Predicting prothrombotic tendencies in sepsis using spatial clot growth dynamics

Natalia P. Soshitova[a], Sergey S. Karamzin[a], Anna N. Balandina[c,e], Olga A. Fadeeva[a], Anna V. Kretchetova[b], Gennadiy M. Galstian[b], Mikhail A. Panteleev[a,b,c,d] and Fazoil I. Ataullakhanov[a,b,c,d,e]

Inflammation in sepsis is associated with hypercoagulation that may lead to thrombosis and disseminated intravascular coagulation. Conventional diagnostic assays are poorly sensitive to procoagulant changes in sepsis. Objectives of the article is to study changes in hemostatic state of septic patients using spatial clot growth assay (currently being developed under the trademark of thrombodynamics) and to compare the sensitivity of this method with the sensitivity of conventional methods. Sixteen patients with hematological malignancies and sepsis were enrolled in the study. All patients had been surveyed for a month following the infection onset. Spatial clot growth assay monitors fibrin clot development in a nonstirred thin layer of platelet-free plasma activated by immobilized tissue factor. Clotting time tests, thromboelastography, D-dimer assays were also performed. Spatial clot growth revealed hypercoagulation in six patients. D-dimer levels increase (with vein thrombosis in one case) was subsequently observed in five of them. D-dimer levels did not increase when spatial clot growth was normal. At the next time point, after spatial clot growth assay showed hypercoagulation, the mean D-dimer concentration was significantly higher than after a normal analysis (457 versus 234 μg/l; $P<0.05$); there was no such correlation for other assays. The remaining 10 patients had elevated D-dimer levels on the first day; this either decreased gradually or remained elevated. Spatial clot growth showed normalization in survivors and growing hypocoagulation in nonsurvivors. Measuring spatial clot growth dynamics has potential diagnostic utility for the evaluation of thrombotic risk. *Blood Coagul Fibrinolysis* 23:498–507 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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[a]HemaCore LLC, [b]National Research Center for Hematology, [c]Center for Theoretical Problems of Physicochemical Pharmacology, [d]Department of Physics, Moscow State University and [e]Federal Research and Clinical Center of Pediatric Hematology, Oncology and Immunology, Moscow, Russia

Correspondence to Mikhail A. Panteleev, National Research Center for Hematology, 4 Novyi Zykovskii Pr, Moscow 125167, Russia
Tel: +7 495 612 3522; fax: +7 495 612-8870; e-mail: mapanteleev@yandex.ru

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Introduction

Due to systemic inflammation and cell necrosis in sepsis, blood coagulation becomes activated [1,2] forming conditions for the progression of disseminated intravascular coagulation (DIC) [3]. DIC is a frequent complication of sepsis [4] that leads to microvascular thrombosis and potentially fatal organ dysfunction [5]. This makes monitoring and treatment of coagulation critically important in sepsis [6].

However, conventional diagnostic methods are poorly able to identify the systemic activation phase of coagulation in sepsis [7]. An increase in D-dimer levels and systemically circulating thrombin in a novel amidolytic thrombin activity assay is associated with hypercoagulation in sepsis [8]; however, D-dimers show lysis of already-formed thrombi. Clotting time tests generally have low sensitivity to hypercoagulation [9] and are often prolonged in sepsis because of consumption [10,11]. One study found that global assays, such as thrombelastometry and calibrated automatic thrombinography, showed delayed coagulation activation followed by normal or increased propagation [11]. Hypocoagulation by thrombelastometry was more pronounced in patients with severe DIC [12,13], and this was also reported for thrombin generation [14]. These data do not currently allow to draw definitive conclusions with regard to the usefulness of these assays as prognostic markers for prothrombotic risk during the acute phase of DIC.

The spatial clot growth assay is based on videomicroscopic observation of fibrin thrombus growth in plasma after clotting is activated by immobilized activator and was designed to better mimic some of the in-vivo conditions by taking into account both the biochemical reactions of the coagulation cascade and the diffusion processes [15]. This assay was used previously to study coagulation in hemophilias A and B [15,16], and other coagulation disorders [17,18]. It was also useful for detecting procoagulant changes caused by platelet microparticles [19], recombinant activated factor VII [20], or an aptamer antagonist of tissue factor pathway inhibitor [21]. Therefore, we attempted to evaluate this approach in a study of coagulation in sepsis. Our data indicate that

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spatial clot growth is sensitive to the hypercoagulation state in sepsis and has potential as a prognostic marker of thrombotic risk.

