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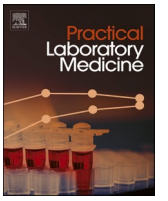

Research Article

Impact of blood centrifugation on the parameters of thrombin generation assay revisited to look for possible revision of the current guidance

Erica Scalambrino [a], Marigrazia Clerici [a], Flora Peyvandi [a,b], Armando Tripodi [a,c,*]

[a] IRCCS Ca' Granda Maggiore Hospital Policlinico Foundation, Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Milano, Italy
[b] Department of Pathophysiolgy and Transplantation, Università degli Studi di Milano, Milano, Italy
[c] Fondazione Luigi Villa, Milano, Italy

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ABSTRACT

Blood centrifugation affects thrombin generation assays (TGA). Current guidance recommends double-centrifugation, which is uncommon in clinical laboratories.

We evaluated the impact of 4 centrifugation speeds on TGA performed with low-triggers (1pM tissue-factor/1.0 μM phospholipids) or high-triggers (5pM tissue-factor/5.0 μM phospholipids). TGA parameters were evaluated in the presence/absence of thrombomodulin.

We included 20 healthy subjects. Centrifugation speeds were: (i)Double-centrifugation: blood at 2,500g(15min) and plasma at 2500(15min) (reference method). (ii)Single-centrifugation at 3,000g(20min). (iii)Single-centrifugation of blood at 3,000g(20min), plasma freezing, then centrifugation of thawed plasma at 10,000g(5min). (iv)Single-centrifugation at 1,700g(10min). Results were also expressed as percentage difference relative to reference centrifugation.

Lag-time was affected when centrifugation speed was relatively slow (1,700g), regardless of low- or high-triggers, presence or absence of thrombomodulin, whereas it was scarcely affected by centrifugation at 3,000g. Peak-thrombin was marginally affected at relatively low-speed (1,700g). ETP was marginally affected at relatively low-speed (1,700g), except when TGA was performed in the presence of thrombomodulin. Peak-thrombin and ETP were not or were poorly affected by centrifugation at 3,000g or 10,000g after thawing, respectively.

In conclusion, slow-centrifugation (1,700g) had a considerable impact on lag-time. This centrifugation speed represents common practice in clinical laboratories and should not be used for TGA, unless controls samples centrifuged at the same speed are used for comparison. Single-centrifugation at 3,000g may be a suitable alternative, which would allow TGA testing without the complex and time-consuming double-centrifugation as recommended by current guidance. We propose that current guidance on plasma preparation for TGA be switched from double-to a more intense single-centrifugation.

1. Introduction

The thrombin generation assay (TGA) is a global coagulation procedure designed to assess the balance of pro-vs-anticoagulants

* Corresponding author. Via Pace 9, 20122, Milano, Italy.
E-mail address: armando.tripodi@unimi.it (A. Tripodi).

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operating in blood [1]. Because of its characteristics, TGA represents a powerful laboratory tool to investigate clinical conditions characterized by hypo- or hypercoagulability [1]. TGA is performed on platelet-poor plasma, prepared by blood centrifugation to remove platelets and blood cells. However, despite its promises, the application of TGA in clinical laboratories is still limited to research purposes and much less to manage patients with coagulation disorders [1]. We speculate that the call for preparing samples by double-centrifugation (i.e., first blood centrifugation followed by plasma centrifugation) is one of the reasons why TGA is poorly used in clinical laboratories.

Among the pre-analytical variables that may affect TGA, centrifugation is one of the most important as it impacts on platelets removal. Platelets are hardly removed from plasma, unless centrifugation speed is relatively high. On the other hand, excess residual platelets in plasma are detrimental to TGA as they are rich of the negatively-charged phospholipids (i.e., phosphatidyl-serine). Phosphatidyl-serine is instrumental to make vitamin K dependent coagulation factors adhering to the surface of activated platelets and speed up thrombin generation. In the TGA the content of phosphatidyl-serine and other phospholipids is optimized by addition of a blend of exogenous synthetic molecules. The effect of residual platelets in plasma is particularly detrimental when TGA is performed for plasma stored frozen. During the cycle of freezing-thawing, platelets are fragmented. Hence, the content of phosphatidyl-serine is heavily and unpredictably increased. Double-centrifugation is typically recommended to remove platelets when preparing plasma samples used for TGA [2,3]. To this end, consensus protocols [3] recommend centrifugation of blood at 2,500g for 15 min to obtain platelet-poor plasma. Then, supernatant plasma is carefully removed, taking care not to disturb the buffy-coat and centrifuged at 2, 500g for 15 min.

