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University of Groningen

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# Thrombin Generation and Cirrhosis: State of the Art and Perspectives

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## Abstract

Epidemiological and laboratory studies performed in the last decades have changed our understanding of coagulopathy in cirrhosis, from a condition at increased risk of hemorrhagic events to one at higher thrombotic risk. However, it is not clear whether the decrease in factors that promote (except factor [F] VIII) versus inhibit coagulation in patients with cirrhosis results in a rebalanced state or in a hypercoagulable phenotype. This issue can be partially addressed using thrombin generation assays (TGA), which unlike routine clotting tests (prothrombin time or activated partial thromboplastin time) are sensitive to both procoagulant factors and coagulation inhibitors. However, many preanalytical issues and variable analytical methodologies used in TGAs complicate data analysis and interlaboratory comparisons. The introduction of TGAs in which activators of the protein C pathway (particularly soluble forms of thrombomodulin [TM]) are added has allowed detection of a reduced anticoagulant effect of TM or even a hypercoagulable phenotype as judged by endogenous thrombin potential. However, inter- and intra-assay variability may be greater with this TGA variant compared with “standard” TGAs. TGAs also allowed identifying main determinants of the hypercoagulability phenotype in the presence of TM: acquired antithrombin and protein C deficiencies, and elevated FVIII levels. The aim of this narrative review is to summarize the preanalytical and methodological variables of TGAs and also the findings of the main studies that have evaluated TGAs in patients with cirrhosis. The review also provides some propositions for future studies and outlines some perspectives on the potential implementation of this promising tool in clinical practice for the study of coagulation in patients with cirrhosis.

## Keywords

- ▶ cirrhosis
- ▶ coagulation
- ▶ hemostasis
- ▶ liver
- ▶ thrombin generation

Several chronic liver diseases can lead to cirrhosis that is characterized by diffuse nodular regeneration surrounded by dense fibrotic septa. This may lead to parenchymal extinction and liver structure collapse that together cause pronounced distortion of the hepatic vascular architecture.<sup>1</sup> The resulting reduced capacity of the liver to synthesize proteins is associated with several changes in the hemostatic system, notably in the coagulation system.<sup>2</sup> Historically, bleeding events were considered the main clinical manifestation of cirrhosis-related coagulopathy, in line with the prolongation in traditional coagulation parameters (e.g., prothrombin time [PT], activated partial thromboplastin time [aPTT]) as used in daily practice). However, recent studies have provided convincing data about a new hemostatic balance in patients with cirrhosis. It is now commonly accepted that many bleeding events are not caused by defective hemostasis in these patients, but rather by portal hypertension, local vascular abnormalities, and mechanical factors, such as vessel wall puncture during invasive procedures. The rupture of esophageal or gastric varices, secondary to portal hypertension, could be considered the most common major bleeding event in compensated and decompensated patients with cirrhosis.<sup>3–5</sup> Moreover, several studies found that patients with cirrhosis are not protected but rather have a significantly increased risk of thrombotic events, particularly venous thromboembolism and portal vein thrombosis.<sup>6–9</sup> In addition, studies of the coagulation system of patients with cirrhosis using thrombin generation assays (TGA) demonstrated that the PT and aPTT do not accurately reflect the coagulation potential of these patients. The measurement of the amount of thrombin activity (potential) generated in plasma over time under defined experimental conditions is often referred to as “thrombin generation,” although it is not restricted to the amount of the conversion of prothrombin to thrombin. With such an approach, we have access to a more comprehensive assessment of the coagulation system. In this narrative review, we will discuss TGA methodological aspects and show how, despite inter-laboratory differences in methodologies and data presentation, TGAs have allowed us to better understand the coagulopathy of patients with cirrhosis. For this review, we selected studies with sufficient reporting of methodological details that performed TGA in plasma of patients with cirrhosis. After a short description of coagulation dysregulation in cirrhosis, we will then discuss the different TGA preanalytical and analytical conditions. Subsequently, we will provide some propositions for using TGA in research and for its potential implementation in clinical practice.

## Coagulopathy and Thrombin Generation Assays in Patients with Cirrhosis

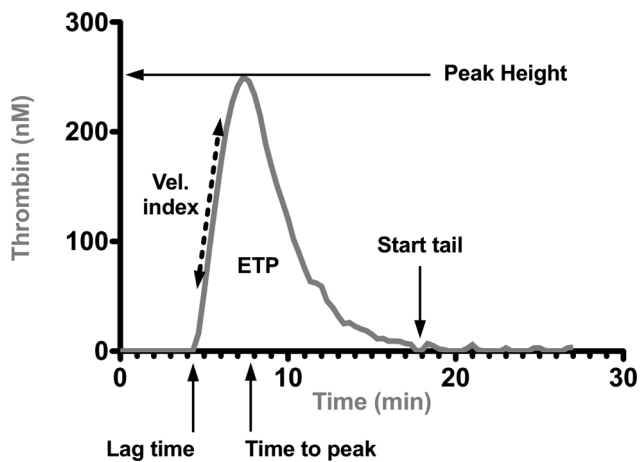
Many hemostasis processes are altered in patients with cirrhosis. Primary hemostasis, plasma coagulation, and fibrinolysis are modified, affecting the assays performed in hemostasis laboratories.<sup>2,10</sup> Although the net effects of the different hemostatic changes in patients with cirrhosis appear to be balanced, distinct hyper- and hypocoagulable features are also observed. The decreased capacity of the

diseased liver to synthesize proteins results in decreased plasma levels of all procoagulant proteins with the exception of factor (F) VIII, which is by contrast elevated. Despite elevated FVIII, there is a prolongation of classical coagulation assays, such as the PT and aPTT.<sup>11</sup> For years, this has been linked to bleeding events occurring in patients with cirrhosis, but this has been strongly challenged in the last two decades by epidemiological and laboratory studies. Indeed, as the routine coagulation assays only evaluate the first traces of thrombin generated by the procoagulant factors of the tissue factor (TF) or contact pathways, they do not assess the overall coagulation process.<sup>12</sup> Furthermore, PT and aPTT are insensitive to coagulation inhibitors (antithrombin [AT], protein C [PC] and protein S [PS], and tissue factor pathway inhibitor [TFPI]), and consequently they cannot give an accurate representation of the overall coagulation phenotype. Therefore, the study of coagulation in patients with cirrhosis is challenging because of the simultaneous changes in factors promoting and inhibiting thrombin generation.

