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Published in:
Thrombosis Research

DOI:
10.1016/j.thromres.2014.12.015

Publication date:
2014

Document Version
Early version, also known as pre-print

Thrombosis Research xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Large external quality assessment survey on thrombin generation with CAT: further evidence for the usefulness of normalisation with an external reference plasma

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ARTICLE INFO

Article history:
Received 1 October 2014
Received in revised form 25 November 2014
Accepted 9 December 2014
Available online xxxx

Keywords:
Thrombin generation tests
Normalisation
Standardisation
Reference plasma

ABSTRACT

Background: Calibrated Automated Thrombography (CAT) has been widely used to assess in vitro thrombin generation as an informative intermediary phenotype of coagulation. Interlaboratory exercises have documented a worrisome poor reproducibility. There are some data on the normalisation with an appropriate external reference plasma (RP). This multicentre study of the French-speaking CAT Club aimed at providing further evidence for the usefulness of such a normalisation.
Materials and Methods: Lyophilised aliquots of a RP along with 3 plasmas (P1 = normal; P2 = hypo-; P3 = hypercoagulable) were sent to 34 laboratories (corresponding to 38 instruments). CAT was studied using 1 and 5 pM tissue factor and other dedicated reagents. Normalisation with the local RP in use in the laboratory could also be performed. Interlaboratory CVs were calculated for each plasma before and after normalisation.
Results: Regarding endogenous thrombin potential, a good discrimination between the 3 plasmas was achieved in all laboratories but there was no overlap after normalisation only. CVs were generally not reduced with the use of local RP but were generally improved with normalisation using the external RP, often becoming lower than

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http://dx.doi.org/10.1016/j.thromres.2014.12.015
0049-3848/

10%. Regarding P2 however, the benefit of normalisation was poor, and there were analytical difficulties as well, some laboratories being unable to get a useable signal.

Conclusions: We confirm that normalisation of CAT results with a suitable external RP is useful in "real life" practice as it often permits an acceptable level of interlaboratory variability. In case of frank hypocoagulability, further improvements are required to get reliable, potentially clinically relevant results.

Introduction

There has been a growing interest for thrombin generation tests (TGTs) [1,2]. Such tests allow a comprehensive and potentially clinically relevant *in vitro* phenotyping of blood coagulation. They take into account thrombin generation beyond the physical phenomenon of clotting as well as the entire process of thrombin inhibition as a whole, allowing the detection and quantification of both hypo- and hypercoagulable states.

Among TGTs, the method developed by Hemker et al., namely Calibrated Automated Thrombography (CAT) [3] has been extensively studied for the last decade since: (i) the use of a calibrator allows an accurate measurement of the actual amounts and activities of generated thrombin; (ii) the detection of fluorescent signals allows the measurement in cellular milieus (except red blood cells), for instance platelet-rich plasmas [4] or leucocyte-rich plasmas [5].

Data have been published with the CAT method, consistent with a potentially high added value in clinical practice, in settings such as diagnosis and management of bleeding disorders [6–8], detection of hypercoagulability [9,10], characterization/monitoring of anticoagulant drugs including non-vitamin K antagonist oral anticoagulants [11,12] or even in acquired complex coagulation disorders [13]. Most of the data come from single-centre studies and must be confirmed in prospective, large, multicentre studies. However the method still suffers from an important inter-centre variability and a persistent deficiency in standardisation [14–17], which makes difficult the design of multicentre studies unless performing TGT in a centralised laboratory. Normalisation of raw results using a normal plasma (pooled or lyophilised) has been proposed for that purpose [14–18]. Recently, a standardized protocol with normalisation of results using a reference plasma (RP) has been shown to reduce lab-to-lab variability [19]. Though promising, such data, obtained in a relatively small number of reference laboratories and using a single batch of reagents, remain distant from real life practice. We report the largest multicentre study to date on lab-to-lab variability and improvement by normalisation of data using a single and suitable externally provided RP.

