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Development of the FibWave, an improved clot waveform analysis for the assessment of hemostasis

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Development of the FibWave, an improved clot waveform analysis for the assessment of hemostasis

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Development of the FibWave, an improved clot waveform analysis for the assessment of hemostasis

Thromboembolic and hemorrhagic disorders are a global public health problem. Over the last 50 years, research has focused on developing strategies to reduce the mortality and morbidity associated with these diseases. Many studies have been conducted on the assessment of thromboembolic and hemorrhagic risks and the adaptation of patient treatment. In this context, the accessibility of clinical tests is essential to improve the prevention of these disorders.

Currently, many assays, reagents and coagulation analyzers are on the market. Although reagents and assays are readily available, they display various sensitivities. The sensitivity of the reagents depends on the composition of the reagent including the type of activator, the origin and composition of the phospholipids. For coagulation tests, turbidimetry or mechanical detection, viscoelastometry, resonance, visual or fluorometric monitoring are part of the methods available in the laboratory.

The present PhD thesis describes different global hemostasis assays and their potential in clinical applications. Based on some coagulation assays, we aimed to develop a new coagulation method evaluating the fibrin formation kinetics. The method, named the FibWave, allows assessing the global coagulation process by measuring turbidity changes during the fibrin clot formation. An idea of the clot waveform can be depicted in real time and, in addition to obtaining a clotting time, additional coagulation parameters can be extracted.

A detailed description of four global hemostasis assays such as the clot waveform analysis (CWA), global hemostasis potential (OHP), thrombin

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generation assay (TGA), and viscoelastic methods will be introduced. Then, a description of the FibWave and a proof of concept in procoagulant and anticoagulant states will be provided. Finally, the perspectives according to the new in vitro diagnostic medical devices regulation (IVDR) as well as the validation and standardization of FibWave will be discussed.

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Développement du FibWave, une analyse optimisée de la forme d'onde du caillot pour l'évaluation de l'hémostase

Les maladies thromboemboliques et hémorragiques constituent un problème mondial de santé publique. Au cours des 50 dernières années, la recherche s'est concentrée sur le développement de stratégies visant à réduire la mortalité et la morbidité associées à ces maladies. De nombreuses études ont été menées sur l'évaluation des risques thromboemboliques et hémorragiques et l'adaptation du traitement des patients. Dans ce contexte, la disponibilité de tests cliniques est indispensable pour améliorer la prévention de ces maladies.

Actuellement, de nombreux tests, réactifs et analyseurs de coagulation sont disponibles sur le marché. Bien que les réactifs et les tests soient facilement disponibles, ils présentent des sensibilités différentes. La sensibilité des réactifs dépend de leur composition, notamment du type d'activateur, de l'origine et de la composition des phospholipides. Pour les tests de coagulation, la turbidimétrie ou la détection mécanique, la viscoélastométrie, la résonance, le monitoring visuel ou fluorométrique font partie des méthodes disponibles au laboratoire.

Cette thèse de doctorat décrit différents tests d'hémostase globale et leur potentiel dans les applications cliniques. Sur base de certains tests de coagulation, nous avons cherché à développer une nouvelle méthode de coagulation évaluant la cinétique de formation de la fibrine. Cette méthode, appelée FibWave, permet d'évaluer le processus global de coagulation en mesurant les changements de turbidité pendant la formation du caillot de fibrine. Une idée de la forme d'onde du caillot peut être représentée en

temps réel et, en plus d'obtenir des résultats sous la forme d'un temps de coagulation, d'autres paramètres de coagulation peuvent être extraits.

Une description détaillée de quatre tests d'hémostase globale, tels que l'analyse de la forme d'onde du caillot (CWA), le potentiel d'hémostase globale (GHP), le test de génération de thrombine (TGA) et les méthodes viscoélastiques, sera présentée. Ensuite, une description de FibWave, son développement, y compris les méthodologies, et la preuve de concept dans des conditions procoagulantes et anticoagulantes seront fournis. Enfin, les perspectives selon la nouvelle réglementation sur les dispositifs médicaux de diagnostic in vitro (IVDR) ainsi que la validation et la normalisation du FibWave seront abordées.

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Merci à Christelle avec qui j'ai dispensé les travaux pratiques en sciences pharmaceutiques à mon arrivée au département. C'était un plaisir de travailler avec toi. Tu as toujours été de bons conseils et je garderai un très bon souvenir de nos échanges. Merci également à Romain qui m'a beaucoup aidé dans la résolution des divers problèmes auxquels j'ai pu faire face, et ce depuis le début de cette thèse.

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Mes plus sincères remerciements vont à mes parents, ma famille et mes amis pour leur soutien et leurs encouragements, et ce depuis le début de mon parcours universitaire.

Finalement, j'aimerais remercier Louise pour sa présence, son soutien et ses encouragements. Merci d'être là et de me supporter dans les bons moments comme dans les moments plus difficiles.

Table of contents

List of abbreviations

(d)TT	(diluted) thrombin time
APTT	Activated partial thromboplastin time
CAT	Calibrated automated thrombogram
CW	Clot waveform
CWA	Clot waveform analysis
Delta	Difference between absorbance maximum and minimum
DIC	Disseminated intravascular coagulation
DOACs	Direct oral anticoagulants
DRVVT	Dilute Russell's Viper venom time
DTI	Direct thrombin inhibitors
ECA	Ecarin chromogenic assay
ECT	Ecarin clotting test
ETP	Endogenous thrombin potential
HA/HB	Hemophilia A/B
HIL interferences	Hemolysis (i.e. hemoglobin), icterus (i.e. total bilirubin) and lipemia interferences
IVD	In vitro medical devices
IVDD	IVD Directive
IVDR	IVD Regulation
LA	Lupus anticoagulant
Max1	Maximum velocity of coagulation
Max2	Maximum acceleration of coagulation
Min2	Maximum deceleration of coagulation
mVRI	Mean velocity rate index
PPS	Padua Prediction Score
PPP	Platelet-poor plasma
PT	Prothrombin time
PRP	Platelet-rich plasma
RT	Reptilase time
sTF	Small (amount of) tissue factor
TEG/ROTEM	Thromboelastography/thromboelastometry
TG	Thrombin generation
TGA	Thrombin generation assay
Time to Max1	Time to reach the maximum velocity
Time to Max2	Time to reach the maximum acceleration
tPA	Tissue plasminogen activator
Ttpeak	Time to peak
VKA	Vitamin K antagonists
VTE	Venous thromboembolism
Min1	Maximum velocity of fibrinolysis
TFib	Time to (fibrino)lysis
TFib – Ttpeak	Time between the time to lysis (time to reach the Min1) and the time to peak (time to reach the Max1)
TEG/ROTEM	Thromboelastography/thromboelastometry
2G/3G CHC	Second/third generation combined hormonal contraception

Global hemostasis assays

1. Clot waveform analysis (CWA)

I. History

The clot waveform analysis (CWA), initially reported in 1997, allows of the in-depth evaluation of the activated partial thromboplastin time (APTT) and prothrombin time (PT) with the light transmission (**Figure 1**).(Braun, Givens et al. 1997) The APTT and PT tests are standard laboratory screening tests, named traditional, routine or conventional assays in literature and measure the time for plasma to clot by activating the intrinsic or extrinsic pathway, respectively (**Annex 2, Annex 3**).(Bates and Weitz 2005) The APTT- and PT-CWA are the global hemostatic assessment that evaluates the clot formation kinetics during routine clotting tests. The optical detection system measures the coagulation process by measuring changes in turbidity created during the clot formation process.(Luddington, Peters et al. 1997, Downey, Kazmi et al. 1998) Photo-optical reading, in absorbance or transmittance, is continuously recorded over time, creating a graph that reflects the whole process of clot formation, called the clot waveform analysis (CWA). When APTT or PT is assessed, the optical coagulation analyzers automatically generate APTT-/PT-CWA data and therefore visualize the clot waveform from APTT or PT.(Toh, Downey et al. 2000, Oka, Wakui et al. 2020) The qualitative examination as well as the quantitative parameters provided by the clot waveform give additional information to the classic clotting time usually reported for APTT and PT. The first findings were obtained from standard APTT measurements, but PT assay has also been used in various studies.(Shima, Matsumoto et al. 2002, Su, Braun et al. 2002, Toh and Giles 2002, Ruberto, Marongiu et al. 2018)

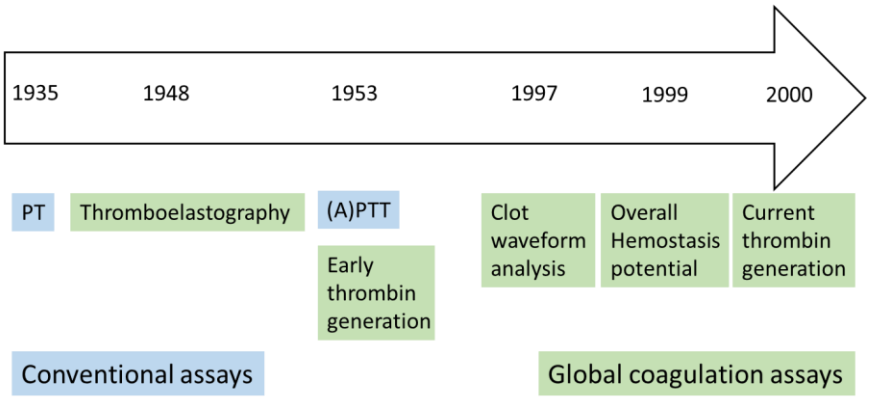


Figure 1: History of functional coagulation tests based on thrombin and fibrin formation.
PT: prothrombin time; APTT: activated partial thromboplastin time.

II. Principle

With the clot waveform analysis, the coagulation process can be depicted and categorized into 3 phases: i) the pre-coagulation phase, matching to signal of the onset of coagulation, ii) the coagulation phase, where absorbance increases over time and defined by a slope in the waveform and iii) the post-coagulation phase, where absorbance tends to stabilize (**Figure 2**).

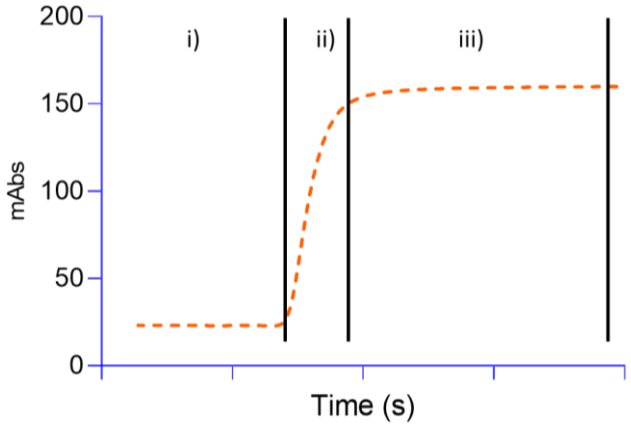


Figure 2: The coagulation process from the APTT-based clot waveform analysis (CWA).
i) pre-coagulation phase, ii) coagulation phase, iii) post-coagulation phase

To perform the CWA, an analyzer using an optical detection system is required. The coagulation analyzers are usually equipped with software that automatically computes absorbance or transmittance data and processes them into CWA parameters. Alternatively, raw data can also be exported and processed by external software to get other parameters such as the Delta parameter. The Delta parameter represents the difference between the top (maximum absorbance or minimal transmittance) and the bottom (minimum absorbance or maximal transmittance) of the curves. This correlates with fibrinogen level and can inform on the quantity of fibrin formed during the coagulation process in the test cuvette. The software algorithm also processes the raw data of fibrin formation and generate two other curves, the first and the second derivative curves. The first derivative curve gives information on the velocity of coagulation. The peak of the first derivative, the maximum value, is considered as the maximum velocity. The second derivative informs on the acceleration and the deceleration of coagulation. The maximum and minimum value of the second derivative, positive and negative peak, are considered as the maximum acceleration and the maximum deceleration. The denomination of these parameters varies depending on the recorded signal, transmittance or absorbance. In the case of absorbance, the maximum velocity of the coagulation is called “Max1”, the maximum acceleration, “Max2” and the maximum deceleration, “Min2”. These parameters are automatically exported in addition to the clotting time from routine assays. The pattern of the transmittance is the reverse of the absorbance pattern. Therefore, the maximum velocity, acceleration and deceleration are called “Min1,” “Min2” and “Max2,” respectively.(Sevenet and Depasse 2017)

According to the activation pathway, i.e. intrinsic or extrinsic pathway, the parameters provided by the software may be different. For PT test,

parameters computed by the software are Max1 and the time to Max1, considered as the PT clotting time. For APTT test, the software provides the Max1, Max2, Min2 and time to Max2 considered as the APTT clotting time by the instrument (**Figure 3**).

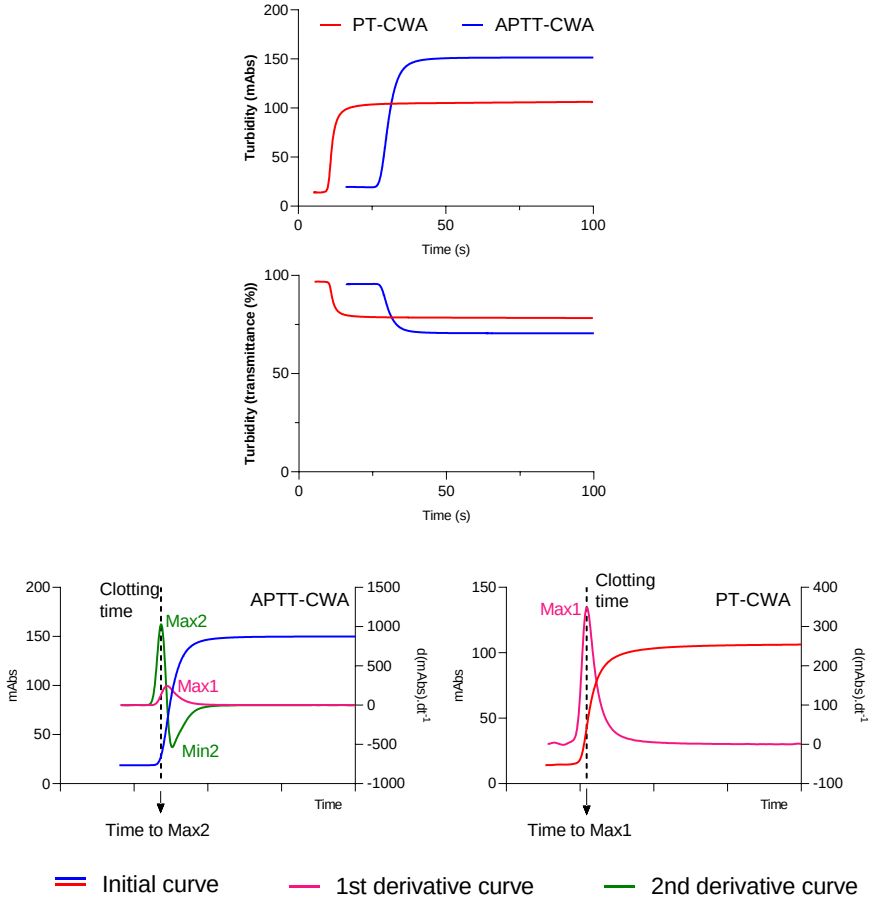


Figure 3: Schematic curve and coagulation parameters of APTT- and PT-based clot waveform analysis.

III. Potential clinical application of clot waveform analysis

a. Bleeding disorders

- Factor deficiencies

The clot waveform analysis showed to be sensitive to mild factor deficiencies (FXII, X, IX, VIII, VII, V, II and I). In factor deficiencies of intrinsic pathway (FXII, FXI, FIX), the pre-coagulation phase was prolonged and changes in slope of first derivative were different.(Shima, Thachil et al. 2013, Lance 2015)

The CWA is able to give information on the quantity of fibrin formed during the test and the difference between the pre-coagulation and post-coagulation phases correlated well with fibrinogen level.(Arai, Kamijo et al. 2020) According to Shima *et al.*, the CWA could reflect the thrombin generation (TG) since fibrinogen is the substrate of thrombin in the coagulation cascade.

In routine laboratory, the functional quantification of plasma fibrinogen levels can be assessed by Clauss fibrinogen assay (CFA), based on thrombin time, and PT-derived (optical method). Fibrinogen defects may be quantitative (hypo- or hyper-fibrinogenemia) or qualitative (dysfibrinogenemia). The CFA is the diagnostic tool of choice when diagnosing or treating patients with low fibrinogen levels but provides only information on functional fibrinogen. The immunological fibrinogen antigen determination allows evaluating the amount of fibrinogen but not performed in all laboratories due to high cost and low throughput.(Mackie, Kitchen et al. 2003, Mackie, Cooper et al. 2013) These both assays are relevant because of their detection methods but differentiating low levels of functional fibrinogen (qualitative defects) from low levels of fibrinogen antigen (quantitative defects) is not possible with CFA or immunological determination alone. For the investigation of congenital dysfibrinogenemia, both functional and immunological fibrinogen antigen assays should be

performed to identify any discrepancy between results.(Mackie, Kitchen et al. 2003)

The CWA in Clauss fibrinogen assay (CFA-CWA) has recently been studied for providing a marker for fibrinogen antigen or for detecting functional fibrinogen abnormalities without additional measurement of fibrinogen antigen. The preliminary results showed that the maximum velocity of CFA-CWA was strongly associated with fibrinogen antigen in normal and fibrinogen disorders. The introduction of an estimated fibrinogen antigen (eAg) levels used as an alternative of the immunological fibrinogen antigen determination and a ratio between fibrinogen activity and eAg (Ac/eAg ratio) could be used to identify quantitative and qualitative abnormalities of fibrinogen. The original method required to export data from analyzer and recently, an automated analysis software has been validated.(Shima, Matsumoto et al. 2008, Sevenet and Depasse 2017, Suzuki, Suzuki et al. 2019, Suzuki, Suzuki et al. 2022)

- Direct oral anticoagulants (DOACs) management

Dabigatran, an oral direct thrombin inhibitor (DTI), apixaban, edoxaban and rivaroxaban, oral direct inhibitors of factor Xa (FXa), constitute the first-line therapy used for the prevention of stroke and systemic embolism in non-valvular atrial fibrillation (NVAF), the treatment and secondary prevention of venous thromboembolism (VTE), and the prevention of VTE after orthopedic surgery.(Douxfils, Mullier et al. 2012, Douxfils, Chatelain et al. 2013, Douxfils, Tamigniau et al. 2014, Jackson and Becker 2014, Fujimori, Wakui et al. 2016, Wright, Brown et al. 2017, Dunois 2021) These agents are referred as direct oral anticoagulants (DOACs). Contrary to vitamin k antagonists (VKA), routine monitoring of the anticoagulant activity of DOACs is generally not necessary, but the measurement of DOAC plasma levels can be required in some situations such as drug accumulation in long-term treatment, overdosage,

thrombotic or bleeding events, trauma, or emergency surgery. Even if inter-patient variability is observed in case of hepatic and renal insufficiency and comedication, DOACs present a more favorable pharmacokinetic and pharmacodynamic profile than VKA. An easy-to-use oral medication with fixed dose and limited food and drug interactions are other advantages of DOACs.(Gustafson, Saunders et al. 2019, Coons, Albert et al. 2020, Douxfils, Adcock et al. 2021, Siriez, Dogne et al. 2021)

The impact of DOACs on conventional coagulation assays such as APTT, PT and TT have been largely studied.(Douxfils, Tamigniau et al. 2014, Adcock and Gosselin 2015, Cuker and Husseinzadeh 2015, Douxfils, Ageno et al. 2017, Favaloro, Pasalic et al. 2017, Gosselin, Adcock et al. 2018, Siriez, Evrard et al. 2019, Dunois 2021) APTT, PT and TT are based on the classical concepts of intrinsic, extrinsic and common cascade mechanisms and reflect the coagulation in a non-physiological environment.(Wakui, Fujimori et al. 2019) They measure only the end-point clotting time, corresponding to formation of 5% of total thrombin, and therefore, reflect the initial coagulation process under supraphysiological triggers.(Duarte, Ferreira et al. 2017) They are widely available in clinical laboratories conferring an important benefit in the case of emergencies but these assays were showed to be relatively insensitive to the effect of DOACs, be reagent- and method-dependent. Furthermore, they do not provide a complete overview of hemostasis compared to assays evaluating coagulation in detail like e.g. thrombin generation tests and viscoelastic tests.(Pernod, Albaladejo et al. 2013, Lance 2015)

Besides these conventional assays, specific laboratory tests, calibrated to report results in ponderal concentration, i.e. in ng/mL, have been developed. The diluted thrombin time (dTT), the ecarin clotting time (ECT), the ecarin chromogenic assay (ECA) and chromogenic anti-FII assays are used to

estimate the concentration of dabigatran in the plasma while anti-FXa are used to estimate the concentration of factor Xa inhibitors. For anti-FXa assays, there are one-stage and two-stage chromogenic assays, both based on the inhibition of FXa by FXa inhibitors. The anti-Xa chromogenic assays are robust but can be influenced by heparins. The Biophen® Direct Factor Xa Inhibitor, a two-stage assay, is performed in conditions of high ionic strength (pH 7.9 buffer) where heparins cannot interact with antithrombin. Therefore, the assay is specific for direct FXa inhibitors. Finally, these different tests correlate very well with the reference standard method, liquid chromatography/tandem mass spectrometry but are more expensive than routine APTT and PT tests (**Table 1**).(Douxfils, Ageno et al. 2017, Favaloro and Lippi 2017, Jaffer, Chan et al. 2017, Wright, Brown et al. 2017, Siriez, Alpan et al. 2020, Dunois 2021, Wiesen, Fietz et al. 2021)

	Drugs				
	Vitamin K antagonist	Dabigatran	Apixaban	Edoxaban	Rivaroxaban
Routine assays (screening for DOACs)					
APTT	↑↑	↑↑	↑	↑	↑
PT/INR	↑↑↑	↑	↑↑	↑↑	↑↑
TT	↔	↑↑↑	↔	↔	↔
Quantifying assays (measuring levels of DOAC)					
dTT	↔	↑↑↑*	↔	↔	↔
ECT/ECA	↔	↑↑	↔	↔	↔
Anti-FXa activity**	↔	↔	↑↑↑	↑↑↑	↑↑↑
Anti-FIIa activity	↔	↑↑↑	↔	↔	↔

Table 1: How coagulation assays are affected by oral anticoagulants.
**Some methodologies require specific calibrators for plasma concentration (< 50 ng/mL).*
***Anti-Xa chromogenic assays using specific apixaban, edoxaban or rivaroxaban calibrators.*
↑: prolongation; ↔: no effect. ECT: ecarin clotting time; ECA: ecarin chromogenic assay.

Besides routine and quantifying assays, the CWA showed a good ability in assessing the effects of DOACs. Wakui *et al.* demonstrated blockage of thrombin-positive feedback by dabigatran and FXa inhibitors and observed differences between them on fibrin generation. According to results, dabigatran suppresses activation of FV and FVIII by thrombin via direct inhibition of thrombin while FXa inhibitors do that via the inhibition of thrombin generation.(Wakui, Fujimori et al. 2019, Wakui, Fujimori et al. 2019, Wakui, Fujimori et al. 2020)

Parameters of the APTT-CWA have been evaluated in orthopedic patients on edoxaban or warfarin. The temporal parameters (time to Max1 and time to Max2) were prolonged with edoxaban, delaying the initiation of clot formation while warfarin decreased the peak of the first and second derivatives (Max1 and Max2), inhibiting the velocity and acceleration of clot formation. Further studies comparing chromogenic assays with CWA are needed because CWA parameters was not well correlated with anti-FXa activity, thus preventing to use the CWA for monitoring direct oral anticoagulant levels.(Hasegawa, Wada et al. 2018, Matsumoto, Wada et al. 2018)

- Hemophilia

In normal condition, FVIII and FIX play a key role in coagulation cascade to provide a strong and amplified fibrin clot.(Wolberg and Campbell 2008) These both factors become activated and form a complex to activate the FX. The activation of FX generates thrombin from prothrombin and thrombin cleaves fibrinogen into fibrin, consolidated by activated FXIII. Therefore, FVIII or FIX deficiencies jeopardize FX activation, leading to insufficient thrombin generation and inadequate fibrin clot.(Vehar, Keyt et al. 1984)

Hemophilia A (HA) and hemophilia B (HB), discovered in 1952, are congenital bleeding disorders caused by mutations in the genes encoding factor VIII (FVIII) and factor IX (FIX), respectively. Deficiency of coagulation factor XI (FXI) is defined as hemophilia C. The prevalence of HA and HB are 1 in 5,000 and 1 in 30,000 in the men population, respectively.(Stefanovic, Bozovic et al. 1956, Berntorp and Shapiro 2012).

The classification of severity is based on the amount of FVIII or FIX activity: severe (<1 international unit (IU)/dl), moderate (1–5 IU/dl) or mild (6 IU/dl to <40 IU/dl).(Stonebraker, Bolton-Maggs et al. 2010, Stonebraker, Bolton-Maggs et al. 2012) HA and HB have similar symptoms, characterized by

bleeding into large joints such as elbows, knees and ankles. In moderate hemophilia, mild bleeds are common although serious bleeding may also occur in response of trauma or surgery (dental extractions) while in severe hemophilia, more spontaneous bleeding are common, although in some cases, mild bleed may occur.(Fischer, Ljung et al. 2014) According to the literature, approximately 10%-15% of severe HA patients present with mild bleeding phenotype. On the other hand, 25% of moderate HA patients can suffer from frequent hemorrhage.(den Uijl, Fischer et al. 2009, Santagostino, Mancuso et al. 2010, Pavlova and Oldenburg 2013, Tarandovskiy, Balandina et al. 2013, Humphries, Mathew et al. 2017, Milos, Coen Herak et al. 2020)

Typically, an isolated prolonged APTT can be the sign of factor deficiencies (hemophilia) or factor inhibitors (lupus anticoagulant or anticoagulant) in patients. To differentiate a factor deficiency from inhibitory substance, mixing tests with normal plasma are performed at 2 incubation time intervals (0 and 2 hours). Then, specific measurement of factor, such as FVIII clotting activity, is performed as definite diagnosis (**Figure 4**). These specific measurements for hemophilia A and B can be carried out using one-stage or chromogenic assays.(Tiede, Collins et al. 2020) The first assay is the most used, the cheaper and is based on APTT test. The determination of levels of FVIII or FIX is made by comparing APTT measured in test plasma with that measured in serial dilutions of standard reference plasma. The second, i.e. the chromogenic assay, is based on the measurement of the amount of FXa generated in plasma, which is proportional to the level of functional FVIII or FIX. In this assay, the concentration of FXa is quantified using a chromogenic substrate for FXa, where the intensity of the color generated is proportional to the level of FXa. (Marlar, Strandberg et al. 2020) The one-stage is mainly used for diagnosis of hemophilia but in some situations, the chromogenic

assay is used for the accurate diagnosis of non-severe HA.(Kitchen, Blakemore et al. 2016)

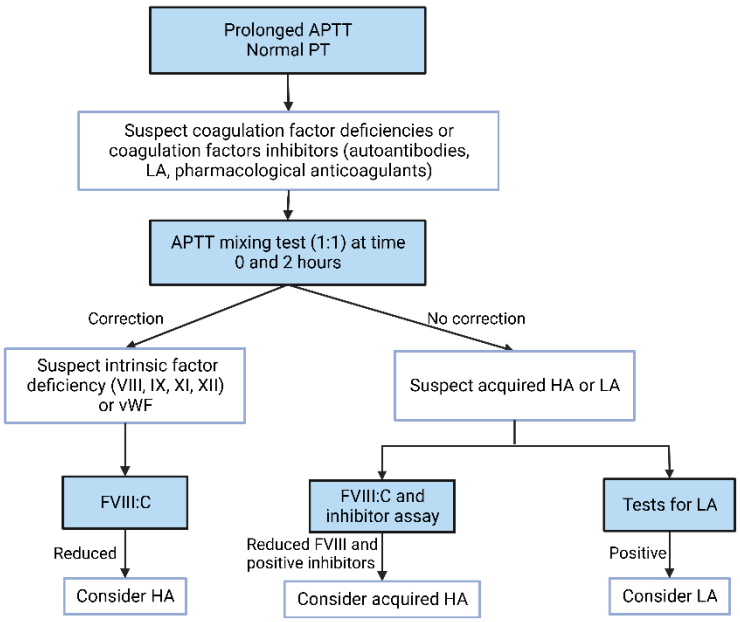


Figure 4: Example of diagnostic algorithm of acquired hemophilia.

Although interesting, these tests are limited by their sensitivity at very low levels (<1 IU/dL) and by their inability to provide an overview of the patient's coagulation system. The discrepancy between levels of clotting factor activity and clinical symptoms has been showed, where mild HA demonstrated frequent episodes of spontaneous bleeding.(Matsumoto, Shima et al. 2006) In addition, development of inhibitors against FVIII or FIX is one of the major complications and the monitoring of hemophilia patients with inhibitors is complicated with standard assays evaluating only a part of the coagulation system.(Lenting 2020) In this way, the priority should be given to developing global assays, able to provide a complete picture of coagulation system and evaluate, as much as possible, the in vivo coagulation, such as the thromboelastography (TEG/ROTEM), thrombin generation assay (TGA) and

clot waveform analysis.(Aghighi, Riddell et al. 2020, Berntorp, Fischer et al. 2021)

Recent studies demonstrated the potential of the CWA in hemophilia. It allowed to differentiate non-severe from severe HA patients as well as patients with or without inhibitors directed against the substitution therapies used in these pathological conditions and to investigate the patients' phenotype. Matsumoto *et al.* compared and evaluated the correlation between the CWA and thrombin generation parameters in the measurement of both FVIII and FIX in the concentration range of 0–1.0 and of 1.0–100.0 IU.dL^{-1}. They highlighted that CWA was more sensitive than TGA for the detection of the hemostatic effect for levels of FVIII or FIX at <1.0 IU dL^{-1}. The clinical severity was also better correlated with CWA parameters than FVIII levels. Other confirmed that CWA and especially velocity (Max1) and acceleration (Max2) may be more appropriate to assess low clotting factor activity.(Shima, Matsumoto et al. 2002, Matsumoto, Shima et al. 2006, Shima, Matsumoto et al. 2008, Matsumoto, Nogami et al. 2017, Aghighi, Riddell et al. 2020, Milos, Coen Herak et al. 2020, Dave, Geevar et al. 2021)

Milos *et al.* showed that their "DELTA" parameter (**Figure 5**), defined as the difference between the onset of coagulation and time at point of inflexion, reflecting the fibrin formation velocity, was significantly correlated with FVIII activities and clinical parameters and permitted to discriminate mild, moderate and severe HA patients.(Nair, Dargaud et al. 2010, Milos, Coen Herak et al. 2014)

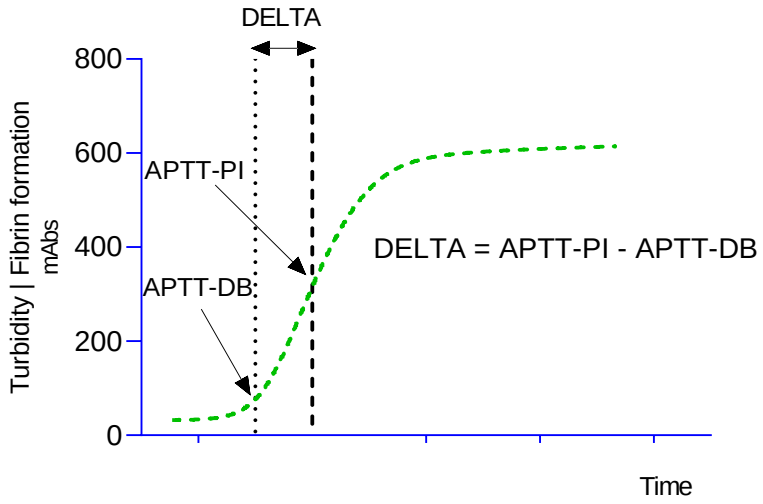


Figure 5: Illustration of DELTA parameter according to Milos et al.
APTT-DB: APTT obtained by drifting baseline evaluation mode; APTT-PI: APTT obtained by point of inflexion evaluation mode.