TGAs were initially developed in the 1950s, but it took some time to improve the assays so that they could provide a better understanding of the *in vivo* coagulation process by taking into account not only the procoagulant factors but also the inhibitors of coagulation.<sup>13,14</sup> Currently, the most widely used TGA method is the calibrated automated thrombogram (CAT) developed by Hemker et al,<sup>14</sup> but there are also other commercially available and in-house systems.<sup>15</sup> Regarding patients with cirrhosis, all TGA studies performed according to CAT method were based on the use of a small fluorogenic thrombin substrate, the cleavage of which leads to an increase in fluorescence, and of a calibrator to calculate the free active thrombin, after subtracting the part bound to  $\alpha 2$  macroglobulin.

The CAT method allows measuring thrombin generation in platelet-poor plasma (PPP) and also in platelet-rich plasma (PRP) under clotting conditions after initiation of coagulation by the addition of TF, phospholipids (PL), and calcium. The generated thrombin is detected and measured during several minutes by using a fluorogenic substrate. After the required calibration and calculations,<sup>16</sup> the software provides the thrombogram (the tracing representing the course of generated active thrombin over time) with its quantitative parameters (► **Fig. 1**): peak height (PH) and endogenous thrombin potential (ETP). ETP represents the total amount of thrombin activity generated by the studied plasma sample over time under well-defined experimental conditions, and can thus be considered as the main TGA parameter; its association with both thrombotic and bleeding events has been reported.<sup>17</sup> Other parameters are lag time (LT), time to peak (TTP), start tail (ST), and velocity index (vel index), which are calculated with the following formula:  $PH/(TTP-LT)$ ; they provide a quantitative assessment of the kinetics of thrombin activity potential. As a general rule, the slower the process, the lower the potential.

*In vivo*, PC activation by thrombin requires thrombomodulin (TM), a transmembrane endothelial protein that is usually absent in plasma. When TM is not present in the reaction mixture, TGA does not measure this regulatory pathway. As changes in PC pathway components can be important,



**Fig. 1** Parameters of the thrombogram. The temporal parameters of the thrombogram are the lag time, the time to peak, and the start tail. The maximal amount of thrombin generated is represented by the peak height. The area under the curve represents the endogenous thrombin potential (ETP).

particularly for the study of coagulopathy in patients with cirrhosis, modified TGAs that account for the PC pathway have been developed. However, these modifications have possibly introduced additional sources of assay variability, which is a concern for a test already known to have substantial inter- and intralaboratory variability.

## Preanalytical and Analytical Conditions for Thrombin Generation Assays in Patients with Cirrhosis

### Preanalytical Variables

TGA results can be influenced by several preanalytical variables, such as blood sampling devices, contact phase inhibition, sample transportation, and preparation. Some recommendations for standardized preanalytical conditions were provided by the scientific and standardization committee of the International Society for Thrombosis and Haemostasis (ISTH).<sup>18</sup> **Table 1** summarizes the main preanalytical conditions used in studies that specifically evaluated thrombin generation in plasma samples from patients with cirrhosis. Vacuum tubes (with some differences in citrate concentration) are the most frequently used blood collection system; only one study used syringes for blood sampling.<sup>19</sup> Of note, ETP and PH were higher in plasma of patients with hemophilia when blood was collected using a vacuum tube system compared with a syringe.<sup>20</sup> This has implications also for studies in patients with cirrhosis. The use of a contact phase inhibitor has been debated. Among the published studies on patients with cirrhosis, only one used corn trypsin inhibitor (CTI) to inhibit contact phase activation on foreign surfaces used to collect, process, and store plasma from withdrawn blood, and during TGA.<sup>19</sup> However, it seems that CTI is only required if the final TF concentration is lower than 1 pM,<sup>18,21,22</sup> and all studies on patients with cirrhosis were performed at TF concentrations high enough to initiate coagulation before contact phase comes into play. Plasma sample transportation and preparation also are important. Most studies reported a time interval

between blood sampling and centrifugation below 2 hours, which is probably sufficiently quick to avoid any effect of prolonged storage.<sup>23</sup> The transportation type from the blood sampling site to the laboratory (e.g., pneumatic tube system or manual transport) can also modify the amount of thrombin generated,<sup>24</sup> potentially due to preactivation events, release of procoagulant PL and microvesicles by platelets, and release of TF by leucocytes, but it is not usually reported. Plasma preparation can include a single or a double centrifugation. The ISTH recommends a double centrifugation with an intermediary decantation of the supernatant.<sup>18</sup> Some studies on patients with cirrhosis used one single centrifugation,<sup>25–31</sup> and others a double centrifugation.<sup>19,32–41</sup> The centrifugation conditions were not always specified (e.g., temperature was sometimes missing), with differences in terms of *g* force, time, and temperature. Only one study used filtration (0.22  $\mu$ m pore diameter) after a single centrifugation to exclude residual platelets and large cellular debris,<sup>25</sup> but filtration could also remove adhesive proteins such as von Willebrand factor, and thus lowers FVIII. After sample preparation, PPP is usually frozen. In most studies, samples were stored at  $-70^{\circ}\text{C}$  or more, and in only one at  $-45^{\circ}\text{C}$ .<sup>19</sup> The thawing and post-thaw mixing conditions are not frequently reported and could represent an additional cause of variability.<sup>42,43</sup> Lastly, the interval between plasma collection/storage and TGA is not always specified. However, this information is important for TGA because the stability of frozen plasma samples has not been precisely evaluated. Nevertheless, it has been established that coagulation proteins are stable for at least 2 years at  $-80^{\circ}\text{C}$ .<sup>44</sup>

