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Abstract: Introduction: Renewed interest has arisen in the use of thromboelastography/thromboelastometry in evaluating coagulation kinetics. The test medium, type of activator and its concentration may influence the interpretation of coagulation kinetics. This study aimed to investigate methodological influences of activator and test medium on thromboelastometric parameters of coagulation kinetics. Methods: Dynamic clot formation was evaluated by thromboelastometry using whole blood (WB), platelet rich plasma or platelet poor plasma employing different concentrations of extrinsic (tissue factor) and contact activator (synthasil) and with variable concentrations of phospholipids. Results: Plasma samples displayed prolonged clot initiation and enhanced clot propagation compared to WB. Clot firmness was markedly reduced in platelet poor plasma as compared to platelet rich plasma and whole blood. Increasing concentration of activator shortened the clot initiation and increased the velocity of clot propagation whereas terminal clot firmness remained unaffected. Platelets accelerated clot propagation and raised clot firmness. Phospholipids shortened the time of clot initiation and increased velocity of propagation, while clot firmness remained unchanged. Conclusion: Our results demonstrate that evaluation of coagulation kinetics using thromboelastometry vary according to the composition of the test medium, type- and concentration of activator, as well as the presence and concentration of phospholipids in the test reagent.

Response to Reviewers: Response to reviewers.

First and foremost, thank you very much for the positive and constructive review. We have tried our best to answer all questions and revised the manuscript accordingly.

Reviewer #1: This is a nice methodological paper about the ROTEM device, which is well written by an experienced group.

Minor comments

- 1) P3, line 41: physiologically - corrected
- 2) P4, line 28: significantly - corrected

3) Please indicate molar concentrations of synthasil and Innovin, if possible

Unfortunately, it is not possible to use molar concentrations. We are working on a detailed (including molar concentrations and activity) examination of various tissue factor sources, however that reached beyond the scope of the present study.

4) P9: followed - corrected

5) P11, line 38: please add . "in vitro [33] as well as coagulation activation in vivo, when the blood is only re-calcified [PMID: 16420574]"

Thank you for this excellent point, we have added accordingly.

6) I am not sure whether I could reproduce the phospholipid preparation, maybe a graphical scheme could be helpful

We have slightly rephrased

7) As many users of the ROTEM probably will not have the extra software, please consider providing graphical presentations of the CT and /or alpha angle in addition to MaxVel, which is a derived variable

CT has been included. We found it too excessive to also show alpha.

8) Refs 21 and 26 appear identical to me

We have corrected the reference list accordingly

Reviewer #2: The paper by Sorensen et al. is interesting as it covers an important issue when using thrombelastography in the clinical setting, i.e. the possible influence of changes in preanalytical conditions on the results obtained.

The paper is well written. However, I think some issues need further consideration.

1) From a practical point of view, the authors should explain why they choose to compare platelet rich or platelet poor plasma with whole blood; whole blood is the material that is currently most frequently used in the clinical setting.

In the introduction we have mentioned that several studies reporting coagulation kinetics using PPP or PRP. In order to demonstrate the obvious differences between PPP, PRP and WB we performed the comparison

2) Today, thrombelastography is most frequently used in the perioperative setting; however, it is not clear whether the results observed here are relevant in that setting (of increased activation).

The results may be helpful in understanding differences in contact activation and tissue factor activation. Furthermore, it demonstrates the explicit importance of platelets for coagulation dynamics.

3) The authors should also try to clarify whether the changes observed might be clinically relevant, i.e. whether the differences observed might lead to differences in judging the results.

The main aim of the present study was to demonstrate similarities and differences in use of platelet poor plasma, platelet rich plasma as well as whole blood as test medium in thromboelastometric evaluation of coagulation kinetics. Secondly, experiments were carried out to describe the influence of platelets and phospholipids on coagulation kinetics as well as the impact of different types and concentrations of activators. Thus, we didn't aim to target particular clinical scenarios.

4) The discussion seems somewhat excessive. I would suggest to shorten the discussion, to stick with the given results and to abstain from speculations.

We have slightly revised the discussion, however it has been difficult to reduce the length considerably.