Materials and methods

Patients

Sixteen patients (age 19–60 years, mean 41, median 46; three women and 13 men) from the ICU of the National Research Center for Hematology who had hematological malignancies, myelotoxic agranulocytosis [Table S3, http://en.hemacore.com/ (Supplementary digital content can be downloaded from the authors' website http://en.hemacore.com/ or requested from the authors)] and sepsis were enrolled in the study within 48 h of the infection symptoms appearance. The protocol was approved by the National Research Center for Hematology Ethics Committee. Upon inclusion, all patients were surveyed from the first through the fifth day, and once weekly for 28 days, thereafter. Some received anticoagulation therapy [antithrombin III, activated protein C (APC), and/or heparin]. Spatial clot growth assays, clotting time tests, thromboelastography (TEG) and D-dimer levels were studied [Table S1, http://en.hemacore.com/ (Supplementary digital content can be downloaded from the authors' website http://en.hemacore.com/ or requested from the authors)]. Additionally, levels of coagulation factors and anticoagulants were measured [Table S2, http://en.hemacore.com/ (Supplementary digital content can be downloaded from the authors' website http://en.hemacore.com/ or requested from the authors)].

Reagents

The following materials were obtained from the sources shown in parentheses: polyethylene imine (ICN Biomedicals, Irvine California, USA), 10% lactic acid (LabChem, Moscow, Russia), factor rVIIa (Novoseven, Novo Nordisk, Bagsværd, Denmark), thromboplastin (Renam, Moscow, Russia), chromogenic substrate for fXa S-2765 (Chromogenix, Lexington, Massachusetts, USA), glutaraldehyde (TED Pella, Redding, California, USA), fX (Enzyme Research Laboratories, South Bend, Illinois, USA), and Pathromtin SL, Thromborel S, Test Thrombin Reagent, and D-dimer PLUS (all from Dade Behring, Germany). All other reagents were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). The specific fXIIa inhibitor corn trypsin inhibitor (CTI) was prepared from corn seeds essentially as described [22].

Plasma preparation

Blood was drawn into 3.8% sodium citrate buffer (pH 5.5) at a 9 : 1 blood : anticoagulant volume ratio. The blood was processed by centrifugation at 1500*g* for 15 min to obtain platelet-poor plasma, then at 10 000*g* for 5 min to obtain platelet-free plasma. Before the experiments, the pH of the platelet-free plasma was set to 7.2–7.4 with lactic acid [23].

The assays

The following tests were performed using fresh platelet-poor plasma samples: activated partial thromboplastin time (APTT), prothrombin time (PT; the Quick percentage value was calculated), thrombin time (TT), and D-dimer test. All tests were performed according to the respective manufacturer's instructions using a Sysmex CA-1500-automated analyzer (Sysmex Corporation, Kobe, Japan). TEG was performed using a TEG 5000 Hemostasis Analyzer System and disposable cups and pins with heparinase (Haemonetics Corporation, Baltimore, Massachusetts, USA). The assays were performed between 10 and 40 min after blood collection using citrated blood samples (340 μl) recalcified with 20 μl of 0.2 mol/l $CaCl_2$. The reaction time (R), clot formation time (K), alpha angle (α), and maximum amplitude (MA) were calculated.

Spatial fibrin clot growth dynamics

The spatial clot growth assay was performed using an experimental device provided by HemaCore LLC (Moscow, Russia) and described earlier [21]. Plasma clotting was activated with surface-immobilized tissue factor (TF) [24] and propagated into the bulk of the plasma (Fig. 1a). Three hundred microlitre of platelet-free plasma was supplemented with 12 μl of 10 mg/ml CTI solution and incubated at 37°C for 10 min. Plasma was recalcified with 6 μl of 1 mol/l $CaCl_2$ and placed into prewarmed thin flat experimental chamber. The activator was placed into the chamber to initiate clotting. Fibrin was detected by imaging light scattering every 15 s for 60 min.

Data analysis

On the basis of the image series, the parameters of clot growth were determined for each experiment. Clot profiles (Fig. 1b) as plots of mean light scattering (based on pixel intensity) versus distance from the activator were calculated. For each profile, clot size was determined as the coordinate of half-maximal light-scattering intensity [17]. The following parameters were calculated from clot size versus time plots (Fig. 1c, right): lag time, initial clot growth velocity and stationary velocity. For each experiment, three values were obtained at different areas of activator and averaged. To describe spontaneous activator-independent clotting, mean light scattering in the area of spontaneous clot formation was calculated and plotted versus time (Fig. 1c, left), and the time to spontaneous clot appearance (i.e., to reach 5% of the maximal light-scattering intensity) was calculated. To estimate normal ranges, this test was performed on plasma that was obtained from 15 healthy donors (age 23–62 years, mean 29, median 26; eight women and seven men). The ranges were accepted as means ± S.D.