The double-centrifugation procedure is time consuming and contrasts with the centrifugation commonly performed in clinical laboratories engaged in routine coagulation testing. Blood samples that are collected for coagulation tests are typically subjected to single (relatively slow) centrifugation and should not be used for TGA. A typical example is TGA when used for research purposes in epidemiological studies to establish whether it is a good predictor of the occurrence of cardiovascular diseases or mortality [4,5]. Blood samples collected during epidemiological studies are generally centrifuged at relatively low speed that inevitably results in variable residual platelets. This could be one of the reasons to explain the variability of results and conclusions stemming from recently published epidemiological studies that employed TGA. For example, von Paridon et al. [4] in their study of nearly 5000 healthy subjects from the population-based Gutenberg Health Study, who were followed up for a median of 9 years concluded that there was a significant association between the overall mortality and increased endogenous thrombin potential (ETP). In contrast, in a recent study de Laat-Kremers et al. [5] while investigating the prospective Moli-sani cohort showed that mortality is associated with decreased rather than increased ETP. While plasma samples in the study of van Paridon et al. [4] have been prepared with a single-centrifugation (2,000g for 10 min), in the study of de Laat-Kremers et al. [5] the centrifugation procedure was not specified. Whether the different plasma preparation explains the contrasting results between the two studies is unknown, but it is likely that it might play a role.

Studies on the effect that residual platelets, negatively charged phospholipids [6] or centrifugation speed [7–9] may have on TGA parameters have been reported, but in some of these studies single triggers' concentrations were used and the addition of thrombomodulin was not considered.

With this as background, we undertook a study aimed to evaluate the impact on TGA parameters of single-centrifugation performed at different speeds versus double-centrifugation taken as reference.

2. Methods

Blood samples from 20 healthy donors [14 males, median age 38, (min-max) 27–60 years] recruited in small groups over 10 days were collected into vacuum-tubes containing 1/10 volume of 109 mM trisodium citrate (Vacuette, Greiner Bio-One International, Kremsmünster, Austria). The study was approved by the institutional review board. It does not require formal approval of ethics committee as it is considered a work done within the frame of activity of calibration of local diagnostic equipment. Healthy donors gave informed consent to participate. We investigated four different plasma preparation stemming from various centrifugation speeds detailed below. These centrifugation procedures may encompass different situations related to residual platelets numbers and associated negatively-charged phospholipids. To allow for fair comparisons of results obtained with different centrifugation procedures, plasma samples for all procedures were prepared by the same operator.

A. Double centrifugation: blood at 2,500g for 15 min following by centrifugation of plasma at the same speed and time. This centrifugation procedure represents (and is called throughout the manuscript) the reference centrifugation.
B. Single centrifugation at 3,000g for 20 min. This was pragmatically chosen, as it combines longer time and relatively high-speed centrifugation that could limit residual platelets without modifying substantially the laboratory practice.
C. Single centrifugation of blood at 3,000g for 20 min followed by plasma freezing (−70 °C) and subsequent centrifugation of thawed plasma at 10,000g for 5 min. This procedure was chosen based on the following rational. During the cycle of freezing and thawing, residual platelets in plasma are fragmented. Hence, plasma samples presumably contain variable and unpredictable amounts of platelet debris that are rich of negatively-charged phospholipids. The plasma centrifugation at high-speed after thawing and before testing could (hopefully) eliminate the platelet debris and associated phospholipids.
D. Single centrifugation at 1,700g for 10 min. This procedure is representative of the centrifugation routinely performed in clinical laboratories engaged in coagulation testing.