Materials and methods

Reagents

Reagents and lyophilised plasmas were supplied from Diagnostica Stago (Asnières, France): PPP Reagent LOW™ and PPP Reagent™, Thrombin Calibrator™ and FLUCA kit™, as well as 4 lyophilised plasmas - plasma 1 ("normocoagulable"); plasma 2 ("hypocoagulable" – heparinised plasma); plasma 3 ("hypercoagulable" – plasma deficient in protein S); and external reference plasma (external RP) for normalisation of results. This external reference plasma is a lyophilised plasma selected to have a thrombin generation profile as close as possible to a fresh frozen normal donor plasma pool. It has been demonstrated to be suitable for such use by Dargaud et al. [19].

Study design

The study aimed to evaluate in a large multicentre survey: (i) the lab-to-lab variability of TGT using CAT method; (ii) the benefit of normalisation of results using a lyophilised external RP and, depending on the ongoing practice of each centre, the local reference plasma.

Each plasma (numbered 1, 2 and 3 as stated above) had to be tested using CAT method in an independent run over 3 days. In parallel, the reference plasma was studied in each run. Instructions were given to participants regarding use of dedicated reagents, test plasma in triplicates and adherence to manufacturer's instructions for plasma/reagent reconstitution and CAT performance. No instruction was given regarding preheating plates at 37 °C. Of note also, the locally implemented version of Thrombinoscope™ software was used (2 different versions). Thus 6 batches of initiating reagents (PPP Reagent™ and PPP Reagent LOW™) were used; concerning Thrombin Calibrator™ and FLUCA kit™, a single batch was provided, but each participant could use its own reagent batch.

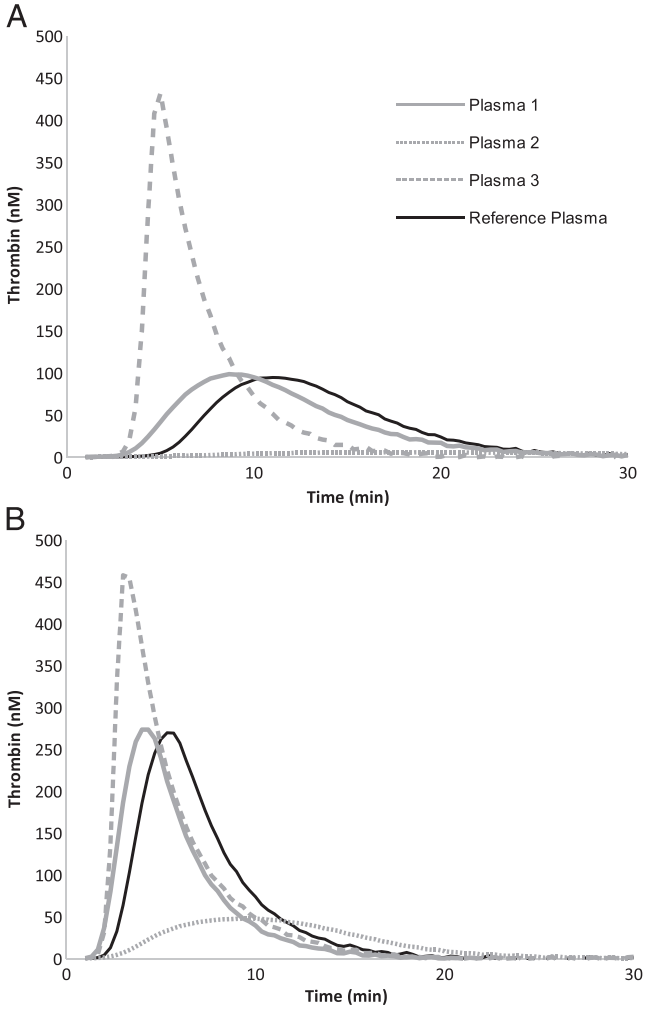

Fig. 1. Typical thrombin generation profiles of the 4 plasmas used in the study, obtained with (A) 1 pM TF – PPP Reagent LOW ™; (B) 5 pM TF – PPP Reagent™.

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Table 1
Robust means (see text) and associated lab-to-lab CVs with 1 pM TF as initiating condition (PPP Reagent LOW ™). Bold characters indicate CVs ≤ 10%. Symbols (↑, =, ↓) illustrate changes in CVs as compared with raw data. Normalised results are expressed as a percentage of a RP, except for temporal data (lag time, start tail and time to peak) which are expressed as ratios.