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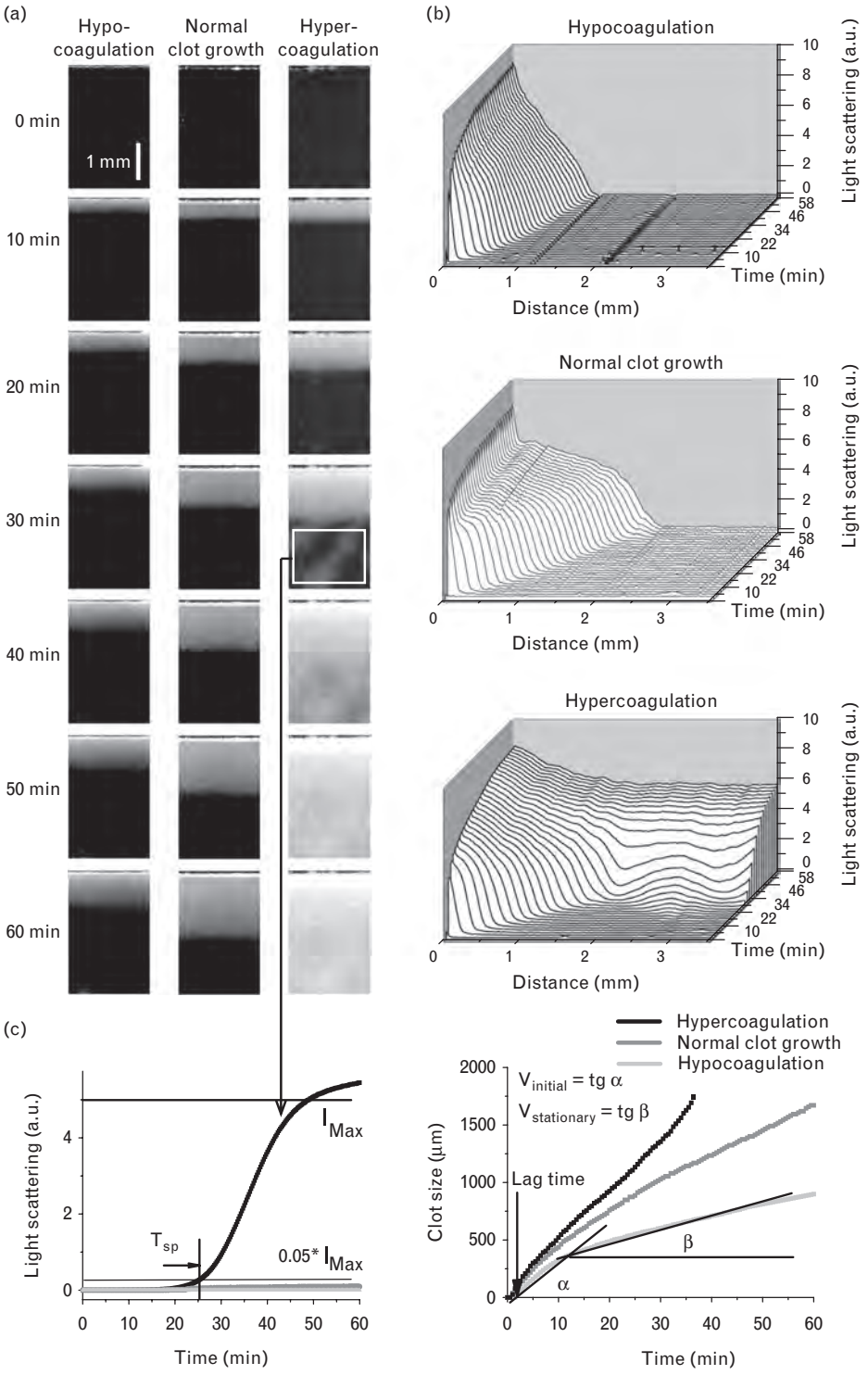
Fig. 1



Spatial clot growth dynamics: sensitivity to the different states of hemostasis. (a) Typical light-scattering time-lapse images of clot growth initiated by immobilized tissue factor (TF) in healthy donor plasma and in plasma from patients. The TF-coated activator is seen as a horizontal black strip at the top of each image. The white rectangle shows an area of spontaneous clot formation. (b) The distribution of light-scattering intensity is plotted against the distance from the activator versus time for the experiments shown in (a). (c) Plots of the average light-scattering intensity in the spontaneous clot formation area (left) and clot size (right) versus time for the experiments shown in (a). The panels also show the following parameters that were used for the experiment analysis throughout the study: lag time, that is time of clot growth initiation; $V_{initial}$ (tg α), that is initial clot growth velocity (mean slope over the first 10 min); $V_{stationary}$ (tg β), that is stationary velocity (slope over the subsequent 30 min) and T_{sp}, that is time of spontaneous clot appearance.

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Statistical analysis

The analyses of statistical differences were performed using the Mann-Whitney U-test with the statistical significance threshold P of 0.05. For the correlation analysis, the Spearman correlation coefficient was calculated.

Results

Spatial clot growth in normal and abnormal cases

Figure 1a shows typical images of clot growth in plasmas of healthy donors and patients with hypocoagulation or hypercoagulation. The fibrin clot began to form after a short time delay (the lag time) on the activator and then gradually propagated into the plasma. In the normal plasma sample, clot grows only from the activator. In hypercoagulation, we observed activator-independent appearance of fibrin clots in plasma. Hereafter, we will call this phenomenon and these clots spontaneous clotting and spontaneous clots, respectively. Based on our investigation of spatial clot growth in healthy donors, the following normal ranges were estimated: 0.4–0.8 min for lag time, 41–51 μm/min for initial clot growth velocity and 22–28 μm/min for stationary velocity. Spontaneous clots did not appear in normal plasma.