### Analytical Conditions

#### Plasma and Reagents

The analytical conditions, such as type of plasma (PPP or PRP), source and final concentrations of PL, TF concentration and detection methods, also influence TGA results. **Table 2** summarizes the various TGA experimental conditions used in the selected studies that specifically evaluated the cirrhosis-related coagulopathy. The concentration of TF is crucial, since it will determine not only the kinetics that the involvement of the intrinsic tenase (made of antihemophilic factors) as well. Of note, when a TF concentration is mentioned, the way it was estimated is seldom described; accurate measurement of active TF in a reagent is a well-known persisting vexing issue. Although the interassay variability can be reduced by normalization with a reference plasma,<sup>45</sup> variability is possibly introduced by the use of homemade reagents and mixtures, especially when PC pathway activators are added to the system.

#### Measuring Protein C Contribution

The standard CAT method with TF and PL (from various sources) does not take the PC inhibitory pathway into account. This pathway can be activated by introducing several different modifications to the “unmodified” TGA. The most common strategy is to directly add soluble TM (rabbit lung TM or human soluble TM, generally) to the reagent mixture.<sup>46</sup> Another strategy previously used is to

**Table 1** Preanalytical conditions used in the selected studies on TGA in patients with cirrhosis

	CTI (final concentration)	Type of tube	Anticoagulant (concentration)	Time between blood sampling and centrifugation	First centrifugation	Second centrifugation	Storage
Tripodi et al <sup>25</sup>	No	Vacuum tubes	Citrate (105 mM)	Within 30 min	2,000 g, 15 min, RT	No	-70°C
Tripodi et al <sup>32</sup>	No	Vacuum tubes	Citrate (105 mM)	Within 30 min	2,000 g, 15 min, RT for PPP 150 g, 15 min, NP for PRP	12 rpm, 5 min, NP	-70°C
Tripodi et al <sup>26</sup>	No	Vacuum tubes	Citrate (109 mM)	Within 30 min	2,880 g, 15 min, RT	No	-70°C
Delahousse et al <sup>33</sup>	No	Vacuum tubes	Citrate (109 mM)	NP	2,500 g, 15 min, 18°C	2,500 g, 15 min, 18°C	-80°C
Gatt et al <sup>19</sup>	Yes (20 µg/ mL) No for Protac assay	Syringe for unmodified TGA Vacuum tubes for Protac assay	Citrate (106 mM) Citrate (109 mM)	NP	2,000 g, 12 min, 4°C	13,000 g, 2 min, NP	-45°C
Tripodi et al <sup>27</sup>	No	Vacuum tubes	Citrate (109 mM)	Within 30 min	2,880 g, 15 min, RT	No	-70°C
Tripodi et al <sup>28</sup>	No	Vacuum tubes	Citrate (109 mM)	Within 30 min	2,880 g, 20 min, RT for PPP 150 g, 15 min, NP for PRP	No	No
Tripodi et al <sup>29</sup>	No	Vacuum tubes	Citrate (109 mM)	Within 30 min	2,880 g, 20 min, RT	No	-70°C
Youngwon et al <sup>30</sup>	No	Vacuum tubes	Citrate (109 mM)	Within 2 h	1,550 g, 15 min, NP	No	-80°C
Kleinegris et al <sup>34</sup>	No	Vacuum tubes	Citrate (105 mM)	Within 1 h	2,000 g, 15 min, 18°C	11,000 g, 10 min, 18°C	-80°C
Potze et al <sup>35</sup>	No	Vacuum tubes	Citrate (3.8%)	NP	2,000 g, 10 min, NP	10,000 g, 10 min, NP	-80°C
Chaireti et al <sup>36</sup>	No	Vacuum tubes	Citrate (0.13 mM)	Within 2 h	2,500 g, 10 min, RT	2,500 g, 10 min, RT	-80°C
Tang et al <sup>31</sup>	No	Vacuum tubes	Citrate (109 mM)	Within 2 h	2,500 g, 15 min, RT	No	-80°C
Kremer et al <sup>37</sup>	No	Vacuum tubes	Citrate (3.2%)	NP	2,821 g, 10 min, NP	2,821 g, 10 min, NP	-80°C
Lebreton et al <sup>38</sup>	No	Vacuum tubes	Citrate (109 mM)	Within 2 h	2,500 g, 15 min, RT	2,500 g, 15 min, RT	-80°C
Sinegre et al <sup>39</sup>	No	Vacuum tubes	Citrate (109 mM)	Within 2 h	2,500 g, 15 min, RT	2,500 g, 15 min, RT	-80°C
Russo et al <sup>40</sup>	No	Vacuum tubes	Citrate (3.2%)	Within 1 h	1,500 g, 10 min, RT	1,500 g, 10 min, RT	-80°C
Bos et al <sup>41</sup>	No	Vacuum tubes	Citrate (3.2%)	NP	2,000 g, 10 mins, NP	10,000 g, 10 min, NP	-80°C

Abbreviations: NA, not applicable; NP, not provided; PPP, platelet-poor plasma; PRP, platelet-rich plasma; rpm, revolutions per minute; RT, room temperature; TGA, thrombin generation assay.