Plasma preparation, including blood drawing, centrifugation and plasma storage (see below) was completed within 2 h. Plasma samples stemming from the above centrifugation procedures were aliquoted in plastic capped tubes, immersed in liquid nitrogen and

stored frozen at −70 °C until testing that was performed no longer than six months from blood collection and plasma preparation. To limit the effect of the methodological variability equal numbers of plasma samples stemming from each of the centrifugation speeds were included in each test session. TGA was evaluated using two reagents' compositions by means of a previously described homemade method [10] according to Hemker et al. [11]. The first reagents' composition involved the activation of coagulation upon addition to plasma of small amounts of human recombinant tissue factor (1 pM) (Recombiplastin 2G, Werfen, MA), a blend of synthetic phospholipids (1.0 μM) (AvantiPolar, AL) and calcium chloride. This is called t**hroughout** the manuscript as low-triggers. In the second reagents' composition, coagulation was initiated using higher concentrations of tissue factor (4 pM) and synthetic phospholipids (5 μM) (called high-triggers). TGA parameters were measured using a synthetic fluorogenic substrate, Z-Gly-Gly-Arg-AMC HCl (417 μM) (Bachem, Switzerland), and a dedicated fluorimeter, Fluoroskan Ascent (ThermoLabsystem, Helsinki, Finland), equipped with a dedicated software, Thrombinoscope (Thrombinoscope, Maastricht, The Netherlands). The software generates the thrombin generation curve and calculates the parameters that describe the dynamic of thrombin generation and decay. We recorded the following parameters. (i) Lag-time, defining the time (minutes) elapsing from the addition of the triggers and the initiation of thrombin generation. (ii) Peak-thrombin concentration (nM). (iii) Endogenous thrombin potential (ETP), expressed as nM × minutes, which is the area under the thrombin generation curve and represents the total amount of thrombin that can be generated under the experimental conditions based on the driving forces of the procoagulants as opposed by the anticoagulants. To mimic the conditions operating in vivo, TGA was also performed in the presence of 2 nM soluble rabbit thrombomodulin (Haematologic Technologies, VT). Thrombomodulin is the physiological activator of protein C and is located on the membranes of endothelial cells and much less in plasma. Thrombomodulin concentration in the assay system was adjusted to achieve a 50 % reduction of the ETP of a homemade pooled normal plasma when tested with low-triggers. The same concentration was used for plasma tested with high-triggers.

2.1. Statistical analysis

Results were reported as median and interquartile range (IQR). Median values for each of the investigated parameters and centrifugation speed were compared with those obtained for the same subjects using the reference centrifugation, and analyzed with non-parametric Wilcoxon signed-rank tests for paired data. Analysis for multiple testing correction has not been performed. P values $<$ 0.05 were considered statistically significant. Results for each TGA parameter obtained with each of the centrifugation speeds were also expressed as percentage difference relative to reference centrifugation. Analyses were performed using the SPSS statistical package (SPSS, IL).

3. Results

Table 1 shows the median and interquartile range (IQR) of TGA parameters obtained when testing plasma samples stemming from the four different centrifugation speeds. Fig.s 1-3 show the actual distribution of results from individual subjects obtained with the four centrifugation speeds. To make easier the evaluation of the impact that centrifugation may have on TGA parameters, results for each parameter obtained with each of the centrifugation speeds were expressed as percentage difference relative to reference centrifugation. Table 2 shows the median percentage (IQR) difference obtained for each TGA parameter when compared with the standard centrifugation.

Lag-time. Inspection of the graphs shows that the inter-individual variability of results was the largest when plasma samples were tested with low-triggers, regardless of the centrifugation speed (Fig. 1). In contrast, the inter-individual variability was the smallest

Table 1
Comparison of thrombin generation assays parameters for plasma from heathy subjects prepared by different centrifugation speeds. Results are reported as median and interquartile range (IQR) and analyzed with non-parametric tests. Median values for each of the investigated parameters were compared with those obtained for blood from the same subjects centrifuged by the reference procedure.
p-values <0.05 were considered as statistically significant. *,**, ***, p < 0.05, <0.01, <0.001.