The dynamics of the clotting tests parameters for patients with sepsis or septic shock

Figure 2 shows the dynamics of the typical clotting test parameters of a patient with septic shock. This patient had left subclavian vein thrombosis that developed during medical maintenance on the fifth day of supervision. The patient received APC therapy from the onset of infection. On the last day of this therapy, thrombosis occurred, after which heparin therapy (from day 5 through day 22) and low molecular-weight heparin therapy (LMWH, from day 23 through day 27) were initiated.

Spatial clot growth revealed the presence of hypercoagulation (with the appearance of spontaneous clots) in this patient as early as day 4, that is 1 day before thrombosis occurred. Stationary velocity was more sensitive to hypercoagulation state than initial velocity. The D-dimer level rose only on day 5; parameters of APTT, TT, PT and TEG on the day of thrombosis were within their normal ranges. Following the heparin therapy initiation, spatial clot growth registered hypocoagulation, as well as APTT and TEG. On the day of the anticoagulant therapy break, the spatial clot growth assay again revealed a hypercoagulation state; at the end of the observation period (i.e., 1 month after the start of the infection), its parameters returned to normal values. The parameters of APTT, PT, and TT were in the normal range or reflected hypocoagulation over the survey period in agreement with the published data [10]. A similar pattern of clotting times and TEG dynamics was observed for all patients in the study [Table S1, http://en.hemacore.com/ (Supplementary digital content can be downloaded from the authors' website http://en.hemacore.com/ or requested from the authors)]. As the patients get into ICU with drastically different states of coagulation system and received different anticoagulant therapy, we analyzed their data individually. The results of the spatial clot growth method show that 36 of 116 (31%) of the samples were in the hypercoagulation state, and 33 of 36 (92%) of the analyses showed a rise in the velocity of clot growth accompanied by spontaneous clots.

Spatial clot growth and D-dimer in the patients with growing hypercoagulation

The spatial clot growth assay revealed growing hypercoagulation in six of the patients. Hypercoagulation was confirmed in five of these patients by a subsequent increase in D-dimer levels (Fig. 3a). In contrast, D-dimer levels did not rise dramatically when the spatial clot growth parameters in the same patients were in their respective normal or hypocoagulation ranges. Figure 3b shows a statistical comparison of the data sets of D-dimer levels that correspond to the hypercoagulation state versus the normal or hypocoagulation state using the spatial clot growth assay (made on the basis of 39 data pairs of D-dimer levels and stationary velocity values obtained by observing six patients). On the left, the D-dimer levels are divided into two groups based on the stationary velocity values that were measured on the same day. There is no statistically significant difference between the groups. The mean D-dimer level in the data set that corresponded to V_{st} more than 29 μm/min is 402 μg/l, and the level that corresponded to V_{st} less than 29 μm/min is 359 μg/l. On the right, D-dimer level values are divided into two groups based on the stationary velocity values at the time of the previous survey. These groups are significantly different ($P < 0.05$). The mean D-dimer levels are 457 μg/l and 234 μg/l in the data sets that correspond to the hypercoagulation state based on the spatial clot growth assay ($V_{st} > 29$ μm/min) or the normal or hypocoagulation state ($V_{st} < 29$ μm/min), respectively. Thus, spatial clot growth method can identify the hypercoagulation state earlier than an increase in D-dimer levels.

Statistical difference between D-dimer levels in hypercoagulation or normal state versus hypocoagulation as measured by activated partial thromboplastin time and thromboelastography

APTT, PT, TT and TEG did not show hypercoagulation with one or two exceptions. Thus, we compared the D-dimer level datasets that corresponded to the hypercoagulation or normal state versus hypocoagulation state as measured by APTT or TEG only (parameters R and α). The groups of D-dimer levels were divided according to the values of APTT or TEG (parameters R and α) on the same day or at the time of the previous survey. None of the datasets significantly differed (Fig. 4).