Lately, Shimonishi *et al.* developed a new algorithm, the "inhibitor index", defined as ratio of CWA clotting time, Min1, or aspect ratio (Ar10), a weighted average-related parameter, at two times of incubation, to differentiate HA with and without inhibitors, and lupus anticoagulant. They showed that the properties of the CWA allowed the differentiation of these diseases with an incubation period of 12 minutes.(Shimonishi, Ogiwara et al. 2021, Shimonishi, Ogiwara et al. 2021)

To date, it remains difficult to assess the effects of bypass therapy in patients with inhibitors because there is no close relationship as observed with level of FVIII activity in HA patients without inhibitors. Haku *et al.* showed that CWA clotting time, velocity (Max1), and acceleration (Max2) could provide useful quantitative information for the monitoring of hemostasis during treatment with recombinant FVIIa (rFVIIa) and activated prothrombin complex concentrate (APCC) in HA patients with inhibitors. Interestingly,

they used a combined reagent of tissue factor and ellagic acid (TF/Elg), initiating extrinsic and intrinsic pathway, and showed a good correlation between laboratory parameters and clinical status. This new TF/Elg mixture associated to method, sTF/FIX-CWA, has been used for assessing coagulation potential in HA patients treated with emicizumab. These encouraging findings suggest that CWA may be useful for monitoring APCC, rVIIa or emicizumab therapy in these patients.(Haku, Nogami et al. 2014, Nogami, Matsumoto et al. 2018, Wada, Matsumoto et al. 2020)

- Cirrhotic disease

The liver is responsible for the production of the majority of factors in coagulation process. A normal hemostatic balance is maintained by an equilibrium between procoagulants and anticoagulants. In patients with cirrhosis, the decrease of liver-dependent clotting factors (FII, V, VII, IX and X), endogenous anticoagulants (protein C, protein S and antithrombin) with the concomitant increase of FVIII and von Willebrand factor (vWF) are observed. A thrombocytopenia, caused by splenic sequestration of platelets secondary to portal hypertension, a decreased production of thrombopoietin (TPO), and increased platelet destruction, is common in cirrhosis. Decreased levels of fibrinogen are also observed in 40% of patients with cirrhosis. Being the substrate of clot formation, its absence could increase bleeding risk. Due to complex interactions resulting in a disruption of the pro- and anticoagulants balance but also a rebalanced system of hemostasis, cirrhotic patients may suffer from bleeding and thrombosis. According to rebalanced hemostasis model, a new balance, more vulnerable, between procoagulant, anticoagulant and fibrinolytic systems occur, which leading to bleeding or thrombosis depending on circumstantial risk factors. (Tripodi, Primignani et al. 2009, Tripodi, Primignani et al. 2013, Lisman and Ariens 2016, Shah and

Caldwell 2016, Flores, Trivedi et al. 2017, Ruberto, Marongiu et al. 2018, Sigal, Sherman et al. 2020)

The traditional laboratory tests (APTT/PT/INR) do not fully reflect the impaired hemostasis and are unreliable for predicting the bleeding risk in patients with cirrhotic disease. The PT and INR assess only procoagulant factors without considering the anticoagulants effect. In cirrhosis disease, the assays can be abnormal because reduced procoagulant factors, but a prolonged PT/INR does not predict bleeding.(Kujovich 2015)

Ruberto *et al.* previously investigated the relationship between APTT-based CWA and the history of bleeding. The APTT-CWA parameters were lower in patients with positive history of bleeding than patients without history, indicating a weakness of the coagulation.(Ruberto, Marongiu et al. 2017) The PT-based CWA has also been investigated as predictive factor for bleeding risk in cirrhosis. In these patients, a delayed clot formation and a weakness in the clot formation, assessed by velocity and acceleration, were observed and associated with the severity of cirrhosis.(Kleinegris, Bos et al. 2014, Ruberto, Marongiu et al. 2018) These information show that the PT-CWA allows to evaluate the procoagulant effect but also the ability to form a clot. The area under the curve of the first and second derivative of PT, not automatically obtained by analyzers and expressing the whole process of clot formation, have also been evaluated. A prolonged clotting time ratio together with a reduced area under the first derivative also correlated well with the hemorrhagic score, demonstrating the potential of PT-CWA to predict bleeding risk and evaluate the global hemostatic function of patients.(Kleinegris, Bos et al. 2014, Ruberto, Marongiu et al. 2018)

b. Thrombotic disorders

- Venous thromboembolic risk

The venous thromboembolism (VTE), which includes both deep venous thrombosis and pulmonary embolism, is a common medical condition with an incidence rate of approximately 1-2 per 1000 person-years. Hospitalized patients for an acute medical illness have about an 8-fold increase in relative risk of developing VTE.(Prandoni and Samama 2008, Ruberto, Marongiu et al. 2018) The VTE has multiple hemostatic factors and can be broadly described in Virchow's triad, i.e. alteration of blood flow, endothelial injury and hypercoagulability of the blood. Therefore, the risk of VTE requires a combination of biomarkers and needed to identify patients at risk to prevent VTE with appropriate prophylaxis as recommended.(Barbar, Noventa et al. 2010, Ruberto, Marongiu et al. 2018, Lim, O'Malley et al. 2019) The increasing age and inherited or acquired hypercoagulability (thrombophilic mutations, cancer, obesity), cardiac or respiratory failure, prolonged immobility, major surgery, multiple trauma are risk factors in development and recurrence of VTE. For predicting the recurrence of VTE, data suggest measuring individual laboratory parameters such as D-dimer, APTT, thrombin activatable fibrinolysis inhibitor (TAFI), FVIII, FIX, thrombin generation. The most extensively studied laboratory parameter associated with VTE recurrence is the D-dimer where elevated levels of D-dimer are associated with an increased risk of VTE recurrence.(Palareti, Legnani et al. 2002, Anderson and Spencer 2003, Tripodi, Legnani et al. 2008, Ay, Dunkler et al. 2010, Barnes, Kanthi et al. 2015, Barco, Konstantinides et al. 2018, Riva, Vella et al. 2018, van Dam, Boon et al. 2021)

The association between APTT-based CWA, fibrinogen, D-dimer, and the Padua Prediction Score (PPS), score used for the assessment of VTE in patients, has been investigated. A positive relationship was observed

between CWA, fibrinogen, D-dimer, and PPS where density (Delta), velocity and acceleration of clot formation were increased in patients with high PPS (score ≥ 4). All of these parameters were also increased, to a lesser degree, in patients with low PPS (score < 4). A recent report showed that patients with acute VTE had significantly higher CWA parameters than controls without VTE. The CWA parameters were also evaluated as diagnostic screening test for acute VTE. It emerged that Delta parameter demonstrated a poor sensitivity (47%) but good specificity (93%), positive (72%) and negative (81%) predictive values. These studies suggest that CWA could be a useful marker in the diagnosis of acute VTE and its inclusion to PPS score could be better identify patients at VTE risk.(Bjøri, Johnsen et al. 2017, Ruberto, Marongiu et al. 2017, Wen Tan, Cheen et al. 2019)

- Cirrhotic disease

As stated above, the coagulopathy of liver cirrhosis is complex and involves prohemorrhagic and prothrombotic changes and due to imbalance of pro- and anti-coagulant factors, cirrhotic patients may suffer from thrombotic episodes and have a relative risk of venous thromboembolism nearly two-fold greater than that of the general population. As for bleeding risk, the APTT and PT are inadequate for assessing the thromboembolic risk. Prolonged APTT and PT/INR can suggest that cirrhotic patients are auto-anticoagulated, and potentially at risk of bleeding, but showed an increased risk of thrombosis.(Tripodi, Primignani et al. 2013, Ruberto, Sorbello et al. 2017, Sigal, Sherman et al. 2020, Zermatten, Fraga et al. 2020)

As observed with PT-CWA, the density, velocity, and acceleration of APTT-CWA were significantly lower in cirrhotic patients compared to normal subjects, suggesting the weakness of blood coagulation activity. However, these same parameters were increased in the cirrhosis group with high PPS score, suggesting a hypercoagulable state. (Ruberto, Sorbello et al. 2017)

The differences observed between cirrhotic patients with low and high PPS score may indicate a relationship between a hypercoagulable state and prothrombotic phenotype. Ruberto *et al.* confirmed that the APTT-clotting time was inadequate for evaluate thrombotic risk, where clotting time did not distinguish between cirrhotic patients with low PPS or high PPS score. The weakness observed in the clot formation, evaluated, in the APTT and PT-based CWA, including the density, velocity and acceleration, support the concept that CWA can be useful to evaluate the hemostatic state of the patient but also its ability to form an appropriate clot.

- Antiphospholipid syndrome (Lupus anticoagulant)

Antiphospholipid syndrome is autoimmune disease, characterized by autoantibodies directed against phospholipid-binding proteins and phospholipids attached to cell membrane receptors, mitochondria, oxidized lipoproteins, and activated complement components. When antibodies bind to these complex antigens, cells are activated and the coagulation and complement cascades are triggered, culminating in thrombotic events and obstetrical morbidity that further define the syndrome. The phospholipid-binding proteins most often involved are annexins II and V, β2-glycoprotein I, prothrombin, and cardiolipin. The official classification criteria for antiphospholipid syndrome recognize three distinct antiphospholipid antibodies: antibodies against the β2-glycoprotein I, against the combination of the phospholipid cardiolipin (CL) and the β2-glycoprotein I, and the "lupus anticoagulant". Lupus anticoagulant is not a single entity but rather a family of antibodies directed against complex antigens consisting of β2-glycoprotein I, and/or prothrombin bound to negatively charged phospholipids. The presence of LA in patients is described to prolong phospholipid-dependent clotting times and to be associated to increased risk of developing blood clots.(Bertolaccini, Amengual et al. 2014, Pengo, Bison

et al. 2014, Sciascia, Baldovino et al. 2016, Molhoek, de Groot et al. 2018) According to the SSC-ISTH, two assays are recommended, the diluted Russel Viper Venom (DRRV) time that investigates the common coagulation pathway with variable phospholipid concentration and an LA-sensitive APTT, with silica as activator and LA-sensitive phospholipids including hexagonal phase phosphatidylethanolamine.(Devreese, de Groot et al. 2020)

Many different observations have been made in patients with LA with APTT- and PT-based clot waveform analysis, and additional studies are still required to confirm first results and provide evidence of the interest of CWA in this diagnosis. Luddington *et al.* observed abnormal aPTT waveform in 25% of antiphospholipid syndrome patients while Su *et al.* showed that the slope of waveform of PT, i.e. Max1, was abnormal in 61.5% of patients with antiphospholipid syndrome. These results highlighted reagent-dependent effect, where silica-reagent allowed to better differentiate LA from normal patients compared to ellagic acid.(Luddington, Peters et al. 1997, Su, Braun et al. 2002, Solano, Zerafa et al. 2011) Shimonishi *et al.* developed a new algorithm, "inhibitor index" and showed to differentiate HA with and without inhibitors, and lupus anticoagulant. They demonstrated that the properties of the CWA allowed the differentiation of these diseases with an incubation period of 12 minutes.(Shimonishi, Ogiwara et al. 2021, Shimonishi, Ogiwara et al. 2021)

- Disseminated intravascular coagulation (DIC) and sepsis-induced coagulopathy (SIC)

Disseminated intravascular coagulation (DIC) is a devastating clinical condition characterized by the systemic activation of blood coagulation, which generates fibrin in the circulation leading to a microvascular thrombosis associated with thrombocytopenia, a bleeding tendency with hyperfibrinolysis and multiple organ dysfunction. DIC is not a disease by itself

but occurs secondary to a complication of infections, solid cancers, hematologic malignancies, trauma, liver diseases…(Taylor, Toh et al. 2001, Toh and Alhamdi 2013, Wada, Thachil et al. 2013, Levi and van der Poll 2014, Suzuki, Wada et al. 2019) The basic mechanism underlying the onset of DIC is the marked activation and consumption of the coagulation factors followed by the activation of fibrinolysis.(Wada, Matsumoto et al. 2014)

DIC is divided into two major phenotypes, fibrinolytic and thrombotic, and several clinical subtypes, including asymptomatic, bleeding, organ failure and complication types such as thrombotic microangiopathy.(Wada 2004) The sepsis is considered as organ failure subtype associated with a thrombotic phenotype, characterized by elevated fibrinogen and suppression of fibrinolysis induced by endothelial function while the trauma, leukemia or aneurysm are considered as bleeding subtype associated with a fibrinolytic phenotype.(Hayakawa 2017, Iba, Arakawa et al. 2018, Iba, Levy et al. 2019)

Based on systemic activation of blood coagulation, the diagnosis as well as the treatment of DIC must consider the underlying etiologic features.(Wada, Thachil et al. 2013, Wada, Matsumoto et al. 2014, Toh, Alhamdi et al. 2016, Iba, Levy et al. 2019) Three different guidelines for diagnosis and management of DIC, the British Committee for Standards in Haematology (BCSH), the Japanese Society of Thrombosis and Hemostasis (JSTH), and the Italian Society for Thrombosis and Hemostasis (SISET) have been published in the literature.(Levi, Toh et al. 2009, Di Nisio, Baudo et al. 2012, Thachil, Toh et al. 2012, Wada, Takahashi et al. 2017) The International Society of Thrombosis and Haemostasis Scientific and Standardization Sub-Committee (ISTH-SSC) attempts to harmonize the three guidelines to facilitate earlier diagnosis and treatment of DIC.(Wada, Matsumoto et al. 2014, Iba, Levy et al. 2019) The first diagnostic criteria for DIC, created by the Japanese Ministry of Health and Welfare (JMHW) in 1983, included platelet count, prothrombin

time (PT) ratio, fibrin/fibrinogen degradation products (FDPs), fibrinogen and clinical features as diagnostic criteria. The ISTH-SSC added D-dimer to FDPs. The Japanese Association for Acute Medicine (JAAM) DIC diagnostic criteria document is used in Japan for the diagnosis of DIC and for the initiation of anticoagulant therapy. Compared to other guidelines, the JAAM DIC scoring algorithm includes specific criteria for evidence of systemic inflammatory response syndrome (SIRS).(Iba, Arakawa et al. 2018) As reported by criteria in different guidelines, there is no "gold" standard assay for the diagnosis of DIC, and no single test can accurately diagnose DIC.(Toh, Alhamdi et al. 2016) In 2017, the ISTH SSC proposed a new category identifying an earlier phase of DIC, called "sepsis-induced coagulopathy" (SIC).(Iba, Nisio et al. 2017) The SIC diagnostic criteria include platelet count, PT-INR and SOFA score (**Table 2**). The SOFA score helps to confirm the sepsis but does not reflect the severity. It was found that almost all patients with DIC also met criteria for SIC, and that SIC preceded DIC in every case.(Iba, Levy et al. 2019, Lambden, Laterre et al. 2019) Based on this, ISTH- and Perioperative and Critical Care-SSC proposed a two-step diagnostic approach for the early detection of DIC. It consists of screening first for the SIC score and if SIC criteria are met, calculating the DIC in the second step. This new approach could facilitate early recognition of DIC and potentially hasten intervention (Table 2).(Wada, Thachil et al. 2013, Iba, Levy et al. 2019)

Tests	DIC	SIC	Score
Platelet count	<50 x 10^9/L ≥50 - <100 x 10^9/L	<100 x 10^9/L ≥100 - <150 x 10^9/L	2 points 1 point
FDPs/D-dimer	Strong increase Moderate increase*		3 points 2 points
Prothrombin time (PT) ratio	≥ 6s ≥ 3s - < 6s	> 1.4 > 1.2 - ≤ 1.4	2 points 1 point
Fibrinogen	< 1 g/L		1 point
SOFA score		≥ 2 1	2 points 1 point
Score	**≥5 [Max score 8]** **An ISTH DIC score of 5 or more, equates to a diagnosis of DIC**	**≥4 [Max Score 6]** **An ISTH SIC score of 4 or more equates to a diagnosis of SIC**	

Table 2: Criteria defining the ISTH-DIC and ISTH-SIC algorithms.
**Three sets of D-dimer cut-off values have been published for moderate and severe rise. Cut-off values of 1 µg/mL and 3 µg/mL; 0.39 µg/mL and 4 µg/mL demonstrated a same sensitivity and specificity. Cut-off of 2.4 µg/mL and 22.0 µg/mL increased the specificity but decreased sensitivity.(Horan and Francis 2001, Dempfle, Wurst et al. 2004, Hatada, Wada et al. 2012, Gao, Yang et al. 2020)*

Recently, the clot waveform, based on APTT or PT, has been shown to predict poor outcomes in severe infections with DIC.(Tan, Wong et al. 2020, Wada, Matsumoto et al. 2020) An atypical biphasic curve was observed in patients developing sepsis, trauma, cancer or other conditions predisposing to DIC.(Toh, Samis et al. 2002, Smith, Charles et al. 2004) This abnormal waveform was defined by an initially steep slope before the clot formation and caused by the formation of calcium-dependent precipitates of C-reactive protein (CRP) complexed with very low-density lipoprotein (VLDL) (**Figure 6**).(Downey, Kazmi et al. 1998, Toh, Samis et al. 2002) This biphasic waveform had higher sensitivity (97.6%) and specificity (98.0%) compared to tests

routinely used in the diagnosis of DIC.(Toh and Giles 2002) In patients in intensive care settings, the presence of this biphasic waveform could be used as an early marker of DIC and was correlated with an increased risk for sepsis and mortality.(Downey, Kazmi et al. 1998, Toh and Giles 2002, Smith, Charles et al. 2004, Matsumoto, Wada et al. 2006, Suzuki, Wada et al. 2019) Photo-optical analyzers, using waveform analysis technology, could potentially flag the presence of abnormal waveform while running routine aPTT or PT tests.

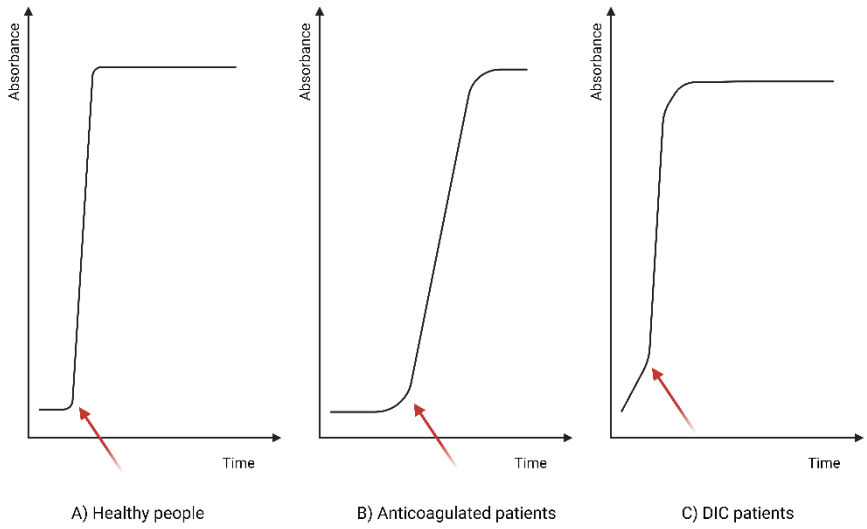


Figure 6: Overview of clot waveform analysis from (A) healthy people, (B) patients with anticoagulant and (C) disseminated intravascular coagulation (DIC).

Suzuki *et al.* analyzed the APTT-based CWA in patients suspected of having DIC and compared the relationship between the outcome and the parameters of the CWA. They demonstrated that the peak of first (Max1) and second (Max2) derivative were increased in infectious patients without DIC compared to healthy volunteers. In DIC patients, the biphasic patterns were observed and the time to Max1 and time to Max2 were significantly longer in DIC patients than without DIC showing time parameters could be used as

a diagnostic tool for DIC. The widths of the derivative curves were also significantly larger in DIC patients than healthy volunteers which may suggest the presence of underlying diseases of DIC. The heights of Max1, Max2 and Min2 were reduced in DIC patients compared to those without DIC. In one study, the DIC group was divided into 2 subgroups, the DIC with and without hypofibrinogenemia. DIC with hypofibrinogenemia might be a more severe bleeding-subtype than DIC without hypofibrinogenemia where the peak of the 1st derivative (Max1) and peaks of the 2nd derivative (Max2 and Min2) were reduced. The different peaks of derivative curves were significantly lower in patients with Hb < 8 g/dl than in those with Hb ≥ 8 g/dl, suggesting that a reducing Max1, Max2 and Min2 might indicate an increased risk for severe bleeding.(Ruberto, Marongiu et al. 2018) According to the results, the peak of the first and the second derivative could be useful for DIC diagnosis and prediction of bleeding risk in patients with DIC.(Suzuki, Wada et al. 2019)

c. Improvements in traditional CWA

- Small tissue factor as physiological coagulation

The most results of CWA are obtained from APTT measurements, reflecting the coagulation in a non-physiological environment. Consequently, the use of classic APTT reagents is a major issue because the physiological blood coagulation process starts after small amounts of tissue factor (sTF) and activated FVII activate FIX which, in association with FVIII, activates FX and generates fibrin. Blood contacting medical devices are known to trigger the coagulation process by activating FXII. Therefore, for investigating the coagulation process, initiated by sTF, it is important to limit the effects of artificial surfaces on contact pathway. The corn trypsin inhibitor, widely used to block the contact pathway, due to its potent FXIIa and moderate FXIa inhibitor effect, can be used.(Josso and Prou-Wartelle 1965, Yau, Stafford et

al. 2011, Jaffer, Fredenburgh et al. 2015, Hamad, Pathak et al. 2017, Wada, Shiraki et al. 2020)

The effects of platelet and phospholipids on clot formation activated by sTF (TF concentration < 0.1 pg/mL) have been studied on platelet-poor plasma (PPP) and platelet-rich plasma (PRP). The results demonstrated that the PRP had a shorter clotting time (time to Max1 and time to Max2) and a higher Max1 and Max2 than PPP, showing an effect of platelets on clot formation kinetics. The use of sTF in CWA may detect platelet count abnormalities.(Hasegawa, Tone et al. 2021)

- Clot-fibrinolysis waveform analysis (CFWA)

Recently, the clot-fibrinolysis waveform analysis (CFWA), which is an extension of clot waveform analysis using $CaCl_2$ containing tissue plasminogen activator to trigger the fibrinolytic process, has been developed. The CFWA is interesting because it permits simultaneous to assess the coagulation and fibrinolysis processes. Preliminary results suggested its usability to assess DOAC effects but could help for understanding the hemostatic potential in patients with bleeding tendencies (cirrhosis, trauma, postpartum hemorrhage, DIC).(Nogami, Matsumoto et al. 2019, Oka, Wakui et al. 2020)

IV. Advantages and disadvantages

The CWA-APTT, and to a lesser degree CWA-PT, is suggested in multiple conditions such as DIC diagnosis, monitoring hemophilia or evaluating hemostasis state, but a large standardization of reagents, methodologies and parameters are required to confirm the potential role as diagnosis tool.

Pre-analytical issues, including hemolysis, icterus and lipemia (HIL) interferences and use of colorless reagents can impact the turbidity

measurements.(Lippi, Plebani et al. 2013, Shima, Thachil et al. 2013, Lance 2015, Nagant, Rozen et al. 2016, Sevenet and Depasse 2017, Wada, Matsumoto et al. 2020) The CWA evaluates the fibrin formation in PPP, sometimes in PRP, using APTT and PT reagents. In these conditions, the platelet function and physiological coagulation cannot be assessed. An evolution is the use of small amount of tissue factor as activator, consistent with the concepts of cell-based mechanisms, allowing to evaluate the coagulation process in physiological environment. Despite interest of CWA, the use of whole blood, more representative of physiological hemostasis, is not possible yet.

V. Conclusion

The clot waveform analysis is gaining more and more attention in last decades. Although the CWA is expanding, easy and economically convenient to perform, the quantitative parameters, although important in various coagulation disorders, are poorly known and then, more data regarding clinical correlations with parameters must be collected. Because its ability to evaluate the clot formation kinetics in its entirety during routine clotting tests, the clot waveform analysis can be considered as a global hemostasis assay.[1]

[1]The CWA, overall hemostasis potential (OHP), thrombin generation assay (TGA) and viscoelastic assay (TEG/ROTEM) are considered as “global” hemostatic/coagulation assays.
An assay that could provide a global assessment of hemostasis would consider the interaction between the protein of the coagulation and fibrinolytic pathways, blood cellular components and the vessel wall. Such an assay does not currently exist, but refinements of old techniques with updated technology and the development of new global assays have brought improvements in this regard. The CWA, OHP, TGA and TEG/ROTEM have the ability to provide an overview of the patient's coagulation and/or fibrinolytic system.

2. Overall hemostasis potential (OHP)

I. History

The overall hemostasis potential (OHP), reported in 1999, was designed for screening the overall hemostatic potential in hypo- and hypercoagulable states. Initially, the OHP was based on fibrin-aggregation curve performed in platelet-poor plasma, in which small amounts of thrombin (0.04 IU/mL), tissue plasminogen activator (tPA, 300ng/mL) and $CaCl_2$ (17 mM) were added. The replacement of thrombin by recombinant tissue factor with phospholipids as trigger has been proposed to provide a more physiologically relevant assay. As for the CWA, the assay is based on measurement of turbidity changes, i.e. absorbance. The generation of fibrin over-time and the area under the curve allow reflecting the balance between the formation and degradation of fibrin. Supplementary parameters extracted from OHP, the overall coagulation potential (OCP) and the overall fibrinolysis potential (OFP), allowed the detection of alterations in the coagulation and the fibrinolytic processes.(He, Bremme et al. 1999, He, Antovic et al. 2001, Antovic 2010)

II. Principle

In absence of tissue plasminogen activator, the curve defines the fibrin generation, which represents the conversion of fibrinogen into fibrin by thrombin. In presence of tPA, the curve can be divided into 2 phases: the first, until the absorbance peak, is the fibrin generation and the second is the fibrin degradation, where plasminogen is converted into plasmin by tPA. The level of absorbance indicates the level of fibrin and the area under the curve reflects the balance between coagulation and fibrinolysis.

Briefly, two fibrin curves are generated and two area under the curves can be calculated: the overall coagulation potential (OCP, without tPA) and the overall hemostasis potential (OHP, with tPA). Then, the overall fibrinolysis potential (OFP) can be calculated by the following formula: [(OCP-OHP)/OCP]*100% (**Figure 7**).

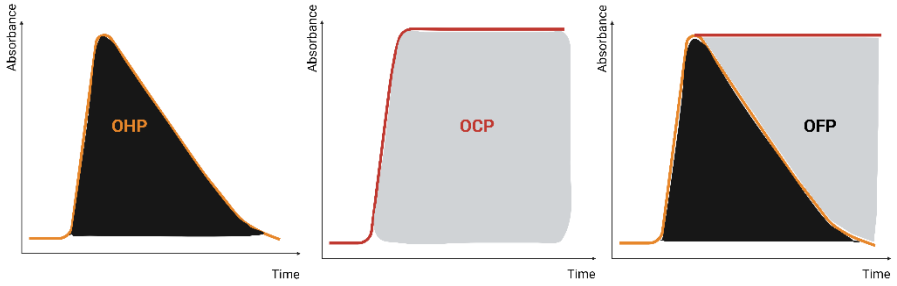


Figure 7: Overview of overall hemostasis potential (OHP) (A), overall coagulation potential (OCP) (B) and overall fibrinolytic potential (OFP) (C).

III. Potential clinical application of OHP

The overall hemostasis potential is the least known method and data are not very expanded. Compared to the clot waveform and clot-fibrinolysis waveform analysis, which provide qualitative examination and quantitative parameters, the OHP method estimates the areas under the curve of the clot formation. The first data showing an interest of the overall hemostasis potential revealed its potential utility in the diagnosis of hypercoagulable states.(He, Bremme et al. 1999) A modification of original OHP using Protac or heparin pentasaccharide, potentiating the inhibitors of coagulation, allowed the detection of major thrombophilia.(Andresen, Iversen et al. 2002) Recently, OHP was able to monitor treatment in anticoagulated or hemophilia patients.(Antovic 2010, Curnow 2017, Milos, Coen Herak et al. 2020) The OHP was also investigated in patients suspected VTE and

compared to D-dimer. In these patients and compared to D-dimer, the OHP, OCP and OFP were impacted, but not significantly to discriminate patients with and without VTE.(Farm, Antovic et al. 2020)

IV. Conclusion

The overall hemostasis potential gives an estimate of global coagulation and fibrinolysis, is also easy to perform, inexpensive and “fast” (60 minutes from sampling to result). However, the methodology is not well developed and known, which limiting its clinical practice. Standardization steps, an implementation on analyzers and automation of calculations could make the OHP useful as diagnosis tool.

3. Thrombin generation assay

Besides APTT and PT, which provide clotting time and information about the beginning of coagulation process, and the clot waveform analysis, which evaluates the clot formation kinetics, the thrombin generation assay (TGA) evaluates the thrombin generation and inhibition over-time, resulting from the action of procoagulant and anticoagulant factors.

I. History

In the 1950s, several research groups reported the measurement of thrombin generation in plasma and blood samples with triggers as tissue factor or cephalin and calcium. It was a time-consuming technique and non-suitable in clinical practice.(Macfarlane and Biggs 1953, Pitney and Dacie 1953) Many years later, Hemker improved the thrombin generation measurement. The first and biggest changes were the replacement of fibrinogen by a synthetic chromogenic substrate specific for thrombin, the use of defibrinated plasma by a snake venom and the use of software to calculate different parameters from the thrombin generation curve.(Hemker, Willems et al. 1986) Then, the chromogenic substrate has been replaced by fluorogenic substrate. The fluorogenic substrate does not require the defibrination of plasma samples as observed with chromogenic substrate and allows the evaluation of the procoagulant function of platelets in platelet-rich plasma.(Hemker, Giesen et al. 2000) Although TGA is a sensitive tool, the turn-around time and the lack of standardization between the different technologies on the market limit its routine clinical use in patients. To further evaluate hemostatic potential and make test more representative of the coagulation process in vivo, a new version of thrombin generation has been developed in whole blood, but still poor information on

its practical application.(Ninivaggi, Apitz-Castro et al. 2012) Recently, the ST-Genesia, a fully automated thrombin generation analyzer was reported to provide enhanced reproducibility compared to calibrated automated thrombogram and designed for the introduction of TG into the clinical routine.(Douxfils, Morimont et al. 2019, Pfrepper, Metze et al. 2020)

II. Principle

Currently, there are different instruments available on the market, the calibrated automated thrombogram (CAT; Diagnostica Stago), Innovance ETP (Siemens Healthcare) and Technothrombin TGA (Technoclone), able to monitor the thrombin generation. These instruments differ by technique, reagents and software.(Kintigh, Monagle et al. 2018) The following detailed principle of TGA will be based on the CAT system.

Initially, plasma sample and intermediate reagent such as tissue factor, phospholipids is added into the well of microplate. A solution of calcium chloride containing fluorogenic substrate is added to well to trigger the coagulation process. The fluorescent substrate will be cleaved by thrombin releasing the fluorophore, which is recorded over-time and converted into signal, in relative fluorogenic units (RFU), by the software. The RFU is proportional to the amount of thrombin generated in the assay and is converted into thrombin concentration by using an internal thrombin standard. This thrombin calibrator is composed a known concentration of alpha-2macroglobulin-bound thrombin (α2M-T). To avoid high values in thrombin generation, the CAT method uses a correction algorithm that subtracts the fluorescence generated by α2M-T activity from the total thrombin activity (i.e. free thrombin plus α2M-T). During the coagulation process, the software traces a thrombogram, i.e. thrombin generation curve, and calculates parameters. The thrombin generation curve can be divided

into 3 phases: the initiation, amplification or propagation and resolution phase. These different phases are defined by thrombin generation parameters. The different parameters are the lag phase or lag time, representing the time between the addition of calcium chloride plus fluorogenic substrate solution and the start of thrombin generation. The lag time can be defined as the time until the thrombin generation signal increases with two standard deviations from baseline or as the time to reach one sixth of the peak height. This parameter corresponds to the time point when the fibrin clot is formed in standard clotting tests. Although time results are different between thrombin generation assay (in minutes) and standard clotting tests (in seconds), some authors agree that the lag time is equivalent to the clotting time but is considered as more sensitive because it is performed in a system closer to physiological hemostasis.(Kim, Kim et al. 2013, Tripodi 2016, Duarte, Ferreira et al. 2017, Bloemen, Zwaveling et al. 2018, Depasse, Binder et al. 2021) The thrombin peak represents the highest thrombin concentration. The time to peak represents the time to reach the thrombin peak, and may, with thrombin peak, represent hypo- or hypercoagulable states. The endogenous thrombin potential (ETP) defined as the area under the curve. The ETP represents the total quantity of thrombin formed during the test, resulting from the action of procoagulant and anticoagulant factors. The last parameter is the mean velocity rate index (mVRI), the slope of thrombin generation curve (**Figure 8, Table 3**). (Matsumoto, Shima et al. 2006, Salvagno and Berntorp 2017, Tripodi 2020, Evrard, Morimont et al. 2021, Verhagen, Valke et al. 2022)

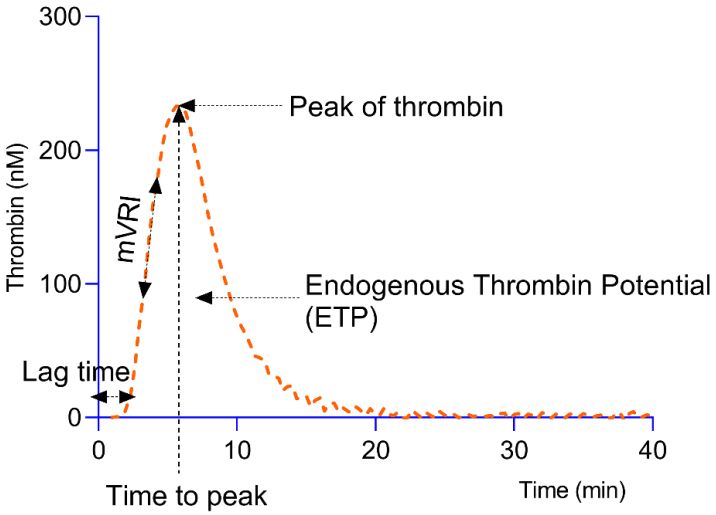


Figure 8: Thrombin generation assay (TGA) assessed by the calibrated automated thrombogram (CAT).

Hypocoagulability	**Hypercoagulability**
Prolonged lag time	Short lag time
Long time to peak	Short time to peak
Low peak	High peak
Low ETP	High ETP
Low mVRI	High mVRi

Table 3: Significance of thrombin generation parameters.

III. Potential clinical application of thrombin generation assay

Thrombin is the key enzyme of the coagulation cascade, able to down-regulate its own generation by cleaving factor Va and VIIIa through the protein C/S complex and by increasing the clot stability by activating thrombin activatable fibrinolysis inhibitor and factor XIII. It was therefore rational to measure its concentration as the ultimate expression of the global hemostatic balance. TGA has been widely studied as a research tool and is helpful to elucidate coagulation mechanisms in various clinical conditions (**Table 4**).(Macfarlane and Biggs 1953, Rotteveel, Roozendaal et al. 1993, Dargaud, Béguin et al. 2005, Tripodi, Martinelli et al. 2007, Brummel-Ziedins, Whelihan et al. 2009, Marchetti, Diani et al. 2012, Brinkman 2015, Lenting, Denis et al. 2017, Pike, Cumming et al. 2017, Dargaud, Lienhart et al. 2018, Rigano, Ng et al. 2018, Pauline, Marina et al. 2019, Douxfils, Morimont et al. 2020, Pfrepper, Metze et al. 2020, Vermeiren, Vandevelde et al. 2022)

Help elucidate mechanisms regulation thrombogenesis
Laboratory diagnosis of disorders of hemostasis
Monitoring treatment with pro-hemostatic agents
Monitoring treatment with antithrombotic drugs
Risk assessment of recurrent VTE

Table 4: Major applications of thrombin generation.

a. Bleeding disorders

- Factor deficiencies and hemophilia

As the thromboelastography (TEG)/rotational thromboelastometry (ROTEM) and the CWA, TGA is useful for understanding and assessing imbalanced in hemostasis linked to bleeding disorders.(Hemker, Giesen et al. 2002, Prior, Mann et al. 2018) The relationship between parameters of thrombin

generation, concentration of clotting factor and severity of bleeding has been investigated in patients with deficits in prothrombin, FV, FVII, FVIII, FIX, FX and FXI.(Al Dieri, Peyvandi et al. 2002, Binder, Depasse et al. 2021)

In factor XI (FXI) deficiency, clinical tests to identify bleeding tendency are poor and FXI:C levels, recommended by guidelines, are not predictive of bleeding risk in FXI deficiency.(Bolton-Maggs, Perry et al. 2004, Duga and Salomon 2013) The therapeutic option consisting on replacing FXI involve the administration of fresh frozen plasma, i.e. solvent-detergent fresh frozen plasma (SD-FFP), plasma-derived FXI concentrate, or rFVIIa. TGA was investigated to measure effect of treatment with SD-FFP, FXI concentrate or rFVIIa in FXI-deficient patients. The weakness of correlations between FXI:C levels and changes in TGA parameters supported that FXI:C levels may not be the ideal parameter to monitor treatment in FXI deficiency. In case of severe FXI deficiency and anti-FXI inhibitor, low doses of rFVIIa were sufficient to normalize thrombin generation suggesting its use without increasing the risk of thromboembolic events.(O'Connell, Riddell et al. 2008, Pike, Cumming et al. 2017, Biguzzi, Siboni et al. 2020)

Investigations of TGA in HA have been more extensive than in HB. According to the results, TGA appears extremely sensitive in both HA and HB patients, with qualitative and quantitative observations.(Matsumoto, Shima et al. 2006, Lenting 2020) Brummel-Ziedens and other groups investigated thrombin generation as a potential marker of bleeding frequency, which proved to be more accurate than FVIII levels in HA patients. It has also been reported that the peak and ETP were interesting markers in monitoring patients with FVIII inhibitors, bypassing agents, and in the prediction of bleeding in cardiac surgery.(Matsumoto, Shima et al. 2006, Brummel-Ziedins, Whelihan et al. 2009, Dargaud, Lienhart et al. 2010, Dargaud, Lienhart et al.