Evaluation of coagulation kinetics using thromboelastometry - methodological influence of activator and test medium

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Key words: Thromboelastography, thromboelastometry, coagulation kinetics, whole blood, platelet poor plasma, platelet rich plasma, tissue factor, contact activation, phospholipid, platelets

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Word count: 4242

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Abstract

Introduction: Renewed interest has arisen in the use of thromboelastography/thromboelastometry in evaluating coagulation kinetics. The test medium, type of activator and its concentration may influence the interpretation of coagulation kinetics. This study aimed to investigate methodological influences of activator and test medium on thromboelastometric parameters of coagulation kinetics.

Methods: Dynamic clot formation was evaluated by thromboelastometry using whole blood (WB), platelet rich plasma or platelet poor plasma employing different concentrations of extrinsic (tissue factor) and contact activator (synthasil) and with variable concentrations of phospholipids. **Results:** Plasma samples displayed prolonged clot initiation and enhanced clot propagation compared to WB. Clot firmness was markedly reduced in platelet poor plasma as compared to platelet rich plasma and whole blood. Increasing concentration of activator shortened the clot initiation and increased the velocity of clot propagation whereas terminal clot firmness remained unaffected. Platelets accelerated clot propagation and raised clot firmness. Phospholipids shortened the time of clot initiation and increased velocity of propagation, while clot firmness remained unchanged. **Conclusion:** Our results demonstrate that evaluation of coagulation kinetics using thromboelastometry vary according to the composition of the test medium, type- and concentration of activator, as well as the presence and concentration of phospholipids in the test reagent.

Introduction

During the past decade, renewed interest has arisen in the use of thromboelastography or thromboelastometry for evaluation of coagulation kinetics [1-12]. These viscoelastic analyses benefit by providing a continuous visualization of physical changes occurring during blood coagulation. Thus, kinetic properties of the entire process of clot formation can be derived by combining traditional parameters, such as clotting time and maximum clot firmness with new dynamic parameters such as maximum velocity of clot formation and the time to maximum velocity of clot formation [8,13]. Interpretation of the rate-specific characteristics of clot formation requires a critical evaluation of experimental conditions and profound insight of the biological functions of coagulation proteins and blood cells on the overall regulation of coagulation. Additionally, it may be important to consider the importance of phospholipids supporting the enzymatic properties of coagulation factors [14]. Thus, assembly of coagulation factors by calcium mediated complex bindings between gamma-carboxylated coagulation factors and phospholipids seems crucial for the overall coagulation kinetics. Most commercially available reagents for thromboelastography/thromboelastometry contain activators such as celite, kaolin or tissue factor preparations in protocols developed for study of whole blood coagulation. In whole blood it seems rational to anticipate that platelets and natural phospholipids are present in physiologically relevant concentrations. However, some studies used platelet poor plasma as test medium in a set-up utilizing reagents designed to activate whole blood coagulation. Moreover, the type of activator (tissue factor versus factor XII activator) may constitute an important determinant in interpretation of coagulation kinetics using thromboelastography/thromboelastometry. As of today, the tissue factor pathway is largely acknowledged as the physiological trigger for in vivo generation of a sufficient haemostatic plug [15,16]. Nevertheless, several interesting studies adopted traditional contact activation [17-20]. The final concentration of the activating reagent, whether tissue factor or contact activator, may also influence the characteristics of coagulation kinetics in various coagulopathies. This is exemplified by the completely normal coagulation pattern of factor VIII deficient blood if activated by

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4 a high concentration of tissue factor reagent, while low concentrations of tissue factor
5 results in a distinctly pathologic and compromised coagulation profile characteristic of
6 severe Haemophilia A [8, 21].
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10 The objectives of the present study was to demonstrate similarities and differences in
11 use of platelet poor plasma, platelet rich plasma as well as whole blood as test
12 medium in thromboelastometric evaluation of coagulation kinetics. Secondly,
13 experiments were carried out to describe the influence of platelets and phospholipids
14 on coagulation kinetics as well as the impact of different types and concentrations of
15 activators. We challenged the hypotheses that; *i*) in blood from healthy individuals,
16 thromboelastometric parameters of dynamic coagulation kinetics differ according to
17 the use of platelet poor plasma (PPP), platelet rich plasma (PRP) or whole blood (WB),
18 *ii*) addition of natural platelet-derived phospholipids to PPP would induce significant
19 changes in the thromboelastometric parameters of coagulation kinetics, and *iii*) the
20 type and concentration of activator significantly influences the thromboelastometric
21 parameters of dynamic coagulation.
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32 ***Materials and Methods***