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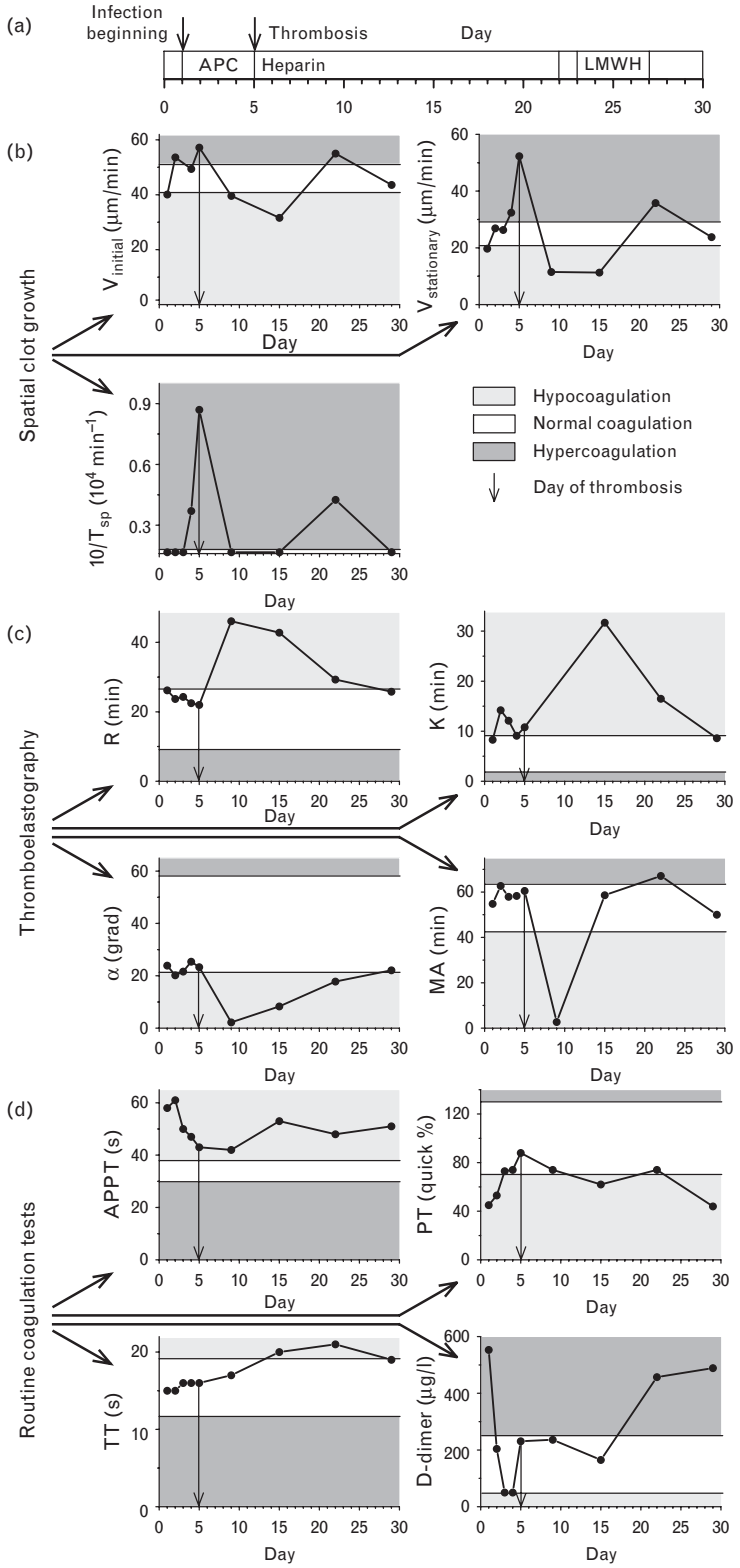
Fig. 2



The dynamics of the clotting tests parameters of a patient with septic shock. (a) Time scale showing the periods of anticoagulant therapy over the time of supervision of the patient from the onset of infection. (b–d) The parameters of the following clotting assays are plotted against time: (b) spatial clot growth, (c) thromboelastography, and (d) clotting time tests and plasma D-dimer level assay. APC, activated protein C; LMWH, low molecular-weight heparin therapy; MA, maximum amplitude; PT, prothrombin time.