**Table 2** Analytical conditions used in the selected studies on TGA in patients with cirrhosis

Publication	Method	Source of tissue factor	Tissue factor, final concentration (pM)	Type of phospholipids	Phospholipids, final concentration (µM)	TM type	TM final concentration (nM)	Other reagent than TM, with concentration	Software
Tripodi et al <sup>25</sup>	CAT	Human relipidated recombinant TF (Recombiplastin)	1	DOPS, DOPE, DOPC (20/20/60)	0.5	Soluble	4	No	Thrombinoscope
Tripodi et al <sup>26,27,32</sup>	CAT	Human relipidated recombinant TF (Recombiplastin)	1	DOPS, DOPE, DOPC (20/20/60)	1	Soluble	4	No	Thrombinoscope
Delahousse et al <sup>33</sup>	CAT	Human relipidated recombinant TF (Innovin)	Procedure A: 5 pM Procedure B: 1 pM	PS, PC, PE (20/60/20)	Procedure A: 4 µM Procedure B: 1 µM	Soluble rabbit lung	Procedure A: 15 nM Procedure B: 4 nM	No	Thrombinoscope
Gatt et al <sup>19</sup>	CAT	Human relipidated recombinant TF (Innovin)	1	NP	NP	No	NA	No	Thrombinoscope
Gatt et al <sup>19</sup>	CAT	Human relipidated recombinant TF (Innovin)	5	NP	4	NA	NA	Yes 0.3 IU/mL	Thrombinoscope
Tripodi et al <sup>27</sup>	ThromboPath	NP	NP	NP	NP	NA	NA	Yes	NA
Tripodi et al <sup>28,29</sup>	CAT	Human relipidated recombinant TF (Recombiplastin)	1	NP	NP	Soluble rabbit lung	6	No	Thrombinoscope
Youngwon et al <sup>30</sup>	CAT	PPP reagent	5	PPP Reagent	4	Soluble	5	No	Thrombinoscope
Youngwon et al <sup>30</sup>	CAT	PPP low reagent	1	PPP low reagent	4	Soluble	2.5	No	Thrombinoscope
Tripodi et al <sup>29</sup>	ThromboPath	NP	NP	NP	NP	NA	NA	Yes	NA
Kleinegris et al <sup>34</sup>	CAT	PPP low reagent	1	PPP Low reagent	4	Recombinant soluble	0.56	No	Thrombinoscope
Kleinegris et al <sup>34</sup>	CAT	PPP reagent	5	PPP reagent	4	Recombinant soluble	0.56	No	Thrombinoscope
Groeneveld et al <sup>47</sup>	CAT	PPP low reagent	1	PPP low reagent	4	Soluble rabbit lung	0, 1, 2, 4, 8	No	Thrombinoscope
Groeneveld et al <sup>47</sup>	CAT	PPP reagent	5	PPP reagent	4	Soluble rabbit lung	0, 1, 2, 4, 8	No	Thrombinoscope
Potze et al <sup>35</sup>	CAT	Thrombinoscope reagents	5	Thrombinoscope reagents	4	Soluble	NP (thrombinoscope reagents)	No	Thrombinoscope
Chaireti et al <sup>36</sup>	CAT	PPP reagent	5	PPP reagent	4	Soluble rabbit lung	4	No	Thrombinoscope
Tang et al <sup>31</sup>	ThromboPath	NP	NP	NP	NP	NA	NA	Yes	NA
Potze et al <sup>48</sup>	CAT	Thrombinoscope reagents	5	Thrombinoscope reagents	4	Soluble	NP, (thrombinoscope reagents)	No	Thrombinoscope
Kremers et al <sup>37</sup>	CAT	PPP reagent	5	PPP reagent	4	No	NA	No	Thrombinoscope
Kremers et al <sup>37</sup>	CAT	PPP reagent	1	PPP low reagent	4	Recombinant soluble	0.56	No	Thrombinoscope
Lebreton et al <sup>38</sup>	CAT	PPP reagent	5	PPP reagent	4	Rabbit lung	4	APC 1 nM	Thrombinoscope
Sinegre et al <sup>39</sup>	CAT	PPP reagent	5	PPP reagent	4	Rabbit lung	4	No	Thrombinoscope
Russo et al <sup>40</sup>	CAT	PPP low reagent	1	PPP low reagent	4	Rabbit lung	1.5	No	Thrombinoscope
Bos et al <sup>41</sup>	CAT	PPP reagent	5	PPP reagent	4	Soluble	NP	No	Thrombinoscope

Abbreviations: CAT, calibrated automated thrombogram; DOPC, 1,2 dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2 dioleoyl-sn-glycero-3-phosphoethanolamine; DOPS, 1,2 dioleoyl-sn-glycero-3-phosphoserine; NA, not applicable; NP, not provided; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TGA, thrombin generation assay.

add Protac, a snake venom that can directly activate PC in a thrombin-independent manner. Protac, a snake venom protein that directly activates PC, has also been used with CAT at a final concentration of 0.3 IU/mL.<sup>19</sup> Finally, addition of activated PC (APC) bypasses the need of PC activation, and allows assessing the contribution of the PC pathway, independently of PC concentration. All of these different strategies have been successively employed for studying the PC pathway in patients with cirrhosis.