33 **Study subjects**

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36 Following informed consent, blood samples were withdrawn from 3 healthy adult
37 volunteers. All study subjects enrolled were within the normal range of platelet
38 count, prothrombin time, activated partial thromboplastin time, thrombin time, D-
39 dimers as well as levels of fibrinogen and antithrombin. None of the subjects had used
40 acetyl-salicylic acid or non-steroid anti-inflammatory drugs during 7 days prior to
41 blood sampling.
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51 ***Thromboelastometry whole blood coagulation analysis***

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53 Continuous dynamic clot formation profiles was recorded using a ROTEM®
54 Thromboelastometry Coagulation Analyzer (ROTEM® Thromboelastometry, Pentapharm
55 GmbH, Munich, Germany) consistent with our method described elsewhere [8]. In
56 brief, the citrated WB rested for 30 minutes at ambient temperature. The
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thromboelastometry analysis was performed by incubating 300 μ l of blood mixture with 20 μ L of activator (human recombinant tissue factor or a contact activator). Coagulation was initiated by addition of 20 μ l of 200 mM CaCl_2 and all analyses were processed in duplicate and recordings were allowed to proceed for at least 90 minutes. The digital signal from the ROTEM Analyzer was imported into a software program (DyCoDerivAn GOLDTM; AvordusoL, Risskov, Denmark) that calculates dynamic velocity profiles of coagulation as well as derived parameters: maximum velocity (MaxVel) [$\text{mm} \cdot 100/\text{sec}$] of clot formation and the time to MaxVel (t, MaxVel) [sec] [8]. In the present study the whole blood clotting process was characterised as clot initiation, defined as CT, clot propagation phase as defined by MaxVel, and clot firmness defined as MCF.

Activators

Extrinsic activator; Recombinant human tissue factor; (Innovin[®], Dade Behring, Marburg, Germany). Factor XII/contact activation; (Synthasil[®], 0020006800, Instrumental Laboratory Company - Lexington, MA, USA).

Test media

Whole blood

Using minimum stasis and a 21 gauge butterfly needle, blood samples for coagulation analyses were drawn into citrated plastic tubes (VenoJect[®] tubes (Terumo Europe, Leuven, Belgium, trisodium citrate 0.129 mol/L: 3.2 W/V %)), at a volume ratio of 1:10. In addition, 3 ml of EDTA blood were collected for blood cell count. Citrated WB rested for 30 minutes at ambient temperature prior to analysis.

Preparation of freeze-stabilized phospholipid concentrate

A concentrate of natural phospholipids was prepared from an outdated well-blended platelet pool. A mixed portion of 100 mL was transferred to a tube and mixed with 100 ml refrigerated TBS buffer (Tris-buffered saline 50/150 mM, Bie & Berntsen, Herlev, Denmark). The platelet-buffer mixture was distributed in small aliquots to 20 plastic tubes and each tube was centrifuged (177 x g, 15 min at 4°C). The supernatants were

transferred to new plastic tubes and approximate 0.5 ml of the sediment containing fragments of red blood cells was discharged. Hence, the supernatants were centrifuged (2800 x g, 15 min at 4°C). The new supernatants were discharged and the pellet was thoroughly re-suspended in 10 ml TBS buffer. The latter centrifugation and re-suspension was repeated 4 times. Following the final centrifugation, the pellet was dissolved in 1 ml TBS buffer. Platelet count was determined and corrected to $300 \times 10^9/L$ by adding a balanced amount of TBS buffer. The platelet/phospholipids concentrate was stored frozen at -80°C in 500 µl aliquots.

Preparation of platelet poor plasma (PPP)

WB was centrifuged for 25 min at 2800 x g. PPP used for titration experiments with activator and addition of phospholipids was stored in aliquots at -80 °C for at least 1 hour, thawed for 10 min in a 37 °C water bath and finally ultra centrifuged at 13,800 x g for 3 minutes.