Parameters		Raw data		Normalised data Local plasma			Normalised data External reference plasma		
		Robust mean	CV	Robust mean	CV		Robust mean	CV	
Plasma 1	ETP	1167 nM.min	17%	122%	28%	↑	98%	**10%**	↓
	Lag time	3.6 min	15%	0.79	17%	↑	0.68	**8%**	↓
	Peak	108 nM	21%	118%	42%	↑	87%	17%	↓
	Start tail	34.5 min	**9%**	1.1	16%	↑	1.08	**9%**	=
	Time to peak	9.0 min	**9%**	0.93	12%	↑	0.89	**7%**	↓
	Velocity index	20 nM/min	29%	113%	50%	↑	77%	27%	↓
Plasma 2	ETP	119 nM.min	38%	11%	41%	↑	10%	56%	↑
	Lag time	3.8 min	31%	0.92	32%	↑	0.77	21%	↓
	Peak	5 nM	35%	5%	43%	↑	4%	42%	↑
	Start tail	52.5 min	17%	1.6	32%	↑	1.6	26%	↑
	Time to peak	15.7 min	18%	1.7	23%	↑	1.6	12%	↓
	Velocity index	0.43 nM/min	46%	2%	53%	↑	2%	43%	↓
Plasma 3	ETP	1929 nM.min	16%	187%	26%	↑	156%	**9%**	↓
	Lag time	3.2 min	13%	0.70	19%	↑	0.60	**6%**	↓
	Peak	440 nM	**10%**	444%	50%	↑	336%	18%	↑
	Start tail	22.3 min	12%	0.68	20%	↑	0.68	12%	=
	Time to peak	4.9 min	**10%**	0.52	18%	↑	0.48	**6%**	↓
	Velocity index	247 nM/min	15%	1421%	67%	↑	937%	34%	↑

Thrombin generation test and data analysis

Briefly, 80 μL plasma samples were mixed with 20 μL initiating reagent (PPP Reagent™ or PPP Reagent LOW™) in a 96-well plate. Coagulation was started by adding 20 μL FLUCA kit™ containing calcium chloride and the fluorogenic substrate (Z-Gly-Gly-Arg-AMC). Upon splitting by thrombin, the fluorescent AMC (7-amino-4-methylcoumarin) is released and measured with a 390-nm-excitation and a 460-nm-emission filter set in an Ascent Fluoroskan™ (ThermoLabsystems, Helsinki, Finland). All samples were run in triplicate. For each plasma sample, the fluorescence signal was corrected for substrate consumption, plasma colour variability and inner filter fluorescence effect by running in parallel 3 calibrating wells where 80 μL plasma were mixed with 20 μL Thrombin Calibrator™. As mentioned above, data were analysed using the locally used version of Thrombinoscope™ software (Diagnostica Stago – Asnières, France).

The following thrombin generation parameters were studied: endogenous thrombin potential (ETP); lag time; thrombin peak; time to peak; velocity index; start tail. Once performed, participants had to enter the data in the Qualiris by Stago® website. This on-line platform collects all the results and provides laboratories with comparative reports including, for each parameter, the "robust mean" calculation (statistical method not affected by outliers, see ISO Standard 13528). Results are expressed in standard units (raw result), as a percentage or as a ratio of external or local reference plasma ("normalised result"). Lab-to-lab coefficients of variation (CV) were then determined for both raw and normalised results with the 3 assayed plasmas.

Results

Typical thrombin generation profiles of the 4 plasmas are shown in Fig. 1.

Thirty-four participants sent data corresponding to 38 CAT instruments; among them, 21 centres sent data using their local reference plasma in addition to the external reference plasma.

Raw data & lab-to-lab variability

Under both initiating conditions (1 or 5 pM TF) and with the 3 plasmas, lab-to-lab variability was important (Tables 1 and 2), most

Table 2
Robust means (see text) and associated lab-to-lab CVs with 5 pM TF as initiating condition (PPP Reagent™). Bold characters indicate CVs ≤ 10%. Symbols (↑, =, ↓) illustrate changes in CVs as compared with raw data. Normalised results are expressed as a percentage of a RP, except for temporal data (lag time, start tail and time to peak,) which are expressed as a ratio.

