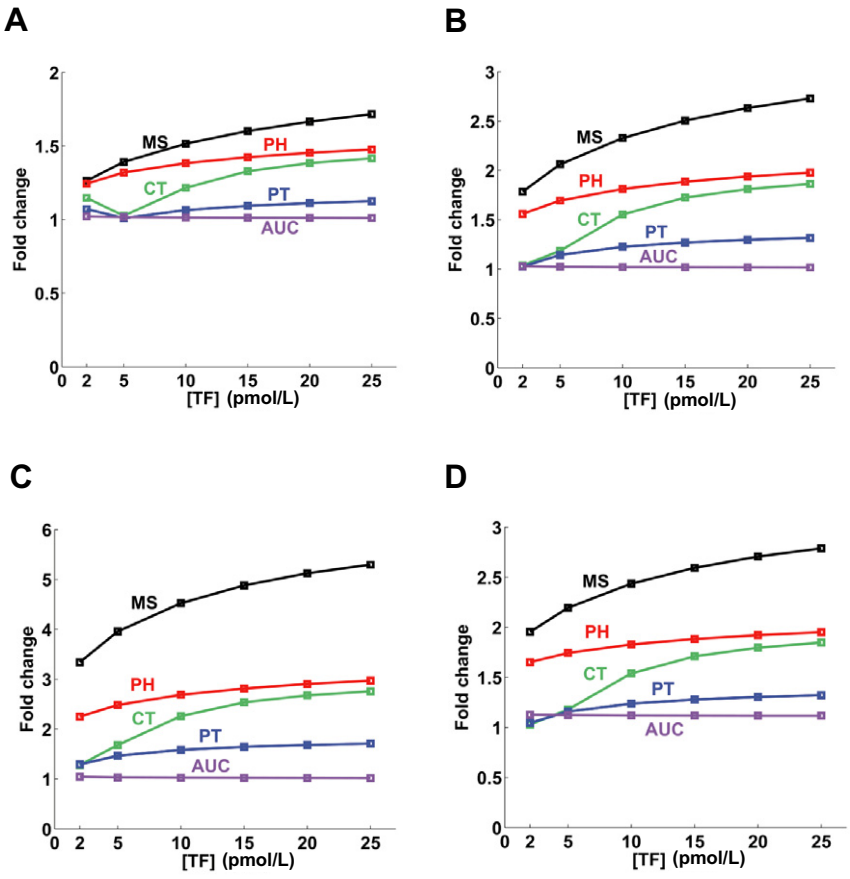

Fig. 4. FC in the thrombin generation parameters in the average subject as a function of TF concentration. The markers designate the computed values for different TF concentrations. (A) Twofold dilution; (B) threefold dilution; (C) fivefold dilution; (D) unequal dilution.

they were decreased by dilution in all of the subjects and for all considered TF concentrations. In contrast, CT and PT were predominantly increased by dilution when [TF] > 2 pmol/L and the degree of dilution was greater than twofold, whereas for [TF] = 2 pmol/L and twofold dilution both of these parameters decreased in more than 94% (SE, <1.01%) of the subjects. AUC decreased upon dilution in every subject. The pattern FC(AUC) < FC(*X*), where *X* represents any other thrombin generation parameter, was detected in more than 90% (SE, <1.40%) of the subjects for all dilution scenarios and all considered TF concentration values, with some exceptions found only for [TF] = 2 pmol/L and [TF] = 5 pmol/L (Table 4).

Model assessment

Full quantitative validation of our modeling predictions would require the determination of thrombin generation curves for all the 472 subjects in our study group. However, such measurements (as well as measurements of thrombin generation curves for another subject group of comparable size) are not available. Yet, an assessment of our modeling approach can still be carried out by comparing our predictions with the published experimental results characterizing the effects of dilution on thrombin generation parameters.

Comparisons with the available experimental data suggested that the computationally predicted effects were in line with in vitro and in vivo data obtained in blood (plasma) dilution experiments, thereby supporting the validity of our modeling strategy. Indeed, in agreement with our predictions for threefold and fivefold proportional dilution and in vivo–type unequal dilution (Figs. 2 and 3, Tables 2 and 3), dilution has been reported to increase prothrombin time, which approximately corresponds to the CT that can be estimated from thrombin generation kinetics.[43-46] The predicted ability of dilution to significantly decrease PH (Figs. 2 and 3, Tables 2 and 3) was confirmed by recent in vitro experiments with human and animal blood, when dilution was performed in vitro and in vivo.[19,20,44,46-48] In agreement with our predictions (Table 4), in vitro experiments showed that PH was affected by dilution noticeably more than PT[17,20,40] or CT.[47,48] As predicted (Figs. 3 and 4, Tables 2-4), the impact of dilution on AUC was reported to be noticeably smaller than its impact on PH.[19,47]