Median (IQR) p-value vs A (Reference)	Low-triggers			Low-triggers + Thrombomodulin		
	Lag-Time (min)	Peak (nM)	ETP (nM x min)	Lag-Time (min)	Peak (nM)	ETP (nM x min)
A, Reference	8 (6–9)	345 (301–368)	2046 (1834–2303)	10 (9–11)	330 (267–372)	1538 (1271–1712)
B	7 (6–8)***	310 (263–346)**	1991 (1831–2300)	9 (7–10)***	280 (242–345) *	1407 (1212–1703).
C	8 (6–8)**	298 (252–333)**	1861 (1746–2202)**	9 (7–11)**	233 (195–324)***	1149 (895–1413) ***
D	6 (5–6)***	353 (334–377)**	2154 (1980–2409)**	7 (6–7) ***	341 (299–374)	1839 (1621–1994) ***
	High-triggers			**High-triggers + Thrombomodulin**		
A, Reference	4 (3–4)	470 (423–499)	2227 (2090–2458)	4 (4–5)	464 (430–487)	2073 (1878–2325)
B	3 (3–4)***	433 (406–482)**	2218 (2018–2462)	4 (3–4)***	442 (403–479)***	2065 (1802–2290).
C	4 (3–4)*	443 (418–498)	2170 (1996–2444)**	4 (4-4)**	439 (406–479)**	2006 (1746–2178)***
D	3 (3-3)***	420 (402–474)***	2265 (2071–2531)	3 (3–4)***	411 (398–461)***	2104 (1872–2297)

A. Double centrifugation: Blood at 2,500g (15min) and plasma at 2500 (15 min) (reference method).
B. Single-centrifugation at 3,000g (20min).
C. Single centrifugation of blood at 3,000g (20min), plasma freezing, then plasma centrifugation at 10,000g (5min) after thawing.
D. Single centrifugation at 1,700g (10min).

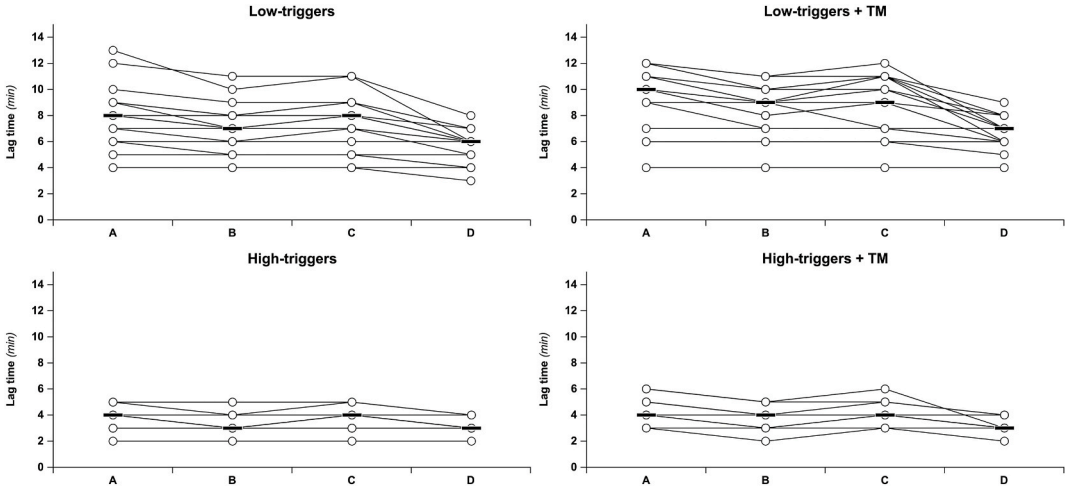

Fig. 1. Distribution of the individual lag-time values for healthy subjects when measured for plasma prepared by different centrifugation speeds. Testing was performed with low- or high-triggers' reagents concentrations, absence or presence of thrombomodulin (TM). **A.** Double-centrifugation: Blood at 2,500g (15min) and plasma at 2500 (15 min) (reference centrifugation). **B.** Single-centrifugation at 3,000g (20min). **C.** Single-centrifugation of blood at 3,000g (20min), plasma freezing, then plasma centrifugation at 10,000g (5min) after thawing. **D**. Single-centrifugation at 1,700g (10min). Bold horizontal bars represent median values. For statistical significance see Table 1.