Preparation of platelet rich plasma (PRP)

WB was centrifuged for 15 min at 114 x g. Platelet count was measured using a counting chamber (Thoma, assistant, Sondheim, Germany) and phase-contrast microscopy. PRP was adjusted to the pre-determined platelet count by adding PPP.

Laboratory protocol

Titration experiments with activators

Whole blood, PPP or PRP were transferred to pre-warmed ROTEM plastic cups using volumes of 300 µl. Twenty µl buffer (HEPES 20 mM, NaCl 150 mM pH=7.4) or 20 µl tissue factor or synthasil diluted was added providing final tissue factor/synthasil concentrations at 1:17, 1:170, 1:1700, 1:17000 and 1:170000. Coagulation was initiated and re-calcified by addition of a calcium chloride containing buffer (HEPES 20 mM, NaCl 150 mM and CaCl 200 mM, pH=7.4).

Platelet count titration studies

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4 PRP with fixed platelet counts at $500 \times 10^9/L$, $250 \times 10^9/L$, $125 \times 10^9/L$, $64 \times 10^9/L$, 32
5 $\times 10^9/L$, $16 \times 10^9/L$, $8 \times 10^9/L$ and $0 \times 10^9/L$ was transferred to pre-warmed ROTEM
6 plastic cups at volumes of 300 μ l. Twenty μ l buffer (HEPES 20 mM, NaCl 150 mM
7 pH=7.4) was added and coagulation was initiated by the addition of 20 μ l undiluted
8 tissue factor or synthasil (final concentration 1:17) or tissue factor or synthasil diluted
9 in a calcium buffer (HEPES 20 mM, NaCl 150 mM and CaCl 200 mM, pH=7.4) at final
10 reaction mixture concentrations of 1:17000.
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19 *Phospholipid titration studies*

20 PPP was transferred to pre-warmed ROTEM plastic cups at volumes of 300 μ l. Twenty
21 μ l buffer (HEPES 20 mM, NaCl 150 mM pH=7.4) or phospholipid stock giving was added
22 at final reaction mixture concentrations of 1:170, 1:340 1:680 and 1:1360. Coagulation
23 was initiated by the addition of 20 μ l undiluted tissue factor or synthasil (final
24 concentration 1:17) or tissue factor or synthasil diluted in a calcium buffer (HEPES 20
25 mM, NaCl 150 mM and CaCl 200 mM, pH=7.4) at final concentrations of 1:17000.
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33 **Data interpretation**

34 Data is presented using qualitative and semi-quantitative descriptive statistics.
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38 **Results**

39 *Non-activated blood*

40 Following re-calcification only (e.g. omitting activator), WB had the shortest clot
41 initiation, followed by PRP and PPP (Table 1). The MaxVel and MCF were highest in
42 PRP, followed by WB and PPP, respectively (Table 1).
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50 *Titration experiments with activators (Figure 1 panel A-C)*

51 In PPP, PRP, and WB increasing concentrations of activator shortened the time of clot
52 initiation. Noteworthy, following activation with lower concentrations of the contact
53 activator, the CT initially remained stable, however, at higher concentrations the CT
54 shortened although not reaching the levels seen following activation with tissue factor.
55 The propagation phase of clot formation was accelerated following increasing
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4 concentrations of activator. However, the pattern of the changes was distinctly
5 different following activation with TF as compared with the contact activator. Thus,
6 with increasing concentration of TF, the MaxVel grew to reach a maximum value; with
7 excess amounts it seemed to decline. The finding was consistently observed in PPP,
8 PRP, as well as WB. At the selected concentrations of the contact activator, the
9 MaxVel revealed a concentration dependent increase. The MCF was highest in PRP,
10 followed by WB and PPP, likely reflecting the total platelet count. Following activation
11 with either TF or the contact activator at various concentrations the MCF was
12 unchanged.
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22 *Platelet count titration (Figure 2 panel A-C)*

23 The concentration of platelets is of crucial importance for the rate specific
24 characteristics of clot formation. In particular, the MaxVel of clot formation revealed
25 concentration dependent changes; the higher the platelet count the higher the
26 maximum rate of clotting. Platelet counts below $100 \times 10^9/L$ caused a prolongation of
27 the clot initiation. The changes in clot initiation were mainly visible following
28 activation with low concentrations of contact activator, although also noticeable
29 following activation with low concentrations of TF. The MCF increased independently
30 of the type and concentration of activator and reached a plateau at platelet counts
31 greater than $200 \times 10^9/L$.
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42 *Phospholipid titration studies (Figure 3 panel A-C)*