Further experimental evidence supporting our modeling approach was obtained from a porcine model of dilutional coagulopathy induced in vivo.[49] In that work, blood dilution induced an approximately 1.3-fold increase in the median prothrombin time, an approximately 1.9-fold decrease in the median PH, and no significant change in the median AUC. Our computations for the unequal blood plasma dilution model predicted the following changes: an approximately 1.2-fold increase in the median CT, an approximately 1.7-fold decrease in the median PH, and an approximately 1.1-fold decrease in the median AUC. In a study by Brummel-Ziedins and colleagues,[50] the effects of dilution on TF-induced thrombin generation in whole blood were studied by measuring the level of thrombin-antithrombin (TAT) complex at 20 minutes after clotting initiation. While the TAT kinetics are not described by the standard thrombin generation curves (Fig. 1) analyzed in our study, it can be expected that the accumulation rate and amount of TAT would be correlated

TABLE 4. Frequencies of dominant patterns detected in the subject group for varying concentrations of TF and dilution scenarios*

Dilution scenario	TF concentration (pmol/L)				
	2	5	10	15	20	25
Twofold dilution	63.77 (2.21); 86.86 (1.55)	97.46 (0.72); 55.72 (2.29)	100.00; 91.53 (1.28)	99.79 (0.21); 99.58 (0.30)	98.52 (0.56); 99.79 (0.21)	97.03 (0.78); 100.00
Threefold dilution	99.58 (0.30); 46.61 (2.30)	100.00; 94.07 (1.09)	100.00; 100.00	98.94 (0.47); 100.00	97.67 (0.69); 100.00	95.13 (0.99); 100.00
Fivefold dilution	100.00; 95.55 (0.95)	100.00; 100.00	99.79 (0.21); 100.00	98.09 (0.63); 100.00	94.92 (1.01); 100.00	92.16 (1.24); 100.00
Unequal dilution	100.00; 7.42 (1.21)	100.00; 65.47 (2.19)	100.00; 96.40 (0.86)	99.79 (0.21); 99.79 (0.21)	98.31 (0.59); 100.00	96.40 (0.86); 100.00

* Data are presented as frequency (SE); both frequencies and their SEs are shown as percentages. In each table cell, the first of the two data entries gives the percentage of subjects for whom the pattern FC(MS) > FC(PH) > FC(X), where X = CT, PT, or AUC, was detected, whereas the second data entry gives the percentage of subjects for whom the AUC was the thrombin generation parameter with the smallest FC. When the percentage equals 100, the SE is 0 (not shown).

with our parameters MS and PH, respectively. Thus, based on our results (Fig. 3), it can be predicted that both accumulation rate and amount of TAT would be decreased by dilution and that the decrease would be larger for higher degree of dilution. These predictions agree with the reported experimental results.[50]

DISCUSSION

Modern transfusion approaches to massive hemorrhage involve almost exclusive use of blood component and crystalloid therapy, which may lead to severe dilutional coagulopathy during transfusion.[51] However, because dilution impacts *both* pro- and anticoagulant components of the blood coagulation system, the net outcome of thrombin generation will depend on the balance between these two opposite effects.[1] Here we performed a comprehensive computational analysis of dilution-induced effects on thrombin generation. Our analysis has shown that, while there is substantial intersubject variability in the quantitative parameters of thrombin generation, the effects of dilution can be characterized by robust patterns, which hold in the vast majority of the subjects for different dilution scenarios and different TF concentrations. Specifically, robust patterns can be initially identified in thrombin generation analyses for the average subject, that is, the subject with average levels of coagulation factors (Fig. 1B, Fig. 4, Table 2).

In our computations, dilution always decreased MS, PH, and AUC (Fig. 3 and Table 3). CT and PT were often increased by dilution, but also could sometimes be shortened (i.e., shifted toward a higher hemostatic potential) in some of the subjects (Fig. 3). Yet, because such situations were rather rare (for higher degrees of dilution), and because the magnitude of FC in MS and PH was typically larger than those in CT and PT (Figs. 3 and 4, Table 4), our results are consistent with the notion that dilution generally tends to decrease the hemostatic potential of blood plasma. Notably, our results indicate that dilution induces a prohemorrhagic coagulation state by impacting both the initiation and the propagation phases of thrombin generation. Of the five thrombin generation parameters, MS was affected by dilution the most, and PT was the second most sensitive parameter (Figs. 3 and 4, Tables 2-4). AUC was the parameter that was often the least affected (virtually unaffected) by dilution (Figs. 3 and 4, Tables 2-4). These patterns demonstrated persistence under variations in TF concentration.