Tripodi et al were the first to perform TGA in the presence of TM.<sup>25</sup> They used a diluted PT reagent (Recombiplastin) containing human relipidated recombinant TF at an estimated final concentration of 1 PM. They also added a PL mixture that included 20% of 1,2 dioleoyl-sn-glycero-3-phosphoserine, 20% of 1,2 dioleoyl-sn-glycero-3-phosphoethanolamine, and 60% of 1,2 dioleoyl-sn-glycero-3-phosphocholine, with final concentrations of 0.5<sup>25</sup> or 1 μM.<sup>26–29</sup> In the quoted studies, soluble rabbit TM at a final concentration of 4 nM<sup>25–27,32</sup> or 6 nM<sup>28,29</sup> was used. Delahousse et al<sup>33</sup> used a different diluted PT reagent as a source of TF. Commercially available reagents, which allow a better assay standardization, have been used since the study by Youngwon et al.<sup>30,34–39,47,48</sup> The PPP low reagent and the PPP reagent (Stago) have different final (nominal) concentrations of TF, 1 and 5 PM, respectively. Of note, details on the PL composition in these reagents are not provided by the manufacturers, or how TF concentration was estimated. Various final concentrations of TM (from sources not always accurately mentioned) have been used, ranging from 0.56 to 8 nM. The final TM concentration is normally the concentration that can lower the ETP of healthy controls not only by ~50%<sup>38,39</sup> but also by 75%.<sup>40</sup> All studies with the CAT method developed by Hemker et al used the Thrombinoscope software (Diagnostics Stago, Inc.), but there are different versions of this software, with undisclosed differences. In one study, TGA was performed in the presence of APC at a final concentration of 1 nM, which reduced the ETP of healthy controls by ~50%.<sup>38</sup>

The ThromboPath assay (Instrumentation Laboratory) measures the decrease in thrombin generation induced by Protac in the presence of TF and PL, using a chromogenic substrate for thrombin.<sup>49</sup> The exact analytical conditions (TF, PL, and Protac final concentrations) in this assay are not provided by the manufacturer. Above all, the thrombin kinetics is recorded over a very limited amount of time only (45 second), which makes it questionable to consider ThromboPath as a genuine TGA.

In conclusion, the various methods and reagents used to activate the PC pathway make it difficult to interpret and compare the results of different studies, despite the almost widespread use of CAT. Nevertheless, several general findings are consistent, as discussed in the next section.

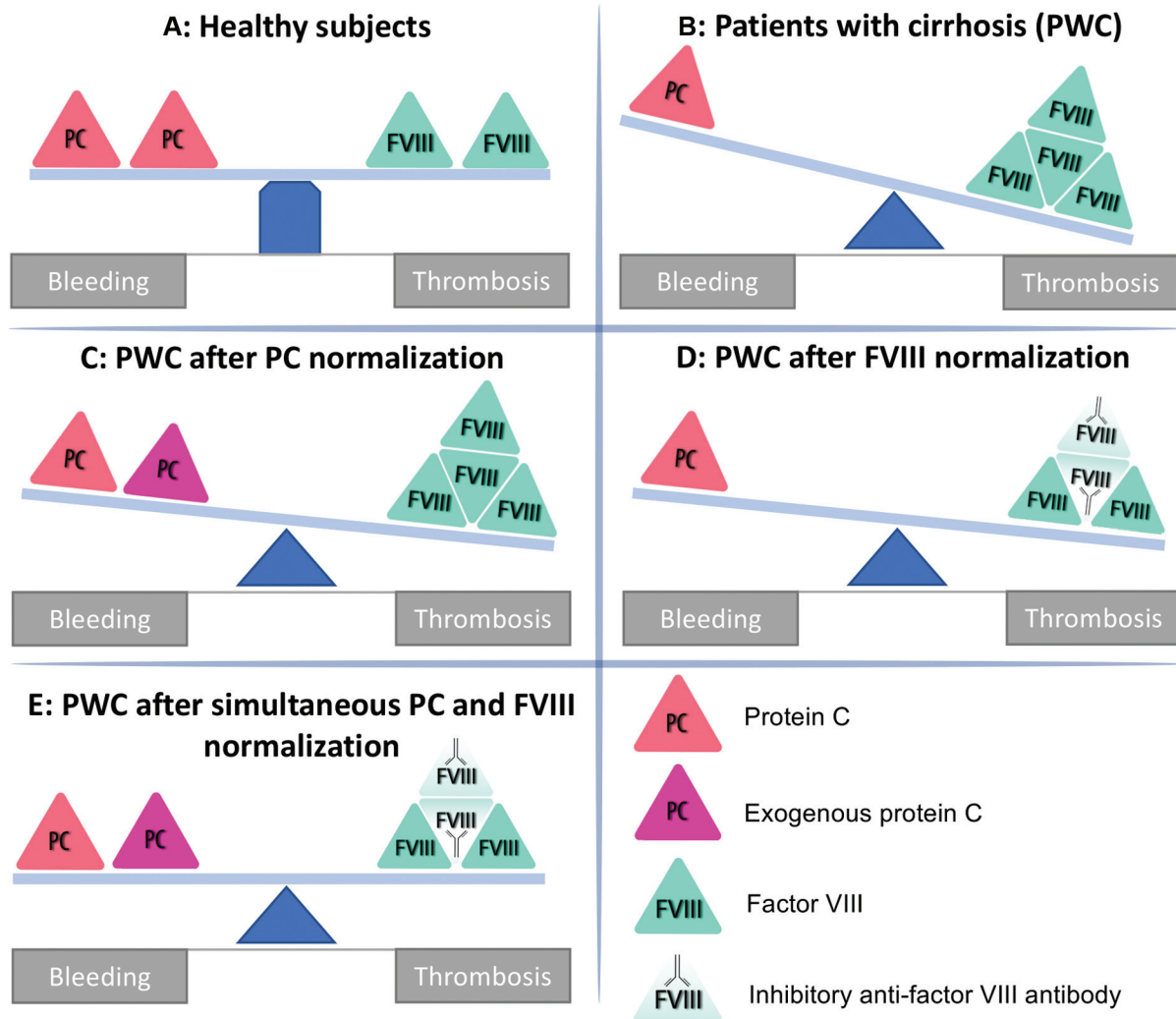
## Result Interpretation

Initially, ETP was proposed as the main thrombin generation parameter and is still often used to describe the results of the test in general. Compared with healthy subjects, a decrease or an increase in ETP indicates hypocoagulability or hypercoagulability, respectively. In some studies, results have been