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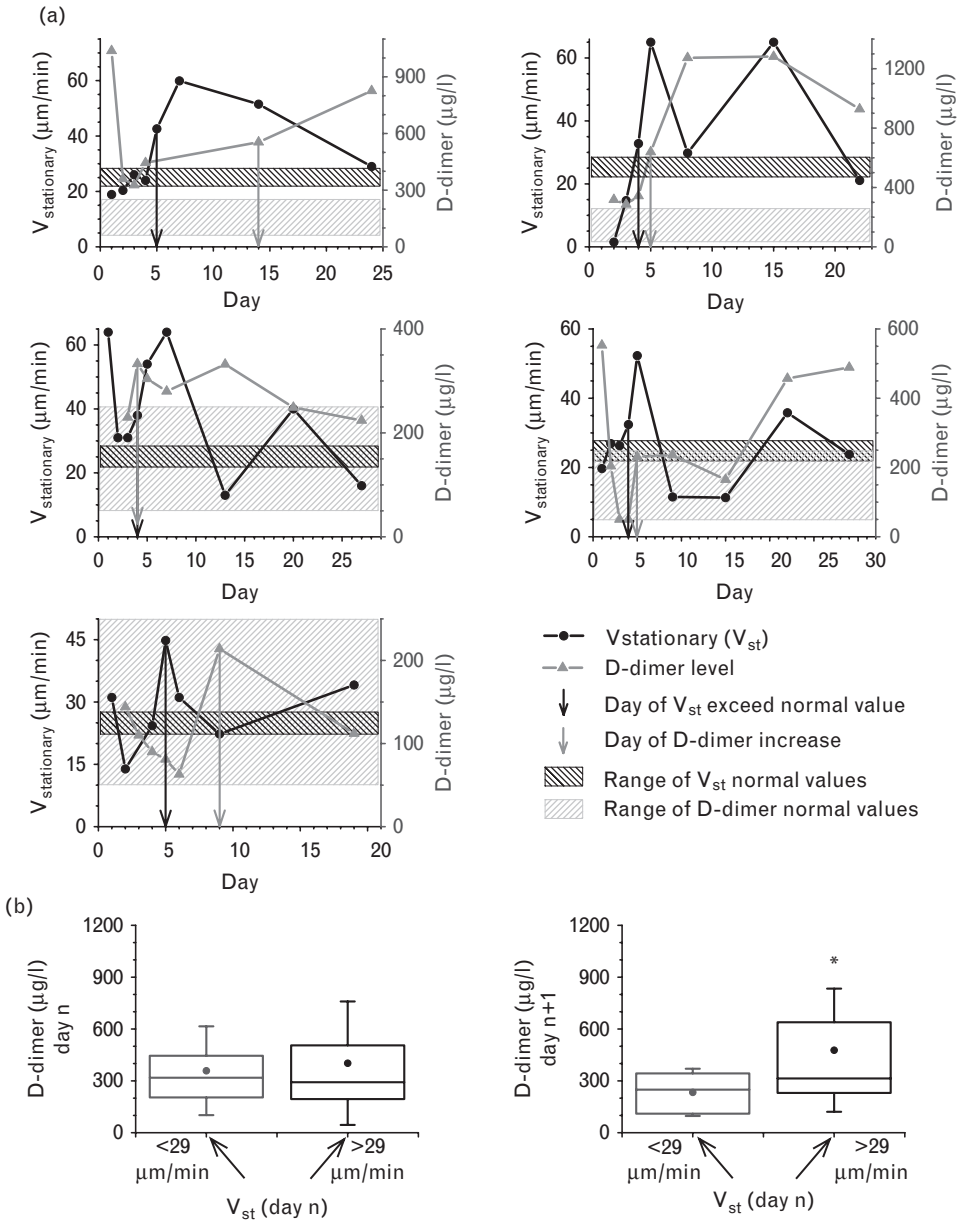
Fig. 3



Comparison of the dynamics of stationary velocity and D-dimer levels. Panel (a) shows the dynamics of stationary velocity and D-dimer levels in five patients over the study period. (b) Statistical comparison of D-dimer levels grouped by the stationary velocity values that were measured on the same day (left), or at the moment of the previous survey (right). The box plots indicate the following parameters: the mean value (the dot inside the box), SD (the ends of the whiskers), the 25th and 75th percentile (the bottom and top of the box, respectively) and the median (the horizontal line inside the box). The asterisk indicates a significant difference between the two groups of D-dimer level values as determined by the Mann-Whitney U-test. The statistical comparison was made on the basis of 39 pairs of data of D-dimer levels and stationary velocities that were obtained from the observation of six patients with grown hypercoagulation.

Dynamics of the coagulation system state in patients without growing hypercoagulation

Another 10 patients had an elevated D-dimer level on the first day of survey. After this day, the level either was gradually reduced or remained elevated. The spatial clot growth assay showed normalization of the coagulation system state in survivors and growing hypocoagulation in the nonsurvivors [Table S1, http://en.hemacore.com/ (Supplementary digital content can be downloaded from the authors' website http://en.hemacore.com/ or requested from the authors)]. Growing hypocoagulation and elevated D-dimer levels in the nonsurvivors are likely to result from progressive consumption coagulopathy in DIC.

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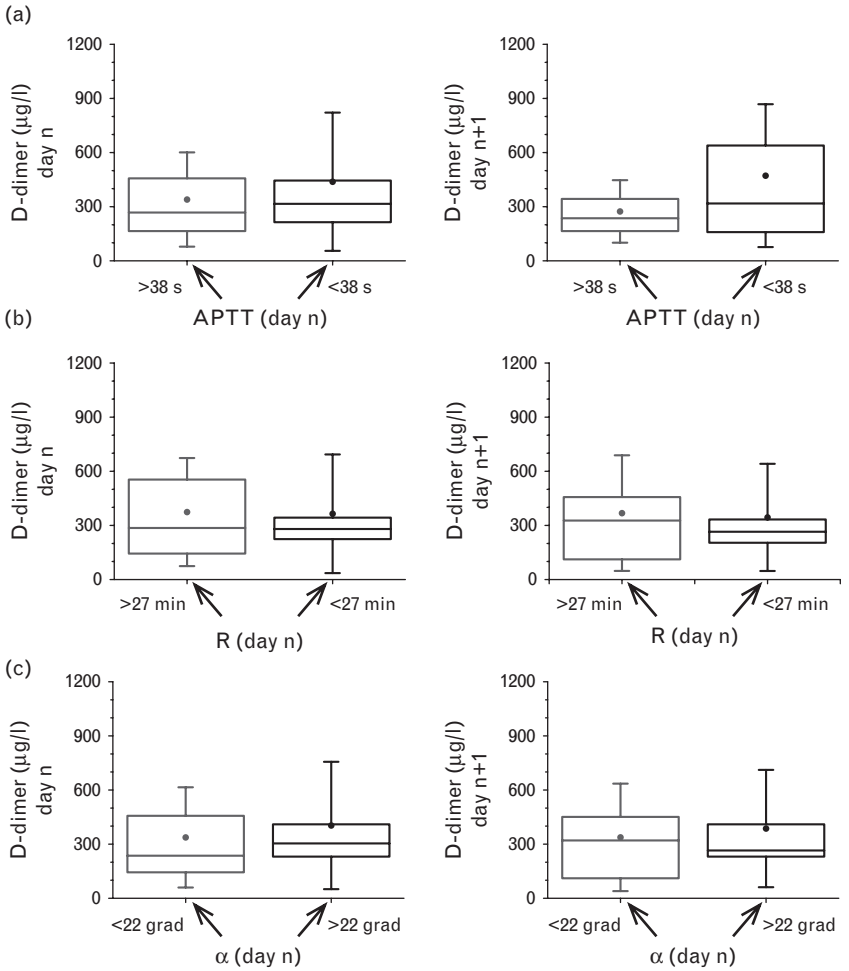
Fig. 4