2018, Chen, Jani et al. 2019, Negrier, Shima et al. 2019, Binder, Depasse et al. 2021)

- Anticoagulant management

Multiple studies investigated the effects of anticoagulants on thrombin generation.(Petros, Siegemund et al. 2006, Robert, Ghiotto et al. 2009, Bloemen, Zwaveling et al. 2018, Rigano, Ng et al. 2018, Pfrepper, Metze et al. 2020)

Qualitative and/or quantitative observations enabled by thrombin generation profiles in presence of anticoagulants are a very interesting and useful approach. The TGA provides an interesting insight into the drugs mechanism of action and visualize how the anticoagulants modify the thrombin generation over time.

Heparin (low molecular weight heparins, LMWH, and unfractionated heparin, UFH) facilitates the interaction between antithrombin and FXa by allosteric changes in the antithrombin and by facilitating the approach of thrombin to antithrombin via the "sliding" along the heparin molecule.(Hemker 2016, Hemker, Al Dieri et al. 2019) The important part of the heparin necessary for optimal activity is a five sugars sequence, named the high affinity pentasaccharide, HA5. This HA5 is responsible of anti-FXa activity, found in about one third of UFH and smaller proportion in LMWH. Then, the combination of HA5 with 12 sugars, named C-domain, gives the heparin molecule a complete anti-thrombin activity.(Walker and Royston 2002, Hemker 2016, Hemker, Al Dieri et al. 2019)

According to the paradigm of Hemker, heparin acts by decreasing the amount of active thrombin formed in clotting plasma and this activity is due to the anti-thrombin activity and not to the anti-FXa activity. This may explain why the anti-FXa activity does not reflect the pharmacodynamics effect of heparin. The LMWH catalyzes predominantly the inactivation of FXa and

thrombin by antithrombin (anti-FXa/anti-thrombin ratio of 3.8). The LMWH limits mostly the amplification/propagation phase and enhance the termination phase of thrombin, observed by a dose-dependent reduction in peak and ETP and prolongation in the lag time. The observed effects of UFH are more pronounced on thrombin generation parameters than those of LMWH, prolonging temporal parameters and reducing peak, ETP and mVRI. The difference in effects between UFH and LMWH can be explained that in LMWH heparins, there will be many HA5 domains, only responsible of anti-FXa activity, but less C-domain and therefore a reduced anti thrombin activity.(Tanaka, Szlam et al. 2007, Robert, Ghiotto et al. 2009, Xu, Wu et al. 2013, Hemker, Al Dieri et al. 2019, Vermeiren, Vandevelde et al. 2022)

Vitamin K is involved in the synthesis of vitamin K-dependent coagulation factors (FVII, IX, X, prothrombin) and protein C and S. Vitamin K antagonists (VKAs) (warfarin, acenocouman, and phenprocouman) prevent this process and cause a decreased activity in these factors. VKAs have a narrow therapeutic window, several food and drugs interactions, and variable anticoagulant response, leading to the need for periodical anticoagulation monitoring and dose adjustment. The effects of VKAs are usually measured with PT test, and expressed as INR.(Walker and Royston 2002, Douxfils, Tamigniau et al. 2014, Nilsson, Strandberg et al. 2018) PT/INR and TGA are sensitive for detecting effects of VKA but with the ability to provide a complete overview of coagulation, TGA could better detect VKA anticoagulation than INR.

Data showed significant associations between TG parameters (lag time, ETP, peak and mVRI) and INR where VKAs appear to limit the initiation and amplification/propagation phases of TG. Indeed, VKAs block upstream production of FVII, IX, X and prothrombin and thus, limit the coagulation system to generate thrombin, observed by prolonged lag time and reduced

peak and ETP (Dale, Eikelboom et al. 2013, Brinkman 2015, Herpers, van Rossum et al. 2015, Nilsson, Strandberg et al. 2018, Schmidt, Chaireti et al. 2019) As TG parameters showed interesting responsiveness to VKA anticoagulation, these parameters could be useful for the assessment of anticoagulation reversal by the prothrombin complex concentrate (PCC) and rFVII. It was observed that PCC better restored the thrombin generation than rFVIIa in VKA samples. Indeed, rVIIa replaces defective FVII, with effect shortens on PT/INR while PCC restores functional prothrombin, FIX and FX. The use of TGA as additional means to guide PCC dosing strategies was showed to be superior to INR where ETP and peak of thrombin presented a linear relationship to the administered PCC dose. However, in absence of thrombomodulin (TM) in TGA, it is not possible to explore protein C pathway, whose activity is decreased by the use of VKAs. It was suggested that the effect of VKAs on TGA could be overestimated in absence of TM.(Tanaka, Szlam et al. 2008, Al Dieri, Ten Cate-Hoek et al. 2011)

Dabigatran, a direct and reversible thrombin inhibitor, is known to delay the initiation phase of TGA. Dabigatran prolongs concentration-dependent lag time and time to peak but counterintuitively, increases the peak and ETP.(Douxfils, Mullier et al. 2012, Artang, Anderson et al. 2017, Bloemen, Zwaveling et al. 2018) These effects were already reported by several authors and may occur due to its in vitro interference with TGA method. According to Bloemen *et al.*, this interference may be caused by two mechanisms. On one hand, the CAT method is based on a correction algorithm in which the fluorescence generated by alpha-2-macroglogulin-bound thrombin (α2M-T) activity is subtracted from the total thrombin activity (free thrombin + α2M-T). On the other hand, dabigatran, a low molecular weight compound, can interact with free thrombin as well as α2M-T. As the algorithm does not take into account the inhibition of α2M-T by dabigatran, it results in an

overestimation of thrombin activity in assay, expressed by increasing peak and TP.(Gribkova, Lipets et al. 2016, Bloemen, Zwaveling et al. 2018)

The addition of idarucizumab (6 µM), a reverse agent able to completely neutralize dabigatran, allows to overcome this interference and to evaluate the TG as if dabigatran was absent.(Bloemen, Zwaveling et al. 2018) Despite this opportunity, only lag time and time to peak can give accurate information and been suggested to better estimate the anticoagulant effect of dabigatran.

Direct FXa inhibitors, apixaban, edoxaban and rivaroxaban, are known to delay, in a concentration-dependent manner, the initiation phase, demonstrated by prolongation of lag time, but also to delay and reduce the amplification/propagation phase, showed by a prolongation of time to peak and reduction of peak of thrombin and mVRI. The impact of FXa inhibitors is less pronounced on ETP.(Douxfils, Mullier et al. 2012, Douxfils, Chatelain et al. 2013, Morishima and Kamisato 2015, Douxfils, Chatelain et al. 2016, Salta, Papageorgiou et al. 2018, Douxfils, Evrard et al. 2021) The thrombin peak has been suggested as a better estimation of the anticoagulant effect of FXa.(Samama, Contant et al. 2013) Among those drugs, rivaroxaban appears to be the most potent TG inhibitor, followed by apixaban and edoxaban with a similar TG inhibitory activity. The less pronounced effect observed with apixaban and edoxaban compared to rivaroxaban might be due to a different inhibition constant (Ki) on FXa.(Jourdi, Siguret et al. 2015, Evrard, Hardy et al. 2021) Several studies have investigated the FXa inhibitors effects on TGA. Schenk *et al.* observed in 20 in-vitro and in-vivo patient samples that rivaroxaban was significantly correlated with thrombin generation parameters.(Schenk, Würtinger et al. 2016) Tripodi *et al.* observed that apixaban affected all thrombin generation parameters.(Tripodi, Padovan et al. 2015) Samama *et al.* demonstrated concentration-dependent effects of

edoxaban in spiked patient samples.(Samama, Mendell et al. 2012) Pfrepper *et al.* included 380 samples from patients taking apixaban, dabigatran, edoxaban, or rivaroxaban and correlated anti-Xa measurements with TG parameters.(Pfrepper, Metze et al. 2020) Similar to these studies, Meihandoest *et al.* observed changes in TG measurements, assessed on Ceveron t100 analyzer (Technoclone), in patients treated with apixaban, edoxaban and rivaroxaban but correlation between TG parameters and drug concentrations was weak to moderate.(Meihandoest, Studt et al. 2021) Except for the Meihandoest *et al.* study's, TGA appears to be a sensitive assay to monitor the pharmacodynamic and pharmacokinetic properties of FXa inhibitors. TGA is sensitive enough to detect anticoagulants and has been proposed to aid clinical decision making by offering a more detailed profile of the patient's coagulation status.(Rigano, Ng et al. 2018, Pfrepper, Metze et al. 2020, Binder, Depasse et al. 2021, Metze, Klöter et al. 2021)

b. Thrombotic disorders

Thrombin generation is able to give important information and has shown to be sensitive enough to evaluate the thrombotic disorders. It has been reported that the increase of thrombin generation (ETP, thrombin peak and to a lesser degree the mean velocity rate index) was associated to an increased risk of venous thrombosis and may be useful as a predictive marker for evaluating thrombosis.(Hron, Kollars et al. 2006, Tripodi, Martinelli et al. 2007, Binder, Depasse et al. 2021, Depasse, Binder et al. 2021)

- Healthy population

Several studies showed that thrombin generation was sex-specific and increased with age and obesity, indicated by shorter lag time and higher peak and ETP.(Haidl, Cimenti et al. 2006, Dielis, Castoldi et al. 2007) Sex-specific differences can be explained by the influence of female endogenous sex

hormones on coagulation, where high levels of fibrinogen and low levels of protein S, antithrombin and protein C were observed in females compared to males.(Rotteveel, Roozendaal et al. 1993, Middeldorp, Meijers et al. 2000, Pauline, Marina et al. 2019)

- Activated protein C (APC) resistance

Thrombophilia is defined by a predisposition to thromboembolic illness and a tendency to thrombosis. Patients with an inherited thrombophilia such as antithrombin, protein C or protein S deficiency, activated protein C resistance due to factor V Leiden or prothrombin G20210A mutation are at higher risk of thrombosis. In inherited thrombophilia, thrombin generation allowed to assess the risk of thrombosis recurrence. The APC resistance may also occur in the absence of a mutation of the FV, called acquired APC resistance. This phenomenon can be observed in women with the use of contraceptives like combined hormonal contraceptives, in presence of antiphospholipid antibodies (lupus anticoagulant) and hyperhomocysteinemia.(Tripodi, Martinelli et al. 2007, Lim, O'Malley et al. 2019, Douxfils, Morimont et al. 2020, Morimont, Bouvy et al. 2020, Binder, Depasse et al. 2021)

- Venous thromboembolism (VTE)

The role of TGA in VTE diagnosis is still under debate. Studies assessed the interest of thrombin generation for predicting VTE occurrence and recurrence. Compared to D-dimer, TGA appears to be less influenced by comorbidities such as cancer, infectious and cardiovascular diseases.(Farm, Antovic et al. 2020) High values of peak and/or ETP were observed in acute VTE and could be predictive of VTE recurrence. A shortened lag time was also associated with increased risk of VTE recurrence, and the time to peak showed to differentiate between patients at high and low risk for VTE.

Tripodi *et al.* indicated patients with thrombin peak > 193 nM had 4.57-fold increased risk of recurrence VTE compared patients with thrombin peak < 115 nM.(Tripodi, Legnani et al. 2008) These results were consistent with those reported by Hron *et al.* where patients with a peak of thrombin less than 300 nM had a lower risk of VTE recurrence compared with patients with a peak thrombin greater than 400 nM.(Hron, Kollars et al. 2006) The addition of thrombomodulin, to thrombin generation assay, better reflected the activity of proteins involved in the protein C pathway and showed higher association between peak/ETP and the risk of recurrent VTE, compared to assay without thrombomodulin.(Hron, Kollars et al. 2006, Eichinger, Hron et al. 2008, Tripodi, Legnani et al. 2008, Tripodi 2016, Park, Spears et al. 2017, Wexels, Dahl et al. 2017)

Besides, the Leiden Thrombophilia Study (LETS) did not observe association between ETP and risk of recurrent thrombotic event. This discrepancy might be due to the amounts of TF used in the assay (≤5 pM in Brummel-Ziedins *et al.*, Tripodi *et al.* vs 15 pM in Van Hylckama *et al.*) and differences in the population. This emphasizes that TGA with a low concentration of TF trigger may be better in predicting the risk of a first and recurrent VTE. Riva *et al.* reported prolonged lag time and time to peak but no impact on ETP in patients with confirmed VTE, suggesting a limited application of TGA in the diagnosis of VTE. In another study, the ETP and the lag time were increased in acute VTE but these parameters did not allow to discriminate between patients with and without VTE, as observed with D-dimer.(Riva, Vella et al. 2018, Farm, Antovic et al. 2020)

- Antiphospholipid syndrome (lupus anticoagulant)

The thrombin generation and acquired APC resistance-based thrombin generation assay have been investigated as potential marker of hypercoagulability and to assess the thrombotic risk in antiphospholipid

syndrome. Studies showed an association between the unbalance in thrombin generation and thrombotic risk in antiphospholipid syndrome where ETP was lower or comparable to healthy subjects, whereas lag time and time to peak was prolonged. An increased APC resistance in antiphospholipid syndrome subjects was also reported.(Regnault, Beguin et al. 2003, Liestøl, Sandset et al. 2007, Zuily, Ait Aissa et al. 2012, Billoir, Duflot et al. 2019)

Devreese *et al.* showed that the TG-normalized peak height/lag time ratio could identify LA in plasma with high sensitivity, measured in 1:1 plasma mixtures.(Devreese, Peerlinck et al. 2009) Efthymiou *et al.* suggested that TG might be able to identify an ongoing prothrombotic state in VKA-treated antiphospholipid syndrome patients.(Efthymiou, Lawrie et al. 2015) Initially, it was not recommended to perform LA testing in patients on anticoagulation as they have prolonged basal clotting times and LA reagents can have different sensitivities to anticoagulants.(Keeling, Mackie et al. 2012, Murer, Pirruccello et al. 2016) However, Cohen *et al.* showed that rivaroxaban could be an effective and safe alternative to warfarin treatment in patients with thrombotic antiphospholipid syndrome. Indeed, even if rivaroxaban was inferior to warfarin in terms of anticoagulation intensity (ETP), the thrombin peak, lag time and time to peak showed significant benefit for rivaroxaban. Clinical outcomes and in-vivo coagulation activation markers showed no difference between rivaroxaban and warfarin. Additionally, in-vitro studies established that antiphospholipid antibodies did not interfere with the anticoagulant action of rivaroxaban.(Cohen, Hunt et al. 2016) According to results from Velasco-Rodriguez *et al.*, the TG appears to quite different in LA subjects compared to healthy volunteers. A prolongation of lag time and time to peak, while higher peak of thrombin, mVRI and ETP were observed in LA subjects. LA subjects showed also a resistance to APC, suggesting that TGA

can help by confirming the procoagulant status and stratify LA patients according to their risk profile, especially in strong LA individuals.(Velasco-Rodríguez, Laso et al. 2020, Radin, Barinotti et al. 2022)

- Cardiovascular diseases

Arterial diseases remain the leading cause of death and disability in elderly people. Thrombin acts as strong cell-signaling molecule, activating several complex processes, and is involved in thrombus formation, platelet activation and atherosclerosis. Thrombin also plays a role in the development and progression of arterial disease, caused by damaged atherosclerotic plaque.(Lutsey, Folsom et al. 2009, Kim, Grodstein et al. 2014, Loeffen, Winckers et al. 2015, Attanasio, Marcucci et al. 2016, Loeffen, van Oerle et al. 2016, Ten Cate and Hemker 2016)

In order to improve the understanding of the pathophysiological mechanisms of cardiovascular diseases and the effects of cardiovascular risk factors, studies investigated the relationship between thrombin generation and cardiovascular events, but results are contrasted.

On one hand, studies reported an increased thrombin generation in acute coronary event or ischemic stroke.(Orbe, Zudaire et al. 2008, Carcaillon, Alhenc-Gelas et al. 2011, Loeffen, Winckers et al. 2015) On the other hand, a prolonged lag time and lower peak and ETP were observed in patients with cardiovascular diseases.(Smid, Dielis et al. 2013, Lowe, Peters et al. 2022) Another study showed no association between thrombin generation and coronary stenosis severity.(Elad, Koren et al. 2020) In the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER), Loeffen *et al.* showed that cardiovascular risk factors and inflammatory markers had no significant effect on relationship between thrombin generation and cardiovascular diseases.(Loeffen, Winckers et al. 2015) Van Paridon *et al.* observed positive associations between ETP with anticoagulant activity and lag time with

mortality in cardiovascular diseases. Results showed that age, obesity and dyslipidemia are the most important clinical factors related to higher thrombin generation. In their population, age and lag time were positively correlated, obesity and dyslipidemia were associated to prolonged lag time, high ETP and thrombin peak. Finally, study highlighted that both lag time and ETP could be potential biomarkers for increased mortality risk, supporting the PROSPER results where a prolonged lag time was observed in cardiovascular diseases.(Pauline, Marina et al. 2019)

IV. Advantages and disadvantages

Although conventional tests such as APTT and PT are widely available in laboratory, these assays lack of sensitivity and do not evaluate the global hemostatic status. TGA has the advantage to measure the formation and inhibition of thrombin over-time, reflect the effect of pro- and anticoagulant factors and may be informative on bleeding or thrombotic tendency.

The most common methodology of thrombin generation is the monitoring of thrombin in platelet-poor plasma. In addition to being time-consuming, the platelet-poor plasma (PPP) does not allow investigating the interactions between platelets and coagulation factors in plasma. Most of research groups use PPP in order to eliminate these artefacts. The use of platelet-rich plasma, also time-consuming, allowed investigating the contribution of platelets and in closer to in vivo mechanisms. The whole blood thrombin generation assay allowed to investigate the contribution of cellular components of blood and reproduce the in vivo conditions better, more representative of physiological hemostasis. However, the whole blood requires different excitation and emission wavelengths from PPP and PRP because red blood cells attenuate the resulting fluorescence.(Tappenden,

Gallimore et al. 2007, Vila, Aznar et al. 2013, Prior, Mann et al. 2018, Depasse, Binder et al. 2021)

V. Conclusion

Despite improvements of pre-analytical steps, use of reference plasma, standardized reagents or development of fully automated analyzer, its clinical practice continues to be limited. In major applications, its clinical use in decision-making needs to be tested in larger prospective studies. The use of fully automated analyzer requiring minimal technical skills should increase repeatability and reproducibility and expand its clinical use.

4. Thromboelastography (TEG) – thromboelastometry (ROTEM)

I. History

Thromboelastography (TEG), first described in 1948, measures the entire life cycle of a clot.(Hartert 1948) Since then, several reviews reported clinical applications and efficacy of viscoelastic methods in the management of bleeding in clinical applications such as liver transplant, cardiac surgery and trauma. The algorithms associated to viscoelastic assays have been developed to interpret the results and guide blood transfusion strategy.(Shore-Lesserson, Manspeizer et al. 1999, Whiting and DiNardo 2014) The thromboelastography and rotational thromboelastometry (ROTEM), an evolution of TEG, are two point-of-care devices that monitor clot formation and dissolution in real-time in whole blood by measuring viscoelastic changes. These tests are able to provide a visual assessment of the process of clot initiation, strength (formation), stability (fibrinolysis) under low shear stress. They also allow to provide individualized coagulation therapy and help for patient management, by reducing or avoiding transfusion of blood cells, fresh frozen plasma and platelets, and thus reduce hospital costs.(Whiting, Al et al. 2015, Wikkelsø, Wetterslev et al. 2016, Korpallová, Samoš et al. 2018, Agarwal and Abdelmotieleb 2020, Bugaev, Como et al. 2020, Grottke, Mallaiah et al. 2020) The use of whole blood allows better reflecting the physiologic conditions in vivo and thus consider interactions between coagulation factors, inhibitors, red blood cells, platelets, and anticoagulants on clot formation and fibrinolysis.(Luddington 2005, Jackson, Ashpole et al. 2009, Lance 2015)

II. Principle

In TEG, the whole blood sample is incubated at 37°C in a heated cup. The temperature can be adjusted allowing evaluating coagulation in patients with hypothermia. The cup oscillates around a submerged torsion pin at ±4°45' every 5 seconds. The TEG system consists of two cups simultaneously examining samples in duplicate to reduce the risk of sampling, measurement errors and requiring manual pipetting. When the blood coagulation starts, the adherence of blood on the cup and pin increase and the movement of the cup induces motion on the pin. The magnitude of pin motion is proportional to the clot strength. At the end of coagulation process, the fibrinolytic process begins. The clot structure, composed of fibrin and platelets, begins to dissolve. The contact between the pin and clot decreases and induces less motion.(Tynngård, Lindahl et al. 2015)

The rotational thromboelastometry or ROTEM was developed to be simpler and used in larger range of clinical setting: simultaneously analyze of four samples, automated pipetting and minimized sensitivity to agitation. The testing can be more easily performed at the bedside and by clinicians outside the laboratory. In the ROTEM system, the movement is initiated from pin, at ±4°75' every 6 seconds, and the cup remains fixed (**Figure 9**).(Jackson, Ashpole et al. 2009, Theusinger, Nurnberg et al. 2010)

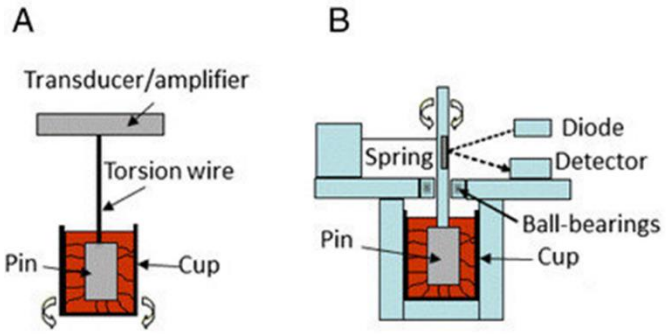


Figure 9: The measuring principle of TEG (A), ROTEM (B).

A newer version, TEG 6s, employing resonance method rather than cup rotation method has been developed. The blood sample is subjected to vibration and the vertical movement of the blood meniscus is measured under LED illumination. The system uses pre-measured cartridges that do not require pipetting and allows the simultaneous performance of four blood tests.(Gill 2017, Brill, Brenner et al. 2021)

The thromboelastogram, i.e. a visual representation from start to end of the clot process, is generated and the analysis of TEG includes the measurement of five major parameters (**Figure 10**).

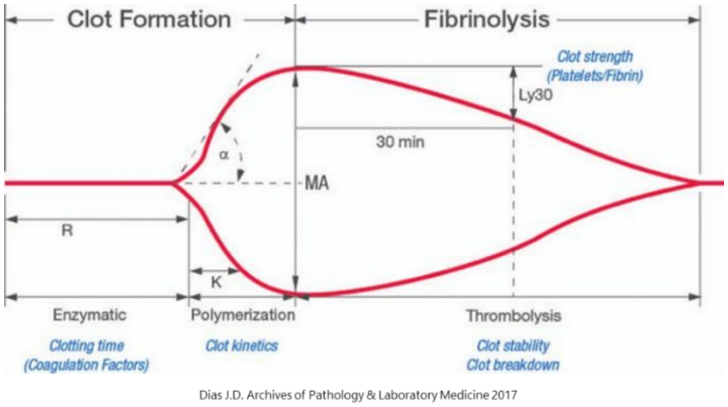


Figure 10: Illustration of a thromboelastography (TEG) tracing and major parameters.

The clotting time or reaction time (R) representing the time between the addition of calcium solution and the start of clot formation. The reaction time corresponds to the clot initiation phase and is dependent of coagulation factors. The clot kinetics (K time) describes the time between the start of clot formation and the moment where an amplitude of 20 mm of the clot is reached. Alpha angle (α) evaluates the rate of fibrin clot formation and represents angle between the baseline and the point of the maximal clot formation, closely related to K. The R time, K time and alpha angle parameters measure fibrinogen contribution to clot formation, therefore dependent of fibrinogen level, and define the amplification and propagation (thrombin burst) phases, respectively. The maximum amplitude (MA) is the highest amplitude (width of the tracing) of the TEG graphic, corresponding to the global stability of the clot, and is influenced by platelets. These three parameters, K time, alpha angle and MA, can represent the overall maximum clot strength. The time, from the beginning to MA, refers to coagulation process. The clot lysis at 30 minutes after maximum clot strength (LY30) is the standard measure of fibrinolysis and defines the percentage decrease in amplitude at 30 minutes post-MA. The MA and percentage of LY30 measure the platelets contribution.(Bose and Hravnak 2015, Hochleitner, Sutor et al. 2017) The parameters measured with ROTEM are similar but with different nomenclature (**Table 5**).

	Parameters	TEG	ROTEM	Biological meaning	Contribution to clot formation
Coagulation process	Reaction time	R	CT	*Initiation phase* The time from the start of test to start of clot formation	Fibrinogen
	Clot kinetics / clotting time	K	Clot formation time (CFT)	*Amplification phase* Time between the start of clot formation and the time taken to reach an amplitude of 20 mm Speed of clot formation	
	alpha angle	α	α	*Termination phase* Rate of clot formation, angle between the baseline and the slope of curve	
	Global maximum clot strength	Maximum amplitude (MA)	Maximum clot firmness (MCF)	*Propagation phase* Width of tracing	Platelets
Fibrinolysis process	Lysis after 30 minutes	LY30	CLY30	*Fibrinolysis phase* Percentage decrease in amplitude after 30' from MA	

Table 5: Nomenclature of TEG and ROTEM parameters.

Initially, the coagulation process was triggered by addition of $CaCl_2$ (without added activators) in the native TEG. The native TEG was well-established for its role in point-of-care resuscitation but presented challenges due to the delay in result time (up to 30 minutes).(Coleman, Moore et al. 2018) Then, the clot reaction was triggered by addition of kaolin-cephalin reagent, initiating the intrinsic coagulation pathway by activating Factor XII. In Rapid TEG, the kaolin-cephalin reagent is replaced by tissue factor and kaolin, allowing to perform the test within 15 minutes. This improvement is of interest in the management of massive transfusions in trauma patients.(Pezold, Moore et al. 2012) The TEG platelet mapping measures the MA of clot strength and allows evaluating the effects of antiplatelet drugs on platelets and function platelet. The assay assesses the platelet function

through direct activation of adenosine diphosphate (ADP) and arachidonic acid receptors. The comparison of TEG platelet mapping curve to the patient's hemostatic potential, obtained with the standard kaolin test, allows the quantification of platelet aggregation and inhibition.(Agarwal, Coakley et al. 2006, Bochsen, Wiinberg et al. 2007, Reikvam, Steien et al. 2009, Barker, Saini et al. 2019) The contribution of functional fibrinogen to clot strength is determined using a potent platelet glycoprotein IIb/IIIa inhibitor, abciximab. The platelet function being inhibited, the fibrinogen contribution to clot strength can be studied. The kaolin with heparinase is used for assessing the presence of heparin on coagulation. In ROTEM, some different activator reagents are used for investigating same components of the coagulation (**Table 6**). Larsen *et al.* demonstrated that the use of panel of reagent is more efficient than the use of kaolin alone, which allow to improve the quality and speed of the diagnosis.(Larsen, Fenger-Eriksen et al. 2011)

Description	TEG	ROTEM
Native	Native - Citrated whole blood + $CaCl_2$	NATEM - Non-citrated whole blood + $CaCl_2$
Intrinsic pathway	Kaolin-TEG - Kaolin-cephalin	INTEM - Ellagic acid
Extrinsic pathway		EXTEM - Tissue factor
Intrinsic and extrinsic pathway	Rapid-TEG - Tissue factor-kaolin	N/A
Assessment of contribution of fibrinogen (blocking platelet contribution)	Functional fibrinogen-TEG - Tissue factor + glycoprotein (GP) IIb/IIIa inhibitor (abciximab) - Comparison with kaolin-TEG	FIBTEM - Tissue factor + actin polymerization inhibitor (cytochalasin D) - Comparison with EXTEM
Assessment of fibrinolysis	N/A - Assess LY30 from kaolin-TEG or rapid-TEG	APTEM - Tissue factor + aprotinin - Comparison with EXTEM
Neutralize heparin	HTEG - Kaolin + lyophilized heparinase - Comparison with kaolin-TEG	HEPTEM - Ellagic acid + lyophilized heparinase - Comparison with INTEM
Function platelet and effect of antiplatelet drugs	Platelet mapping TEG - ADP or arachidonic acid, activates platelets depending on receptors - Heparin, eliminate thrombin activity - Activator F (mixture of reptilase and FXIII), induces a fibrin clot without thrombin - Assay involves four separate analyses	ROTEM platelet mapping system - ARA-TEM for cyclooxygenase inhibitors - ADP-TEM for ADP receptor blocker - TRAP-TEM for glycoprotein IIb-IIIa receptor blockers

Table 6: Nomenclature and description of TEG and ROTEM assays.*(Coleman, Moore et al. 2018, Selby 2020) N.A: not applicable*

III. Potential clinical application of TEG/ROTEM

TEG and ROTEM are widely used to evaluate the patient's global hemostatic function in three major clinical situations: cardiac surgery, liver transplantation, and trauma. Burns, postpartum hemorrhage, neonates are other pathologies where TEG and ROTEM are well-positioned to be helpful during the blood products transfusion.(Huissoud, Carrabin et al. 2009, Afshari, Wikkelso et al. 2011, Solomon, Collis et al. 2012, Tibi, McClure et al. 2021)

- Liver transplantation

One of the first clinical applications was in liver transplantation where the TEG was used to guide the transfusion of hemostatic blood components and thereby reduce bleeding.(Kang, Martin et al. 1985, Stravitz and Lee 2019) The hepatic function failure leads to impaired coagulation and fibrinolysis with risk for bleeding and thrombosis. TEG and ROTEM can be performed at three different stages of liver transplantation: 1) before removing the organ, 2) after removal and before the replacement of new organ, and 3) after placement of new organ. Several studies showed that TEG or ROTEM were superior and may replace traditional laboratory-based diagnostic methods (D-dimer, thrombin/anti-thrombin complexes, tissue-type plasminogen activator antigen) in different stage of liver transplantation. Tissue factor test-ROTEM was more sensitive than contact activated K-TEG in identifying a hyperfibrinolysis in liver transplantation patients. The use of contact pathway activator in K-TEG can explain the lower sensitivity in hyperfibrinolysis. In liver transplantation management, the FIBTEM was also investigated, which showed a superior sensitivity to EXTEM and K-TEG. The use of FIBTEM (ROTEM) could allow a better detection of hyperfibrinolysis in liver transplantation, which may improve intervention with anti-fibrinolytic

agents in cases of clinically significant hemorrhage. (Wang, Shieh et al. 2010, Abuelkasem, Lu et al. 2016)

- Cardiac surgery

Cardiac surgery often results in perioperative bleeding requiring the use of blood transfusion. The most common acquired condition in cardiac surgery is induced coagulation impairment that occurs due to contact between blood and the extracorporeal circuit. The use of anticoagulant or antithrombotic therapy contribute to derangement of coagulation. The POC monitoring is important to assess the cause of bleeding and provide best therapies where a massive blood loss increases the rates of massive transfusion and re-exploration. Transfusion of hemostatic blood products in cardiac surgery was traditionally based on conventional laboratory tests. However, these tests are limited because of poor predictive value of bleeding tendency and not allow evaluating interaction between different components of hemostasis as TEG and ROTEM.(Aoki, Sugimoto et al. 2012, Fleming, Redfern et al. 2017, Agarwal and Abdelmotieleb 2020, Tibi, McClure et al. 2021)

Compared to conventional tests, viscoelastic assays are more sensitive and specific for identifying patients at risk of bleeding in cardiac surgery and should be used for guiding transfusion therapy. The viscoelastic devices are recommended to manage and monitor hemostasis during and after cardiac surgery, which was associated with a significant reduction in transfusion needs. TEG and ROTEM have been investigated before a cardiac surgery but did not predict the risk of bleeding. The ROTEM was nevertheless able to identify patients who were not at risk of bleeding, with high negative predictive value (~90%).(NICE 2014, Meesters, Burtman et al. 2018)

Transfusion of hemostatic blood products may be guided by clinical judgement, standard laboratory tests, TEG/ROTEM, or a combination of these in a treatment algorithm. Studies showed that the use of TEG/ROTEM

in cardiac surgery decreases the number of patients exposed to blood products transfusion. TEG/ROTEM methodologies-based algorithms were associated to reduction blood components transfusions, reduction risks associated with transfusions and improvement in patient outcome.(Ak, Isbir et al. 2009, Afshari, Wikkelso et al. 2011, Sun, Jeleniowski et al. 2014, Deppe, Weber et al. 2016, Hans and Besser 2016, Li, Zhao et al. 2019, Schmidt, Israel et al. 2019, Meco, Montisci et al. 2020, Rali, Salem et al. 2021, Tibi, McClure et al. 2021)

- Trauma-induced coagulopathy

Trauma-induced coagulopathy (TIC) is a clinical situation at high risk of death that can occur after trauma injury. TIC results from uncontrolled release of TF, consumption of clotting factors and hyperfibrinolysis. Massive and uncontrolled bleeding is the major cause of preventable death in patients following trauma(MacLeod, Lynn et al. 2003, Cap and Hunt 2015, Hofer, Schlimp et al. 2021) The diagnosis of TIC is initially performed with conventional coagulation testing, i.e. APTT, PT, platelets and fibrinogen counts. However, their turnaround time and the focus on single part of clotting cascade (focus on count rather functional activity) prevent them from being ideal tests for diagnosing TIC. Therefore, the use of TEG/ROTEM in this clinical situation may provide an alternative way to assess coagulation status, initiate or guide specific treatment. TEG/ROTEM demonstrated to better identify coagulopathy in trauma patients and guide the need for massive transfusions than conventional coagulation testing.(Schochl, Nienaber et al. 2010, Holcomb, Minei et al. 2012, Sankarankutty, Nascimento et al. 2012, Durila and Malosek 2014) It was observed that the use of viscoelastic assays in bleeding patients with and without trauma leaded to improved survival and reduction of blood transfusions.(Da Luz, Nascimento et al. 2014, Wikkelsø, Wetterslev et al. 2016)

- Antiplatelet therapy

TEG platelet mapping, evaluating the effects of antiplatelet drugs on platelets and function platelet, has showed to have good correlation with the clinical standard assay, i.e. the light transmission aggregometry (LTA). According studies, the TEG platelet mapping may help to guide timing of surgery, to prevent bleeding in cardiac and non-cardiac surgery, identify platelet dysfunction, or even predict postoperative bleeding in cardiac surgery under antiplatelet therapy.(Mahla, Prueller et al. 2016, Malm, Hansson et al. 2016, Task Force on Patient Blood Management for Adult Cardiac Surgery of the European Association for Cardio-Thoracic, the European Association of Cardiothoracic et al. 2018, Agarwal and Abdelmotieleb 2020)

- Anticoagulants management

Monitoring of anticoagulant therapy is desirable for adjusting the dose and diagnosing poor therapeutic compliance. Compared to TEG, EXTEM, extrinsic pathway of ROTEM, was adequate for identifying patients on warfarin.(Schmidt, Holmström et al. 2015) Although TEG/ROTEM are not recommended for DOAC monitoring, studies showed that these could detect the presence of DOACs. The K time from Kaolin-TEG was dose-dependently affected by apixaban and dabigatran but not rivaroxaban, while the clotting time from Rapid-TEG was sensitive to apixaban, rivaroxaban and dabigatran. In ROTEM, significant prolongations of the EXTEM-clotting time, EXTEM-clot formation time, EXTEM-alpha angle and INTEM-clotting time values were observed with rivaroxaban. However, the ROC curve analysis showed that APTT was the most suitable parameter for predicting rivaroxaban-induced anticoagulation than EXTEM-CT and INTEM-CT.(Casutt, Konrad et al. 2012, Solbeck, Meyer et al. 2014, Dias, Norem et al. 2015, Hans and Besser 2016) The HTEG/HEPTEM assays are used for investigating coagulation in

heparinized patients or evaluate the efficacy heparin reversal in patients after protamine administration. Recently, the clotting time from INTEM/HEPTEM ratio was explored for identifying the presence of low or no heparin activity.(Meesters, Lancé et al. 2015, Katz, Leffert et al. 2022)

- Antiphospholipid syndrome (lupus anticoagulant)

Studies with viscoelastic assays in antiphospholipid syndrome are limited and reported discordance between traditional assays (prolonged) and TEG (normal) values in patients with antiphospholipid syndrome.(Breen and Hunt 2010, Wallace, Dumont et al. 2016) In a case-report of pregnant woman with antiphospholipid syndrome who developed septic shock, results from APTT and TEG were similar (clotting time, R-time and K-time prolonged), suggesting a low coagulation state.(Zhou, Tao et al. 2020) On the other hand, Rezoagli *et al.* reported discrepancies between APTT (prolonged clotting time) and TEG (normal coagulation state) in septic patient with antiphospholipid syndrome.(Rezoagli, Barzaghi et al. 2019) Harnett *et al.* showed that in LA patients receiving heparin therapy, APTT were prolonged while R-time of TEG, sensitive to traces of heparin, was in the normal range, suggesting that the elevated APTT were due to the LA and not the effect of heparin.(Harnett and Kodali 2006) According to these diverging results, TEG might not be a suitable method to assess coagulation in patients with LA.