expressed as the ETP with/without TM ratio. An increase in this ratio represents a lesser anticoagulant effect of TM, often referred to as “resistance to TM,” but the phrase “procoagulant imbalance” also has been used to describe this condition.<sup>50</sup> In 2005, Tripodi et al demonstrated that the ETP (analyzed by TGA in the presence of TM in PPP) was comparable between patients with cirrhosis and healthy controls.<sup>25</sup> A subsequent study then showed that plasma from patients with cirrhosis is resistant to the anticoagulant action of TM, with a gradual increase in the ETP ratio from patients with mild (Child–Pugh A) to severe (Child–Pugh C) cirrhosis, without reporting the actual ETP values.<sup>26</sup> In subsequent studies, also by other workers, TGA consistently highlighted a reduced effect of TM (based on the ETP ratio) that was proportional to the disease severity.<sup>30,33,34,37,38,40,47,51</sup> Many recent studies have reported a hypercoagulable phenotype in plasma from patients with cirrhosis, based on ETP values in the presence of TM, that increases with the disease in severity, in contrast to the initial observations by Tripodi et al. The growing consensus is that patients with cirrhosis, even with severe disease, display a hypercoagulable phenotype when tested by TGA with TM.<sup>36,38,40,41,48,52</sup> This hypercoagulable phenotype was found using different analytical conditions, such as low or high TF concentrations, and various TM concentrations.<sup>47</sup> The hypercoagulable phenotype was also detected when Protac was used as activator of the PC pathway. Specifically, Protac-induced coagulation inhibition percentage was consistently lower in patients with cirrhosis compared with healthy controls.<sup>19,27,29,31</sup> No hypercoagulable phenotype was found by TGA when APC was added to the test mixture, consistent with the hypothesis that the hypercoagulability determinants are upstream of APC.<sup>38</sup>

The identification of the determinants of cirrhosis-related hypercoagulability has been the focus of much research. Rapidly, the acquired PC deficiency and FVIII increase have been suspected, and it was shown that the FVIII/PC ratio is correlated to the extent of resistance to TM and Protac.<sup>27</sup> Tripodi et al demonstrated that resistance to TM and Protac could be reduced or normalized in the majority of patients with cirrhosis by spiking their plasma with fixed amounts of exogenous PC. Sinégre et al confirmed the role of PC deficiency, and provided evidence that the increase in FVIII plays a significant role in this hypercoagulable phenotype.<sup>39</sup> **Fig. 2** schematically represents the coagulation system in patients with cirrhosis assessed with TGA in the presence of TM as a balance of pro- and anticoagulant factors. As mentioned above, most studies reported that in plasma of patients with cirrhosis, TGAs performed in the presence of TM found a hypercoagulable phenotype, proportional to the disease severity. Normalization of PC levels alone (by addition of exogenous PC) or of FVIII levels alone (by addition of an inhibitory anti-FVIII antibody) is not sufficient to rebalance the system. Only the simultaneous normalization of PC and FVIII restores ETP to the values observed in healthy controls. Importantly, these findings do not rule out that AT, PS, or TFPI also could influence the fragile coagulation phenotype in patients with cirrhosis. For instance, normalization of AT levels also rescues thrombin generation.<sup>53</sup> The role of these proteins deserves additional





**Fig. 2** Role of protein C (PC) and factor VIII (FVIII) in the fragile coagulation balance assessed by thrombin generation assay in the presence of thrombomodulin (TM). In healthy controls (A), the coagulation system is well-balanced and stable. In patients with cirrhosis, a hypercoagulable phenotype is found, in relation with the acquired PC deficiency and FVIII increase (B). PC normalization alone after in vitro addition of exogenous PC (C) or FVIII normalization alone after in vitro addition of an anti-FVIII inhibitory monoclonal antibody (D) is not sufficient to rescue the fragile equilibrium. Only the simultaneous normalization of PC and FVIII (E) can rescue the coagulopathy of patients with cirrhosis.<sup>39</sup>

studies, particularly prothrombin and AT, because the balance between thrombin formation and direct inhibition influences the coagulation profile of patients with cirrhosis, according to some authors.<sup>37,53</sup> There is still another issue to be properly addressed, which is the extent of the stability of the phenotype over months if patients are stable, or the worsening of hypercoagulability for one given patient due to the increased severity of cirrhosis.

### Propositions for Future Studies

As mentioned before, the different preanalytical and analytical conditions make it difficult to compare results of TGA studies in patients with cirrhosis among published reports. In addition to the usual specifications about blood sampling for coagulation studies,<sup>18,54</sup> we suggest that specific preanalytical and analytical parameters should be provided in the methods section of future studies using TGA in patients with cirrhosis (► **Table 3**).

Although the tube type and citrate concentration are usually specified, how samples are transported to the laboratory (pneumatic tube system or hand-carrier) should also be reported because of the influence of the pneumatic tube systems on TGA.<sup>24</sup> Furthermore, the use (or not) of a contact phase inhibitor should be clearly mentioned, although it seems that CTI could be avoided for the TF concentrations used in studies on patients with cirrhosis.<sup>18,21,22</sup> The interval between sampling and centrifugation should also be specified (ideally less than 1 hour to enable the greatest sample integrity and preventing loss of labile factors such as FV and FVIII).<sup>55</sup> To the best of our knowledge, no study specifically compared single or double centrifugation in TGA with TM. Nevertheless, double centrifugation is suggested as a cautious approach, to minimize the influence of platelets that may be lysed after freeze/thawing. The specific centrifugation conditions also should be provided (including the temperature in the centrifuge).<sup>18</sup> As samples for TGA are usually frozen until use, the storage period should be provided. Samples are probably stable for at least