Statistical comparison of different data sets of D-dimer levels. (a) The box plots correspond to the data sets of D-dimer levels that are divided according to values of activated partial thromboplastin time (APTT) or (b) the thromboelastography parameters *R* and (c) α on the same day (left) or at the moment of the previous survey (right). The box plots show the following parameters: the mean value (the dot inside the box), SD (the ends of the whiskers), the 25th and 75th percentile (the bottom and top of the box, respectively), and the median (the horizontal line inside the box). The data sets do not significantly differ as determined by the Mann-Whitney U-test.

Correlation between the clotting test parameters

Figure 5 shows the correlation between the various parameters that were measured. The Spearman correlation coefficient between APTT and PT is −0.65 with a level of significance (P) of 6×10^{-13}. The coefficient between the TEG parameters *R* and α was −0.82 ($P = 0$). The coefficient between the initial and stationary velocities was 0.74 ($P = 0$). There was no statistically significant correlation between the parameters of the TEG and clotting times. Although the correlation coefficients were low, the stationary clot growth velocity significantly correlates with all of the analyzed parameters.

Discussion

Our main conclusion is that spatial clot growth in plasma can be used to predict an increase in D-dimer levels in sepsis patients. Specifically, increased clot growth velocity is associated with a subsequent increase in D-dimer levels. This study is the first attempt to evaluate the diagnostic utility of this experimental model initially proposed for research [15–21]. Its underlying idea, which it shares with several other models developed over the past 15 years [25–29], is that clotting is a spatially heterogeneous process, and that coagulation factor diffusion is an important controlling component. To take this into account, clotting is activated not by a homogeneously dissolved tissue factor but rather by a surface with immobilized TF. It is known that the sensitivity of clotting assays is increased as the activation level decreases; it could, therefore, be speculated that hypercoagulation would be more readily observed in a system with localized TF.

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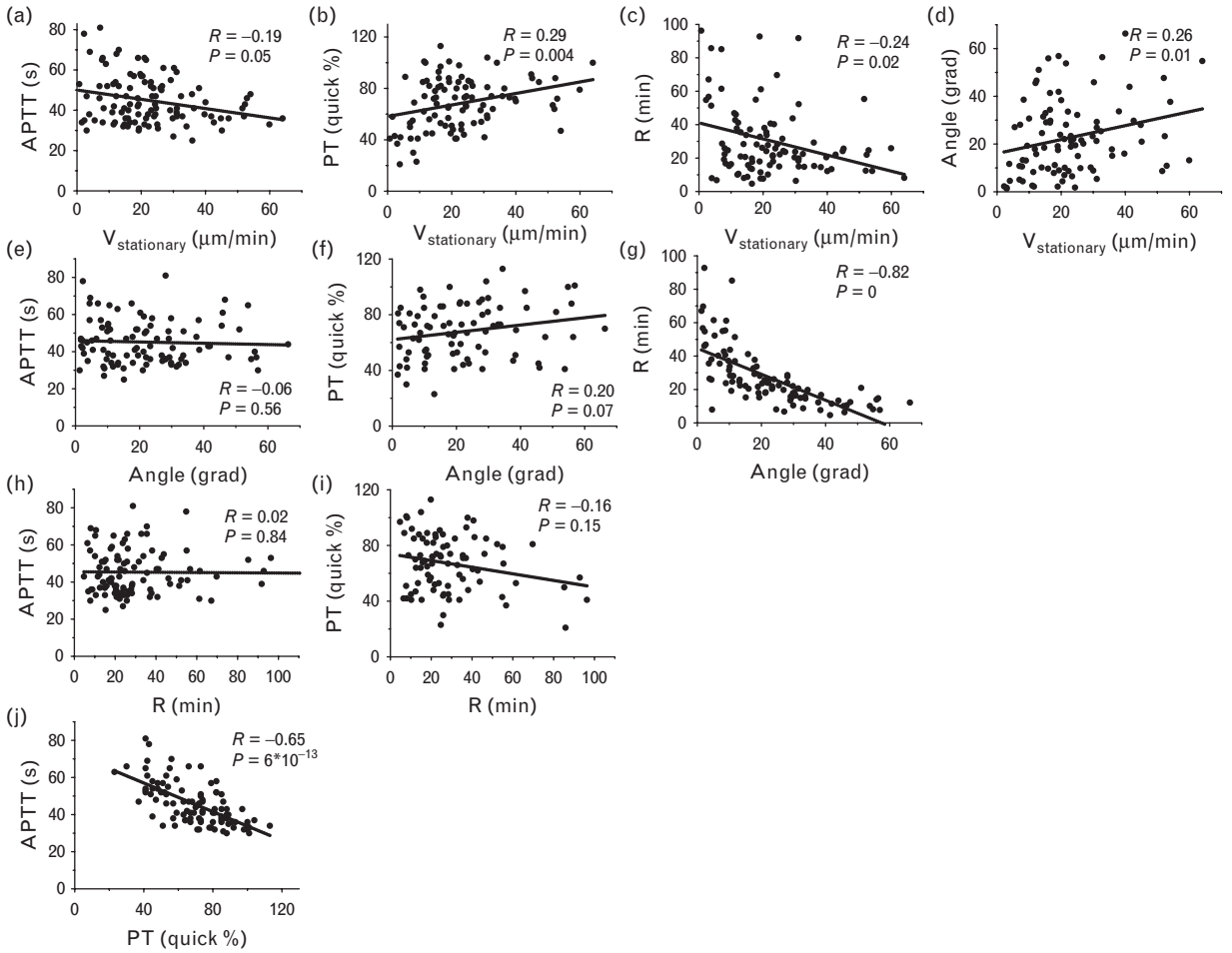
Fig. 5