EXTEM and INTEM (ROTEM) methodologies were investigated in LA patients. Contrary to what was observed in TEG, the clotting time in EXTEM and INTEM were prolonged and correlated with APTT in LA patients. Differences between TEG and ROTEM can be attributed to differences in reagents, kaolin in TEG and ellagic acid in ROTEM. A clotting time prolonged in EXTEM/INTEM and APTT usually indicates deficiencies in intrinsic/extrinsic coagulation pathways. However, in LA patients, an increased risk of bleeding could be misidentified and a treatment for correcting clotting time could increase the

risk for thrombosis. Therefore, ROTEM analysis will be susceptible to misinterpretation in this setting. Other parameters should be investigated and the use of specific tests to confirm or exclude the presence of LA are recommended.(Lee, Narasimhan et al. 2015, Hensch, Kostousov et al. 2018)

IV. Advantages and disadvantages

The most advantage of viscoelastic methodologies, compared to other global hemostasis assays, is to monitor the clot formation and dissolution in real-time in whole blood allowing to investigate the contribution of components of blood under in vivo conditions.(Luddington 2005, Crochemore, Piza et al. 2017)

The conventional methodology of TEG is based on kaolin-cephalin reagent, triggering the intrinsic pathway. The use of tissue factor, triggering the coagulation pathway in a more physiological way, has also been proposed. However, as for other global assays, TF leads to issues such as the significant variability among different sources of TF, the lot-to-lot variability of TF due to its biologic nature and the need to dilute the TF reagent.(Chitlur, Rivard et al. 2014) Even if the use of similar activators potentially generated similar results, TEG and ROTEM are not fully equivalent with not interchangeable results and interpretations.(Nielsen 2007, Sankarankutty, Nascimento et al. 2012, Rizoli, Min et al. 2016) The use different activators or methods may explain the lack of interchangeability between TEG and ROTEM. Moreover, the hemostasis is associated with a large range of normal values due to the variability of patient's hemostatic system. Therefore, before treatment or surgery, each patient should have baseline TEG/ROTEM measurements to be used as individualized reference. Other known limitations are the time measurement, the user-dependent and results variability even repeated on the same subject, which can be as high as 30%.(Gurbel, Bliden et al. 2008,

Anderson, Quasim et al. 2014) In clinical setting, the development of improved technology such as TEG 6s and validation of treatment algorithms for the two devices should improve patient care.(Kitchen, Kitchen et al. 2010)

V. Conclusion

TEG and ROTEM are not routine tests, but in vitro assays performed in physiological conditions in vivo, with results available almost immediately and used as point of care testing to monitor patients in some clinical situations. Viscoelastic assays have been widely investigated in liver transplant, cardiac surgery and trauma. Diagnosis and treatment algorithms incorporating ROTEM or TEG analysis allowed improving bleeding management and optimizing therapy by reducing blood transfusion.

5. The FibWave

The FibWave is a newly developed coagulation assay based on the analysis of the clot formation kinetics. The FibWave allows the evaluation of the global coagulation process by measuring the turbidity changes created during the clot formation process and has been developed to be more sensitive compared to the CWA. The qualitative examination and quantitative parameters provided by the clot waveform give information on the global coagulation process. Currently, four methodologies have been developed.

Initially, the first studies with the FibWave were performed on a microplate reader requiring manual placing of reagents and samples into the wells, which was a time-consuming process. The high inter-experiment variability (> 10%) could be explained by the fact that microplate reader did not provide continuous measurement of absorbance (i.e., the absorbance was measured in every 12 to 16 seconds depending on plate filling). The implementation of the FibWave on an automated coagulometer allowed the continuous measurement of absorbance in every millisecond which is translated into a gain in reproducibility and accuracy.

I. Principle

With the FibWave, the coagulation process can be also categorized in pre-coagulation, coagulation, and post-coagulation phases (**Figure 2**). To perform the FibWave, an analyzer with an optical detection system is needed for collecting absorbance data and processes them in quantitative parameters.

Information and parameters extracted from the clot waveform are equivalent to the CWA method. As for the APTT- and PT-based clot waveform analysis, the initial curve can be mathematically processed by an algorithm

to obtain visually the first and second derivative curves (**Figure 11**). The initial curve, i.e., the clot formation curve, is able to automatically provide the following parameters, depending on the methodologies: clotting time (time to Max2), coagulation velocity (Max1), acceleration (Max2) and deceleration (Min2). The parameters such as the delta, the time to peak (Time to Max1), and the fibrinolysis velocity (Min1) are parameters manually extracted from the initial and first derivative curve, respectively.

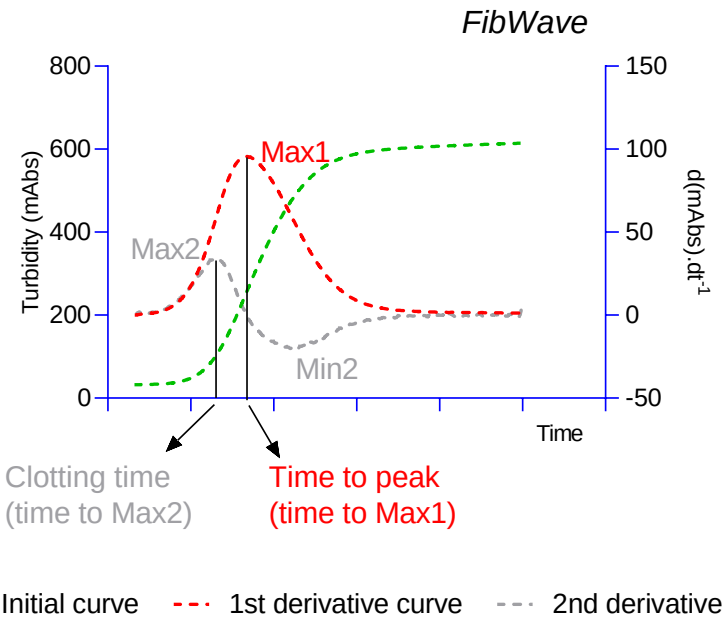


Figure 11: Overview of the initial and derivatives curves and parameters of the FibWave.

II. Methodologies currently developed with the FibWave

a. FibIn and FibEx methodologies

The FibIn and FibEx methodologies allow the exploration of the intrinsic and extrinsic coagulation pathways using a solution of contact activator and phospholipids, or a mixture of low concentration of tissue factor (±5 pM) and phospholipids. The main parameters extracted are the clotting time (time to

Max2), time to peak (time to Max1), velocity (Max1), acceleration (Max2) and deceleration (Min2). The delta parameter can help for giving information on the level of fibrinogen (**Figure 12**).

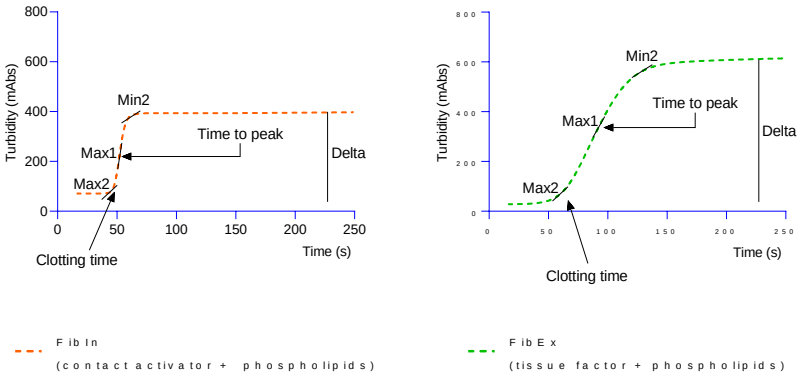


Figure 12: The clot formation and main parameters obtained with the FibIn and FibEx methodologies.

b. FibAPC methodology

The FibAPC methodology explores the protein C pathway, uses the same reagent as the FibEx methodology to which is added a protein C activator extracted from the copperhead snake venom Agkistrodon contortrix (Protac).

The FibAPC methodology has been developed for evaluating the activated protein C resistance (APCr) where the impact of Protac on Max1 is expressed in inhibition percentage using the following **equation 1**. The impact of Protac on Max1 on the initial and the first derivative curves is depicted in the **figure 13**. An optimal concentration of Protac is selected to provide 70% of inhibition of the velocity (Max1) on healthy pooled plasma. The Max1 parameter was chosen because it was the most sensitive and the less variable.

$$Inhibition\ percentage\ for\ Max1\ (\%) = 1 - \left(\frac{Sample\ Max1\ with\ Protac}{Sample\ Max1\ without\ Protac}\right)\%$$

Equation 1: Percentage of inhibition for Max1.
Protac: protein C activator.

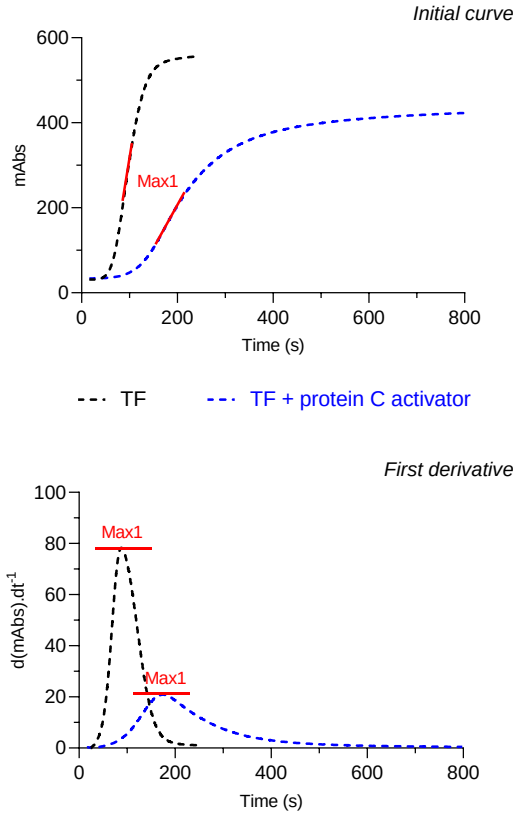


Figure 13: Impact of protein C activator (Protac) on Max1 parameter on the initial and the first derivative curves.
TF: tissue factor.

c. FibLysis methodology

The latest developed methodology is the FibLysis. This methodology assesses the fibrinolytic process, triggered by the tissue plasminogen activator (tPA) added to the reagent of FibEx methodology. The most important point is that

the selected concentration of tPA should not have an impact on the parameters of clot formation, i.e., velocity (Max1), time to peak (time to Max1) and the delta. Therefore, under the suitable conditions, the clot formation and fibrinolysis can be assessed simultaneously.

The parameters extracted from the FibLysis methodology are the maximum fibrinolysis velocity (|Min1|, in fibrinolysis unit), the fibrinolysis time (TFib, in seconds), and the difference between time to lysis and time to peak (TFib–Ttpeak, in seconds) (**Figure 14**).

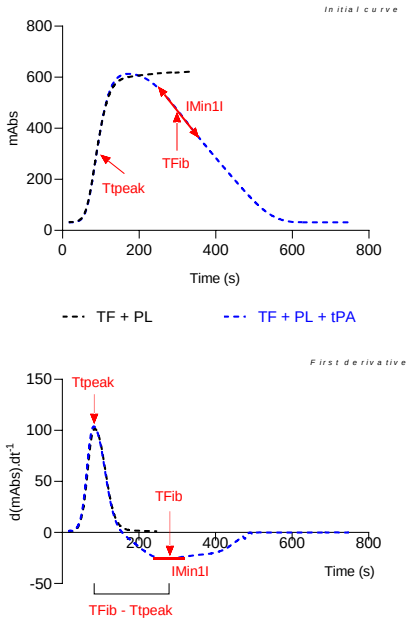


Figure 14: Overview of fibrin formation in absence of tissue plasminogen activator (tPA, black curve) and fibrinolysis in presence of tPA (blue curve).
Min: fibrinolysis velocity, TFib: fibrinolysis time and TFib-Ttpeak: difference between fibrinolysis time and time to peak are reported.

Objectives

The aim of this thesis was to develop a new coagulation assay evaluating the fibrin formation kinetics and evaluate the proof of concept in different coagulation states.

The developed coagulation assay, named the FibWave, allows assessing the global coagulation process by measuring turbidity changes during the fibrin clot formation. An idea of the clot formation can be depicted in real time and, in addition to obtaining a clotting time, additional coagulation parameters can be extracted.

During the development of the FibWave, the choice of type of reagents and volume of plasma and reagents was based on current coagulation assays. We aimed to associate the benefits of two important assays, the conventional assays, and the thrombin generation assay, without their respective drawbacks.

The proof of concept consisted of providing recommendations for performing the analysis of the fibrin clot formation and evaluating and comparing the FibWave with global coagulation assays in anticoagulant and procoagulant states.

Development of the FibWave methodologies

1. FibIn and FibEx methodologies

The conventional assays (APTT and PT) and the CWA reflect the coagulation in a non-physiological way. The conventional assays measure the clotting time in seconds, ratio, or INR while the CWA provides a global picture of the coagulation process using APTT and PT reagents.

The APTT and PT reagents contain surface activator or tissue factor at high concentration (concentrations not provided by manufacturers). The sensitivity between reagents can be due to the composition of the reagent, including the type of activator, the origin and composition of the phospholipids. The APTT reagents are composed of a surface activator, which include ellagic acid, silica or micronized silica, kaolin, polyphenolic activator, and platelets substitute as cephalin or synthetic phospholipids. The PT reagents are composed of a source of tissue factor (e.g. thromboplastin, recombinant tissue factor), phospholipids and calcium ions (**Table 7**). In the conventional assays, a 1/3 plasma ratio is used to standardize the amount of reagent and the assay. This ratio is considered as optimal for APTT and PT tests because it provides consistent and reproducible clotting times (**Table 8**).

Reagents of intrinsic pathway (APTT)	Composition
Actin FS®	Ellagic acid, synthetic phospholipids
SynthAFax®	Ellagic acid, synthetic phospholipids
SynthASil®	Micronized silica, synthetic phospholipids
Cephen®	Activator (equivalent to colloidal micronized silica), cephalin
STA®-PTT-A®	Silica (particulate activator), cephalin
STA®-Cephascreen®	Polyphenolic activator, cephalin
STA®-C.K.Prest®	Kaolin, cephalin
Reagents of extrinsic pathway (PT)	Composition
ReadiPlasTin®	Liquid recombinant human tissue factor, synthetic phospholipids, calcium ions, polybrene
RecombiPlasTin 2G®	Lyophilized recombinant human tissue factor, synthetic phospholipids, calcium ions, polybrene
Innovin®	Recombinant human tissue factor, synthetic phospholipids, calcium ions, a heparin-neutralizing compound
PT HTF®	Lyophilized thromboplastin, calcium ions
PT Excel®	Tissue thromboplastin from rabbit brain, calcium ions
PT Excel S®	Tissue thromboplastin from rabbit brain, calcium ions
STA®-Neoplastin Ci®	Lyophilized thromboplastin prepared from fresh rabbit cerebral tissues, inhibitor of heparin, calcium ions
STA®-Neoplastin Ci +®	Lyophilized thromboplastin prepared from fresh rabbit cerebral tissues, inhibitor of heparin, calcium ions
STA®-Neoplastin R®	Lyophilized thromboplastin prepared from human recombinant tissue factor and from phospholipids, inhibitor of heparin, calcium ions
STA®-NeoPTimal®	Lyophilized thromboplastin prepared from rabbit brain extract, inhibitor of heparin, calcium ions
Reagents of common pathway (TT)	Composition
STA®-DRRV Screen®	Russell's viper venom, phospholipids, inhibitor of heparin, calcium ions
STA®-DRRV Confirm®	Russell's viper venom, phospholipids, inhibitor of heparin, calcium ions
STA®-Reptilase®	Thrombin-like enzyme extracted from the venom of bothrops atrox
STA®-Thrombin®	Titrated calcium thrombin (human) lyophilized
HemosIL® Thrombin Time	Lyophilized bovine thrombin with bovine albumin and buffer
Reagents of thrombin generation	Composition
PPP Reagent Low®	± 1 pM of tissue factor (TF), phospholipids
PPP Reagent®	± 5 pM of TF, phospholipids
PPP Reagent High®	± 20 pM of TF, phospholipids

Table 7: Overview of the composition of the reagents from the intrinsic, extrinsic and common pathway.

The TGA is typically triggered by addition of tissue factor and/or phospholipids. The triggers from the intrinsic pathway can also be used.(Binder, Depasse et al. 2021) Depending on the clinical situation, the type of activator, the amounts of TF and phospholipids need to be selected appropriately. For example, low concentrations of TF (±1 pM) are suitable for detecting hypocoagulability, while higher concentrations of TF (20-50 pM) are suitable for determining the coagulation status of patients on anticoagulant therapy. The concentration of activator or TF is lower than that of the APTT and PT reagents. In TGA, a 2/3 plasma ratio is used for optimizing the sensitivity of the test and allows for optimal thrombin generation.

During the development of the FibWave, we aimed to associate the benefits of the conventional assays and the thrombin generation assay without their respective drawbacks. For the volume of plasma and reagents, we based on the calibrated automated thrombogram (CAT) system. For the type of reagent, we use APTT reagent for the FibIn and a low concentration of TF (±5 pM) for the FibEx, FibAPC, FibLysis methodologies.

The incubation time is important because it allows the reagents to mix with sample (coagulation factors and inhibitors). The incubation time is the time during which the sample and reagents is under optimal conditions, e.g. controlled temperature (37°C), before the coagulation process is performed. For all FibWave methodologies, we established an incubation time of 5 minutes, intermediate to the conventional (3 minutes for APTT) and TG (10 minutes) assays. There is no incubation time for PT test. PT reagents contain calcium ions and the addition of reagents on plasma immediately triggers the clot process. The 5-minute incubation time has been defined during the development of the FibAPC methodology because the protein C activator used in FibAPC is known to activate FV and FVIII within the first 3 to 5 minutes

of incubation. Therefore, we decided to harmonize the 5-minute incubation time to the other methodologies (**Table 8**).

Name of assays	Plasma volume	Reagent volume	Trigger reagent volume	Ratio (Plasma volume/total volume)	Incubation time
APTT and APTT-based CWA	50 µL	50 µL	50 µL of $CaCL_2$ (20 or 25 mM)	1/3	3 min
PT and PT-based CWA	50 µL	/	100 µL of TF + PL + $CaCl_2$	1/3	/
TGA-based CAT system	80 µL	20 µL	20 µL of $CaCl_2$+fluorogenic substrate (16.7 mM)	2/3	10 min
FibWave	100 µL	25 µL	25 µL of $CaCl_2$ (100 mM)	2/3	5 min

Table 8: Summary of plasma and reagents volume for the conventional assays (APTT, PT), TGA and FibWave.

For the FibWave, the volume of plasma and reagents is the same for the different methodologies. TGA-based CAT system: thrombin generation assay-based on calibrated automated thrombogram system; APTT: activated partial thromboplastin time; PT: prothrombin time; CWA: clot waveform analysis.

Recently, Wada *et al.* demonstrated that physiological coagulation starts when small amounts of TF (sTF) and activated FVII activate FIX. As the APTT and PT reagents include higher PL content and contact activator or tissue factor than physiological coagulation, they cannot sufficiently reflect the FIX activation by a sTF.(Wada, Shiraki et al. 2020) In this way, the FibEx methodology, exploring the extrinsic pathway, uses a low TF concentration, close to the one studied by Wada *et al.* (final TF concentration in test: ± 0.8

pM in FibEx and <0.1 pg/mL in Wada's study), could therefore/potentially adequately reflect the physiological coagulation.

In term of clotting time, the plasma samples from healthy people will coagulate within 25-35 seconds for APTT, 11-13 seconds for PT, 45-50 seconds for FibIn and 65-75 seconds for FibEx (**Figure 15**).

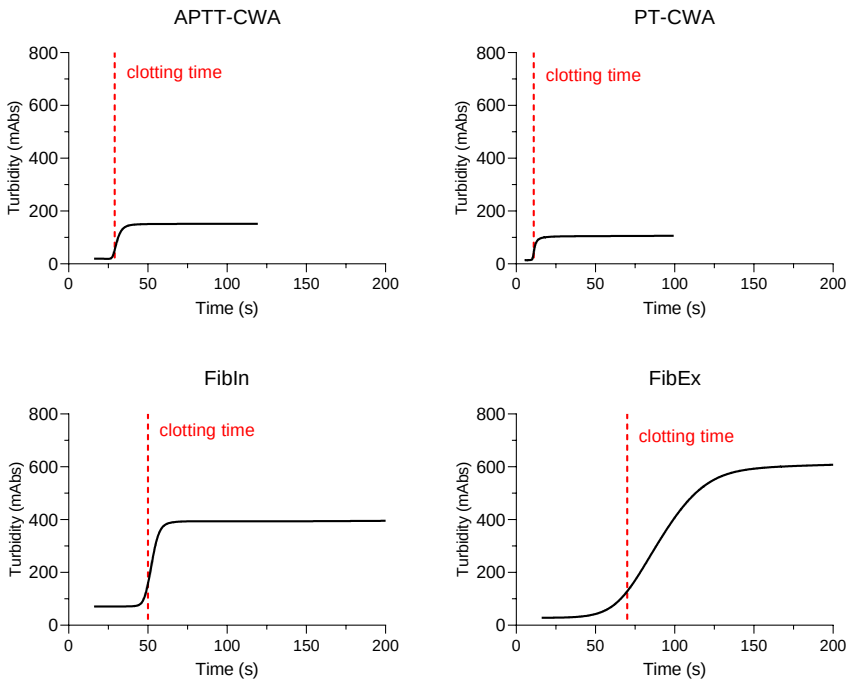


Figure 15: Comparison of clotting time between APTT- and PT-based clot waveform analysis and FibEx and FibIn methodologies.

2. FibAPC methodologies

The FibAPC methodology has been developed for exploring the protein C pathway and evaluating the activated protein C resistance (APCr).

Different methods for the detection of APCr have been developed over years but those recommended by the European Medicines Agency (EMA) guidance for the assessment of steroid contraceptives are the APTT-based APC resistance and the ETP-based APC resistance assay. Some scientists prefer to use thrombomodulin (TM) as the inhibitor agent instead of exogenous APC or protein C activator to assess the resistance.(Dargaud Y, Trzeciak MC et al. 2006, Zia, Callaghan et al. 2015)

During the development of FibAPC, we evaluated the impact of thrombomodulin and APC on fibrin clot formation.

The results showed that in presence of TM, the parameters of FibWave were not significantly impacted but in presence of APC, the clotting time was significantly prolonged and the velocity reduced (**Figure 16**).

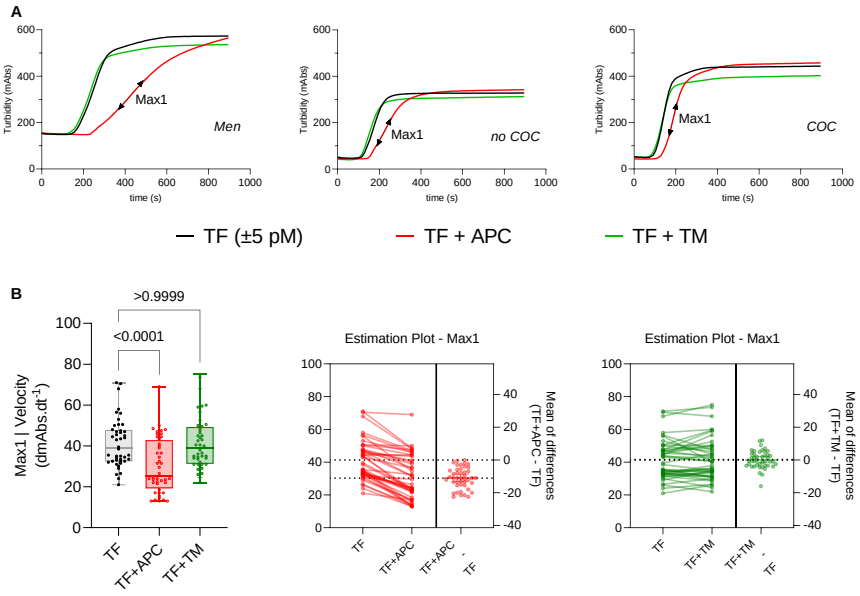


Figure 16: Overview of fibrin formation in presence of tissue factor (black curve), in presence of tissue factor + thrombomodulin (green curve) and in presence of tissue factor + activated protein C (red curve) in 3 populations (men, women without contraception (no COC) and women with contraception (COC) (A). Assessment of the impact of thrombomodulin (green) and activated protein C (red) on Max1 (B).

P < .05 indicates a significant difference between test conditions, determined by a paired t test. TF: tissue factor; TM: thrombomodulin; APC: activated protein C; Max1: velocity of coagulation.

The TM is an endothelial cell thrombin receptor and acts as a cofactor for the thrombin-dependent activation of protein C. The generation of APC by the thrombin-TM complex leads to inactivation of the procoagulant cofactors FVa and FVIIIa, suppressing the thrombin generation. Although studies showed that TM suppresses the thrombin generation, its activity on fibrin formation is limited.(Evrard 2019 (Unpublished results), Binder, Depasse et al. 2021, Depasse, Binder et al. 2021)

The impact of APC and protein C activator (Protac) was also evaluated on FibWave parameters. The Max1 parameter showed a similar sensitivity toward resistance to APC or Protac. As in the ETP-based APC resistance, the

impact of APC or Protac on Max1 is expressed in percentage of inhibition. In FibAPC studies, an optimal concentration of APC or Protac was selected to provide 70% of inhibition of the Max1 on healthy pooled plasma (**Figure 17**).

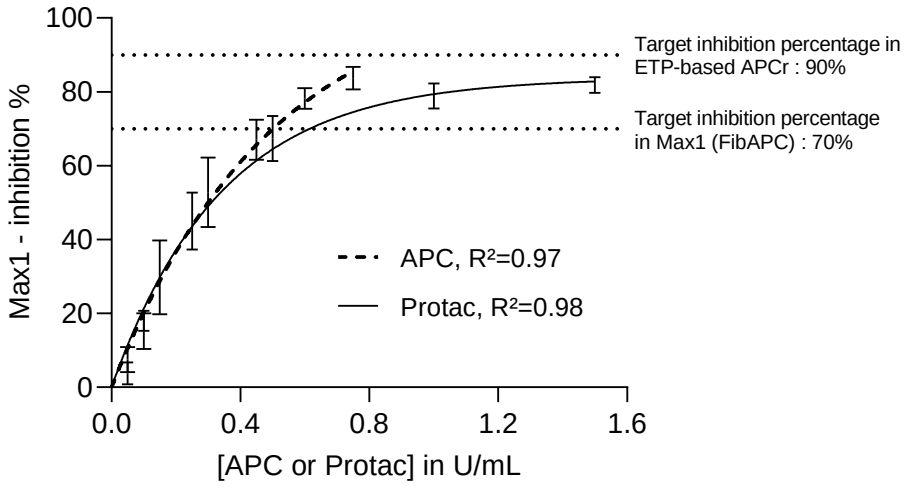


Figure 17: Dose-response curves in presence of exogenous APC or Protac.
Coefficients of determination (R^2) are reported for both conditions. The APC and Protac concentrations in FibAPC are defined to obtain 70% of inhibition of Max1. It was not possible to have 90% inhibition of the Max1 with the Protac.

3. FibLysis methodologies

The FibLysis methodology was developed for evaluating and quantifying the fibrinolytic process, triggered by the tissue plasminogen activator (tPA) added to the TF.

Laboratory investigations using assays of specific plasma factors have been used for many years to measure the coagulation and fibrinolytic activity. However, the levels of individuals factors may not always reflect the clinical phenotype. Therefore, the development of techniques capable of evaluating and quantifying the overall fibrinolysis activity of sample appear to be particularly useful.

Currently, some global coagulation assays, including viscoelastic assays (TEG, ROTEM), TGA, OHP and CWA, have been investigated in clinical practice to reflect the comprehensive processes of coagulation and/or fibrinolysis. Recently, the thrombin and plasmin generation assay (T/PGA) and the clot-fibrinolysis waveform analysis (CFWA) have been described to investigate the interplay between coagulation and fibrinolysis.(Matsumoto, Nogami et al. 2013, Saes, Schols et al. 2019)

Based on the conditions of the fibrinolytic assays (T/PGA and CFWA), we aimed to adapt the FibWave for measuring the coagulation and fibrinolytic processes. The T/PGA uses a reagent consisting of a mixture of TF and tPA while CFWA uses APTT reagent and $CaCl_2$ containing (recombinant) tPA (rTPA) (**Table 9**).(Oka, Wakui et al. 2020, Evrard, Maloteau et al. 2021) We have experimented different conditions, added tPA in calcium solution, tPA in APTT reagent and finally chose to add tPA in low TF (**Figure 18**). The coefficients of variation in these experiments ranged between 10-30% for $CaCl_2$-tPA, 5-20% for APTT-tPA and 4-15% for TF-tPA conditions for the fibrinolysis velocity (Min1).

Name of assay	Type of reagent	Type of trigger reagent
T/PGA	TF + tPA Final concentration: 1 pM TF and 3.3 nM tPA – **Matsumoto *et al.* 2013** Final concentration: ± 0.3 pM TF and 193 IU/mL tPA – *Saes et al. 2019*	Fluorescent thrombin-specific substrate and fluorescent plasmin-specific substrate + **100 mM** or *16.7 mM* $CaCl_2$
CFWA	APTT	$CaCL_2$ + tPA or rtPA Final concentration: 1.67 µg/mL tPA or 0.63 µg/mL rtPA
FibLysis	TF + tPA Final concentration: ±0.8 pM TF and ≤ 0.8 µg/mL tPA	100 mM $CaCl_2$

Table 9: Summary of plasma and reagents volume for the thrombin and plasmin generation assay, clot-fibrinolysis waveform analysis and FibLysis methodologies.
T/PGA: thrombin and plasmin generation assay; CFWA: clot-fibrinolysis waveform analysis; rtPA: recombinant tissue plasminogen activator.