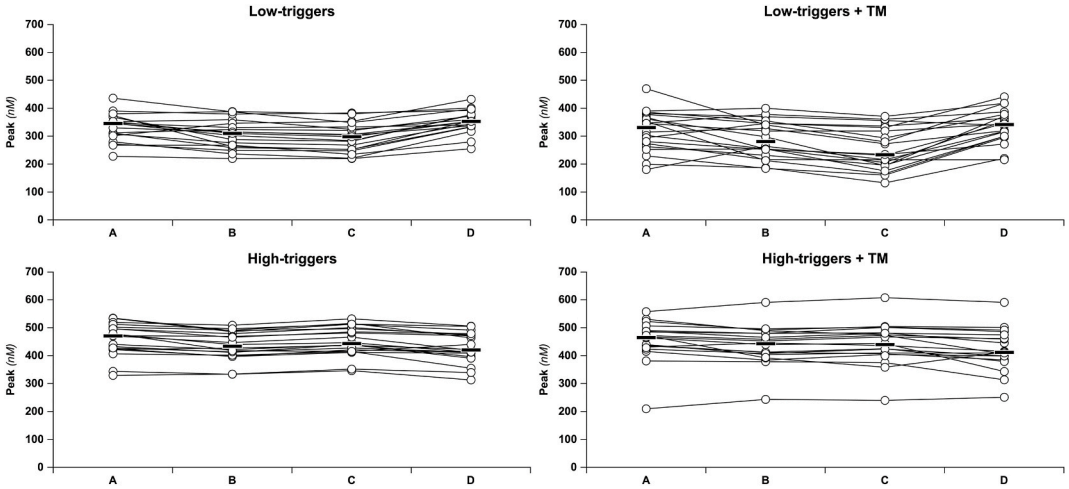

Fig. 2. Distribution of the individual peak-thrombin values for healthy subjects when measured for plasma prepared by different centrifugation speeds. See also legends to Fig. 1.

when testing was performed with high-triggers (Fig. 1). These results were similar regardless of whether testing was performed in the presence or absence of thrombomodulin (Fig. 1).

The median lag-time obtained for the four centrifugation speeds (in the presence of low- or high-triggers' concentrations, with and without thrombomodulin) showed statistically significant differences compared to the reference centrifugation in all centrifugation conditions (Table 1, Fig. 1). However, the average percentage difference for each centrifugation speed relative to the standard centrifugation was relatively small, ranging from 8 % to 11 % when using single centrifugation at 3000*g* for 20 min and low-triggers' concentrations in the absence or presence of thrombomodulin or 10 % when using high-triggers concentrations regardless of thrombomodulin. The average percentage differences were much smaller (i.e., 2–6 %) when plasma samples were centrifuged at high-speed after thawing. Finally, percentage differences were much larger (16–36 %) when plasma samples were centrifuged at relatively low-speed (1,700g) (Table 2).

Peak-thrombin. The inter-individual variability of results was similar regardless of testing with low- or high-triggers, presence or absence of thrombomodulin and centrifugation speed (Fig. 2).