Dilution impacts the delicate balance of multicomponent, nonlinear feedback interactions in the blood coagulation system.[33,52] Therefore, the relative sensitivities of the thrombin generation parameters to dilution are shaped by complex, quantitative mechanistic factors. Yet, it is possible to suggest an explanation for some of our results. Indeed, the considerable sensitivity of PH to dilution

could perhaps be anticipated because dilution decreases the concentration of thrombin's precursor, prothrombin, thereby decreasing the maximum amount of thrombin that can be generated in the system. Furthermore, because the rate of biochemical reactions is determined by the reactant concentrations, a decrease in prothrombin concentration leads to a decreased rate of prothrombin conversion into thrombin. This gives AT more time to inhibit thrombin, thus contributing to a lower PH. The AUC is known to be generally rather insensitive to variations in the concentrations of the main coagulation proteins, with the exception of prothrombin and AT, which have opposite effects on AUC.[53] Because dilution decreases the concentrations of both prothrombin and AT, their opposite effects on AUC could be expected to somewhat cancel each other out, resulting in a relatively unchanged AUC. From our analyses of thrombin generation curves (Fig. 1), we note that the "simplest" way to decrease PH without significantly changing AUC (and the shape of the thrombin curve) is to decrease MS, which could thus also be expected to be sensitive to dilution. Without a clear picture of the dependence of CT and PT on dilution, their sensitivities could (naively) be placed somewhere between those of MS/PH and that of AUC. This simple argument can be used to interpret some of our results. Yet, because of the nonlinear feedback effects of the plasma (and blood) coagulation components, it is clearly insufficient to predict the computationally derived patterns, such as FC(MS) > FC(PH) > FC(*X*) (where *X* is CT, PT, or AUC) or that AUC is *not* always the least sensitive thrombin generation parameter (Table 4).

Our computational results are supported by published experimental evidence (see "Model assessment"). Yet, some of the reported experimental results can be in disaccord with findings from other laboratories, as well as with our computations. For example, in contrast to our findings, two published works reported a considerable increase in AUC for moderate in vitro human blood plasma dilutions.[17,21] Moreover, these works reported a dilution-induced increase in PH, which is in contradiction with a number of experimental studies and our own computational predictions. Such disparities in experimental findings are reminiscent of the considerable disparities between in vitro data sets characterizing the effects of recombinant FVIIa, which have been attributed to the differences in experimental protocols used by different laboratories.[16] The recently suggested standardization could be an efficient way to avoid such issues for thrombin generation assays,[22,54] and similar measures will likely be useful for other types of hematology experiments. We believe that, by using a validated computational model of thrombin generation, we have avoided some of the biases that can plague thrombin generation assays. Moreover, we elucidated the dilutional effects that are compatible with the current mechanistic view of thrombin generation biochemistry.[11]

Our study was motivated by the need for improved strategies to diagnose and control dilutional coagulopathy during massive transfusion in surgical and trauma patients. In a clinical setting, coagulopathy is typically detected by performing standard in vitro hematologic tests, such as measuring prothrombin time and thromboplastin time, whose sensitivity may be insufficient for early coagulopathy detection.[40,55] Experimental assays that measure the thrombin generation curve may be attractive alternatives to the traditional coagulation tests.[9,54,56] The results of our work suggest that MS and PH are the most sensitive thrombin generation parameters for the detection of dilutional coagulopathy. Based on our findings, we propose that these two parameters might prove effective as primary predictors of dilutional coagulopathy in massive transfusion and fluid resuscitation. Furthermore, our findings indicate that dilutional coagulopathy could be effectively treated by therapeutics that increase MS and PH in human blood plasma, while affecting the other thrombin parameters to a lesser extent.

The limitations of our study were defined by data availability, as well as by our reductionist approach to coagulation analysis for blood plasma. First, our modeling strategy assumes that the rate constants that govern the biochemical reactions in the thrombin generation network are the same for all subjects. As there are currently no data that could allow us to estimate the magnitude of rate constant variations, we chose to analyze how one of the sources of natural variability—the variability in clotting factor concentrations—affects thrombin generation in human populations. Second, the computational model used in this study does not take into account the action of PLTs and the generation-degradation of fibrin.[14,29] Our choice to use this model was aimed to attempt a realistic yet tractable modeling of a system with a relatively high concentration of active phospholipid surfaces, such as activated PLTs in a PLT plug.[16] Furthermore, this choice was based on the understanding that, while the action of PLTs is of critical importance for blood coagulation in vivo,[10] in biochemical assays PLT activity may be difficult to normalize and quantitate, which can be one of the reasons to use phospholipids in thrombin generation assays.[9,57] Our focus on thrombin, rather than on fibrinogen and fibrin, is a reflection of the functional significance of thrombin, which is the defining factor in the conversion of fibrinogen into fibrin and plays a number of other critical roles in the blood coagulation network.[8,9] Finally, our approach to modeling dilution did not take into account the specific type of liquid that dilutes the blood. While it is known that the type of resuscitation fluid can potentially influence thrombin generation kinetics and clot formation,[50,58] we focused on the dilutional effects that can be attributed to dilution itself and thus may be considered common to all diluents.

Coagulopathy in vivo is often multifactorial, with dilution and consumption being exacerbated by hypothermia and acidosis.[3,41] Yet, both hypothermia and acidosis impact the clotting system by decreasing the biochemical activity of its molecular components.[2,59] It may be expected that decreasing the activity of coagulation factors and reducing the concentrations of fully active coagulation factors could produce similar effects on blood clotting.[60] It is thus conceivable that the suggested markers of dilutional coagulopathy can also be used to diagnose other types of trauma-induced coagulopathic conditions.

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CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

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