43 Increasing amounts of phospholipids induced a marked shortening in the clot initiation
44 and increased clot propagation, in particular when using low concentrations of
45 activator. Furthermore, the importance of phospholipids seemed to be most
46 pronounced following use of contact activator. At high concentrations of the activator,
47 the impact of phospholipids was less prominent. The MCF remained unaffected by
48 increasing concentrations of phospholipids, and similar to observations gained from
49 other experimental conditions the amplitude was independent of the type and
50 concentration of activator.
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Discussion

The present study demonstrates considerable differences in thromboelastometry parameters of dynamic coagulation when comparing PPP with PRP or WB as test medium. Furthermore, the data emphasized characteristic differences depending on the type and concentration of activator. Finally, titration experiments with increasing concentrations of platelets and phospholipids verified that these components play a crucial role in the dynamics of clot formation.

In recent years, several studies have been published on measurement of thromboelastography/thromboelastometry clotting profiles in order to describe dynamic characteristics of the coagulation system in various experimental and clinical settings. Examples include investigations of the contribution of selected coagulation factors [7], the effect of vasoactive agents, platelet agonists, and anticoagulants [22,23], as well as assessment of storage of donor platelets [24]. Some studies have revealed that different types of activators provide different results in various clinical scenarios, such as e.g. the effect of aprotinin on various activators [25].

Data from this study indicate that the dynamic characteristics of coagulation as evaluated by thromboelastometry are highly dependent upon the experimental conditions selected. The choice of test media is important since PPP, PRP or WB, show pronounced differences in dynamic parameters. The maximum velocity is highest in PRP, followed by WB and PPP. Furthermore, the clotting time is longer in PPP as compared to PRP and WB respectively. When a given volume of whole blood is recalcified, the calcium distributes primarily in the plasma volume, which is only a fraction of the total, due to the substantial red cell volume. This would not be the case for PPP or PRP. So, the whole blood samples probably end up with a higher plasma calcium concentration than PRP/PPP. This could explain the shorter clot initiation times of whole blood. The presence of platelets and phospholipids are important determinants for these observed differences. As visualized by the results, PPP as test medium is very sensitive to phospholipids. Thus, contamination with even

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4 small amounts of phospholipids in PPP, such as microparticle contamination, may
5 contribute as a serious confounder and compromise the reproducibility. Furthermore,
6 the choice of activator, and its concentration may seriously change the properties of
7 coagulation kinetics and their correct interpretation. Data further illustrate a
8 considerable diversity in dynamic parameters using different activators. The clotting
9 time and maximum velocity are parameters showing the most pronounced differences,
10 in particular following use of minute (diluted) amounts of activator. Following
11 increasing concentrations of tissue factor or contact activator the clotting time is
12 shortened. However, even following a high degree of activation, there is still
13 significant differences between tissue factor and contact activator. Increasing the
14 intensity of contact activation results in increasing maximum velocity. The greater
15 importance of phospholipid to activation by the contact initiator may be because the
16 tissue factor reagent already has a fairly large amount of phospholipid in it to keep the
17 TF soluble. Paradoxically, it appears that increasing concentrations of tissue factor
18 activation gave place to a maximum value followed by a slight decline. A plausible
19 explanation could be that moderate amount of tissue factor trigger thrombin
20 generation via activation of FX via TF-FVIIa and activation of FX via TF-FVIIa induced
21 activation of FIX. Thus, defects of the intrinsic FIXaFVIIIa complex can be detected in
22 diluted prothrombin time. With the highest concentration of tissue factor, it may be
23 speculated that thrombin generation predominantly gets activated via FXa triggered by
24 TF-FVIIa without involvement of the intrinsic FIXaFVIIIa complex. The FIXaFVIIIa
25 complex is a significantly more potent activation of FX than TF-FVIIa [14]. Another
26 explanation could be that factor VIIa is saturated with high TF. Tissue factor
27 containing phospholipid vesicles could be unoccupied by factor VIIa. If factor X or
28 factor IX bind to these particles, there will not be a productive VIIa/TF complex to
29 activate them.
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54 A potential limitation of the study is the low sample size of n=3. However, the study
55 aimed to describe characteristic changes and not to evaluate inter-individual
56 variability or reference ranges. Another shortcoming of the protocol was that we did
57 not include various sources and formulation of tissue factor and contact activator. It
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has been reported that various sources and formulations of tissue factor may display distinctly different activities as evaluated by a specific double monoclonal antibody fluorescence-based immunoassay [26]. Several other analytical variables have not been investigated. Recent pioneering work by KG Mann et al. have shown that citrate chelation and re-calcification itself may significantly change the dynamics of thrombin generation and thromboelastometry clotting profiles; in particular when the process is activated with minute amounts of tissue factor [27]. Furthermore, our study did not account for changes related to spontaneous artificial contact activation; a process that might have been quenched by using a corn trypsin inhibitor [28]. It is also important to notify that lack of flow in the test-system and the lack of interaction with the vascular endothelium constitutes another important limitation of thromboelastometry. The present study focused on clot initiation, clot propagation, and clot firmness. Increasing numbers of studies have been utilising thromboelastography/thromboelastometry to address dynamic changes of clot strength or resistance against accelerated fibrinolytic activity [29-31], however, that was not addressed in the present methodological evaluation. Finally, it would have been interesting to correlate our findings to direct measurement of thrombin generation, although recent studies have demonstrated that thromboelastometry represents a useful surrogate measure of thrombin generation in whole blood in vitro [32], as well as coagulation activation in vivo, when the blood is only re-calcified [33].