The median peak-thrombin showed statistically significant differences compared to the reference centrifugation when TGA was performed at low- or high-triggers' concentrations, presence or absence of thrombomodulin (Table 1, Fig. 2). However, the average percentage differences from the reference centrifugation were relatively small (4–9 %) when samples were prepared by centrifugation

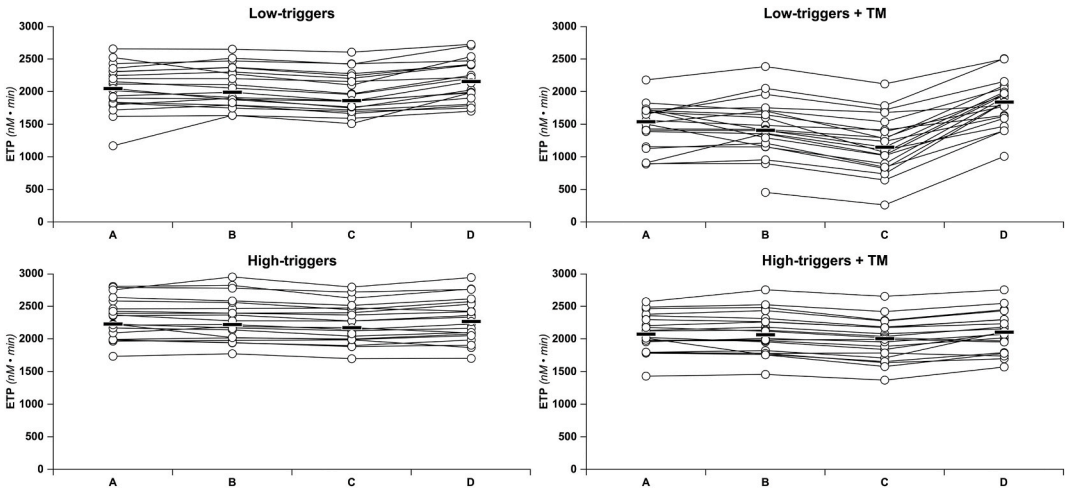

Fig. 3. Distribution of the individual endogenous thrombin potential (ETP) values for healthy subjects when measured for plasma prepared by different centrifugation speeds. See also legends to Fig. 1.

Table 2
Median percentage differences (IQR) for each parameter of thrombin generation assay (TGA) for healthy subjects measured for plasma prepared by different blood centrifugation speeds when compared to the same parameter obtained for plasmas prepared by the reference centrifugation.

TGA reagents	TGA parameter	Centrifugation Speed		
		A vs **B**	**A** vs **C**	**A** vs **D**
Low-triggers	Lag-time	8 (4–14)	2 (0–9)	25 (16–37)
	Peak	9(0–13)	12(4–21)	−5(-11–1)
	ETP	0(-3–4)	3(2–8)	−5(-7–1)
Low-triggers + thrombomodulin	Lag-time	11(6–21)	6(2–17)	36(26–52)
	Peak	9(-2–21)	25(7–39)	−5(-16–3)
	ETP	0(-8–5)	18(6–31)	−21(-37–13)
High-triggers	Lag-time	10(9–14)	2(0–6)	16(11–19)
	Peak	4(2–7)	0(-2–3)	6(2–10)
	ETP	0(-1–2)	3(0–5)	1(0–2)
High-triggers + thrombomodulin	Lag-time	10(7–13)	3(0–7)	20(11–27)
	Peak	4(2–7)	2(0–6)	5(3–14)
	ETP	0(-2–1)	4(2–8)	−2(-4–3)

A. Double centrifugation: Blood at 2,500g (15min) and plasma at 2500 (15 min) (reference centrifugation).
B. Single centrifugation at 3,000g (20min).
C. Single centrifugation of blood at 3,000g (20min), plasma freezing, then plasma centrifugation at 10,000g (5min) after thawing.
D. Single centrifugation at 1,700g (10min).

at high-speed (i.e., 3,000g for 20 min). Percentage differences ranged from 0 to 25 % when plasma samples were prepared by plasma centrifugation at high-speed after thawing.

Endogenous thrombin potential (ETP). The inter-individual variability of results was similar regardless of testing with low- or high-triggers, presence or absence of thrombomodulin and centrifugation speed (Fig. 3).