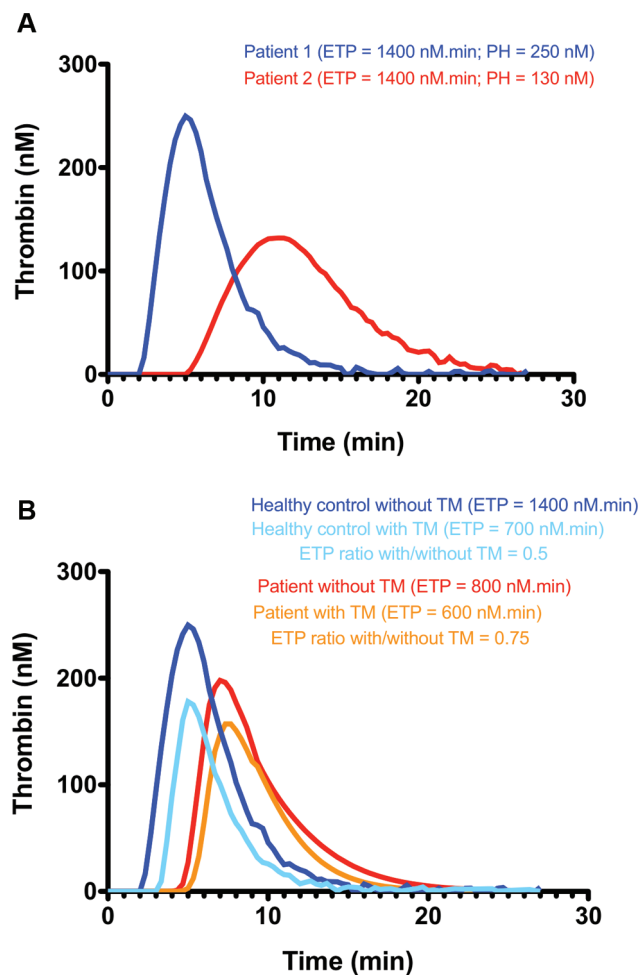
**Table 3** Minimal dataset to provide for further studies

Preanalytical conditions
<ul style="list-style-type: none"> <li>Blood sampling method (tourniquet, type of needle use, etc.)</li> <li>Type of tube, citrate concentration</li> <li>Method of transportation to the laboratory</li> <li>Use (or not) of a contact phase inhibitor</li> <li>Interval between sampling and centrifugation</li> <li>Centrifugation protocol (<i>g</i> force, duration, temperature, single or double centrifugation)</li> <li>Freezing conditions (temperature, storage duration)</li> <li>Thawing and post-thaw mixing conditions</li> </ul>
Analytical conditions
<ul style="list-style-type: none"> <li>Precise if use of in-house / commercially available reagents</li> <li>If available, source and final concentration of tissue factor</li> <li>If available, phospholipids composition and final concentration</li> <li>Source and final concentration of the activator of PC pathway</li> <li>Methodology used to determine the final concentration of the activator of PC pathway</li> </ul>
Results of thrombin generation assays
<ul style="list-style-type: none"> <li>ETP ratio should not be reported alone</li> <li>Provide all the values obtained, including the parameters that reveal the TG curve shape</li> </ul>

Abbreviations: ETP, endogenous thrombin potential; PC, protein C; TG, thrombin generation.

18 months, but the stability of frozen samples has not been specifically evaluated for TGA in the presence of TM. Importantly, when comparing patients with cirrhosis and healthy controls, sample collection and processing should be exactly the same.

The analytical conditions and the presentation of TGA results also are important. The final concentrations of TF and PL should be provided, or when concentrations are not available (e.g., for commercial PT reagents), the dilutions should be specified. How the issue of the actual active TF concentration can be tackled remains unstandardized. The development of commercial reagents improves TGA standardization, but the use of PC pathway activators possibly introduces some variability. The source and type of TM or Protac should be provided, as well as the final concentration and how it was selected (e.g., percentage of ETP reduction). How results are presented also is crucial for TGA interpretation and possibly for the prediction of clinical events. ETP is usually considered as the main parameter because it represents the total amount of potential thrombin activity generated in the sample. When PC pathway activators are added, the ratio of ETP with/ETP without activators could be used, but this method can induce confusion. **Fig. 3A** shows the TGA curves of two patients. Patient 1 has an ETP of 1,400 nM.min and a PH of 250 nM. Patient 2 has the same ETP value (1,400 nM.min), but different thrombin generation kinetics (prolonged LT, and lower velocity and PH). This shows that the thrombogram shape could be important, and that besides ETP, other TGA parameters should be reported. **Fig. 3B** shows the thrombin generation curves of one healthy control



**Fig. 3** Examples of thrombin generation curves in patients with cirrhosis. (A) shows the thrombin generation curves of two patients with cirrhosis. Although they have the same endogenous thrombin potential (ETP) value (1,400 nM.min), the thrombin generation kinetics is different with prolonged lag time and decreased peak height and velocity in patient 2 (red curve) compared with patient 1 (blue curve). (B) shows the thrombograms of one healthy control (dark and light blue curves) and one patient with cirrhosis (red and orange curves), without and with addition of thrombomodulin (TM). The patient's ETP value in the presence of TM (600 nM.min) suggests a hypocoagulability phenotype when compared with the healthy control's ETP value (700 nM.min). Conversely, the ETP ratio with/without TM of the patient (0.75) indicates resistance to TM activity (ratio for the healthy control = 0.5). This shows that for the same patient, the thrombin generation assay conclusion could be opposite if only the ETP value or the ETP ratio with/without TM is considered.