For determining the concentration of tPA, the impact of tPA was evaluated on coagulation phase. Normal plasma was mixed with TF and various concentration of tPA (1.25 – 20 µg/mL). Results showed that high concentration led to decrease the Delta (10 and 20 µg/mL) and reduce the time to peak (20 µg/mL). The Max1 was not impacted at any concentration (**Figure 19**). tPA concentrations from 1 to 5 µg/mL could be appropriate for measuring the fibrinolytic process in a relatively short time (**Figure 20**). Intermediate concentrations of tPA (±5 µg/mL) are expected to improve the sensitivity and reproducibility of Min1 and TFib compared to a low concentration (1 µg/mL). We suggested that the most important point is that the concentration of tPA should not have an impact on the parameters of clot formation, i.e., velocity (Max1), time to peak (time to Max1) and the Delta. Therefore, under the suitable conditions, the clot formation and fibrinolytic processes can be assessed simultaneously in a short time.

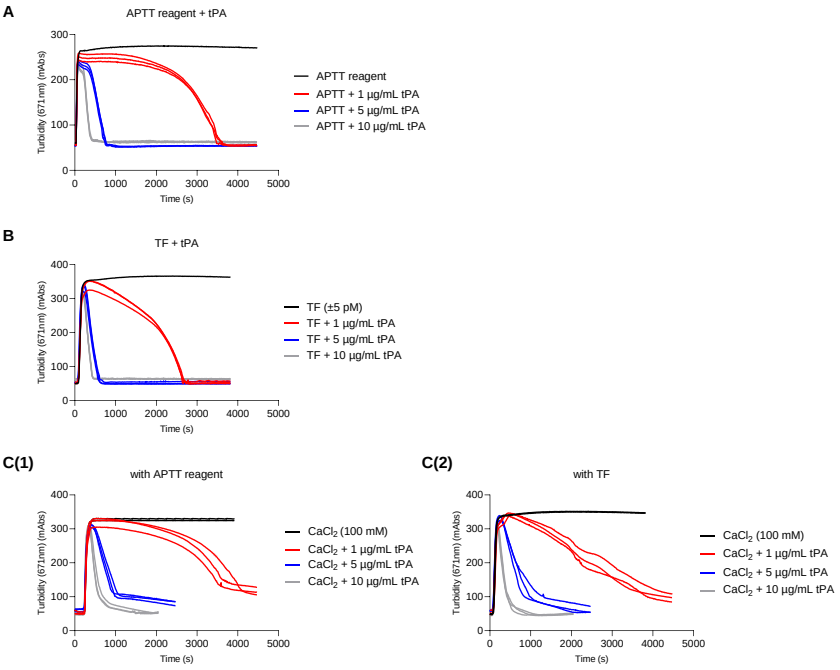


Figure 18: Assessment of the impact of tPA (concentration: 1, 5 and 10 µg/mL) added to different reagents.

The tPA was added to APTT reagent (A), to TF (B) and to calcium solution (100 mM), triggered by APTT reagent (C1) or TF (C2).

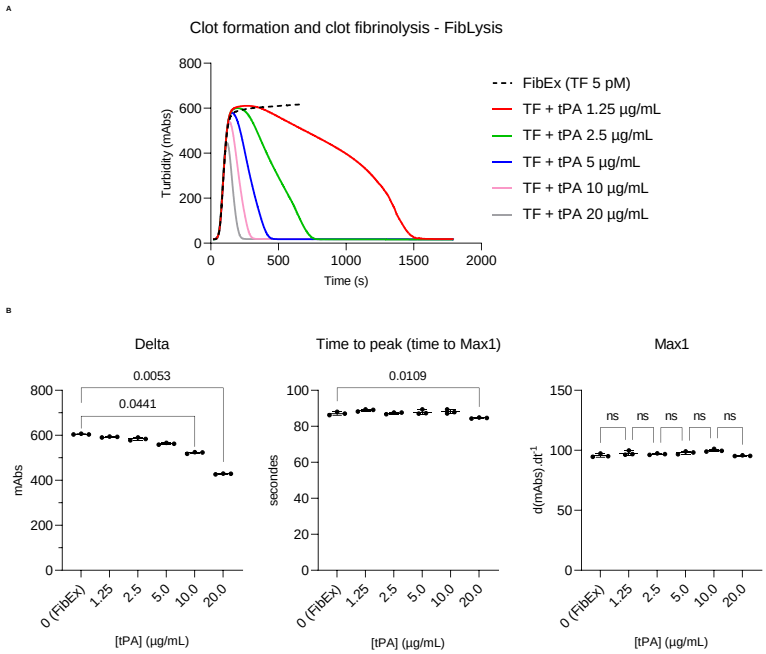


Figure 19: Overview of the clot formation and clot fibrinolysis with various concentration of tissue plasminogen activator (tPA) (A). Assessment of the impact of various concentration of tPA on clot formation parameters (B).

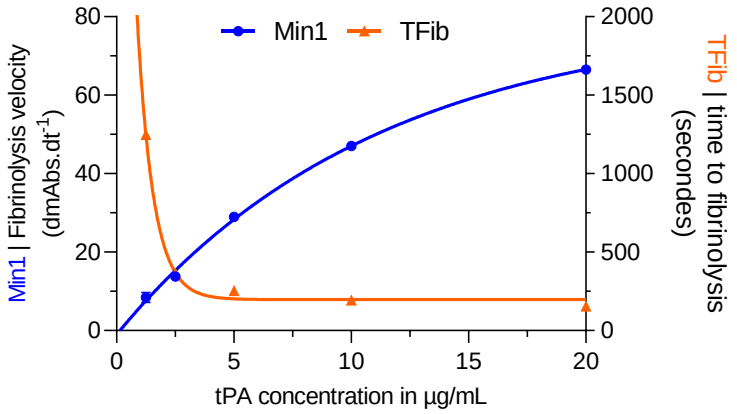


Figure 20: Dose-responses curves performed with various concentrations of tissue plasminogen activator added to the tissue factor.

Proof of concept of FibWave

1. Recommendations in term of wavelengths and reagents for the FibWave

APTT and PT assays are well established but the CWA and related parameters are not well standardized, which limiting its routine use in clinic.(Lippi, Plebani et al. 2013, Shima, Thachil et al. 2013) In particular, the wavelength at which the analysis is performed, the choice of reagent, parameters for analyzing samples or hemolytic, icteric and lipemic (HIL) interferences from plasma samples which may affect interpretation of results.

The FibWave is not yet standardized method but during first steps of development of methodology, we investigated and determined which wavelength was the most sensitive and specific for distinguishing non-clotted and clotted plasma samples, and evaluated the impact of reagents from the intrinsic, extrinsic, and common coagulation pathway on baseline absorbance. (Evrard, Siriez et al. 2019)

I. The most sensitive and specific wavelength

Currently, depending on the analyzer, different wavelengths (340, 405, 635, 575, 660, 671, 705 and 800 nm) can be used to perform CWA. Based on these wavelengths, the plasma was screened at wavelength from 280 to 700 nm to provide absorbance spectra of clotted and non-clotted plasma. The signal-to-noise ratio (clotted plasma absorbance/non-clotted plasma absorbance) was calculated at different wavelengths.

The absorbance spectra of non-clotted and clotted plasma is shown in **figure 21**. The results of clotted and non-clotted plasma samples showed a higher absorbance at 340 nm than other wavelengths, suggesting in a first idea that this wavelength could be optimally used to perform CWA. However, the results also showed that the signal-to-noise ratio was higher at wavelengths

>550 nm (**Figure 22**), suggesting that the most sensitive and specific wavelengths could be >550 nm. Previous studies reported that HIL interferences have specific optical spectra and can significantly interfere with the fibrin coagulation process where samples with apparent hemolysis may undergo premature coagulation activity and disrupt the clot detection.(Favaloro, Funk et al. 2012, Lippi, Ippolito et al. 2013, Magnette, Chatelain et al. 2016, Hernaningsih and Akualing 2017) The current hemostasis analyzers are equipped with a system for detecting the interfering substances in addition to clot formation. Briefly, the HIL control is performed by measuring the absorbance of sample at three different wavelengths, i.e. 671 nm for turbidity, 535 nm for turbidity and cell-free hemoglobin and 405 nm for cell-free hemoglobin and total bilirubin. The measurement at the three wavelengths creates three equations that can then be solved mathematically to determine the levels of these three interfering substances.(Lippi, Ippolito et al. 2013)

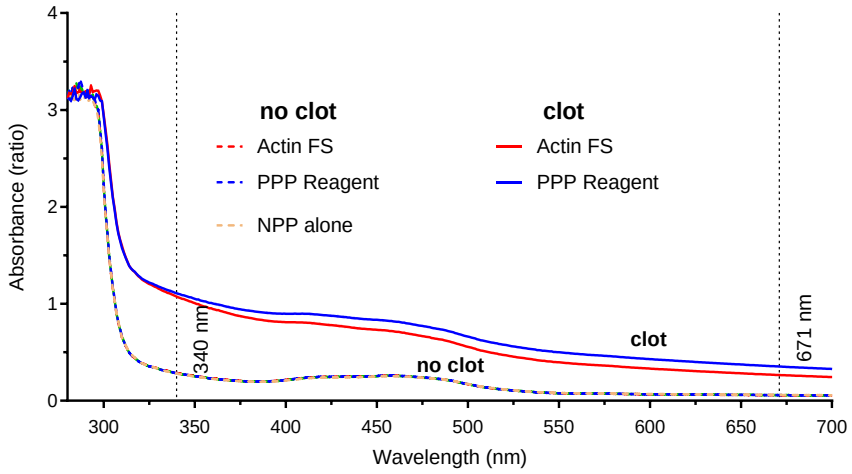


Figure 21: Absorbance spectra of non-clotted and clotted plasma.
The intrinsic pathway was triggered with Actin FS while the extrinsic pathway was triggered with PPP reagent.

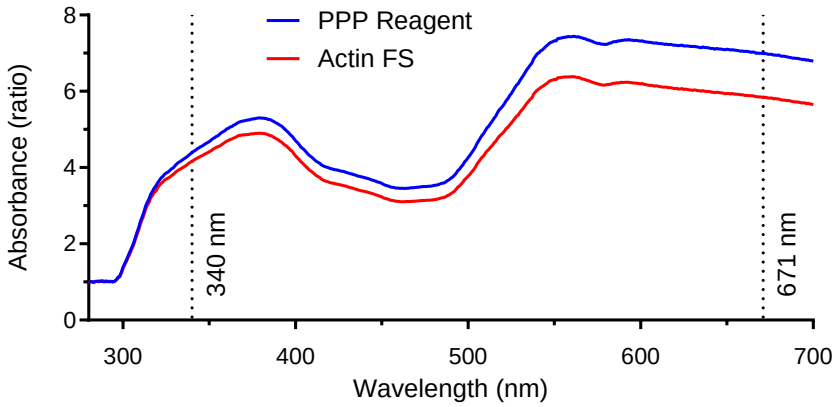


Figure 22: Spectrum of the signal-to-noise ratio.

Thus, according to our results and the data available in the literature, the measurement and the analysis of a clot waveform using an optical detection system should be performed at wavelengths >650 nm for at least two reasons: to avoid HIL interferences and to have a high signal to noise which in definitive will provide a better sensitivity for the parameters that can be evaluated in CWA.

II. The impact of reagents on baseline absorbance

As the CWA is based on photo-optical detection method, the appearance (color) of reagents could, in rare instances, impact the output signal of samples (i.e. minimum absorbance). For investigating the inner-filter effects of reagents exploring the intrinsic pathway (APTT), we mixed plasma with APTT reagents. For investigating the inner-filter effects of reagents exploring the extrinsic (PT) and common (TT, RT, dRVVT) pathway, plasma samples were replaced by serum samples. The addition of one of these reagents, containing calcium ions, on plasma triggers the coagulation process and makes impossible the assessment of the inner-filter effect of these reagents.

The inner-filter effect of APTT reagents assessed on non-clotted plasma and the inner-filter effect of reagents, containing calcium ions, assessed on serum are summarized in **Table 10**. Except for the STA-CKPrest® ($p < .05$) and the STA-NeoPTimal® ($p < .05$ at 340 nm, $p > .05$ at 671 nm), the reagents had no significant effect on the baseline absorbance of plasma or serum.

Among the APTT reagents assessed on plasma only the STA-CKPrest® significantly impacted the output signal. The STA-CKPrest®, composed of kaolin and cephalin, is used on analyzers based on mechanical detection of the clot. The results showed that this reagent present a large variability in absorbance measurements, and we consider that this reagent should not be used to perform CWA. Among the reagents from the extrinsic (PT) and common (TT, RT, dRVVT) pathway assessed on serum, only the STA-NeoPTimal® significantly affected the output signal at 340 nm. No interference was reported for all other reagents, even those which are colored.

In conclusion, despite colorful appearance of some reagents, most of reagents currently on the market could be used to perform CWA. Based on the FibWave methodology, the use of colorless reagent is no longer recommended but it could be interesting to evaluate the inner-filter effect of new reagent before performing a CWA.

Plasma						
	340nm			671nm		
	Mean ± SD	CV (%)	p-value (<0.05)	Mean ± SD	CV (%)	p-value (<0.05)
Reagents of the intrinsic coagulation pathway						
Actin FS®	330.6 ± 4.0	1.20%	0.4798	57.4 ± 0.8	1.33%	0.9525
SynthAFax®	291.9 ± 5.209	1.78%	0.9003	59.1 ± 0.6	0.93%	0.7961
SynthASil®	273.9 ± 4.8	1.74%	0.6079	54.0 ± 1.1	2.04%	0.7232
Cephen®	300.1 ± 1.6	0.53%	0.9585	64.8 ± 0.9	1.32%	0.3409
STA®-PTT-A®	359.5 ± 0.3	0.08%	0.1872	69.6 ± 0.9	1.32%	0.1277
STA®-Cephascreen®	331.8 ± 3.6	1.08%	0.4640	58.4 ± 1.3	2.17%	0.8625
STA®-C.K.Prest®	778.8 ± 36.0	4.63%	<0.001	355.8 ± 38.7	10.88%	<0.001
Reagents of thrombin generation test						
PPP Reagent Low®	267.0 ± 3.5	1.30%	0.5091	53.4 ± 0.7	1.33%	0.6634
PPP Reagent®	269.0 ± 3.0	1.10%	0.5365	53.3 ± 0.9	1.74%	0.6546
PPP Reagent High®	266.2 ± 1.8	0.68%	0.4982	53.4 ± 1.0	1.85%	0.6634
Serum						
	340nm			671nm		
	Mean ± SD	CV (%)	p-value (<0.05)	Mean ± SD	CV (%)	p-value (<0.05)
Reagents of the extrinsic coagulation pathway and common coagulation pathway						
ReadiPlasTin®	416.9 ± 6.9	1.66%	0.7583	67.0 ± 0.7	1.08%	0.8981
RecombiPlasTin 2G®	421.8 ± 1.9	0.44%	0.7188	66.1 ± 1.0	1.51%	0.8584
Dade Innovin®	385.3 ± 1.3	0.34%	0.9724	65.2 ± 0.6	0.85%	0.8161
PT HTF®	437.3 ± 4.8	1.10%	0.5995	82.6 ± 0.6	0.76%	0.4452
PT Excel®	455.6 ± 7.5	1.64%	0.4745	83.0 ± 0.7	0.87%	0.4313
PT Excel S®	449.5 ± 1.6	0.34%	0.5142	83.0 ± 0.3	0.39%	0.4302
STA®-Neoplastin Ci®	477.1 ± 4.4	0.92%	0.3514	91.2 ± 0.1	0.06%	0.2252
STA®-Neoplastin Ci Plus®	478.8 ± 10.9	2.28%	0.3434	91.5 ± 1.6	1.74%	0.2199
STA®-Neoplastin R®	408.8 ± 1.6	0.38%	0.8258	72.1 ± 0.3	0.37%	0.8660
STA®-NeoPTimal ®	560.3 ± 8.2	1.46%	0.0942	116.9 ± 2.9	2.47%	0.0238
DRVV Screen®	410.2 ± 1.8	0.43%	0.8140	70.3 ± 0.5	0.70%	0.9474
DRVV Confirm®	449.8 ± 5.6	1.23%	0.5121	79.0 ± 1.3	1.65%	0.5729
STA®-Reptilase®	395.5 ± 1.5	0.38%	0.9392	66.7 ± 1.8	2.67%	0.8829
STA®-Thrombin®	426.4±3.8	0.89%	0.6819	72.1 ±1.0	1.41%	0.8660
HemosIL® Thrombin Time	478.2 ± 2.6	0.54%	0.3458	77.0 ±0.5	0.60%	0.6521

Table 10: Summary of the inner-filter effect of reagents.

Impact of the reagents on minimum absorbance from the intrinsic, extrinsic, and common pathway on the baseline absorbance of plasma and serum.

2. Investigation of influence of coagulation factors (pro- and anticoagulant) on the FibEx methodology in plasma

The aim of this exploratory study was to investigate and compare the influence of the concentration of specific procoagulant and anticoagulant factors on FibEx and TGA parameters. The study was performed using frozen coagulation factor-deficient human plasma. Different factor levels were obtained by spiking depleted plasma with different amounts of normal plasma.

The FibEx methodology from the FibWave, exploring the extrinsic pathway, was evaluated and compared to the TGA using the same reagent (±5 pM TF), according to the methods previously described (**Table 8**).

I. Effect of factor levels on TGA parameters

Moderate effects of FXI levels were observed on ETP. In the absence of FXI, peak thrombin and mVRI reached values of 36% and 23% of normal plasma. The ETP was slightly affected by FVIII levels. In the absence of FVIII, the peak and mVRI were reduced by about 90%.

When the AT concentration was reduced to 50%, ETP and peak doubled and mVRI reached value of 288% of normal plasma. No results were available for AT concentrations above amount of 70% of deficient plasma due to complete consumption of substrate. No effect of protein S was observed on ETP and peak. A moderate effect was observed on mVRI.

The time to peak and lag time were prolonged with decreasing FXI and FVIII concentrations. Changes in time to peak was higher than in lag time for FVIII. The lag time and time to peak were reduced in absence of protein S or AT, reaching ratio from 0.89 to 0.72 to normal plasma (**Table 11**).

TF (±5 pM)	TGA parameters	Amounts of normal plasma						
		100%	90%	70%	50%	30%	10%	0%
FXI	Lagtime	1.00	1.00	1.11	1.22	1.33	1.41	1.44
	ETP	100%	100%	100%	94%	95%	83%	64%
	Peak	100%	96%	90%	75%	84%	54%	36%
	Ttpeak	1.00	1.07	1.16	1.26	1.23	1.47	1.51
	mVRI	100%	85%	75%	58%	75%	35%	23%
FVIII	Lagtime	1.00	1.04	1.12	1.12	1.12	1.12	1.12
	ETP	100%	96%	84%	71%	57%	40%	27%
	Peak	100%	91%	66%	47%	30%	17%	11%
	Ttpeak	1.00	1.02	1.18	1.29	1.50	1.80	2.00
	mVRI	100%	91%	54%	33%	17%	8%	4%
Protein S	Lagtime	1.00	1.00	1.00	0.89	0.89	0.89	0.89
	ETP	100%	99%	102%	99%	103%	102%	93%
	Peak	100%	103%	109%	114%	119%	128%	126%
	Ttpeak	1.00	0.98	0.91	0.86	0.82	0.77	0.74
	mVRI	100%	107%	131%	137%	156%	193%	211%
AT	Lagtime	1.00	0.84	0.72	0.72	n.m.	n.m.	n.m.
	ETP	100%	118%	151%	215%	n.m.	n.m.	n.m.
	Peak	100%	125%	156%	196%	n.m.	n.m.	n.m.
	Ttpeak	1.00	0.86	0.75	0.70	n.m.	n.m.	n.m.
	mVRI	100%	143%	201%	288%	n.m.	n.m.	n.m.

Table 11: TG results obtained with TF (±5 pM) of the indicated coagulation factors. *Coagulation factor-deficient plasma samples were spiked with different amounts (%) of normal plasma to achieve the indicated concentrations of each factor. The results are expressed as a ratio for the temporal parameters (lag time and time to peak) and a percentage (%) for ETP, Peak and mVRI measured in a normal pooled plasma.*

II. Effect of factor levels on FibEx parameters

In the absence of FXI (100% of deficient plasma), Max1, Max2 and Min2 reached values of 66%, 57% and 40% of normal plasma. Moderate effects of FVIII levels were observed on parameters. In the absence of FVIII, Max1 and

Max2 reached values of 72%-74% of normal plasma, and Min2 reached values of 43% of normal plasma.

When the AT level was reduced to 50%, Max1 and Max2 were not impacted, and Min2 was increased and reached vales of 189% of normal plasma. In the absence of protein S, no effect was observed on Max1. Max2 and Min2 were increased by about 45%.

The time to peak was prolonged with decreasing FXI and FVIII levels. The changes in time to peak was more marked with FXI deficiency. In absence of AT, the time to peak reached values of 50% of normal plasma. The time to peak was no affected by the absence of protein S (**Table 12**).

TF (±5 pM)	FibEx parameters	Amounts of deficient plasma						
		0%	10%	30%	50%	70%	90%	100%
FXI	Ttpeak	1.00	1.10	1.24	1.33	1.43	1.57	1.67
	Max1	100%	96%	87%	81%	81%	73%	66%
	Max2	100%	91%	72%	70%	63%	68%	57%
	Min2	100%	94%	81%	68%	68%	52%	40%
FVIII	Ttpeak	1.00	0.95	1.09	1.14	1.22	1.27	1.31
	Max1	100%	98%	102%	91%	87%	79%	74%
	Max2	100%	99%	103%	90%	85%	79%	72%
	Min2	100%	90%	85%	68%	54%	43%	43%
Protein S	Ttpeak	1.00	1.05	1.09	1.00	1.00	1.00	0.95
	Max1	100%	101%	114%	109%	112%	114%	106%
	Max2	100%	106%	119%	127%	122%	142%	142%
	Min2	100%	101%	127%	124%	133%	144%	147%
AT	Ttpeak	1.00	0.86	0.63	0.55	0.63	0.59	0.50
	Max1	100%	107%	119%	99%	103%	92%	96%
	Max2	100%	153%	156%	115%	131%	128%	139%
	Min2	100%	136%	209%	166%	189%	175%	189%

Table 12: FibEx results obtained with TF (±5 pM) of the indicated coagulation factors. *Coagulation factor-deficient plasma samples were spiked with different amounts (%) of normal plasma to achieve the indicated concentrations of each factor. The results are expressed as a ratio for the time to peak and a percentage (%) for Max1, Max2 and Min2 measured in a normal pooled plasma.*

The aim of this exploratory study was to evaluate and compare the influence of the concentration of procoagulant (FXI, FVIII) and anticoagulant (AT, protein S) factors between FibEx and TGA.

The clot process was triggered by a low concentration of TF in TGA and FibEx. Many authors have shown that at low TF concentrations, the FVII-TF complex activates FIX which, in association with FVIII, activates FX and generates thrombin and fibrin. Results showed that the TGA with low TF was sensitive to different factor levels and make this test an attractive approach for

exploring the FIX and FVIII activation in thromboplastin-dependent coagulation in plasma.(Josso and Prou-Wartelle 1965, Duchemin, Pan-Petesch et al. 2008, Butenas, Orfeo et al. 2009)

Under our experimental conditions, an ETP 64%, peak 36% and mVRI 23% of normal plasma were measured in the absence of FXI, demonstrating the importance of FXI levels as an amplifier of thrombin generation initiated by low TF concentrations.(He, Xiong et al. 2001, Keularts, Zivelin et al. 2001)

In our study, the mVRI and peak of thrombin appeared more sensitive than ETP to FVIII levels, confirming previous observations that these parameters could be useful markers for distinguishing between different types of hemophilia patients (mild vs severe).(Hemker, Giesen et al. 2002, Hemker, Giesen et al. 2003, Matsumoto, Shima et al. 2006)

Increased TG can be caused by an excess of prothrombin, overactive prothrombin conversion (factor V Leiden and deficiency of protein C or S) or by decreased thrombin breakdown (AT deficiency). In terms of coagulation inhibitors, AT had a considerable effect on thrombin generation. Decreasing AT levels caused significant increases in thrombin generation, consistent with the in vivo consequences of AT deficiency.(Kremers, Peters et al. 2015, Luna-Záizar, González-Moncada et al. 2015)

Low plasma levels of the natural anticoagulant protein S are strongly associated with venous thromboembolism. Our results showed that the protein S levels affected more time to peak than lag time, and very few effects was observed on ETP compared to the peak and mVRI. Low levels of protein S caused a reduction in the time to peak and an increase in the peak and mVRI, confirming previous observations.(Luna-Záizar, González-Moncada et al. 2015, Brouns, Tullemans et al. 2022)

The parameters of FibEx appeared to be as sensitive as parameters of TGA for FXI deficient plasma but were not as pronounced as those in TGA with

FVIII. As FVIII is involved in the intrinsic pathway, the initiation of coagulation by TF may not be appropriate for evaluating fibrin formation in FVIII-deficient plasma. Previous studies evaluated the correlation between the APTT-CWA and TGA (20 pM of TF) and highlighted that APTT-CWA was more sensitive than TGA for the detection of the effect for levels of FVIII at <1.0 IU.dL-1.(Matsumoto, Shima et al. 2006) Therefore, further investigations are needed with the FibWave exploring the intrinsic pathway.

Contrary to TGA where all parameters were significantly affected when AT level was reduced to 50%, only Min2 and time to peak were affected in FibEx. The protein S concentrations affected more acceleration (Max2) and deceleration (Min2), suggesting that these parameters could be more sensitive to variations in protein S.

The results obtained from the in-vitro deficient plasmas with FibEx methodology seemed to correlate with those of TGA. The effects of procoagulant and anticoagulant factors were more pronounced on the thrombin peak and mean velocity rate index than other parameters, while with the FibEx, the acceleration (Max2), deceleration (Min2) and time to peak appeared to be the most sensitive to factor variations. However, we could not corroborate our results with samples of patients with congenital coagulation factor deficiency. This study was performed with frozen plasma samples that may not necessarily reflect the in-vivo behavior. An influence due to the plasma preparation process (immunodepletion) cannot therefore be excluded. An immunodepleted deficient plasma is not equivalent to a congenital deficient plasma and results obtained from FVIII-deficient plasma could be very different from results obtained from a severe hemophilia A patient under the same assay conditions. Contrary to FibEx, the use of the same plasma sample as calibration in TGA allows to reduce the inner-filter effect of plasma (**Figure 23**).

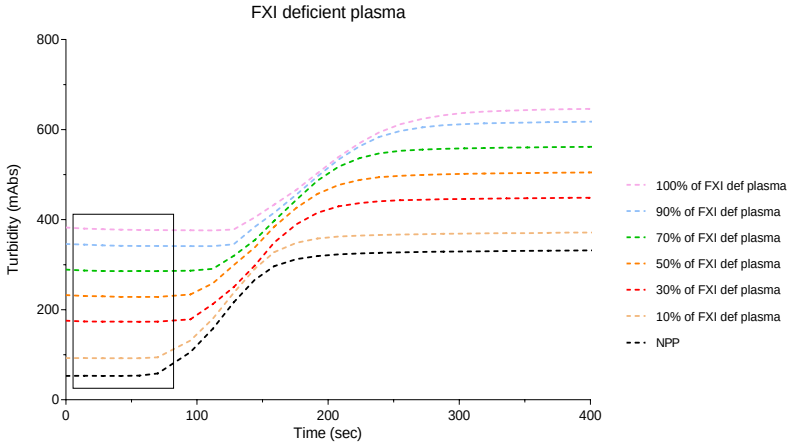


Figure 23: Effect of FXI on fibrin formation (FibEx).
An influence due to the plasma preparation process impacting the inner-filter effect of plasma cannot be excluded.

This exploratory study shows that parameters such as the deceleration and time to peak of FibEx were able to assess the coagulation factor activity with sensitivities close to or slightly lower than those obtained with the TGA. Further studies needed to be performed on samples from patients with congenital coagulation factor deficiency.

3. Anticoagulant state

I. Impact of direct oral anticoagulants on FibWave parameters and comparison with the thrombin generation assay

Several studies have demonstrated the potential of TGA for the assessment of DOACs and the monitoring of reversal therapies.(Bloemen, Zwaveling et al. 2018, Rigano, Ng et al. 2018, Pfrepper, Metze et al. 2020, Metze, Klöter et al. 2021) Although TGA is a sensitive and global assay, it estimates the thrombin concentration over-time, which is not the final endpoint of the coagulation process. Therefore, the assessment of the kinetics formation of fibrin could be of interest to detect coagulation abnormalities beyond thrombin. In first proof-of-concept study, the impact of DOACs was evaluated and compared on fibrin (FibWave) and thrombin (CAT method) formation. The sensitivity of the different FibWave and TGA parameters was also investigated.(Evrard, Morimont et al. 2020)

DOACs (apixaban, edoxaban, rivaroxaban and dabigatran) were spiked in normal pooled plasma at the following final concentrations: 0, 10, 30, 50, 100, 250 and 500 ng/ mL. The fibrin and thrombin formation were assessed by the FibWave and CAT, according to their methodology.

a. DOACs effect on the FibWave and TGA parameters

The results showed that the FibWave was able to assess the anticoagulant activity of DOACs with a sensitivity similar to the one obtained with the TGA (CAT method). In the FibWave, FXa inhibitors prolonged the time to Max1 and reduced the velocity, acceleration and deceleration of fibrin formation while dabigatran prolonged the time to Max1 and had little effect on other parameters (**Table 13**).

As previously observed in TGA, the FXa inhibitors effects were more pronounced on the lag time, the time to peak, the peak and the mVRI than ETP while dabigatran delayed thrombin generation with major effects on the lag time and the time to peak (**Table 13**). Differences observed between the intrinsic and extrinsic pathways enhance the importance of the choice of the activation pathway for evaluating DOACs.(Douxfils, Ageno et al. 2018, Dunois 2021)

FibWave			Time to Max1	Max1	Max2	Min2
DOACs	*Triggering pathway*	*Threshold (ng/mL)*	*Ratio (s)*	*Ratio ($dmAbs.dt^{-1}$)*	*Ratio ($dmAbs.dt^{-2}$)*	*Ratio ($dmAbs.dt^{-2}$)*
Apixaban	Intrinsic	30	1.0	0.9	0.9	0.8
		50	1.1	0.9	0.9	0.8
		100	1.1	0.8	0.9	0.8
	Extrinsic	30	1.1	0.8	0.6	0.7
		50	1.4	0.8	0.6	0.6
		100	1.6	0.7	0.4	0.5
Edoxaban	Intrinsic	30	1.1	1.0	0.9	0.9
		50	1.2	1.0	0.9	0.8
		100	1.4	0.9	0.7	0.7
	Extrinsic	30	1.5	0.7	0.4	0.6
		50	1.6	0.7	0.4	0.6
		100	2.0	0.7	0.4	0.5
Rivaroxaban	Intrinsic	30	1.1	0.9	0.9	0.9
		50	1.1	0.9	0.8	0.7
		100	1.3	0.9	0.8	0.9
	Extrinsic	30	1.7	0.7	0.5	0.5
		50	1.9	0.7	0.5	0.5
		100	2.3	0.7	0.5	0.4
Dabigatran	Intrinsic	30	1.8	1.0	0.8	1.0
		50	2.0	1.0	0.6	1.0
		100	2.5	0.8	0.4	0.8
	Extrinsic	30	2.3	1.1	1.0	1.5
		50	2.8	1.1	0.9	1.5
		100	3.8	1.0	0.9	1.5

Table 13: Ratio obtained for FXa inhibitors and dabigatran for FibWave and TGA parameters with the intrinsic (ellagic acid) and extrinsic (tissue factor) pathways at three relevant thresholds.

Table 13 (continued) **TGA (CAT method)**			**Lag time**	**Time to peak**	**Peak**	**ETP**	**mVRI**
DOACs	*Triggering pathway*	*threshold (ng/mL)*	*Ratio (s)*	*Ratio (s)*	*Ratio (nM)*	*Ratio (nM*min)*	*Ratio (nM/min)*
Apixaban	Intrinsic	30	1.1	1.0	0.7	0.9	0.7
		50	1.1	1.1	0.7	0.9	0.7
		100	1.1	1.1	0.6	0.8	0.6
	Extrinsic	30	1.3	1.3	0.5	0.9	0.4
		50	1.5	1.4	0.4	0.8	0.3
		100	1.7	1.5	0.3	0.7	0.3
Edoxaban	Intrinsic	30	1.1	1.2	0.5	0.9	0.4
		50	1.1	1.2	0.5	0.9	0.4
		100	1.3	1.3	0.4	0.7	0.3
	Extrinsic	30	1.8	1.8	0.5	0.9	0.3
		50	1.9	2.0	0.4	0.9	0.2
		100	2.1	2.3	0.4	0.7	0.1
Rivaroxaban	Intrinsic	30	1.2	1.1	0.6	0.9	0.7
		50	1.2	1.2	0.5	0.8	0.5
		100	1.3	1.2	0.4	0.7	0.4
	Extrinsic	30	1.5	1.6	0.5	0.8	0.3
		50	1.5	1.8	0.4	0.8	0.2
		100	1.9	2.3	0.3	0.7	0.1
Dabigatran	Intrinsic	30	1.4	1.3	1.0	1.1	1.0
		50	1.7	1.5	1.0	1.0	1.1
		100	1.9	1.6	0.9	0.9	1.0
	Extrinsic	30	1.5	1.2	1.1	1.0	1.3
		50	1.8	1.3	1.0	1.0	1.2
		100	2.3	1.5	1.0	0.9	1.4

Table 13 (continued): Ratio obtained for FXa inhibitors and dabigatran for FibWave and TGA parameters with the intrinsic (ellagic acid) and extrinsic (tissue factor) pathways at three relevant thresholds.

According to the literature,(Pernod, Albaladejo et al. 2013, Testa, Legnani et al. 2016) a plasma concentration of 30 or 50 ng/mL of rivaroxaban is not

sufficient to prolong APTT or PT outside the normal range (ratio > 1.2) meaning that traditional assays are not sufficiently sensitive and may provide false reassurance that the DOAC is absent. Our results showed that 30 ng/mL of FXa inhibitors gave a ratio out of the normal limits for the time to Max1 (ratio > 1.2), Max1, Max2 and Min2 (ratio < 0.8) and 30 ng/mL of dabigatran gave a ratio out of normal limits (ratio > 1.2) for the time to Max1. Similar results were observed in TGA. These first results suggest that the FibWave could be sensitive enough to identify patients with low DOAC concentration.

b. Limit of detection for FibWave and TGA parameters

The range of concentration of DOACs and limits of detection (LOD) for FibWave and TGA parameters are reported in **table 14**. Several parameters of the FibWave showed to be of interest for measuring the activity of DOACs in plasma. The TGA and the FibWave provided LOD results within the same range, showing the good sensitivity of our method to assess the effects of DOACs.