So far, there is no agreed consensus on pre-analytical procedures, the preferred test medium, as well as the type and concentration of activator. Current practice allows for considerable variations in the type and concentration of activators used. Consistent with previous observations, our results demonstrate that extrinsic and contact activation exerts different effects on thromboelastometry variables. Several investigations made in our laboratory have suggested that activation with low-dose tissue factor may be advantageous since this pathway appears to represent the physiological initiator of in vivo whole blood clot formation and allows for phenotypic characterization of a broad spectrum of coagulation disorders. However, based on

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4 ongoing and future studies it seems rational to use corn trypsin inhibitor stabilized
5 citrated whole blood [34,35].
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10 In conclusion, the results of the present study suggest that evaluation of coagulation
11 kinetics using thromboelastometry requires critical interpretation based on a series of
12 experimental variables such as test medium, type, and concentration of activator, as
13 well as the presence and concentration of phospholipids in the test reagents.
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Legends

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33 **Table 1** Thrombelastographic parameters in non-activated, re-calcified whole blood,
34 platelet rich plasma (PRP) and platelet poor plasma (PPP). MaxVel; Maximum Velocity,
35 MCF; Maximum clot firmness. Data presented as mean±SE. N=3
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41 Figure 1 Thrombelastographic variables CT (clotting time), MaxVel (maximum velocity)
42 and MCF (maximum clot firmness) employing increasing concentrations of extrinsic
43 (tissue factor (solid black)) or contact activator (synthasil (short dash, dotted
44 symbols)) in three different test media; ● whole blood, ▲platelet rich plasma and ■
45 platelet poor plasma.
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51 Figure 2 Thrombelastographic variables CT (clotting time), MaxVel (maximum velocity)
52 and MCF (maximum clot firmness) in plasma with increasing platelet count activated
53 with tissue factor (solid black) or contact activator synthasil (short dash, dotted
54 symbols) at two different concentrations ▼diluted (final concentration 1:17000) or ◆
55 undiluted (final concentration 1:17).
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6 Figure 3 Thrombelastographic variables CT (clotting time), MaxVel (maximum velocity)
7 and MCF (maximum clot firmness) in platelet poor plasma with increasing amounts of
8 phospholipids activated with tissue factor (solid black) or contact activator synthasil
9 (short dash, dotted symbols) at two different concentrations ▼ diluted (final
10 concentration 1:17000) or ◆ undiluted (final concentration 1:17).
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Table 1

	Whole blood	PRP	PPP
Clotting time (sec)	612±33	771±25	885±65
MaxVel (mm*100/sec)	8.1±1.4	14.1±1.0	5±0.9
MCF (mm*100)	4966±331	6555±146	2261±221