There was no significant difference in the median ETP performed at low- or high-triggers' concentrations in the absence of thrombomodulin between samples centrifuged once at high speed (i.e., 3,000g for 20 min) when compared to the standard centrifugation (Table 1, Fig. 3). The above results were essentially unchanged when testing was performed in the presence of thrombomodulin (Table 1, Fig. 3). However, the median ETP was significantly different from that obtained with the reference centrifugation for samples tested with low- or high-triggers' concentrations, when plasma samples were centrifuged at 10,000g after thawing (Table 1, Fig. 3). Finally, median ETP for plasma samples prepared by slow centrifugation (i.e. 1,700g) was significantly different from that obtained with the reference centrifugation, regardless of low- or high-triggers' concentrations and presence or absence of thrombomodulin (Table 1, Fig. 3). However, the average percentage ETP difference from the reference centrifugation was null (0 %) when plasma samples were prepared by single centrifugation at 3,000g for 20 min and testing was performed with low-triggers, regardless of thrombomodulin (Table 2).

4. Discussion

In this study we examined the impact of blood centrifugation on TGA parameters measured for plasma samples from heathy

subjects with high- or low-triggers' concentrations, absence or presence of thrombomodulin. The impact that centrifugation may have on TGA is extremely important for the application of the procedure for research as well as for diagnostic purposes, especially if one considers the impact that double-centrifugation may have on the presently available total laboratory automation systems that require single-centrifugation.

The conditions of blood centrifugation are one of the most important determinants of the poor comparability of TGA results across laboratories. Additionally, centrifugation by affecting the content of negatively charged phospholipids in the test system is likely to modify the responsiveness of TGA to detect hypo- or hypercoagulability.

The user manual of the Thrombinoscope method (Thrombogram Guide, Thrombinoscope BV) recommends double-centrifugation to prepare platelet poor plasma [12]. There are other reports dealing with the influence that centrifugation speed may have on TGA that have in general shown that residual platelets, white blood cells and microparticles can significantly influence TGA parameters [6–9]. As shown in this and other studies [6–9], the influence is in general greater when testing is performed with low-triggers' concentrations. This is not surprising as low-triggers' concentrations increase the test sensitivity of TGA and consequently the contribution of phospholipids from residual platelets in plasma is likely to result into variable TGA parameters when compared with no or little residual platelets when plasma is prepared by the reference double-centrifugation.

In general, the results of the present study confirm previous findings on the variable effect that centrifugation speed may have on TGA parameters and extend the observations to TGA testing performed in the presence of thrombomodulin. However, considering the actual impact observed when calculating the percentage difference between values obtained under different centrifugation speeds and those obtained using the reference centrifugation, we can derive the following conclusions.

Overall, centrifugation at high-speed (i.e., 3,000g, 20 min) affects TGA much less than the other centrifugation speeds when compared to the reference centrifugation (see Table 2). Moreover, the centrifugation conditions do not affect the main TGA parameters at the same extent. The centrifugation conditions that most affect TGA is low-speed, and/or short duration (i.e., 1,700g for 10 min). This centrifugation impacts much more on the lag-time, regardless of the triggers' concentrations when compared to the other TGA parameters such as thrombin-peak and ETP. Although there are no technical explanations for this different behavior, it is tempting to speculate that the time needed for thrombin generation to start (i.e. the lag-time) is much more dependent from phospholipids than the total amount of generated thrombin that is represented by peak-thrombin and ETP. Whatever the reasons, we believe that low-speed blood centrifugation as it is routinely performed in clinical laboratories to prepare plasma should not be used for TGA solely because one of the most important parameters (i.e. lag-time) is affected. The high-speed plasma centrifugation after thawing impacts on ETP and peak-thrombin to some extent (see Table 2). This is probably due to freezing and thawing cycles that lead to platelet fragmentation, releasing membrane-bound and/or soluble phospholipids and other procoagulants, such as platelet factor 4 and coagulation factors. The above substances that are soluble are probably not completely removed by high-speed centrifugation. This phenomenon is only evident when TGA is performed with low-triggers' concentrations in the presence of thrombomodulin. However, when high-triggers' concentrations are used, the average percentage difference remains consistently 4 % or less.