Correlation between the clotting test parameters. Each dot represents the data of each individual measurement. The lines show the best linear fit of data. Parameters *R* and *P* represent the Spearman correlation coefficient and its statistical significance, respectively.

In our study, we observed increased spatial clot growth closely associated with a subsequent increase in D-dimer levels (and vein thrombosis in one case). A limitation of the conclusions of this study is the relatively small number of included patients. However, each experiment produced a series of data points with a total of 39 pairs, thereby yielding statistically significant preliminary data. We were not able to observe significant hypercoagulation effects in either the routine clotting time tests or TEG measurements, in agreement with previous reports [10,30].

The normal ranges of the spatial clot growth parameters used in our study were obtained with a small selection of young donors and they do not represent true reference ranges. It is likely that older donors may have increased procoagulant activities leading to different reference ranges. This limitation does not affect the main conclusion that the increase in D-dimer levels occurred after stationary velocity in patients exceeded a given level. It is, however, interesting that this level was identical to the upper boundary of the range estimated as 'normal'.

In the spatial clot growth assay, hypercoagulation was characterized by both quantitative and qualitative changes, that is by appearance and growth of spontaneous clots far from the activator. The significance of these clots is currently unclear, but they can be interpreted as evidence of the presence of circulating procoagulant material in the plasma, in agreement with published data regarding coagulation activation in sepsis. In particular, the number of procoagulant microparticles can be increased [31]. The soluble TF isoform can be expressed and released from endothelial cells in response to inflammatory cytokines [32]. Some bacteria release proteinases that can directly activate coagulation [33]. The ultimate cause of the spontaneous clot growth remains to be determined and may vary among patients.

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Conclusions of this study are predominantly based on the comparison of our data with the dynamics of the D-dimer levels; only one thrombosis case was observed. The D-dimer level is an established assay, known to be sensitive to the activation of the hemostasis system in sepsis [30] and to the presence of thrombosis [34,35]; its only drawback is that the D-dimers are products of already-dissolved thrombi. This allows us to use it as a reference to estimate the accuracy of the spatial clot growth method in detecting the hypercoagulation state.

By analogy with thrombin generation and TEG, spatial clot formation is a global coagulation assay; although it is able to determine hypocoagulation and hypercoagulation, it provides limited information about the molecular changes underlying these changes. Additional assays [8] or modifications of this assay [17,18,20,21,36] could be employed to achieve this. In addition, the spatial assay protocol used here is currently labour intensive and inconvenient for wide clinical use. Although it can be used to test diagnostic utility of spatially nonuniform experimental models, a more user-friendly version of the device, software and protocol should be developed.

Additional research is definitely needed to draw reliable conclusions regarding the ability of spatially heterogeneous experimental models to predict thrombosis, and future studies should utilize larger patient cohorts with a sufficient statistics of thrombosis observations. However, the data in the present early study suggest that systems employing clot growth initiated by a surface-immobilized activator could have diagnostic potential and are worth developing.

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Conflicts of interest

There are no conflicts of in N.P.S., S.S.K., O.A.F., M.A.P. and F.I.A. are employees and/or founders of HemaCore LLC that develops the spatial clot growth assay under the name of Thrombodynamics. The work performed by A.N.B. was supported by grants from HemaCore LLC.

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