Parameters		Raw data		External reference plasma Local plasma			Normalised data External reference plasma		
		Robust mean	CV	Robust mean	CV		Robust mean	CV	
Plasma 1	ETP	1467 nM.min	15%	114%	**10%**	↓	94%	**8%**	↓
	Lag time	2.2 min	13%	0.82	13%	=	0.77	**9%**	↓
	Peak	264 nM	19%	121%	20%	↑	99%	**10%**	↓
	Start tail	22.4 min	**10%**	0.99	**8%**	↓	0.93	**6%**	↓
	Time to peak	5.0 min	12%	0.87	12%	=	0.86	**8%**	↓
	Velocity index	95 nM/min	32%	139%	33%	↑	105%	18%	↓
Plasma 2	ETP	567 nM.min	22%	46%	18%	↓	36%	20%	↓
	Lag time	3.0 min	19%	1.16	16%	↓	1.08	**9%**	↓
	Peak	38 nM	37%	18%	31%	↓	14%	39%	↑
	Start tail	38.8 min	17%	1.68	14%	↓	1.63	15%	↓
	Time to peak	10.7 min	13%	1.84	16%	↑	1.86	**10%**	↓
	Velocity index	5.0 nM/min	54%	8%	43%	↓	6%	52%	↓
Plasma 3	ETP	2079 nM.min	18%	155%	14%	↓	129%	**8%**	↓
	Lag time	2.0 min	15%	0.73	**9%**	↓	0.70	**8%**	↓
	Peak	468 nM	**9%**	202%	28%	↑	172%	14%	↑
	Start tail	22.0 min	15%	1.00	14%	↓	0.91	**8%**	↓
	Time to peak	3.6 min	12%	0.62	16%	↑	0.62	**8%**	↓
	Velocity index	283 nM/min	14%	417%	58%	↑	306%	28%	↑

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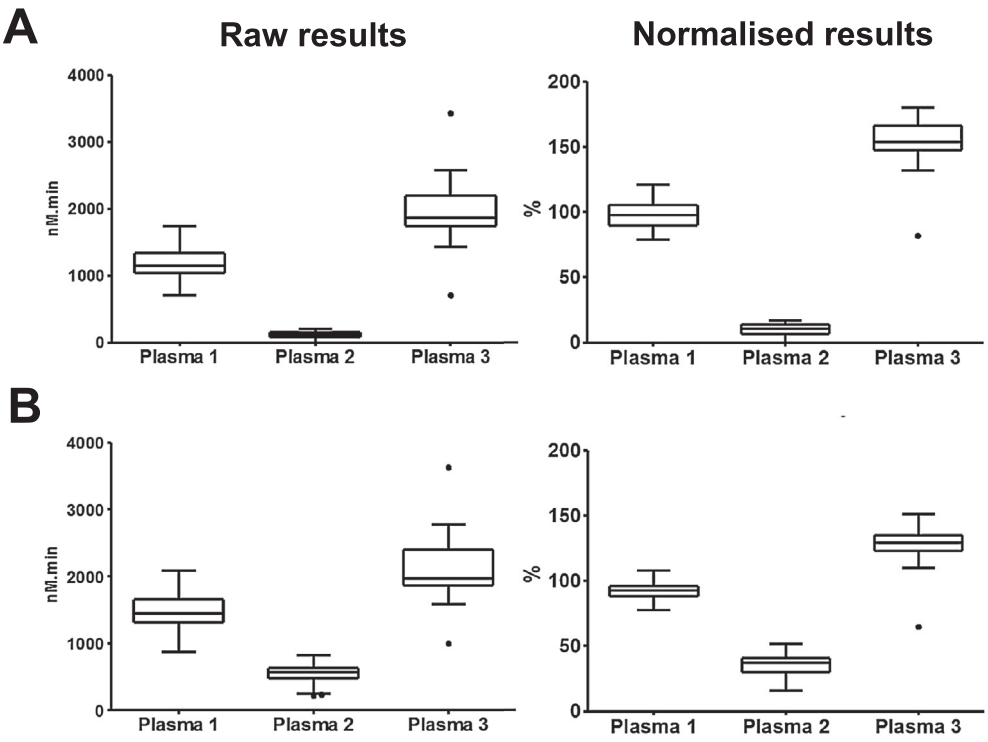

Fig. 2. ETP as raw and normalised results with external RP, obtained with (A) TF 1 pM – PPP Reagent LOW ™ (B) TF 5 pM – PPP Reagent™.