and one patient with cirrhosis, obtained without and with TM. In the healthy control, ETP was 1,400 nM.min without TM, but was reduced by 50% with TM, leading to an ETP ratio with/without TM of 0.5. In the patient, the ETP without TM was 800 nM.min and decreased to 600 nM.min after TM addition, leading to an ETP ratio with/without TM of 0.75. This example demonstrates that opposite conclusions can be drawn by using the ETP value with TM (hypocoagulable phenotype) and the ETP with/without TM ratio (hypercoagulable phenotype, or "procoagulant imbalance"). In reality, the patient was poorly sensitive to the anticoagulant effect of TM, which nevertheless reduced the amount of thrombin. For these

reasons, it seems important to provide all the values obtained by TGA, including the parameters that reveal the TG curve shape. The need of reporting all raw values, and not just the ETP ratios, has been already debated elsewhere.<sup>56,57</sup> In any case, the thrombin generation curve shape deserves more attention,<sup>34</sup> because there is no unique pattern for a single ETP value. Moreover, at some point, TGA might be implemented for routine hemostasis diagnosis, and therefore it is important to specify what TGA parameters need to be reported to the clinician. To date, no TGA parameter has been clearly linked to clinical events in patients with cirrhosis (except the ETP ratio in a retrospective study).<sup>58</sup>

## Perspectives and Conclusion

TGA is a promising tool for the study of the cirrhosis-related coagulopathy. TGA improves our understanding of the complex hemostasis balance, particularly when the PC pathway is taken into account. However, the lack of standardization of TGA methods in the presence of a PC activator is a critical issue for their routine implementation. The preanalytical and analytical conditions of TGA in the presence of PC pathway activators need to be standardized. New recently available tools, such as the fully automated ST-Genesia system developed by Stago, is a first step in this direction. Indeed, ST-Genesia includes a reagent without and with TM (ThromboScreen, Fisher Diagnostics) with a final concentration of TM that reduces the ETP of healthy controls by ~50%.<sup>59</sup> Moreover, the presence of a reference plasma and internal quality controls might help to reduce the large interlaboratory variability of the CAT method.<sup>45,60</sup> These modifications will probably improve TGA standardization and facilitate multicenter studies.

In patients with cirrhosis, standardized TGA measurements could be used to monitor anticoagulant treatment, which is a particular challenge in patients with cirrhosis.<sup>61</sup> Indeed, *in vitro* studies suggest major differences in the anticoagulant potency of commonly used anticoagulant drugs.<sup>35,62</sup> In addition, the pharmacokinetics of anticoagulant agents may be different in patients with cirrhosis. This might be particularly relevant for direct oral anticoagulants that are partially cleared by liver, and dose adjustments may be required in patients with cirrhosis. For instance, Bos et al recently demonstrated that *in vivo*, the anticoagulant activity of the direct Xa inhibitor edoxaban is lower in patients with cirrhosis compared with healthy individuals.<sup>63</sup> Dose adjustments could be guided by TGA to monitor the achievement of the ideal ETP values for patients under anticoagulant therapy. Similarly, TGA could be used to make treatment decisions or monitor procoagulant therapy. For example, clinical observation and laboratory studies using TGA have demonstrated that procoagulant therapy using fresh frozen plasma (FFP) is likely ineffective in patients with cirrhosis both in prophylactic settings and during active bleeds.<sup>64,65</sup> Other more potent procoagulants may benefit from monitoring by TGA. For example, Lisman et al have demonstrated enhanced *in vitro* potency of prothrombin complex concentrates in patients with cirrhosis, suggesting dose-reductions may be required for these types of agents.<sup>62</sup> Moreover, it

would be important to pay attention to a potential tilt of the fragile balance toward (more) hypercoagulability, if no AT is administered (PCC), or if AT is in insufficient amounts in FFP.

TGA performed using whole-blood also is an interesting tool.<sup>66</sup> As most CAT assays are performed with PPP, they do not take all *in vivo* hemostasis components, for instance platelets that play a role in TGA of patients with cirrhosis, into account.<sup>28,32</sup> Whole blood TGA tests might be suitable as point-of-care test that might facilitate treatment decisions in actively bleeding patients.

Whatever assay will prove to be the best and more convenient candidate, it will have to be evaluated in patients with cirrhosis, and one of the persisting challenges is to determine which parameter is best correlated with clinical events. Moreover, thrombin generation represents only one part of the complex puzzle of hemostasis. Associating TGA with other assays, such as platelet function analysis, study of fibrinolytic potential or evaluation of the clot structure,<sup>67</sup> could bring added value for the prediction of clinical events. Therefore, multicenter collaborations and prospective studies on cirrhosis-related coagulopathy are urgently needed to identify the parameter(s) or assay combinations that might help to predict thrombotic and bleeding events in patients with cirrhosis.

Thrombin generation in the presence of TM has helped to better understand the coagulability status in patients with cirrhosis, and it has also been a suitable tool for determining the consequences of complex changes in the coagulation system in other liver diseases and in surgical procedures involving the liver. Future studies on TGA place in the management of bleeding and thrombosis events in patients with acute liver failure and in patients undergoing partial liver resection or liver transplant surgery should follow similar principles, as discussed in this manuscript.<sup>51,68,69</sup> TGA with TM takes the interplay between all pro- and anticoagulants into account, and therefore has a definite added value compared with other whole blood tests of hemostasis, such as thromboelastography and rotational thromboelastometry. This point is relevant for all patients with simultaneous alterations in pro- and anticoagulant proteins. Finally, enhanced thrombin generating potential despite prolonged conventional coagulation tests is observed not only in patients with liver diseases but also in other patient populations with simultaneous changes in pro- and anticoagulant proteins, as has been demonstrated in healthy neonates,<sup>70</sup> and in patients undergoing lung transplant surgery.<sup>71</sup> Future studies in these patient categories would benefit from the standardization procedures we propose here.

## Conflict of Interest

None.

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