Figure 1-3

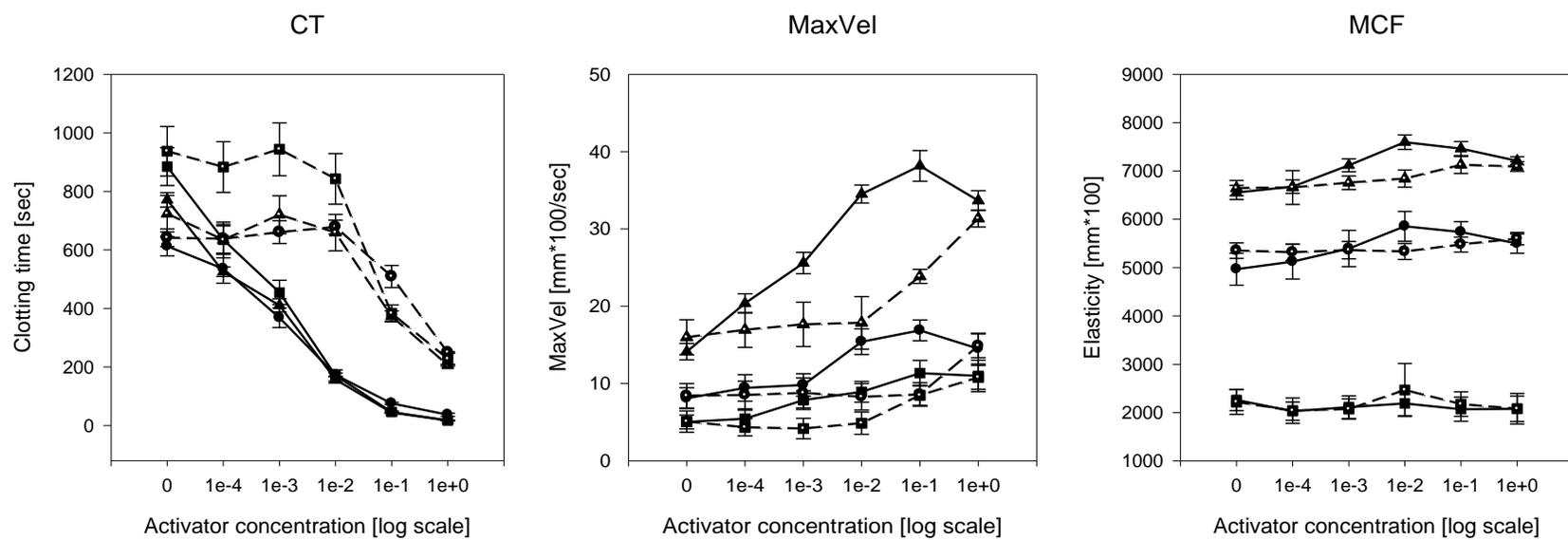


Figure 1

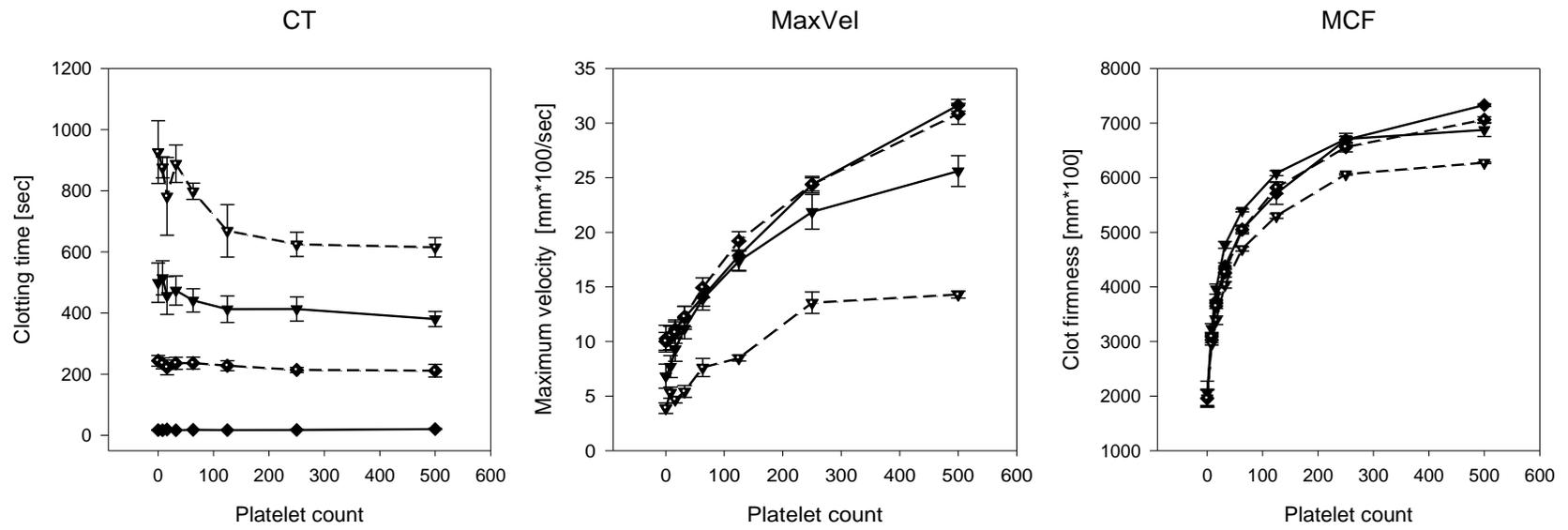


Figure 2