	Dosage and range of concentration ($C_{through}$ - C_{Max} in ng/mL)	**FibWave**				**TGA**				
		LOD (ng/mL)				LOD (ng/mL)				
		Time to Max1	Max1	Max2	Min2	Lag time	Time to peak	Peak	ETP	mVRI
Apixaban	2.5 mg bid (11 – 221)	72	55	23	46	16	16	6	35	21
	5 mg bid (22 – 321									
	10 mg bid (41 – 572)									
Edoxaban	30 mg od (8 – 225)	8	13	5	6	6	4	3	79	10
	60 mg od (10 – 317)									
Rivaroxaban	10 mg od (4 – 273)	22	7	3	9	n.d.	n.d.	6	52	18
	20 mg od (6 – 535)									
Dabigatran	150 mg bid (39 – 275)	11	359	117	583 ǂ	8	27	139	103	478

Table 14: Comparison of limit of detection (LOD) of FibWave and TGA parameters for DOACs with the extrinsic pathway.[2]

These first results on performances of the FibWave are encouraging. The FibWave showed to be sensitive at low concentrations with a reproducibility similar to the one observed on the TGA. The next steps have been the implementation on an automated coagulometer and the comparison of clinical performances with the APTT and PT-based clot waveform analysis in patients treated with edoxaban.

II. Impact of edoxaban on FibWave parameters and comparison with the APTT- PT-based clot waveform analysis

Edoxaban is indicated for stroke prevention in patients with non-valvular atrial fibrillation (NVAF) and for the prevention and treatment of venous

[2] n.d.: not determined. ǂ: extrapolated values out of calibration range.

thromboembolism (VTE) and pulmonary embolism (PE). The monitoring of its anticoagulant activity is generally not required but the measurement of its plasma levels can be necessary in some clinical situations such as before surgery, invasive procedures or antidote administration, during bleeding or thromboembolic events, or to make decision on thrombolytic therapy in stroke patients.(Favaloro, Pasalic et al. 2017, Douxfils, Ageno et al. 2018, Gosselin, Adcock et al. 2018, Douxfils, Adcock et al. 2021)

First investigations on anticoagulants were performed in normal pooled plasma supplemented with DOACs. This next study consisted of confirming our first in vitro experiments and comparing the clinical performances of the FibWave to the APTT- and PT-based clot waveform analysis in a cohort of patients treated with edoxaban.(Evrard, Siriez et al. 2022)

The study population consisted of 57 patients treated with edoxaban representing 71 plasma samples and 45 healthy volunteers representing 45 control samples. For some patients, plasma samples were collected at C_{TROUGH} (i.e. 12 hours after the last drug intake) and C_{MAX} (i.e. approximately 3 hours after drug intake).

The APTT and PT were performed on an ACL TOP 700 CTS (Werfen) and the clot waveform analysis was done using the integrated software. The APTT was performed using the SynthasIL® reagent (Werfen) and the PT was performed with the ReadiPlasTin® reagent (Werfen). For the PT, the software was set up by the manufacturer to provide the time to Max1 (considered as the PT clotting time by the instrument and reported in seconds) and the Max1 ($dmAbs.dt^{-1}$). For APTT, the software was set up to provide the Max1 ($dmAbs.dt^{-1}$), the Max2 ($dmAbs.dt^{-2}$), the time to Max2 (considered as the APTT clotting time by the instrument and reported in seconds) and the Min2 ($dmAbs.dt^{-2}$) (**Figure 3**).

The FibEx, FibIn methodologies of the FibWave were implemented on the same ACL TOP 700 CTS (Werfen) and the setup of the methods allowed extraction of the following parameters from the integrated software, i.e. Max1, time to Max1 (time to peak), Max2, time to Max2 (clotting time), Max2 and Min2 (**Figure 12**).

Between-group comparisons were done using a one-way ANOVA with a Tukey's multiple comparison test. The threshold for significance has been set at 0.05. Receiver operating characteristic (ROC) curves were performed at relevant thresholds for clinical decisions, i.e. 30 and 50 ng/mL for the most sensitive parameters.

The comparisons between edoxaban C_{MAX}, edoxaban C_{TROUGH} and healthy subjects for the different parameters of PT-based clot waveform analysis and FibEx are reported in the **figure 24**.

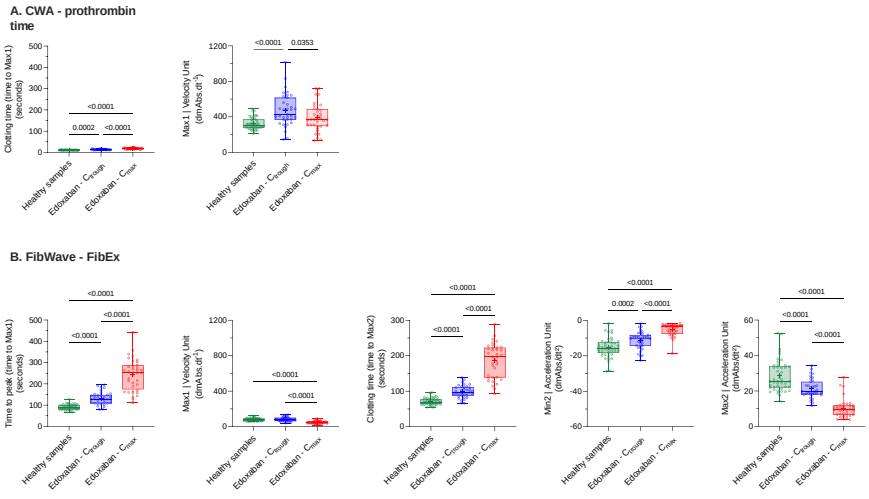


Figure 24: Population comparison of CWA – prothrombin time (panel A) with the FibEx (panel B).

Time to Max2, Max2 and Min2 were not investigated for PT-CWA because these parameters were not extracted from PT procedure.

The comparisons between edoxaban C_{MAX}, edoxaban C_{TROUGH} and healthy subjects for the different parameters of APTT-based clot waveform analysis and FibIn are reported in the **Figure 25**.

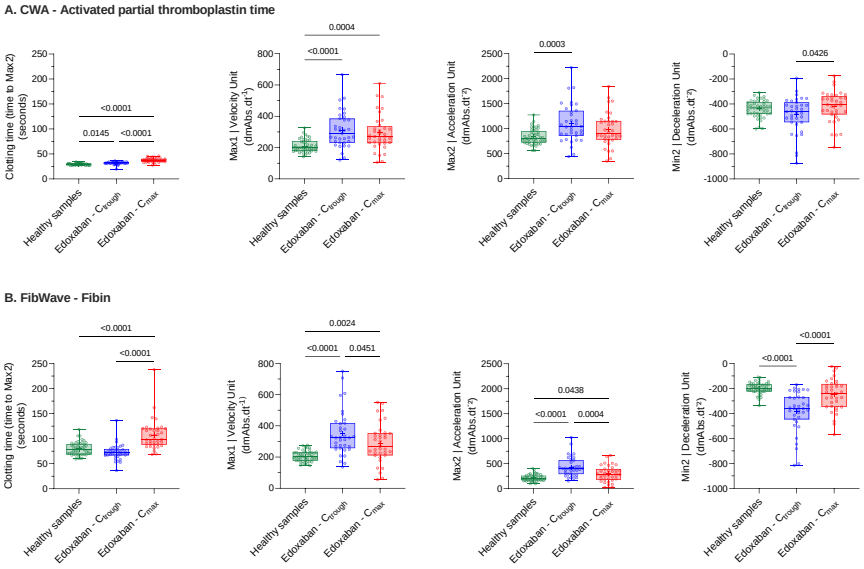


Figure 25: Population comparison of CWA – activated partial thromboplastin time (panel A) with the FibIn (panel B).

The results showed that the FibEx methodology, exploring the extrinsic pathway of the coagulation, is more sensitive than the APTT- and PT-based clot waveform analysis for assessing the anticoagulation status of edoxaban treated patients. All temporal parameters, reflected by the time to Max1 (time-to-peak) and the time to Max2 (clotting time) as well as velocity (Max1), acceleration (Max2) and deceleration (Min2) were better discriminated with the FibEx than the APTT- and PT-based clot waveform analysis. With the FibEx, edoxaban prolonged the time to Max1 and the time to Max2 while the velocity, the acceleration and the deceleration of fibrin

formation were dose-dependently reduced, confirming our in vitro experiments (**Figures 24 & 25**).

In the edoxaban groups, the correlations, Pearson's chi-squared test, between the different parameters and the plasma concentrations were performed. Significant correlations between the APTT or the PT clotting time and the edoxaban concentration were observed (r Pearson = 0.65 and 0.80) (**Figure 26**). Significant correlations between the FibEx clotting time (r = 0.89), time to peak (r = 0.81), Max1 (r = -0.64), Max2 (r = -0.79), Min2 (r = 0.62) and the edoxaban concentration were observed. Correlations between the FibIn clotting time (r = 0.60), time to peak (r = 0.60), Max2 (r = -0.41), Min2 (r = 0.39) and the edoxaban concentrations were also significant. There were no significant correlation between the aPTT, PT and FibIn Max1 parameter and the edoxaban concentration (p-value = 0.96, 0.20 and 0.08, respectively) (**Figure 27**).

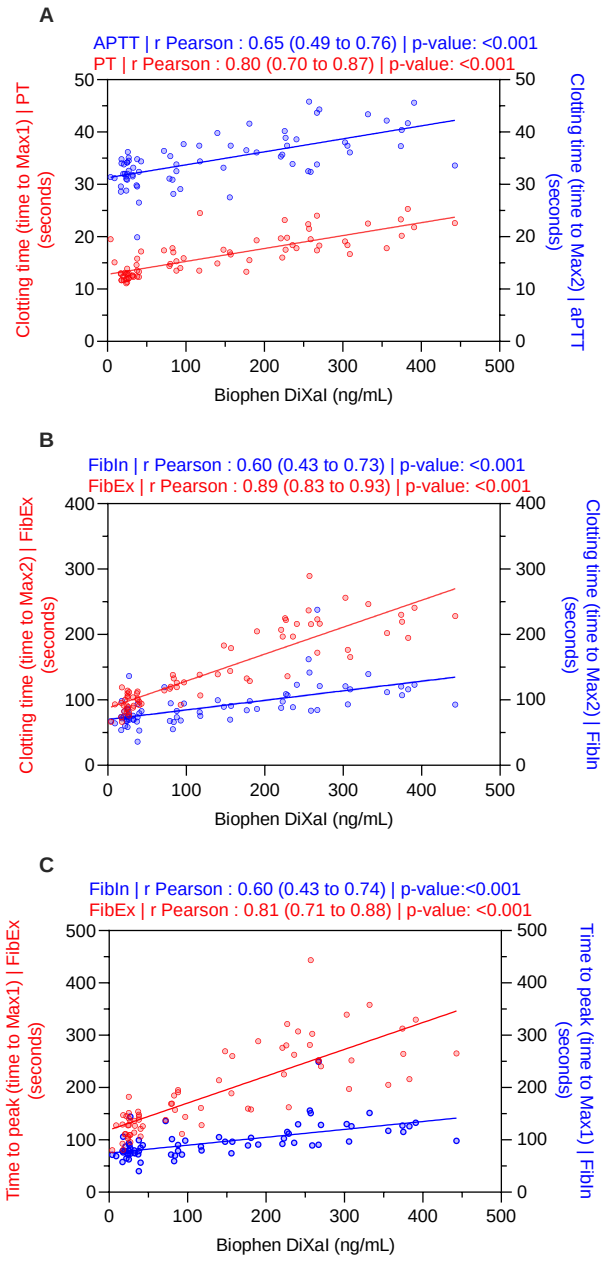


Figure 26: Correlation between Clot Waveform Analysis and FibWave parameters with plasma edoxaban concentrations.

Panel A represents the "clotting time" as reported by the PT and APTT methodologies on the ACL-TOP analyzer which correspond to the time to Max1 for the PT and the time to Max2 for the aPTT. Panel B represents the time to Max2 for the FibEx and the FibIn. Panel C represents the time to Max1 for the FibEx and the FibIn.

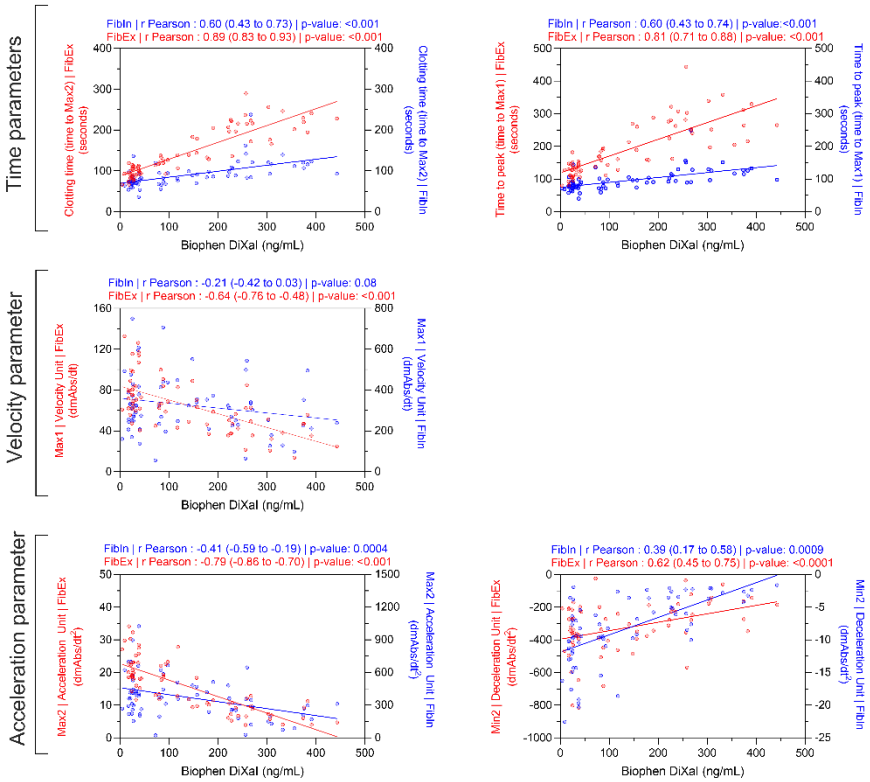


Figure 27: Correlation of the different FibWave parameters with the plasma concentration of edoxaban.

When we compared the performances between PT-based clot waveform analysis and the FibEx, the time to Max1 showed similar correlations for both PT and FibEx (r = 0.80 [95%CI, 70% to 87%] and 0.81 [95%CI, 71% to 88%]). Interestingly, the time to Max2 (FibEx) reported the best correlation with edoxaban concentration (r = 0.89 [95%CI, 83% to 93%]). These results suggest that the FibEx – time to Max2, i.e. clotting time, is able to reliably reflect the intensity of edoxaban without the need for expensive techniques like chromogenic assays specifically calibrated against the drug of interest (**Figure 26**).

Other parameters like the FibEx Max2 (r = -0.79 [95%CI, 70% to 86%]) and the FibEx Min2 (r = 0.62 [05%CI, 45% to 75%]) were also relevant as they were sensitive towards the anticoagulation intensity, but only investigated for FibEx because these parameters were not extracted from PT procedure (**Figure 27**). The Max1 was the less relevant parameter for all methodologies.

Although the FibEx time to Max2 (clotting time) showed good performance for discriminating samples with plasma concentration above 30 ng/mL and 50 ng/mL, the FibEx time to Max1 demonstrated best performance for discriminating samples with significant cut-offs. The Youden indexes revealed that the FibEx performs better than the PT-based clot waveform analysis at these low concentrations (**Figure 28, Table 15**). These preliminary data are already very promising, but the cut-offs need to be challenged in a larger cohort of patients treated with edoxaban and should also be investigated with the other DOACs.

Cut-off = 30 ng/mL	ROC AUC (95%CI)	Youden index cut-off (95%CI)	Sensitivity (95%CI)	Specificity (95%CI)
CWA PT – Clotting time (time to Max1, seconds)	0.967 (0.917 - 0.991)	13.1 (13.0 – 15.1)	0.902 (0.790 - 0.957)	0.954 (0.873 - 0.987)
CWA PT – Max1 ($dmAbs.dt^{-1}$)	0.610 (0.515 - 0.699)	331.9 (164.0 – 408.2)	0.686 (0.550 - 0.797)	0.554 (0.433 - 0.668)
FibEx – Clotting time (time to Max2, seconds)	0.972 (0.923 - 0.994)	96.5 (86.4 – 119.3)	0.902 (0.790 - 0.957)	0.892 (0.794 - 0.947)
FibEx – Time to Peak (Time to Max1, seconds)	0.965 (0.913 - 0.990)	110.8 (105.3 – 136.2)	0.980 (0.897 - 0.999)	0.815 (0.705 - 0.891)
FibEx – Max1 ($dmAbs.dt^{-1}$)	0.763 (0.675 - 0.837)	63.2 (56.3 – 70.6)	0.667 (0.530 - 0.780)	0.815 (0.705 - 0.891)
FibEx – Max2 ($dmAbs.dt^{-2}$)	0.895 (0.824 - 0.944)	18.4 (16.0 – 19.4)	0.780 (0.648 - 0.873)	0.938 (0.852 - 0.976)
FibEx – Min2 ($dmAbs.dt^{-2}$)	0.822 (0.739 - 0.884)	-10.2 (-9.6 – -11.7)	0.840 (0.715 - 0.917)	0.785 (0.670 - 0.867)
Cut-off = 50 ng/mL	**ROC AUC (95%CI)**	**Youden index cut-off (95%CI)**	**Sensitivity (95%CI)**	**Specificity (95%CI)**
CWA PT – Clotting time (time to Max1, seconds)	0.978 (0.931 – 0.996)	13.1 (12.8 – 13.9)	1.000 (0.914 - 1.000)	0.893 (0.803 - 0.945)
CWA PT – Max1 ($dmAbs.dt^{-1}$)	0.565 (0.469 - 0.656)	331.9 (204.8 – 466.6)	0.659 (0.506 - 0.784)	0.507 (0.396 - 0.617)
FibEx – Clotting time (time to Max2, seconds)	0.992 (0.953 - 1.000)	114.2 (111.8 – 119.3)	0.951 (0.839 - 0.991)	0.987 (0.928 - 0.999)
FibEx – Time to Peak (Time to Max1, seconds)	0.982 (0.938 – 0.998)	154.1 (151.6 – 182.2)	0.902 (0.775 - 0.961)	0.987 (0.928 - 0.999)
FibEx – Max1 ($dmAbs.dt^{-1}$)	0.837 (0.757 - 0.899)	58.7 (49.4 – 68.7)	0.659 (0.506 - 0.784)	0.907 (0.820 - 0.954)
FibEx – Max2 ($dmAbs.dt^{-2}$)	0.935 (0.873 - 0.973)	13.4 (12.9 – 19.4)	0.800 (0.652 - 0.895)	0.987 (0.928 - 0.999)
FibEx – Min2 ($dmAbs.dt^{-2}$)	0.872 (0.797 - 0.927)	-10.2 (-9.6 – -12.0)	0.925 (0.801 - 0.974)	0.747 (0.638 - 0.831)

Table 15 Receiver operating characteristic curves analysis at relevant clinical decision thresholds.

ROC curves were performed at the cut-offs of 30 and 50 ng/mL. The area under the curve and its 95% confidence interval have been calculated. The Youden index has been used to determine the best cut-off for the corresponding parameter. Based on this cut-off, the sensitivity and the specificity have then been calculated.

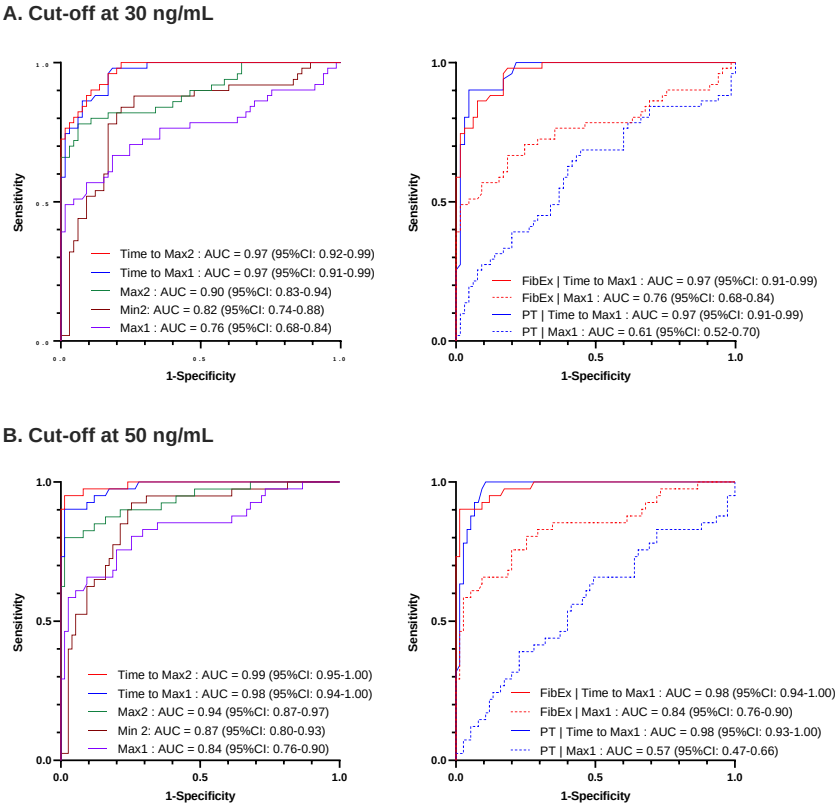


Figure 28: ROC curve of the Clot Waveform prothrombin time and FibEx at the cut-off of 30 and 50 ng/mL.

III. Intended clinical use of FibWave in anticoagulant situations.

The FibWave and the clot waveform analysis are similar to thrombin generation assay, except that the endpoint is not the generation of thrombin but rather the formation of fibrin. For several years, it suggested that assessing the ponderal concentration is not the best approach to assess the intensity of anticoagulation. Relevant concentrations of different DOACs (i.e. 30, 50, and 100 ng/mL) have been performed in TG to assess if a same concentration between different DOACs leaded to the same degree of anticoagulation. It was observed that the TG profiles, i.e. curves, significantly

differed between different DOACs.(Evrard, Hardy et al. 2021) The same experiment was performed to assess if differences, reported with the TGA, could be observed with the FibWave. Results appear to correlate with the TGA where dabigatran presented a thrombin and fibrin formation curves significantly different from FXa inhibitors (**Figure 29**).

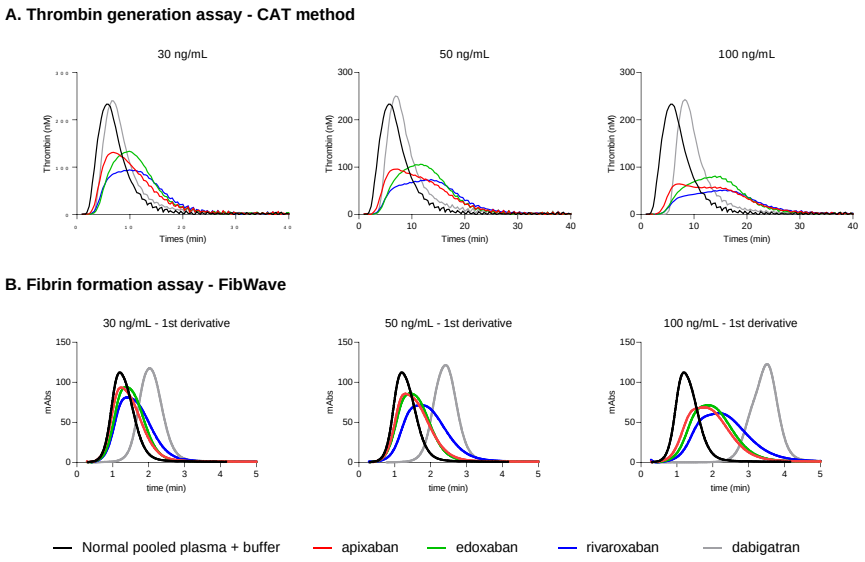


Figure 29: Impact of FXa inhibitors and dabigatran at three relevant concentrations on the thrombin generation assay (CAT method) (Panel A) and FibWave (1st derivative) (Panel B).

These in-vitro results are supported by Metze *et al.* suggesting that global coagulation assays like thrombin generation, viscoelastometric assays or the FibWave could be interesting if they demonstrate sufficient performances at relevant clinical decision-making thresholds. The sensitivity of the time to Max2 and the time to Max1 to predict edoxaban levels < 30 ng/mL were at least equal to the one of thrombin generation parameters. Indeed, according to a study of Pfrepper et al. thrombin generation parameters showed a sensitivity of 90.5% which was similar to the sensitivity of 98% (95%CI, 90% to 100%) of the FibEx – time to Max1. The sensitivity of the FibEx to predict

edoxaban levels < 50 ng/mL was also at least equivalent to the performance of thrombin generation since the sensitivity of the FibEx – time to Max2 at this threshold was above 95% (95%CI, 84% to 99%) compared to a sensitivity ranging from 82.6% to 87.0% of thrombin generation. According to our results, the specificity of FibEx time to Max2, time to Max1 and Max2, for discriminating edoxaban samples from healthy samples, were comparable to PT-clotting time and TG parameters.(Pfrepper, Metze et al. 2020, Metze, Klöter et al. 2021)

In conclusion, the FibWave and especially the FibEx methodology, showed to have a shorter turn-around time, be as sensitive at relevant threshold concentrations as TGA, and be more sensitive and specific to the presence of edoxaban compared to the APTT and PT-based clot waveform analysis. The relevant performance of the FibEx time to Max1 (i.e. time to peak) to discriminate samples at very low plasma concentrations, i.e. 30 and 50 ng/mL, could make this test useful for clinical decision-making.

4. Procoagulant state

Activated protein C (APC) resistance is a well-known risk factor of venous thromboembolism, mainly caused by a factor V genetic mutation, known as the factor V Leiden (FVL) mutation.(Dahlback, Carlsson et al. 1993, Koster, Vandenbroucke et al. 1993, Tans, Van Hylckama Vlieg et al. 2003) However, APC resistance may also occur in the absence of FVL mutation and is called acquired APC resistance.(Tosetto, Rodeghiero et al. 1998) The acquired APC resistance can be due to hormonal effects, acute phase reactions, lupus anticoagulants or other causes. The phenomenon of hormonal APC resistance is mostly observed in women and is related to the hormonal changes they are facing during their life which are the use of combined hormonal contraceptives (CHC) during their fertile period, the pregnancy and postpartum periods and the use of hormone replacement therapy (HRT) during menopause.(Rosing, Tans et al. 1997, Saenz, Johnson et al. 2011, Moore, Van Cott et al. 2019, Douxfils, Morimont et al. 2020)

Different methods for the detection of APC resistance have been developed over years: assays based on APTT, PT, FXa clotting time, Russel Viper venom time, textarin time in presence of APC, or based on TG quantifying the effect of APC on ETP.(Dahlback, Carlsson et al. 1993, Hoagland, Triplett et al. 1996, Nicolaes, Thomassen et al. 1997, Curvers, Thomassen et al. 1999, Visser, Hylckama Vlieg et al. 2005) The methods recommended by the European Medicines Agency (EMA) guidance for the assessment of steroid contraceptives are the APTT-based APC resistance and the ETP-based APC resistance assay. The TGA is more sensitive than APTT for detecting acquired APC resistance and identifying FVL making this test a suitable marker of the prothrombotic tendency of women using combined contraception.(Hoagland, Triplett et al. 1996, Nicolaes, Thomassen et al. 1997, Curvers, Thomassen et al. 1999, Visser, Hylckama Vlieg et al. 2005)

The proof of concept of FibWave has already been demonstrated in anticoagulant effects of DOACs but the performance of the FibWave has not been tested to assess a prothrombotic tendency, as observed with combined hormonal contraception. Thus, we investigated the impact of hormonal contraception on the FibWave parameters and compared the performances of FibWave with the TG, APTT- and PT-based clot waveform analysis assays.

The study consisted of investigating different subgroups of population including men (n=23), women not using hormonal therapy (no CHC) (n=22), women using second-generation combined hormonal contraception (2G CHC) (n=12), women using third-generation CHC (3G CHC) (n=23), as well as subjects carrying heterozygous FVL (n=5) and carrying prothrombin G20210A (PT G20210A) (n=5) mutations.

The FibEx, FibIn (**Figure 12**) and FibAPC methodologies (**Figure 13**), the ETP-based APC resistance(Douxfils, Morimont et al. 2020) and the APTT- and PT-based clot waveform analysis (**Figure 3**) were performed on different cohorts.

A Kruskal-Wallis test and a Dunn's multiple comparison have been used in order to assess the statistical differences between groups. The threshold for significance has been set at 0.05.

I. Impact of hormonal contraception on FibWave parameters

For the FibIn, except for the velocity (Max1), no significant differences were observed in parameters between cohorts. The main differences were observed with the FibEx methodology. An increase of the Delta was observed in women using hormonal contraceptive compared to men and women not using hormonal contraception (**Figure 30**). According to studies with the clot

waveform analysis on other systems (e.g. the MDA, the Destiny Max, the ACL TOP, and Siemens analyzers), the height of delta is related to fibrinogen level and several studies reported that hormonal contraception increased the level of plasma fibrinogen.(Bonnar 1987, Olivieri, Friso et al. 1995) According to our data, the fibrinogen level is positively correlated with the Delta, and reveals that the FibWave is able, to a certain degree, to inform on the level of fibrinogen in a particular patient.

The temporal parameters were reduced in women using hormonal contraceptive. The velocity, acceleration and deceleration were higher in women using hormonal contraceptive than in men and women not using hormonal therapy. The estrogenicity of hormonal contraceptive is known to affect hemostasis by increasing plasma concentrations of clotting factors II, VII, VIII, X, XII, fibrinogen while it also reduces the levels of natural anticoagulants like protein S, antithrombin or tissue factor pathway inhibitor. The combination of all these perturbations observed during contraceptive therapy may be responsible for the increased coagulation velocity, acceleration and deceleration observed with the FibWave. The overall effect on the coagulation system is a shift in favor of clot formation.(Middeldorp, Meijers et al. 2000, Van Cott, Soderberg et al. 2002) Therefore, a hypercoagulable state may be characterized by reduced clotting time and increased Max1 and Max2 and decreased Min2 (**Figure 30**).

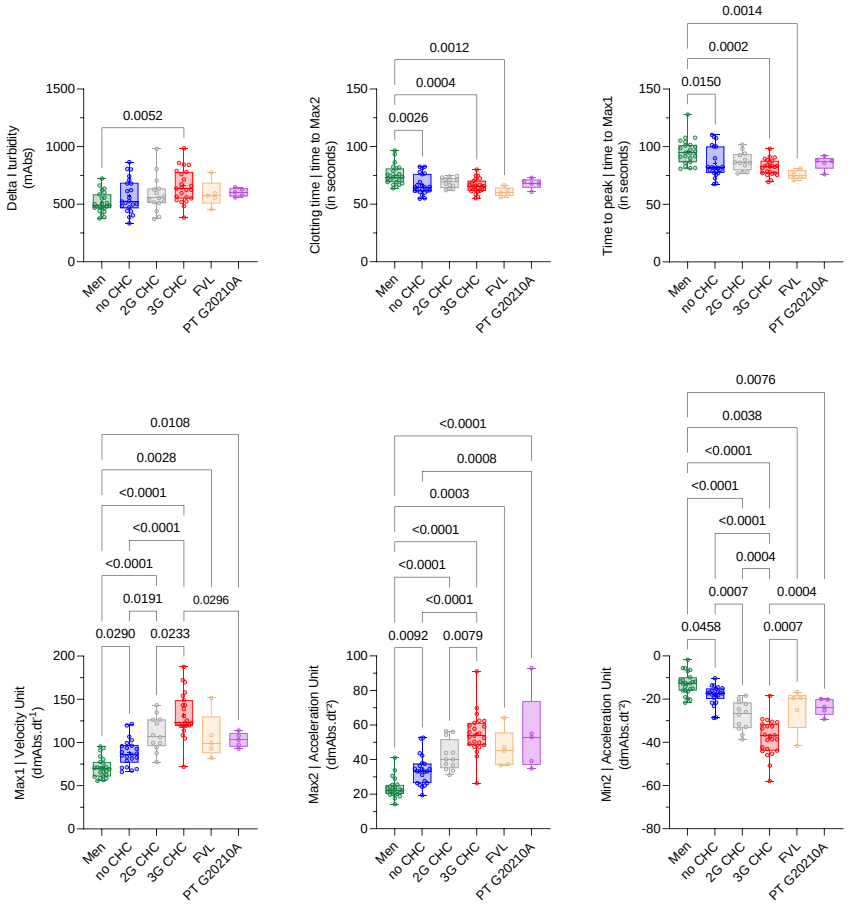


Figure 30: Summary of FibEx parameters in absence of protein C activator. Only significant differences are reported.

Only significant differences are reported.

II. Impact of hormonal contraception on APTT- and PT-based clot waveform analysis and comparison with the FibWave

In APTT- and PT-based CWA, no differences were observed in clotting time, i.e. time to Max2 for APTT and time to Max1 for PT. The additional parameters appeared more sensitive where velocity, acceleration and

deceleration were higher in women using hormonal contraceptive (3G CHC) than men (**Figure 31**).

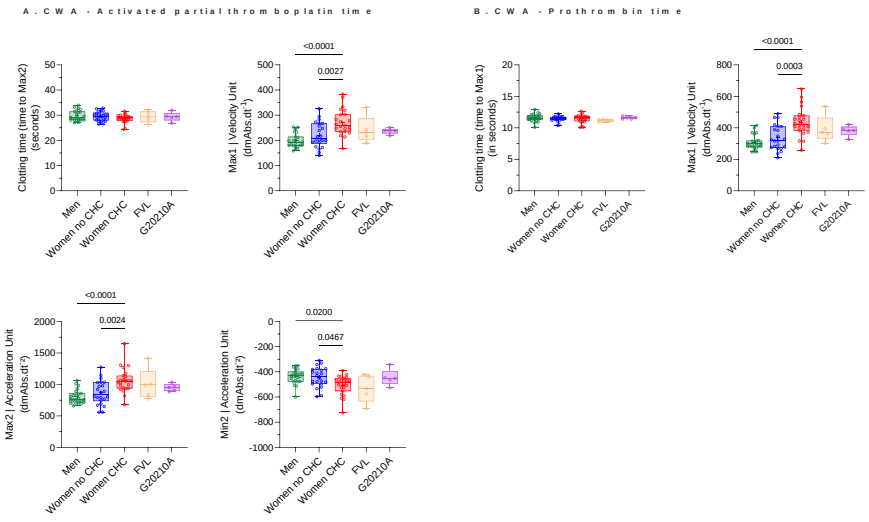


Figure 31: Population comparison of activated partial thromboplastin time (panel A) with the prothrombin time (panel B).
Only significant differences are reported.