Notably, the single, rapid centrifugation for an extended duration (i.e., 3000g for 20 min) is the only centrifugation condition investigated in this study that does not exert a significant influence on TGA parameters, regardless of the triggers' concentrations or the presence of thrombomodulin. As shown in Table 2, TGA parameters obtained with the single rapid centrifugation deviate on average from the double-centrifugation for less than 11 %. These values (although arbitrary) could be taken as an acceptable limit.

Presently, TGA guidance recommends double-centrifugation, but clinical laboratories commonly prepare plasma samples using a single low-speed centrifugation for 10 min. Our and other studies show that this condition impacts on TGA parameters compared to the reference centrifugation. According to our study a single centrifugation at high-speed could be a viable alternative that minimizes the discrepancies of results when compared with the reference centrifugation without the complexity and time demands of double-centrifugation. In fact, in our study, TGA parameters stemming from plasma samples prepared by centrifugation at 3000*g* for 20 min did not differ from the reference double centrifugation for more than 11 % and for most parameters was null or less than 5 % (see Table 2). These differences are acceptable, especially in consideration of the fact that the single centrifugation is much less demanding than double-centrifugation. Hence, we propose that the current guidance on the centrifugation conditions for TGA should be amended calling pragmatically for a single-centrifugation at relatively high-speed (i.e., 3000g for 20 min). This would greatly help the adoption of TGA in the general practice of clinical laboratories, which at the moment lags behind its merits. In fact, a recent search of the literature in PubMed with the key terms "thrombin generation" yielded more than 13,000 reports on the subject that are mainly related to TGA for research purposes and much less for patients' management.

4.1. Potential limitations of our study should be recognized

First, we did not attempt to count residual platelets in the plasma samples stemming from different centrifugation speeds. Counting platelets by means of automated analyzers is not reliable for plasma containing low platelets numbers. In doing so, we assumed a priori that the effect of centrifugation on TGA parameters is mainly due to residual platelets. However, if one considers the technical features of TGA that employs diluted plasma combined with small amounts of tissue factor and synthetic phospholipids and the documented effect that platelets may have on its parameters [10], it is likely that the main determinants of TGA variability are the residual platelets in the test plasma. Finally, Rodgers et al. [8] have shown that residual platelet counts depend on the speed of centrifugation, being relatively high in low speed compared to high speed centrifugation.

Second, we investigated plasma samples from healthy subjects and there is no assurance that the conclusions derived from this study are valid also when using TGA for investigating hypo- or hypercoagulability in other clinical settings. On the other hand, the impact that centrifugation may have for all the clinical settings where TGA is used has never been investigated simply because it is

practically far reaching.

In conclusion, this study confirms and extends previous observations that centrifugation conditions, mainly due to residual platelets in plasma may affect TGA parameters. Since double-centrifugation is not commonly used in routine practice and particularly to prepare plasma collected in large epidemiological studies [5,6], we suggest that plasma samples for TGA should be prepared by single high-speed blood centrifugation (i.e., 3000g for 20 min). This centrifugation condition is not time consuming and does not require gross deviations from routine practice. Furthermore, this centrifugation condition is unlikely to be detrimental for the most common parameters of coagulation other than TGA and could therefore be adopted as the general procedure for coagulation testing in local laboratories. This would be a great simplification in the routine diagnostic workup of coagulation.

Finally, whatever the choice on the centrifugation speed, we suggest that whenever TGA is used for research purposes it is paramount to include along with patients' plasma an adequate number of controls' plasma from healthy subjects that are prepared by centrifugation at the same speed and time as those used for patients' plasma. These controls' plasmas allow for a more correct interpretation of TGA results, regardless of the conditions of centrifugation. Likewise, when TGA is used for diagnostic purposes, it is advisable to establish local reference ranges by measuring TGA parameters for blood from healthy subjects centrifuged at the same speed and time as those used for patients' plasma.

CRediT authorship contribution statement

Erica Scalambrino: Formal analysis, Data curation, Conceptualization. **Marigrazia Clerici:** Investigation, Data curation. **Flora Peyvandi:** Writing – review & editing. **Armando Tripodi:** Writing – review & editing, Writing – original draft, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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