CVs being higher than 10% for almost all parameters, some values even exceeding 20%. As an example, regarding ETP, CVs were comprised between 15 and 35 %. Of note, dispersion of data was more important with plasma 2 or at low TF concentration. In addition, the velocity index stood as the most variable parameter, the CVs being almost always the highest of the series, except for plasma 3.

Normalisation with the local reference plasma

Using the local reference plasma, lab-to-lab variability was still important, as only few CVs fell below 10%, whatever TF concentrations or plasmas. Some CVs were even higher than before normalisation; at low TF concentration, normalisation consistently worsened CVs.

Normalisation with external RP

Except for plasma 2, for which there were analytical difficulties as well (3 laboratories being unable to get a useable signal) and whatever the TF concentration, normalisation of results with external RP obviously generally improved interlaboratory CVs. Noteworthy, for a given parameter, CVs after normalisation, when improved, often decreased below 10%. Furthermore, regarding ETP, even if a good discrimination of the 3 plasmas was achieved in all laboratories, the overlap was only eliminated after normalisation (an outlier value persisting with plasma 3) (Fig. 2). Regarding the "thrombin peak" with plasma 3 however, CV after normalisation was higher than before (18% versus 10% at 1 pM TF). Besides, the normalisation was of poor benefit with some parameters, the more so regarding velocity index, with equal or higher CVs under both TF conditions, with the 3 plasmas.

Discussion

We report the largest study to date regarding thrombin generation with CAT, in terms of number of participants and analysed parameters, on lab-to-lab variability and potential benefit of results normalisation using an external RP. As an example, the European external assessment program ECAT 2013–2 exercise gathered 15 and 20 participants with 1 pM or 5 pM TF as coagulation initiating conditions respectively (see ECAT External quality Control for Assays and Tests foundation, report 2013–02 Thrombin Generation Test).

Three kinds of plasmas were chosen to cover the whole spectrum of thrombin generation measuring range, markedly hypocoagulable, normocoagulable and highly hypercoagulable, as compared to data obtained in healthy subjects under similar conditions [18,20]. For practical purposes it was decided for defective coagulation to supplement normal plasma with heparin. Plasma artificially depleted in protein S was found to be hypercogulable due at least in part to the expected loss of APC-independent anticoagulant effect [21,22]. In agreement with previously published data [19] and ECAT TGT surveys, the lab-to-lab variability of raw results was important whatever the initiating conditions or types of plasma. Indeed, only few CVs were inferior to 10%, a maximal accepted target-value to allow multicentre studies. The variability was more important at low TF concentration or with the hypocoagulable plasma. In a previous multicentre study (5 participants), Dargaud et al. reported a better improvement in CVs than in the present work, under similar initiating conditions (1 pM TF). However, the design of the previous study significantly differed by using a single batch of reagents and frozen plasmas; plasmas were prepared using corn trypsin inhibitor (to eliminate the variable contribution of the contact phase) and were less hypo- or hyper-coagulable.

In the present study, the fact that CVs for ETP (often considered as the most global and important parameter of CAT) were often higher than 15% raises some questions on differences in real-life local practices, since all participants were familiar with the method. As a matter of fact, even if the experimentation protocol was standardised, some local experimental variability is likely to have existed, regarding factors such as the pre-heating or not of samples and/or plates before measurement, or even the reconstitution of reagents and plasmas (the latter being probably more subject to variability than thawing of frozen plasma).

To reduce lab-to-lab variability, normalisation of raw data with an appropriate plasma has been proposed [14,15,19]. In the herein reported study, participants had to normalise their data with external RP

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prepared by Diagnostica Stago and, if applicable, the locally prepared pool of normal plasma.