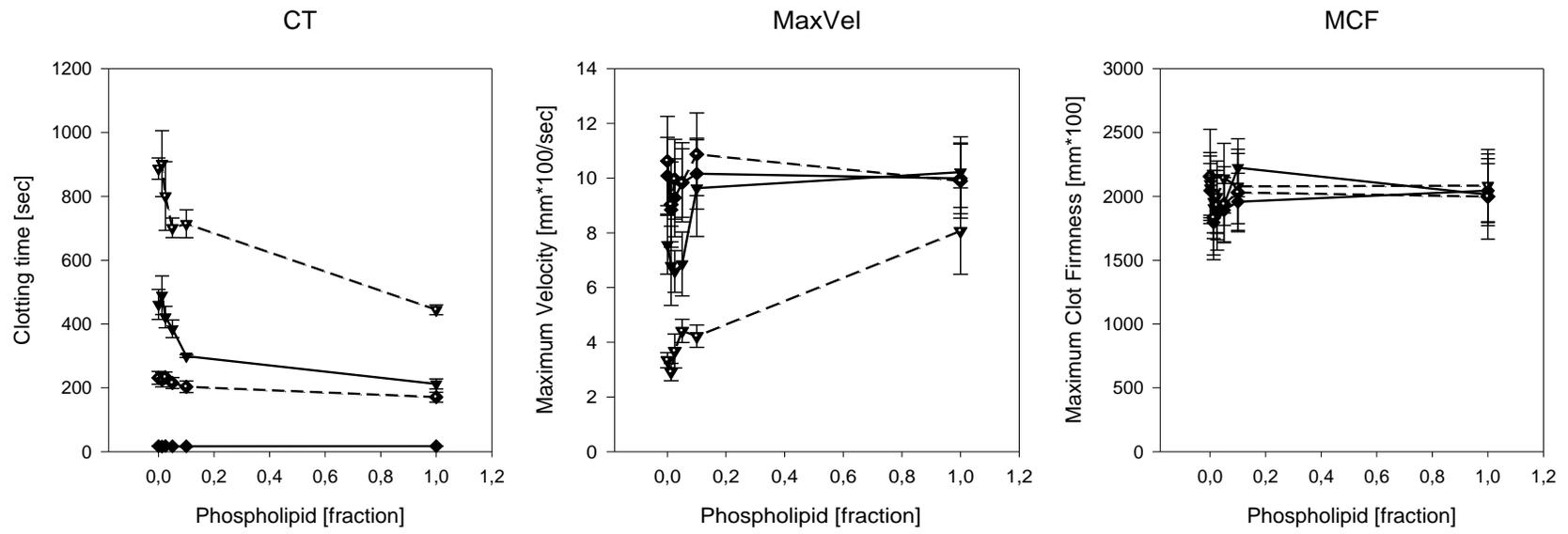


Figure 3

***Conflict of interest**

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