The FibIn is not supportive methodology because none of its parameters allowed to differentiate cohorts while the FibEx, as well as the APTT- and PT-based CWA, showed to distinguish different subgroups of population relative to their hypercoagulable state. The FibEx better discriminated different populations than the CWA (**Figure 32**).

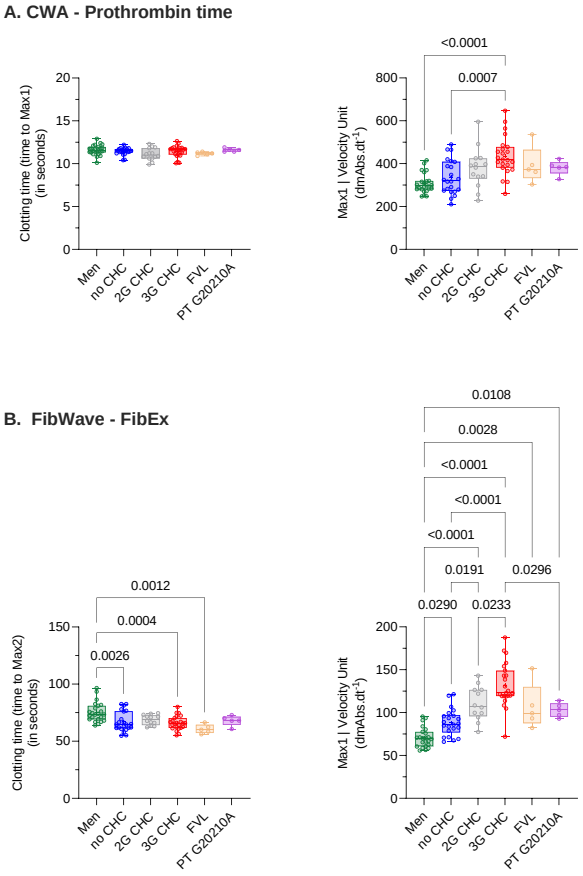


Figure 32:Population comparison of prothrombin time (panel A) with the FibEx (panel B). *Only significant differences are reported.*

It has been suggested that APTT reagents, including high PLs content and contact activator, cannot sufficiently reflect the activation of FIX by the extrinsic tenase complex.(Wada, Shiraki et al. 2020, Hasegawa, Tone et al. 2021) Regarding PT reagents, they can suffer from the same disadvantage because of the large amount of TF and phospholipids. Thus, although widely implemented in laboratories, current routine coagulation tests have several limitations that are due to the poor level of information extracted from complex interactions between several effectors of the coagulation process

and their inhibitors. The FibEx, with a low tissue factor concentration (final TF concentration in test: ± 0.8 µg/mL), seem to be more suitable for assessing the physiological coagulation and hemostasis changes.

III. Impact of hormonal contraception on thrombin generation assay and comparison with the FibWave

The thrombin generation assay, and particularly the ETP-based APC resistance, is known to be a useful marker of the prothrombotic tendency of women on contraception and sensitive to the hypercoagulable state induced by hormonal therapies. The ETP-based APC resistance was performed according to the standardized and validated procedure.(Douxfils, Morimont et al. 2020, Morimont, Bouvy et al. 2020) The impact of APC on ETP and Protac on Max1 is expressed in percentage of inhibition and a low percentage of inhibition (ETP or Max1) corresponds to a high APC resistance.

In absence of APC, we observed that hormonal contraception as well as FVL or PT G20210A mutations increased the ETP, the peak, and the mVRI, and reduced the Ttpeak and a lesser degree, the lag time (**Figure 33**).

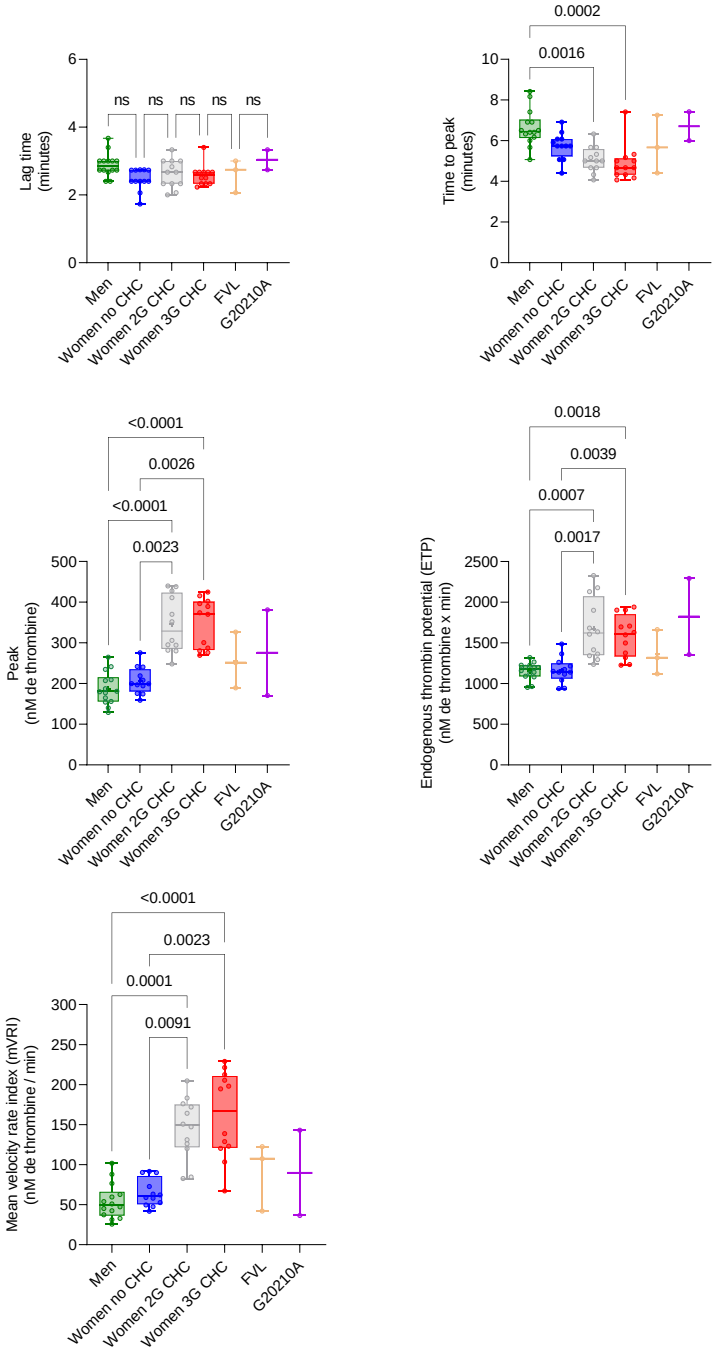


Figure 33: Impact of hormonal contraception on thrombin generation parameters in presence of tissue factor (±5 pM) without APC.

In presence of APC, men showed higher inhibition percentage compared to women not using hormonal therapy ($p < .05$) confirming earlier studies in which women not using hormonal contraception appear slightly more resistant to APC than men.(Koster, Vandenbroucke et al. 1993, Hoagland, Triplett et al. 1996, Rosing, Tans et al. 1997) A lower percentage of inhibition was observed in women using third-generation CHC compared to women using second-generation CHC ($p < .05$) (**Figure 34**). These results are correlated by the fact that the third-generation contraception have a more pronounced effect on the anticoagulant protein C system than second-generation contraception, particularly due to the decrease in protein S level.(Middeldorp, Meijers et al. 2000, Tans, Van Hylckama Vlieg et al. 2003, Douxfils, Morimont et al. 2020, Morimont, Bouvy et al. 2020)

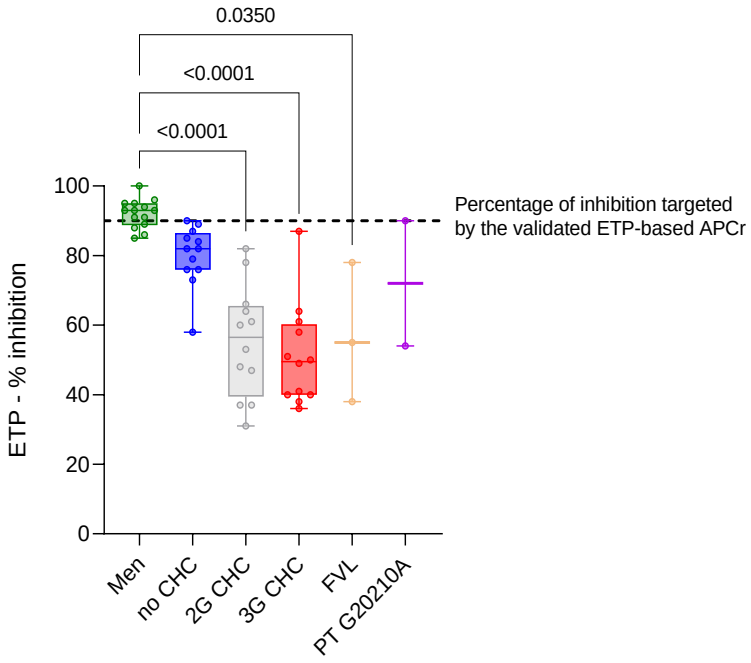


Figure 34: ETP-based APC resistance in healthy people.
Only significant differences are reported ($p < .05$).

The assessment of the ETP-based APC resistance, validated according to regulatory standards, allows comparing different cohorts of healthy subjects. The balance between procoagulant and anticoagulant factors is important for thrombin generation and contraception induced changes on coagulation that are multiple and complex.(Tans, Van Hylckama Vlieg et al. 2003, Zia, Callaghan et al. 2015, Bloemen, Huskens et al. 2017)

The assessment of the APC resistance according FibAPC methodology showed similar results to the ETP-based APC resistance, and was able to discriminate different subgroups of population relative to their hypercoagulable state. With the FibAPC, we were able to not only distinguish men and women not on hormonal therapy from women on hormonal contraception and subjects with coagulation abnormalities (FVL or prothrombin G20210A mutation) but also distinguish men from women not on hormonal therapy ($p < .05$) (**Figure 35**). As observed in the ETP-based APC resistance, men had the highest inhibition percentage of Max1 compared to other groups ($p < .05$), also confirming that women not using hormonal contraception appear slightly more resistant to APC than men. Interestingly, the percentage of inhibition of Max1 for FVL mutation was lower than women with hormonal contraception ($p > .05$), suggesting the ability of FibAPC to detect a FVL mutation and supporting that FVL mutation is more at risk of venous thromboembolism than women on hormonal therapies.(Eppenberger, Nilius et al. 2022)

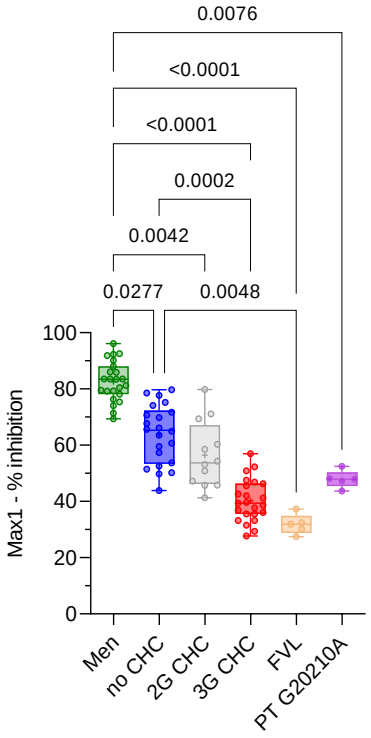


Figure 35: Comparison of inhibition percentage for Max1 in healthy subjects (FibAPC).
Only significant differences are reported (p<.05).

In conclusion, the FibEx proved to be more sensitive and to have better potential to reflect coagulation activity than the APTT- and PT-based clot waveform analysis. The FibAPC proved to be more discriminative than the ETP-based APC resistance in distinguishing different levels of APC resistance. The FibAPC Max1 has shown encouraging results, suggesting its utility as a screening marker for the assessment of thrombogenicity.

5. Fibrinolysis

Besides coagulation assays, methods available for investigating fibrinolysis have lagged. Under normal circumstances, fibrinolysis in normal blood is slow to develop after clotting, which is a major obstacle in assessing the global fibrinolysis activity. Fibrinolysis techniques are often technically more difficult, time consuming, expensive and not fully automated. Therefore, the development of easy-to-use techniques for evaluating and quantifying the overall fibrinolysis is needed. The quantity of fibrinolytic protein can be measured by antigen-based assays, and both quantity and quality can be assessed using functional assays.(Chapin and Hajjar 2015, Kuiper, Kleinegris et al. 2016, Longstaff 2018, Nogami, Matsumoto et al. 2019, Zheng, Mukhametova et al. 2023)

The main goals of fibrinolysis study are to improve understanding of the hemostasis, the diagnosis of fibrinolytic state or the management of therapeutic interventions. Although deficiencies of fibrinolysis inhibitors are not common, they can occur and lead to life-threatening blood loss. Accelerated fibrinolysis has been observed in major trauma or after surgery, especially involving extracorporeal surgery, lung or liver.(Hayakawa 2017, Saes, Schols et al. 2018) Increased fibrinolysis was also seen in cirrhosis, renal failure, menorrhagia and some malignancies.(Ferguson, Helmy et al. 2008, Longstaff 2018) The research and development of a robust and sensitive laboratory assay, taking in wider interactions with hemostasis proteins, is of interest for better diagnostic and therapeutic methods.

At this time, viscoelastic methods as thromboelastography/rotational thromboelastometry (TEG/ROTEM), fluorogenic method as thrombin and plasmin generation assay or photometric methods as clot-fibrinolysis waveform (CFWA) are available for investigating the fibrinolysis.(Kuiper,

Kleinegris et al. 2016, Longstaff 2018, Nogami, Matsumoto et al. 2019) The benefit of the viscoelastic methods is the use of whole blood which permits the contribution of cells and platelets and its use as a point of care.(Nilsson, Tynngard et al. 2013, Lance 2015, Moore, Moore et al. 2015, Gorlinger, Bhardwaj et al. 2016)

In order to investigate the fibrinolysis, the FibWave has been adapted to simultaneously measure the coagulation and the fibrinolysis processes. The impact of edoxaban and hormone-induced changes were assessed on this adapted version of the FibWave, i.e. the FibLysis (**Figure 12**).(Evrard, Maloteau et al. 2021, Evrard, Siriez et al. 2022)

I. Impact of Edoxaban on clot fibrinolysis

Before evaluating the fibrinolytic process, the impact of tPA was assessed on parameters of the clot formation in patients treated with edoxaban. The addition of tPA showed a tendency to enhance anticoagulant effect on clot formation, observed by a decrease in Delta ($p < .05$) and Max1 ($p > .05$) (**Figure 36**). It was assumed that tPA, even at an optimal concentration, can enhance edoxaban effect on coagulation.

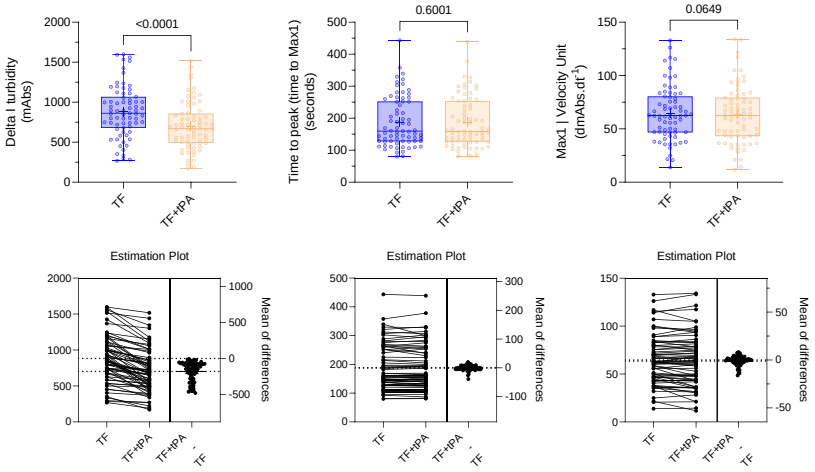


Figure 36: Impact of the addition of tPA to patients treated with edoxaban on clot formation parameters (Delta, time to Max1 and Max1).

A paired t test was used to compare the TF and TF + tPA conditions. P<.05 indicates a significant difference between conditions.

The fibrinolytic process was impaired in a dose-dependent manner by edoxaban. The analysis of fibrinolysis activity showed that patients treated with edoxaban at either C_{TROUGH} or at C_{MAX} had a prolonged time to lyse and a higher fibrinolysis velocity than the healthy population ($p < .05$) (**Figure 37**). It suggests that edoxaban has either an indirect impact on fibrinolytic proteins or that the structure of the clot differs in the presence of edoxaban.(Königsbrügge, Weigel et al. 2018)

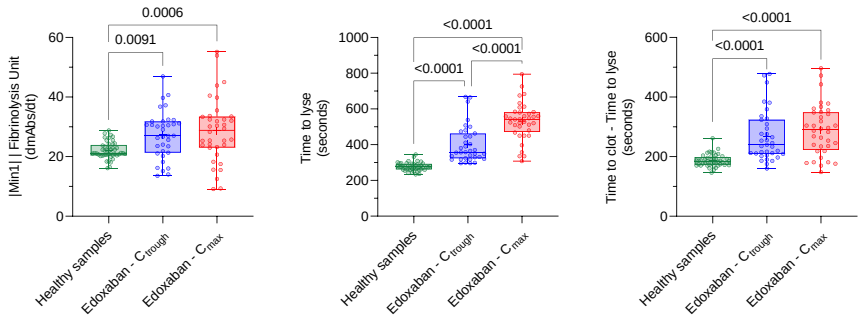


Figure 37: FibLysis parameters in healthy subjects and in samples from patients on edoxaban.

Stratifications have been made between healthy subjects, edoxaban CMAX, and edoxaban CTROUGH. Between-group comparisons were done using a one- way ANOVA with Tukey's multiple. P<.05 indicates a significant difference between groups. CMAX, maximum concentration; CTROUGH, minimum concentration.

Previous results demonstrated that FXa inhibitors and dabigatran promoted the fibrinolysis by increasing the plasmin generation or reducing thrombin activatable fibrinolysis inhibitor (TAFI) activation, respectively.(Ammollo, Semeraro et al. 2010, Semeraro, Incampo et al. 2016) It has been reported that rivaroxaban and apixaban are able to enhance fibrinolysis due to accumulation of FXaβ which catalyzes the activity of tissue plasminogen activator on plasminogen. The consequent accumulation of FXaβ in the plasma of patients treated with edoxaban could result in persistent FXa-derived fibrinolytic activity (**Figure 38**).(Carter, Talbot et al. 2018, Königsbrügge, Weigel et al. 2018, Nogami, Matsumoto et al. 2019)

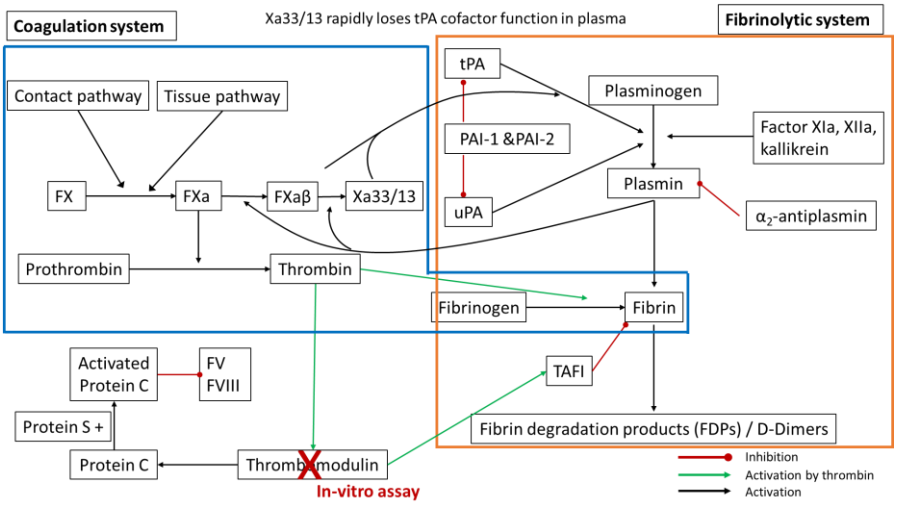


Figure 38: Impact of FXa inhibitors on coagulation and fibrinolysis systems.

Since, our test does not include thrombomodulin, the fibrinolytic activity cannot be explained by a reduction in the activation of TAFI.(Varin, Mirshahi et al. 2013) In addition, the thinner fibrin fibers and larger pores in clots can also be responsible for the accelerated fibrinolysis in edoxaban samples.

The prolonged time to lyse could be explained by the impact of edoxaban on the time to Max1 in the coagulation phase, which was also prolonged, compared to healthy subjects. This therefore prolongs the temporal parameters of the fibrinolysis.

II. Hormone-induced changes on fibrinolysis

Before evaluating the hormone-induced changes on fibrinolytic process, the impact of tPA was assessed on parameters of the clot formation. The addition of tPA to different cohorts showed a reduction in the Delta and the time to Max1, and an increase of Max1 in clot formation ($p < .05$). The differences

observed between TF and TF + tPA conditions were -19.7 ± 18.8 mAbs, -0.94 ± 2.3 second and 2.4 ± 2.9 dmAbs.dt^{-1}, respectively (**Figure 39**).

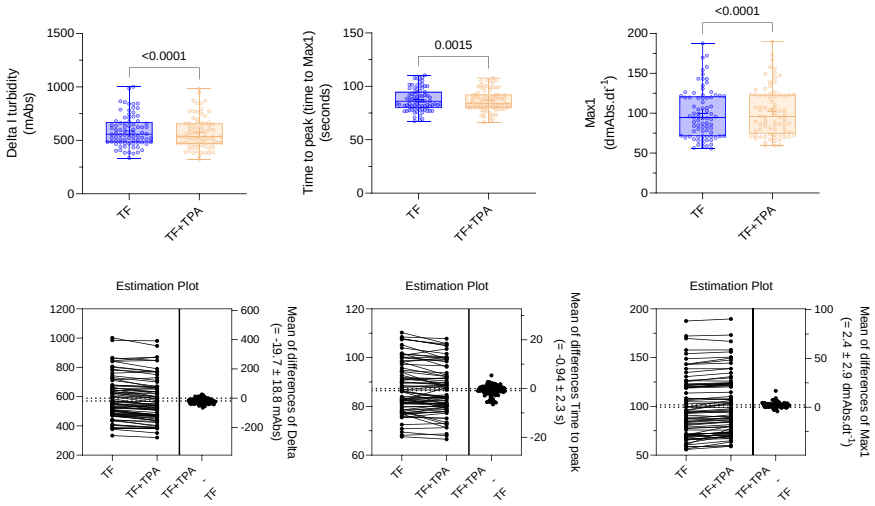


Figure 39: Effect of the addition of tPA to healthy subjects on clot formation parameters (Delta, time to Max1 and Max1).

A paired t test was used to compare the TF and TF + tPA conditions. P<.05 indicates a significant difference between conditions.

The FibLysis showed that fibrinolytic activity was different between subjects. The fibrinolysis velocity (|Min1|) ($p > .05$) in women with and without hormonal contraception was higher while the time to lysis (TFib) and the difference between time to lysis and time to clot (TFib-Ttpeak) were prolonged ($p < .05$) compared to men (**Figure 40**).

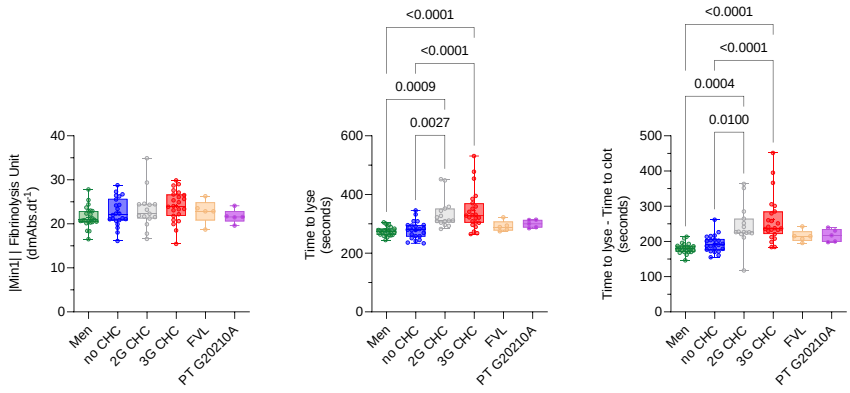


Figure 40: Comparison of FibLysis parameters in population.
Between-group comparisons were done using a one- way ANOVA with Tukey's multiple. P<.05 indicates a significant difference between groups.

The prolonged lysis time and the increased of fibrinolysis velocity could be explained by the use of hormonal contraception. Studies reported that in addition to impairing the coagulation, hormonal contraception affects the fibrinolytic pathway by increasing levels of tPA, plasminogen, plasmin-α2-antiplasmin complexes, thrombin-activatable fibrinolysis inhibitor (TAFI) and reducing plasminogen activator inhibitor-1 (PAI-1) (**Figure 41**). Therefore, the hormone-induced hypercoagulable state should be counterbalanced by an increased fibrinolytic activity.(Meijers, Middeldorp et al. 2000, Krzek, Ciesla-Dul et al. 2012, Chapin and Hajjar 2015) However, in absence of thrombomodulin (TM) (e.g., in vitro assay), the cofactor for the activation of TAFI, it is suggested that the coagulation system downregulates the fibrinolysis, and according to our data, although the fibrinolysis velocity is increased, the fibrinolysis time was prolonged in women using hormonal contraception. This downregulation could also be attributed to a denser fibrin clot observed in women on contraception (Delta in **Figure 30**), which could counteract fibrinolysis, observed by a prolonged lysis time.(Mosnier,

Meijers et al. 2001, Cellai, Lami et al. 2010, Sidelmann, Kluft et al. 2017, Pirog, Piwowarczyk et al. 2019)

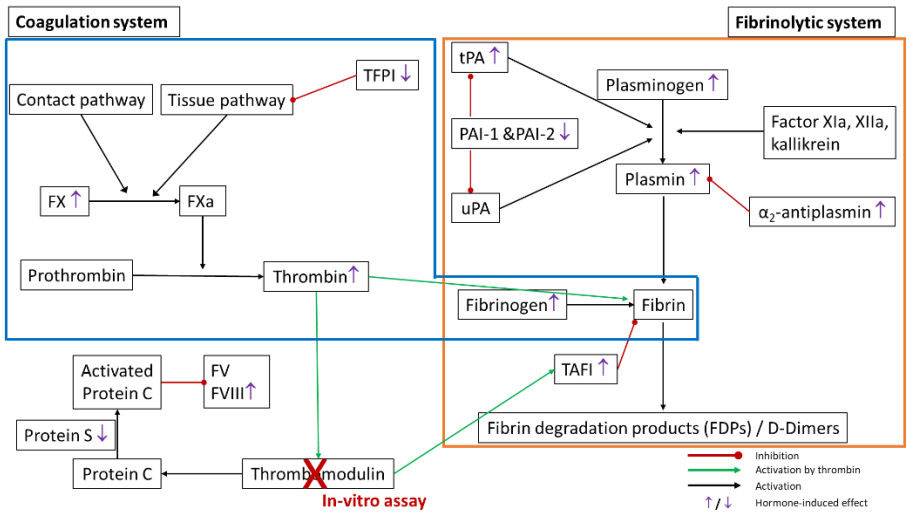


Figure 41: Impact of hormonal contraception on coagulation and fibrinolytic systems.

The FibLysis proved to reflect the fibrinolytic activity (quantity and quality) in patients treated with edoxaban and healthy people.

Thanks to its capacity to assess the clot formation and fibrinolytic processes, the FibLysis could be useful in improving the understanding of the fibrinolytic activity in clinical practice.

Perspectives: implications of new regulation on in vitro diagnostic medical devices (IVD)

The FibWave is an in vitro diagnostic medical device (IVD), similarly to TGA, viscoelastic methods and routine coagulation assays. The FibWave provided relevant results in different conditions, i.e. procoagulant and anticoagulant tendency, but it is important to consider the (new) requirements to implement and validate/standardize the FibWave as a laboratory hemostasis test.

1. Definition and impact of in vitro diagnostic medical device (IVD) on hospital/healthcare costs?

A medical device is defined as any instrument, apparatus, machine, software, material, or related device (including any diagnostic product for in vitro use) that is intended by the manufacturer to be used, alone or in combination, for human beings for the specific purpose. In vitro diagnostic medical devices (IVD) are considered as medical devices and indispensable tools/tests for patient management. The IVD are important for detection, diagnosis, prognosis and monitoring diseases and therapies. They also provide information on physiological and/or pathological conditions and about 70% of clinical decisions are made using the IVD. The term “IVD” gather all tests used on specimens derived from the human body, tissue, urine, blood samples, to detect or assess a disease, monitor progression of disease and efficacy of therapy. They include reagents, calibrators, software and instruments, and form an integral part of effective healthcare systems by providing essential information for medical decision-making.(Bogavac-Stanojevic and Jelic-Ivanovic 2017, Hoffmuller, Bruggemann et al. 2021)

Since the last decade, the number of new laboratory tests has increased, and will continue to grow which creates new challenges for physicians who must find the optimal strategy for monitoring disease.

Compared to medical aids and pharmaceuticals, which represents 5% and 15% of hospital costs, the IVD accounts for less than 5% of hospital cost. Even if costs of IVD are a small part of healthcare costs, 1% to 3%, they have a significant impact on clinical decisions.(Lippi and Mattiuzzi 2013, Rohr, Binder et al. 2016)

2. The importance of IVD market

The IVD market is segmented by product, technology, or test and by application (**Figure 42**).

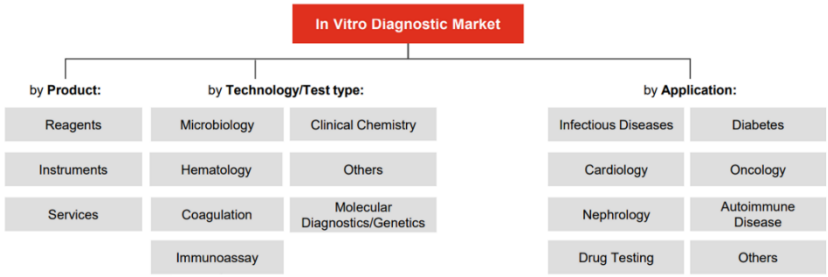


Figure 42: In vitro diagnostic medical devices segmentation.

Growth in the European IVD market was in decline for several years until 2013. Since then, the annual growth rate for the European IVD market was 2.7%, reaching 25% in 2020. This growth can be attributed to increased testing during the Covid-19 pandemic. The European IVD market is estimated at approximately 21.3 billion euros in 2020 and expected to grow at +6.5% annual growth rate in 2020-2024.(Morel, McClure et al. 2016) The development of new automated IVD for laboratories and hospitals to provide efficient, accurate and error-free diagnosis is also expected to support the market growth. It is always foreseen a coexistence between "Conformité Européenne" (CE)-IVD tests, provided by the manufacturers, and laboratory developed test (LDT) or in house-IVD (IH-IVD), developed and used by the academic sector, with complementary roles in the translational research value chain (**Figure 43**).(Lubbers, Schilhabel et al. 2021, Dombrink, Lubbers et al. 2022)

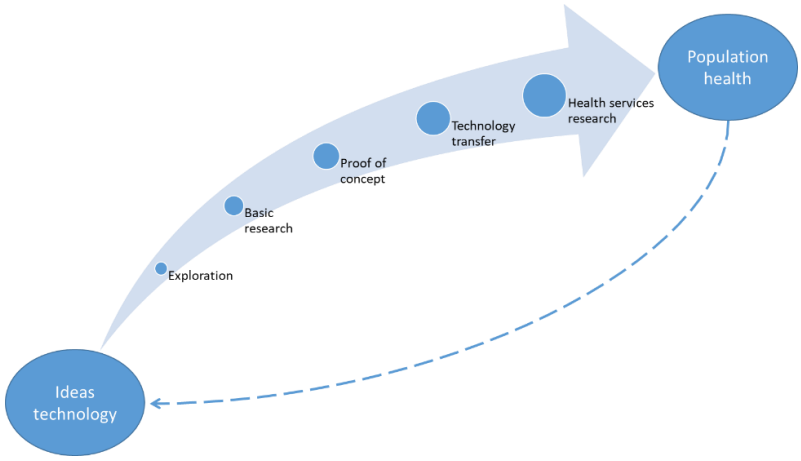


Figure 43: Translational research value chain.

Based on results reported of laboratory tests in the Netherlands and UZ Leuven, a large majority of results (>95%) were performed with CE-IVD tests. However, only 42% of laboratory tests were CE-IVD, nearly 50% were LDT, 11% were off-label CE-IVD and 0.3% were RUO. CE-IVD tests are mainly used in laboratory for routine chemistry and hematology. However, under conditions, when CE-IVD tests are not available, the LDT, often more complex, can still be used but have to demonstrate with a good certification what is the intend of use and the quality of the system.(Vermeersch, Van Aelst et al. 2021) Currently, there is no alternative for almost 70% of LDT, principally immunology tests and chemistry laboratory tests.

3. The new IVD regulation and its impact on the laboratory

It is important for in vitro diagnostic medical devices that results are reliable because the consequence of incorrect results can be life threatening. Therefore, it is essential that IVD are regulated to ensure public and users safety and to achieve the performance, in terms of analytical and diagnostic sensitivity, analytical and diagnostic specificity, accuracy, repeatability, reproducibility.