Strikingly, the local plasma was generally unable to improve lab-to-lab variability whatever the initiating conditions or plasma, even worsening CVs after normalisation. This may be related to the disparities in preparation modalities (sampling, centrifugation, pooling…), as it is accepted that preanalytical treatment largely influences TGT [16]. Therefore, the main interest of such a material is essentially to act as an "internal quality control" to minimise run-to-run intralaboratory variability. Thus a suitable, well-characterised, external reference material is needed.

Normalisation with external RP generally improved CVs, which often dropped below 10% for plasma 1 and 3. In particular, CVs for normalised ETP were acceptable, except for plasma 2. More generally, regarding the markedly hypocoagulable plasma 2, normalisation with RP had poor impact on CVs since the values obtained are at the very low end of the CAT measuring range. Surprisingly, for unclear reasons, normalisation with RP worsened CVs for thrombin peak with the hypercoagulable plasma, under both initiating conditions. Interestingly, despite an outlier value persisting with plasma 3, normalised ETPs allowed a better discrimination of the 3 plasmas at the multicentre scale, where raw ETPs overlapped. This appears very encouraging for the design of large-scale studies.

Our results also highlight that variability is important and not or only poorly corrected by normalisation. This is especially the case for velocity index. An explanation would be that this parameter is calculated using lag time, time to peak and peak, therefore cumulating variability of each individual parameter. Furthermore, the peak is derived from the first derivative of the fluorescent signal, and the noise in a derivative is always higher than in the original signal; thus it is expected that this parameter and those based on it have a high variability. Several concerns have been raised with plasma 2, some centres being unable to get a useable increase in fluorescence or reporting a '0 nM.min' result for ETP. This is a problem because in case of frank hypocoagulability, tiny thrombin generation might be of clinical importance compared to no generated thrombin at all [23,24]. The reasons for this discrepancy between centres are unclear; further improvements are required to get reliable results. The poor results obtained with plasma 2 (markedly hypocoagulable) may also be due to the noise of the fluorescent signal, the impact of which is proportionally more important at low signals, in particular for the determination of the tail of the curve ("start tail"), and this ultimately affects the shape of the final, α2M-corrected thrombin generation curve [25].

Calibration and normalisation combine their effects to reduce lab-to-lab variability. On one hand the use of a calibrator allows to reduce some technical aspects like plasma colour and optical factors (filters, lamp…) [26]. On the other hand, normalisation is very likely to correct batch-to-batch variability of reagents; in addition, it may correct some analytical aspects such as measurement temperature, the pre-heating or not of plates, which is critical [27], or the operator-related variability (reconstitution of reagents or dispensing).

To go one step further, it is interesting to note that the improvement of CVs is the clearest for plasma 1, which is normal, possibly because the external RP has a normal profile as well. The hypothesis that normalisation with a RP close to the studied thrombin generation profile (that is, hypocoagulable RP for hypocoagulable samples; hypercoagulable RP for hypercoagulable ones) would be more successful deserves to be studied.

To conclude, we confirm with the largest multicentre study to date that normalisation of CAT results with a suitable external reference plasma (RP) – which will be commercially available soon - is useful in "real life" practice, with acceptable levels of variability. However, in case of frank hypocoagulability, further improvements are required. In addition, the next step should include different kinds and/or levels of hypo- and hyper-coagulability to confirm the suitability of RP to cover the whole spectrum of thrombin generation disorders. Moreover, in order to further reduce interlaboratory variability, a strict adherence to a well-defined detailed experimental scheme seems also mandatory. Our data suggest that lab-to-lab normalisation using a common and suitable material could facilitate the design of multicentre studies, allowing TGT to be locally performed in each participating centre.

Conflict of interest

None.

Acknowledgements

This study was supported by Diagnostica Stago (Asnières, France).

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Please cite this article as: Perrin J, et al, Large external quality assessment survey on thrombin generation with CAT: further evidence for the usefulness of normalisation with..., Thromb Res (2014), http://dx.doi.org/10.1016/j.thromres.2014.12.015

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Please cite this article as: Perrin J, et al, Large external quality assessment survey on thrombin generation with CAT: further evidence for the usefulness of normalisation with..., Thromb Res (2014), http://dx.doi.org/10.1016/j.thromres.2014.12.015