The IVD European Directive (IVDD, 98/79 EC) was introduced in 1998 and addressed the safety, quality and performance of IVD devices. The aim of the Directive was to ensure that IVDs did not compromise the health and safety of patients, users and achieve the performance levels specified by the manufacturer. Although broadly used in medical laboratories, the IVDD did not regulate the use of laboratory developed tests (LDT) and in-house devices (IH-IVD), representing nearly 50% of laboratory tests. To avoid malfunctions of IVD and guarantee the health and safety of patients and users, the European Parliament and the Council reviewed the previous regulatory, the IVDD 98/79 EC which was unchanged since 1998, to adopt the new regulation (the IVD Regulation, IVDR, EU 2017/746). With this new IVD-Regulation, which entered in application on May 26, 2022, the European Union will set harmonized requirements for IVD for the first time.

This new regulation aims to i) improve quality, safety and reliability of IVD with the new risk-based device classification system, ii) enhance transparency and information for patients and iii) enhance market vigilance and monitoring. Thanks to the introduction of the new risk-based device classification approach, approximately 80% of IVD will need to be approved by notified body, compared to only 7% under IVDD.(Bank, Jacobs et al. 2020, Vermeersch, Van Aelst et al. 2021)

The risk-based assessment approach for IVD uses an internationally accepted classification system that was created by the Global Harmonization Task Force (GHTF). The GHTF created the risk classification system with the purpose that these controls are sufficient for each class to safeguard the health and safety of patients, users and other persons. Although laboratory developed tests (LDT), in-house IVD and research use only (RUO) are not precisely defined in the IVDR, the same classification system is applied and defined into four classes (**Table 16**):

- Class A covers laboratory devices, instruments, accessories, and products for general use.
- Class B is the default ruling were no other rule applies. It represent the majority of IVD on the EU market, i.e. tests performed in clinical laboratories, in health institutions, “near-patient” testing. This class also covers some specific self-testing IVD (pregnancy, detection cholesterol, glucose, leucocytes levels…).
- Class C covers IVD where a failure of a diagnosis could be life threatening, including tests for infectious disease, cancer biomarkers, diagnostics, genetic, monitoring high risk substances, e.g. insulin dose calculation for someone suffering with diabetes.
- Class D represents the highest risk, where the failure of a test could lead to a wide spread threat to public health or an immediate life threatening condition for a patient. The rules in the new EU IVDR cover IVD used to test the safety of blood and blood derivatives, cells, tissues and organs for transfusion or transplantation.

	IVDD classification	IVDR classification			
			Individual health risk	Public health risk	
Low Risk	Others	Class A IVD	Low	Low	Self-certified
	Annex II, list A	Class B IVD	Moderate – low	Low	Notified body
	Annex II, list B	Class C IVD	High	Moderate – low	
High risk		Class D IVD	High	High	

Table 16: IVDD and IVDR classification.

As already mentioned, IVDD did not regulate the use of laboratory-developed tests (LDT) and in-house devices (IH-IVD), but the new IVDR does not use the term "LDT/IH-IVD" either. The IVDR defines what an IVD is and describes LDT/IH-IVD as in vitro diagnostic testing methods performed by using IVD (**Figure 44**).(Spitzenberger, Patel et al. 2022)

'*in vitro* diagnostic medical device' means any medical device which is a reagent, reagent product, calibrator, control material, kit, instrument, apparatus, piece of equipment, software or system, whether used alone or in combination, intended by the manufacturer to be used *in vitro* for the examination of specimens, including blood and tissue donations, derived from the human body, solely or principally for the purpose of providing information on one or more of the following:

(a) concerning a physiological or pathological process or state;

(b) concerning congenital physical or mental impairments;

(c) concerning the predisposition to a medical condition or a disease;

(d) to determine the safety and compatibility with potential recipients;

(e) to predict treatment response or reactions;

(f) to define or monitoring therapeutic measures.

Specimen receptacles shall also be deemed to be *in vitro* diagnostic medical devices;

Figure 44: Definition of in vitro medical device according IVD regulation EU 2017/746.

According to IVDR, the use of LDT/IH-IVD tests are limited to those that are used to assess specific needs of target patient group's that cannot be adequately met by an appropriate device on the market (CE-IVD). With the exception of general safety and performance requirements, the requirements of IVDR shall not apply to LDT if conditions such as no transfer to another legal entity and no manufacture on an industrial scale (…) are met (Chapter II, Article 5, Regulation 2017/746).(Hoffmuller, Bruggemann et al. 2021, Lubbers, Schilhabel et al. 2021) The LDT that are manufactured on a non-industrial scale and used by health institutions, including hospitals, laboratories and public health institutes, can be categorized into four cases (**Table 17**). An IVD become a LDT when the intended use is different from that intended by the manufacturer (reagent substituted, removed, or tests not used in accordance with the manufacturer's instructions for use…).

Scenario 1	Scenario 2	Scenario 3	Scenario 4
An in-house developed and produced test, with no certification	Tests that are labeled “research use only” (RUO) and that are used for diagnostics	A CE-IVD marketed test in which adjustments are made and change the intended use (matrix, volume, intended use, expirations dates…)	A CE-IVD marketed test in which adjustments to the protocol are made without changing the intended use
Examples			
Use of normal pooled plasma	Reptilase time ECA assay	Factor inhibitor. The measurement is out of the previous CE-marked for product	Platelet function test

Table 17: Four major scenarios for the qualification and demarcation of LDT.

4. The FibWave and the IVD Regulation

According to the third rule of classification IVD in the context of the IVDR, "devices, when not classified as Class D according to rules 1 and 2, are to be classified in Class C, irrespective of the indent applied". The rule 3 covers a range of devices and the Rule 3j applies to devices intended for monitoring an analyte with the purpose of adjusting patient management, i.e. it is intended to be used for observing, checking, or keeping a record of the level, activity, presence, absence etc. of an analyte, e.g. APTT and PT. Because of its similarity to the APTT and PT, the FibWave will be classified in Class C and this new regulation requires the approval by notified body allowing its implementation into clinical practice. However, approximatively 80% of IVD currently in use do not meet this regulation and must be certified to ensure their use in laboratory. Therefore, the FibWave is not disadvantaged by the implementation of the IVDR compared to the current assays.

The TGA and viscoelastic (TEG/ROTEM) assays are sensitive and considered as global assays, but their turn-around time (TAT) could limit the routine clinical. The APTT and PT assays are fast, but they reflect only the initial coagulation process (end-point clotting time) and are proven to be relatively insensitive to the effects of DOACs, be reagent- and method-dependent. The FibWave was developed to attempt to combine the advantages of each method without their respective drawbacks. Additionally, the FibWave appears to be easy and economically convenient to perform. At this time, the FibWave is available on one automated coagulometer (ACL-TOP) but could be implemented on other photo-optical coagulometers (**Table 18**).

	APTT and PT-CWA	Overall hemostasis potential (OHP)	Thrombin generation assay (TGA)	Thromboelastography (TEG)/ thromboelastometry (ROTEM)	FibWave
Analysis method	Turbidimetry Fibrin formation and inhibition	Turbidimetry Fibrin formation and inhibition	Fluorogenic, chromogenic thrombin generation (TG) and inhibition	Rheometry Fibrin clot formation and dissolution in whole blood	Turbidimetry Fibrin formation (and inhibition)
Wavelength	Various wavelengths depending on manufacturers	405 nm	390 nm (excitation), 460 nm (emission)	/ (viscoelastic method)	671 nm
Sample type	PPP, PRP	PPP	PPP, PRP, whole blood	Whole blood	PPP, PRP
Analysis time (Without incubation time)	1-3 minutes*	60 minutes*	40 minutes*	15 minutes for Rapid TEG 30 – 50 minutes	5 minutes* (FibEx, FibIn) 15 minutes* (FibAPC, FibLysis)
Cost (Based on analysis method)	€	€	€€	€€	€
(Potential) Clinical applications	Screening for factor deficiencies, vWD, LA Monitoring heparin, DOACs, vitamin K antagonist Screening for common factor deficiencies: DIC, liver disease	Diagnosis of hyper- and hypocoagulable states Diagnosis of hyper- and hypofibrinolytic states Monitoring treatment in anticoagulated and hemophilia patients	Determining patient's coagulation profile (detecting bleeding tendency or thrombotic risk) Monitoring reversal agents, anticoagulants	Point of care (POC) device Assessment of the patient's global hemostatic function in hepatic and cardiac surgery, trauma, burns, postpartum hemorrhage, neonates Guide transfusion strategy (decreasing use of blood products)	Assessment of patient's coagulation profile (hyper- and hypocoagulable state) Assessment of fibrinolytic process Potential clinical applications similar to the APPT- and PT-CWA

Table 18: Table 18: Comparison of methodologies presented in this thesis.

**Blood samples are centrifuged twice (2x15 minutes) for PPP. DIC : disseminated intravascular coagulation; LA : lupus anticoagulant, DOACs :direct oral anticoagulants, vWD: von Willebrand disease; PRP : platelet rich plasma; PPP : platelet poor plasma (Tschaikowsky, Balint et al. 1998, Pepperell, Morel-Kopp et al. 2014, Tripodi 2016, Binder, Depasse et al. 2021)*

5. Validation and standardization of FibWave methodologies

The clinicians have access to a variety of relevant tests to achieve a correct diagnosis for initiating or monitoring treatment. Therefore, hemostasis laboratories are equipped with an arsenal of tests, molecular-based, functional or antigenic, allowing an accurate and complete diagnosis. The new IVDR have an impact on clinical laboratories, which are mandated to use IVD that received CE-IVD certification.

The requirement to regulate the process is required at many stages of the testing process in order to provide the appropriate results. In this way, the use of quality control (internal quality control (IQC) and external quality assurance (EQA)) allows to assess variables, pre-analytical, analytical, post-analytical and interpretative error, to ensure results. The pre-analytical variables are related to collection, transport and handling samples. The analytical variables are associated to reagents (misuse, poor recovery), methodologies and instruments (systems failing). The post-analytical variables are related to the misinterpretation of results.(Favaloro 2007, Favaloro 2007, Bonar, Favaloro et al. 2010, Gosselin and Marlar 2019)

It may be assumed, as the FibWave is close to APTT and PT assays that the issues related to pre-analytical and analytical variables will be quite similar. However, the post-analytical variables will be predominant because the FibWave give qualitative and quantitative information, which are, at present, little known and will require training of laboratory and clinicians.

The next section will be aimed at the validation steps of the FibWave methodologies with reagents previously used, to ensure repeatability and reproducibility of the method over time. The validation steps, first steps necessary to implement this test in routine, will have to be performed according to the clinical and laboratory standard institute document (CLSI-H57-A). Although the validation study has not yet performed, precision

(intra- and inter-repeatability) and reference range, based on previously generated results, can be provided.

I. Reference plasma

Previous publications recommended the use of a reference plasma to reduce the inter-experiment and the inter-laboratory variability. The choice of a lyophilized plasma could be an option but these plasmas have higher thrombin generation capacity than frozen plasma due to the preparation process and additives. Moreover, a degree heterogeneity between the different commercial reference plasma has been reported, which may interfere with the benefit of their use.(Dargaud, Luddington et al. 2007, Dargaud, Wolberg et al. 2012, Perrin, Depasse et al. 2015) The latest updated ISTH guidelines recommend the use of in-house normal pooled plasma constituted from at least 40 normal donors.(Devreese, de Groot et al. 2020, Testa, Meijer et al. 2022) To meet the needs of the new IVD regulations, the choice of commercially available and certified plasma should be more appropriate. However, this point did not clarify by the IVDR, because on one hand, the mixing tests done in laboratory (hemophilia, LA) are not CE-IVD and should fill the criteria for a LDT, however, others suggest that the NPP is considered as “unassayed controls” meaning they are not covered by the IVDR.(Testa, Meijer et al. 2022)

According to the recommendations, an in-house reference plasma, composed of at least 40 normal donors, can be used as reference plasma. At department of Pharmacy, two kinds of pooled plasma are produced during blood campaign. A normal pooled plasma composed of men and women aged from 18 to 65 years. The exclusion criteria for the constitution of normal pooled plasma are history of thrombotic and/or hemorrhagic events,

treatment by antiplatelets or anticoagulants medication or other drugs potentially affecting platelets or coagulation, and carrier of FVL and prothrombin G20210A mutations. The second is a healthy pooled plasma composed only of men and women not using hormonal contraception. For this pooled plasma, the use of hormonal therapy is an additional exclusion criterion to the previous ones. The normal pooled plasma was used as a control for the FibEx, FibIn and FibLysis analysis and the healthy pooled plasma was used as a control for the FibAPC methodology. The **figure 45** shows the fibrin formation curves from healthy and normal plasma, suggesting that they could be used as reference plasma. Mostly, our in-house normal pooled plasma is constituted at least 45 normal donors, which corresponds to the ISTH guidelines, while the healthy pooled plasma is constituted of ± 20 healthy donors.

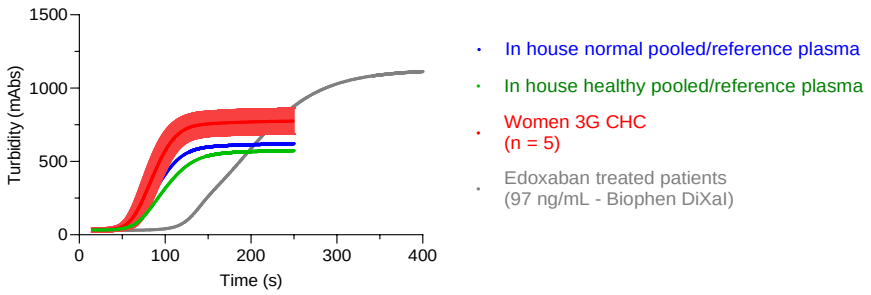


Figure 45: Fibrin formation curves of healthy and normal pooled plasma, women using third-generation hormonal contraception (3G CHC) (n=5) and patient treated with edoxaban.

II. Quality controls

A major weakness in the FibWave studies is the absence of internal and external quality controls. To ensure results and comply the regulations, guidelines or standards issued by relevant bodies, the use internal and external quality controls will be required as for other global coagulation

assays.(Bagot and Leishman 2015, Castellone 2017, Depasse, Binder et al. 2021, Ni, Xue et al. 2021)

To meet regulations, our healthy and normal pooled plasma could be used as internal quality control. The follow-up charts for the normal pooled plasma, from 2021-08-05 to 2022-09-19, for the FibEx clotting time and Max1 are provided as example (**Figure 46**).

Figure 46: Follow-up charts for FibEx-clotting time (A) and FibEx-Max1 (B).

III. Stability of the reagents

The reagent used in FibIn methodology is a CE-IVD marked. According to the manufacturer's recommendations, the reagent, once reconstituted, is stable 7 days at 2-8°C in its original capped vial.

The reagent used in FibEx methodology is RUO product. According to the manufacturer's recommendations, the reagent, once reconstituted, is stable

4 hours at room temperature. To be compliant with the guidelines from IVDR and build the first steps of implementation in routine, we should use a CE-IVD reagent with characteristics similar to the RUO product.

For FibAPC and FibLysis, including protein C activator and tissue plasminogen activator, respectively, it is essential to check that reagents are stable after reconstitution in case of reagent re-use. The stability of reagent has been assessed at 1h, 2h and 3h after reconstitution and stabilization at room temperature.

- The activity of protein C activator on Max1 was variable but was not less than 90% at 3 hours after the preparation.
- The activity of tPA on Min1 has shown to be stable until 3 hours (> 90%). As mentioned by the manufacturer, the RUO reagent used for FibEx, FibAPC and FibLysis, composed of tissue factor and phospholipids, lose activity after reconstitution (**Figure 47**).

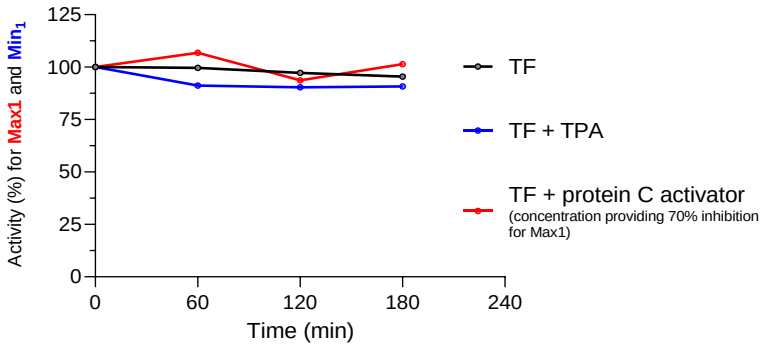


Figure 47: Time influence between preparation of protein C activator and tissue plasminogen activator and their effect on the velocity of coagulation (Max1) and fibrinolysis (Min1), respectively.

The inter-batch management should be carried out on different reagents to ensure recovery between the previous and the new batch of reagents. It was reported that some APTT reagents can present variations between different

batches which may affect the results. For the PT, TGA as well as TEG/ROTEM, a change in reagent lot number could also affect the results.(Shojania, Tetreault et al. 1988, Chitlur, Rivard et al. 2014)

IV. Definition of reference range

Interpretation of laboratory results requires comparison with a reference range or interval. There are guidelines making recommendations about establishment of reference intervals. It is recommended that each laboratory establish its own normal values or laboratory verifies that any manufacturer's stated reference intervals are appropriate for the laboratory's patient population.(Friedberg, Souers et al. 2007)

The reference range should be established by analyzing a representative subset of subjects drawn from the same population as the samples. Because of many factors that influence the levels of hemostatic parameters and factors, and therefore test results, this process is not without difficulty. A practical approach is to select normal subjects and adopt inclusion/exclusion criteria before analysis. The reference interval is usually defined as the interval between the upper and lower reference limits. For most coagulation tests, the limits correspond to two standard deviation (SD) of the mean for a normally distributed population, including 95% of the reference population. It is usually difficult to procure a large number of reference (normal) individuals, required for the construction of reference range. According to the guidelines, the number of normal subjects should be 120 to obtained valid estimates. However, a minimum of 40 individuals is recommended to establish the reference interval.(Solberg and Stamm 1991, Marlar 2013, Kitchen and Makris 2016)

For the FibWave, reference ranges have been defined for all methodologies and parameters. The reference ranges, expressed as mean ± 2SD, are

reported for the most relevant parameters of the FibWave methodologies, obtained from 20 men and 20 women not using hormonal contraception (**Table 19**).

	FibEx	
	Reference range *Mean (-2 SD; + 2SD)*	**Women 3G CHC** *Mean ± SD*
Clotting time (s)	71.9 (52.3 ± 91.4)	65.9 ± 6.1
Time to peak (s)	91.6 (65.3 ± 118.0)	82.2 ± 7.0
Max1 (dmAbs.dt^{-1})	79.9 (46.8 ± 113.0)	131.9 ± 25.4
Max2 (dmAbs.dt^{-2})	28.6 (10.4 ± 46.8)	55.0 ± 12.0
Min2 (dmAbs.dt^{-2})	-15.4 (-4.0 ± -26.8)	-37.8 ± 8.4
	FibIn	
	Reference range *Mean (-2 SD; + 2SD)*	**Women 3G CHC** *Mean ± SD*
Clotting time (s)	56.4 (39.3 ± 73.4)	74.9 ± 11.0
Time to peak (s)	60 (42.9 ± 77.0)	81.7 ± 11.8
Max1 (dmAbs.dt^{-1})	271.6 (144.5 ± 398.7)	262.0 ± 75.7
Max2 (dmAbs.dt^{-2})	433.8 (122.6 ± 745.0)	263.5 ± 132.4
Min2 (dmAbs.dt^{-2})	-413.6 (-188.0 ± -639.2)	-228.8 ± 77.7
	FibLysis	
	Reference range *Mean (-2 SD; + 2SD)*	**Women 3G CHC** *Mean ± SD*
Min1 (dmAbs.dt^{-1})	22.4 (16.7 ± 28.0)	24.1 ± 3.6
Time to lysis (s)	276.6 (227.8 ± 325. 4)	344.7 ± 67.1
Time to lysis – time to peak (s)	186.2 (143.7 ± 228.7)	261.4 ± 66.5
	FibAPC	
	Reference range *Mean (-2 SD; + 2SD)*	**Women 3G CHC** *Mean ± SD*
Inhibition percentage of Max1 (%)	73.6 (47.8 ± 99.5)	40.3 ± 7.5

Table 19: Reference ranges [mean (- 2SD; + 2SD)] for parameters of the FibWave methodologies and mean ± SD for women using third generation of hormonal contraception (CHC).

V. Precision and repeatability

Precision refers to the closeness of agreement between independent test results. This may be reported as the standard deviation (SD) or the coefficient of variation (CV), expressed as a percentage (%). There are two measurements of precision: within-run and between-run. The within-run repeatability is assessed by testing the same sample multiple times in a single run of measurements, e.g. a minimum of 10 replicates of a single sample is tested within a single run. For the between-run precision, run a duplicate QC once daily for at least 5 days.(Mackie, Cooper et al. 2013, Gardiner, Coleman et al. 2021)

The precision steps have not yet been realized according to guidance, but the results obtained during the implementation process and the different studies have made it possible to evaluate the within- and the between-run repeatability criteria. However, criteria such as intermediate repeatability, i.e. with different operators, still need to be validated.

- Within run: five (5) replicates of a single sample were assessed within a single run on different FibWave methodologies. The acceptance criteria (CV < 5%) were established for different parameters.
- Between-run: one replicate was assessed once daily for 3 days on different FibWave methodologies. The acceptance criteria (CV < 10%) were established for different parameters.

Although some parameters exceeded the established acceptance criteria, the results are relevant (**Table 20**). The validation steps, according guidance (CLSI-H57-A) should be carried out with CE-IVD reagents.

Precision – repeatability	Acceptance criteria	Results (expressed as coefficient of variation (CV%))
FibEx		
• Within-run	CV < 5% for clotting time, time to peak, Max1, Max2 and Min2	CV were 1.8%, 1.0%, 1.4%, 3.0% and 1.4%, respectively
• Between-run	CV < 10% for clotting time, time to peak, Max1, Max2 and Min2	CV were 4.1%, 3.4%, 3.4%, 6.6% and 7.3%
FibIn		
• Within-run	CV < 5% for clotting time, time to peak and Max1, **Max2** and Min2	CV were 2.0%, 2.2%, 2.5%, **6.5%** and 4.3%, respectively
• Between-run	CV < 10% for clotting time, time to peak, Max1, **Max2** and Min2	CV were 5.8%, 5.4%, 4.9%, **13.1%** and 6.3%, respectively
FibLysis		
• Within-run	CV < 5% for time to peak, Max1, time to fibrinolysis and **Min1**	CV were 2.2%, 1.4%, 2.7% and **6.0%**
• Between-run	CV < 10% for time to peak, Max1, time to fibrinolysis and Min1	CV were 2.0%, 2.6%, 3.1% and 8.3%
FibAPC		
• Within-run	CV < 5% for Max1 (+ Protac), expressed as percentage of inhibition	CV was 3.3%
• Between-run	CV < 10% for Max1 (+ Protac), expressed as percentage of inhibition	CV was 6.2%

Table 20: Results obtained for within- and between-run criteria for different methodologies of the FibWave.

Conclusion

Compared to end-point clotting tests, APTT and PT, which do not cover all information, the global coagulation assays (CWA, TGA and TEG/ROTEM) have advantage to provide more than one coagulation parameter and to assess the global hemostatic potential. The FibWave, a coagulation assay based on the analysis of clot formation kinetics, was developed to be more sensitive than CWA and also allow the evaluation of the overall coagulation process.

In anticoagulated states, the FibWave, and specially the FibEx methodology, was more sensitive and succeeded in discriminating samples at very low plasma concentration in edoxaban, i.e. 30 and 50 ng/mL than APTT- and PT-based CWA. The FibWave showed also to have a shorter turn-around time and as sensitive as TGA to the DOACs effect.

In prothrombotic states, the FibEx methodology proved to have better potential to assess the hormone-induced changes than the APTT- and PT-based CWA. The FibAPC methodology proved to be more discriminative than the ETP-based APC resistance in distinguishing different levels of APC resistance, suggesting its usefulness as screening marker for the thrombogenicity.

In the same way as viscoelastic assays, the FibLysis methodology was able to investigate simultaneously the coagulation and the fibrinolytic processes and therefore, can be considered as a global coagulation assay.

Additionally, the FibWave appears to be easy and economically convenient to perform and could be implemented on other automated photo-optical coagulometers.

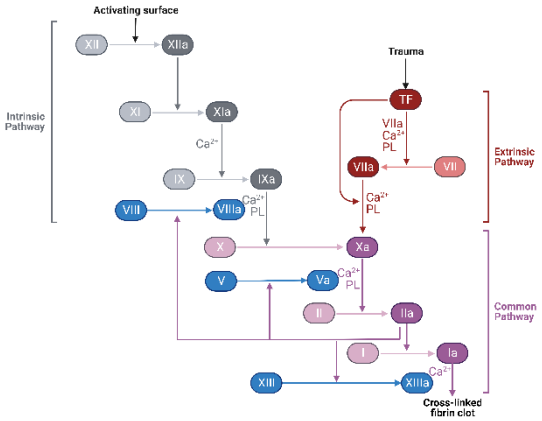
Before being implemented in the laboratory testing, the FibWave should respect the IVDR and further studies evaluating and comparing the clinical performances to other coagulation assays in various clinical situations will should be performed.

Index of coagulation assays

Coagulation consists of three pathways, the extrinsic, intrinsic, and common pathways, that interact together to form a stable blood clot. The extrinsic and intrinsic coagulation pathways both lead into the final common pathway by independently activating FX.

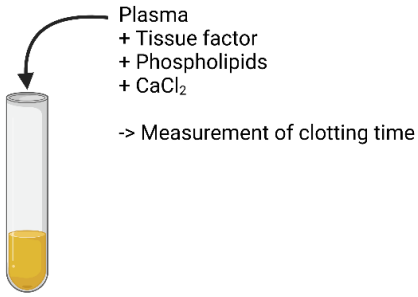
The extrinsic pathway starts with disruption of the endothelium exposing the tissue factor (TF) to FVII/FVIIa. TF bound with calcium, phospholipids and FVIIa, forming the extrinsic tenase complex, activates FX into FXa. Then the FXa uses the FVa and calcium to form the prothrombinase complex which activates prothrombin (FII) into thrombin (FIIa). Thrombin proteolytically cleaves fibrinogen (FI) into fibrin (FIa) and FXIII into FXIIIa. FXIIIa combines with calcium to from cross-links between the fibrin chains, reinforcing the fibrin mesh.

The intrinsic pathway starts when FXII encounters negatively charged phosphates on the membrane of activated platelets or collagen exposed by trauma, and is subsequently activated in FXIIa. FXIIa cleaves FXI into FXIa and with calcium, FXIa activates FIX. FIXa with calcium and FVIIIa, forming the intrinsic tenase complex, activates FX (**Annex 1**).



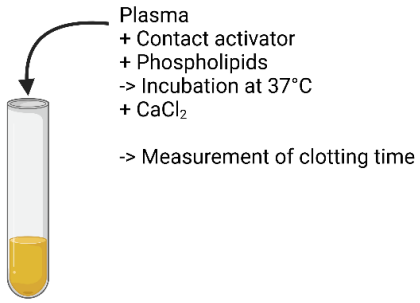
Annex 1: Coagulation Cascade.
TF: tissue factor; PL: phospholipids; Ca^{2+}: calcium ions

Prothrombin time (PT): measures the activity of the extrinsic and common pathway of coagulation. The assay is dependent on the functional activity of FII, FV, FVII, FX and fibrinogen. Plasma is mixed with TF, phospholipids, and a calcium chloride (CaCl2) solution initiating the coagulation. The time, from the addition of CaCl2 to clot formation, is measured (**Annex 2**).



Annex 2: Principle of the prothrombin time test.

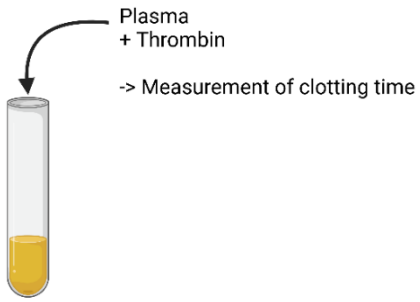
Activated partial thromboplastin time (APTT): measures the activity of the intrinsic and common pathways of coagulation. Plasma is mixed with contact activator and phospholipids. This step leads to the conversion of FXI to FXIa but the remainder of the pathway is not activated. The addition of CaCl2 triggers the coagulation. The time, from the addition of CaCl2 to clot formation, is measured (**Annex 3**).



Annex 3: Principle of the activated partial thromboplastin time test

Thrombin time (TT): the thrombin time measures the fibrin formation caused by the action of thrombin, the final step in the clotting cascade. The principle of the test is that a standardized concentration of thrombin is added to citrated plasma and time to fibrin clot formation recorded in seconds (**Annex 4**). The TT is very useful for detecting the presence of low concentrations of dabigatran in plasma. If TT is in normal range, it excludes presence of dabigatran.

Diluted thrombin time (dTT): assay based on TT principle in which a plasma sample is diluted with saline solution and normal pooled plasma before initiation of coagulation with thrombin.

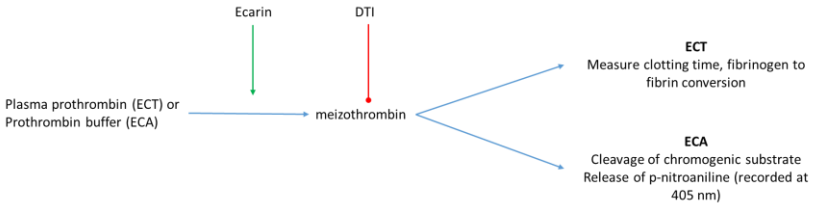


Annex 4: Principle of the thrombin time test

Ecarin clotting time (ECT): an activator of plasma prothrombin, ecarin (snake venom from Echis carinatus) activates prothrombin creating meizothrombin, a thrombin analogue consisting of alpha-thrombin complexed with prothrombin fragment 2, which is generated from cleavage at the Arg323-Ile324 site. The time to clot formation is measured. In presence of DTI, meizothrombin is inhibited and the clot formation is prolonged. The ecarin clotting time is dependent on patient prothrombin and fibrinogen levels, and therefore, may be prolonged with low prothrombin and/or fibrinogen levels.

The ECT is useful for measuring drug activity over the therapeutic range but only for research (RUO).

Ecarin chromogenic assay (ECA): assay based on ECT and developed for quantitative determination of DTI. Ecarin cleaves human prothrombin, contained in excessive amount in prothrombin buffer, creating meizothrombin. The meizothrombin cleaves chromogenic substrate and release p-nitroaniline, recorded at 405 nm. Unlike in the ECT, the sample is diluted in prothrombin buffer, eliminating the effect of low prothrombin levels, and the method is not dependent on fibrinogen conversion, and thus is not affected by low fibrinogen levels.

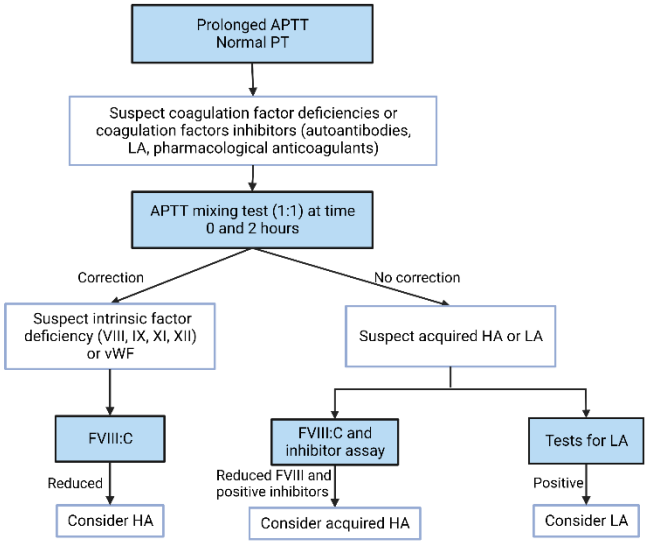


Annex 5: Principle of ECT and ECA

Index of pathologies

Cirrhosis, liver disease or liver dysfunction results in a reduction in production/synthesis of most coagulation factors. A decreased production of coagulation factors can lead to prolonged clotting time.

Hemophilia is an inherited predisposition to bleeding caused by a deficiency in a clotting factor, factor VIII (FVIII) for hemophilia A and factor IX (FIX) for hemophilia B. the severity of hemophilia is based on plasma coagulation factor levels and is classified as severe, moderate, or mild. Patients with severe hemophilia develop spontaneous joint and muscle hematomas, in addition to bleeding after minor injuries, accidents, and surgical procedures. Those with moderate disease develop muscle and joint hematomas after mild trauma and can bleed excessively after surgery and dental extractions. Patients with mild hemophilia do not bleed spontaneously but they bleed after surgery, significant trauma or dental extractions. Coagulation screening tests, APTT and PT, are performed for any patient with a suspected bleeding disorder. If the PT is normal, deficiencies of the extrinsic and common coagulation pathways are excluded and a prolonged APTT suggests an intrinsic or common pathway deficiency, or the presence of a coagulation factor inhibitor. A mixing test is required to differentiate whether the prolonged APTT is due to a factor deficiency or anticoagulants, including a coagulation factor inhibitor. If the APTT is normalized by the presence of coagulation factors from the normal plasma in the mixing test, a factor deficiency is more likely than the presence of inhibitors. Factor activity assays are performed to provide a differential diagnosis of the clotting factor deficiency (**Annex 6**).



Annex 6: Example of diagnostic algorithm of acquired hemophilia.

Disseminated coagulation intravascular (DIC) causes a massive overactivation of the coagulation system in response to trauma, malignancy, obstetric complications or sepsis. There can be release of procoagulant factors promoting clot formation. The coagulation pathway goes into overdrive resulting in widespread clot formation with depletion of platelets and clotting factors, which plugs up blood vessels leading to ischemia, necrosis and eventually organ damage. The clots are also broken down through fibrinolysis and fibrin degradation products are released into the circulation, interfering with platelet aggregation and clot formation.

Antiphospholipid syndrome is a non-inflammatory autoimmune disease defined by the presence of antiphospholipid antibodies in the plasma of patients with venous and/or arterial thrombosis and/or recurrent complications of pregnancy, including anticardiolipin and anti-β2 glycoprotein I antibodies and lupus anticoagulant. Antiphospholipid

antibodies are a heterogeneous group of antibodies that react with a myriad of phospholipids. Significant antigenic targets include β2GPI, tissue plasminogen activator (tPA), phosphatidylserine (PS), plasmin, annexin 2, activated protein C, thrombin, antithrombin III, and annexin V.

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