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Full Length Article

Suggestions for global coagulation assays for the assessment of COVID-19 associated hypercoagulability

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ABSTRACT

Introduction: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) infection is associated with a clear prothrombotic phenotype. Although the exact pathophysiological mechanisms are not yet fully understood, thrombosis is clearly a highly important in the prognosis and outcome of COVID-19. As such, there is a need for diagnostic analysis and quantification of the coagulation potential in these patients, both at diagnosis and follow-up. Global coagulation assays like thrombin generation (TG) and rotational thromboelastometry (ROTEM) might be suitable in estimating COVID-19 associated coagulopathy and thrombosis risk. Therefore, we aimed at validating both assays for samples with high levels of fibrinogen and in the presence of anticoagulant heparins, such as commonly observed for COVID-19 ICU patients.

Materials and methods: Calibrated Automated Thrombography (CAT) was optimized to assess plasma thrombin generation in the presence of heparins. The final conditions with either 10 μg/mL Ellagic acid (EA) or PPP Reagent HIGH (high tissue factor; HPPH) were validated according to the EP5 protocol for within-run and between-run variability. Overall variability was well below 10%. To estimate the influences of heparins and high fibrinogen levels, CAT was performed on spiked plasma aliquots from 13 healthy volunteers. Comparable to the CAT method, tPA-ROTEM was used to validate the effect of high fibrinogen and heparins on clotting time, clot firmness and clot lysis parameters.

Results: Our adjusted COVID-19 assay showed a heparin dose dependent decrease in peak height and endogenous thrombin potential (ETP) for both EA and HPPH triggered variants. High fibrinogen did not alter the inhibitory effect of either LMWH or UFH, nor did it influence the peak height or ETP in any of the conditions. The tPA-ROTEM showed a significant prolongation in clotting time with the additions of heparin, which normalized with the addition of high fibrinogen. MCF was markedly increased in all hyperfibrinogenemic conditions. A trend towards increased lysis time and, thus, decreased fibrinolysis was observed.

Conclusion: Thrombin generation and tPA-ROTEM protocols for measurements in the COVID-19 populations were adjusted and validated. The adjusted thrombin generation assay shows good sensitivity for measurements in heparin spiked plasma. High levels of fibrinogen did not alter the assay or the effectiveness of heparins as measured in this assay. t-PA ROTEM was effective in measurement of both high fibrinogen and heparins spiked samples and was sensitive to the expected relevant coagulant changes by these conditions. No clear fibrinolytic effect was observed in different conditions.

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Table 1
Validation data of the thrombin generation assay in normal pool with within-run and between-run variability for PPP Reagent HIGH (left side) and ellagic acid (right side) triggered thrombin generation respectively.
(CV: Coefficient of variation; EA: Ellagic Acid; LMWH: low-molecular weight heparin; Nadroparin, 0.6 IU/mL; UFH: Unfractionated heparin; Heparin Leo, 0.3 IU/mL).

Trigger	Within-run variation	Between-run variation	Trigger	Within-run variation	Between-run variation
PPP reagent HIGH	%CV	%CV	EA (10 mg/mL)	%CV	%CV
Lag time	3.3%	1.7%	Lag time	6.5%	1.9%
Lag time (+UFH)	3.7%	1.5%	Lag time (+UFH)	7.3%	4.7%
Lag time (+LMWH)	5.0%	1.6%	Lag time (+LMWH)	5.6%	5.8%
ETP	2.3%	4.4%	ETP	1.8%	4.1%
ETP (+UFH)	2.6%	3.6%	ETP (+UFH)	2.1%	3.9%
ETP (+LMWH)	2.4%	4.0%	ETP (+LMWH)	1.8%	3.3%
Peak height	1.3%	2.1%	Peak height	2.4%	1.6%
Peak height (+UFH)	2.4%	2.0%	Peak height (+UFH)	3.7%	5.0%
Peak height (+LMWH)	3.0%	3.5%	Peak height (+LMWH)	4.1%	4.8%

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) is a highly pathologic beta-coronavirus causing the worldwide COVID-19 pandemic. It is suggested that the human to human transmission of the virus depends on close human contact and aerosol particles [1]. Infection mechanisms involve virus particles entering airway epithelial cells in an angiotensin-converting enzyme 2 (ACE2) receptor dependent manner [2–4]. The common clinical presentation includes dry cough, dyspnea, fever and fatigue, but also anosmia and diarrhea are observed. Severity of clinical symptoms range largely, from only mild shortness of breath to severe acute respiratory failure [5]. In addition to the pulmonary symptoms, patients have relatively high rates of venous thromboembolism (VTE) related to disease severity, and probably also higher incidences of arterial thrombotic disease. This thrombotic tendency is also referred to as COVID-19 associated coagulopathy (CAC). Initially, the hypercoagulable state of CAC was thought to have features of systemic coagulopathies like disseminated intravascular coagulation often found in severe systemic inflammation. However, both the clinical phenotype and the biochemical characteristics of CAC are markedly different from other coagulopathies [6–9]. One of the characteristics, highly elevated fibrinogen, increases with disease severity but might also influence global coagulation assays used to assess the coagulopathy [10,11].

Global coagulation assays, such as the plasma thrombin generation (TG) method and whole blood rotational thromboelastometry (ROTEM), have the potential to be applicable for assessment of the hypercoagulable state in CAC [12]. Conceptually, in the setting of COVID-19 management, such global assays could be of use not only to document severity of coagulopathy, but also to guide anticoagulant treatment. However, not all standard conditions of such assays, in particular TG, are suitable to monitor heparinization. Moreover, these assays might be influenced by hyperfibrinogenemia and the frequent use of heparin anticoagulant therapy in the CAC patient population [13,14]. Therefore, we aimed to assess the influence of hyperfibrinogenemia and heparins by means of TG and tPA-ROTEM assays, tailored to be applicable in patients with COVID-19 related coagulopathy, with and without systemic anticoagulation.

2. Materials and methods

2.1. Whole blood and plasma preparation

For thrombin generation and tPA-ROTEM analysis, venous blood was collected from 14 healthy volunteers using a Vacutainer 21-gauge needle (Becton Dickinson, Plymouth, UK). Blood was collected in 3.2% (*w/v*) citrated Vacutainer tubes (Becton Dickinson). Withdrawal of the citrate tubes was preceded by Vacutainer clot activator tube, which was discarded. No contact activation inhibitors were used in blood withdrawal. Platelet poor plasma was prepared according to local protocols by double centrifugation: an initial centrifugation of 2000 ×*g* for 5 min followed by a secondary centrifugation at 10,000 ×*g* for 10 min at room temperature. The platelet-poor plasma (PPP) was subsequently snap-frozen in liquid nitrogen and stored at −80 °C. Before each thrombin generation experiment, PPP was spiked to obtain the following conditions: low molecular weight heparin (LMWH) by means of Nadroparin (Aspen Netherlands BV) or to a final concentration of 0.6 IU/mL, unfractionated heparin (UFH) by Heparin Leo (Leo Pharma BV) to a final concentration of 0.3 IU/mL, fibrinogen using Haemocomplettan P (CSL Behring BV) to an additional 7.5 g/L, and combinations of LMWH with fibrinogen and UFH with fibrinogen.

Whole blood tPA-ROTEM analysis included 10 healthy volunteers for which blood aliquots were spiked to obtain final concentrations of 0.45 IU/mL LMWH and 0.23 IU/mL UFH, corresponding with plasma concentrations of approximately 0.6 IU/mL and 0.5 IU/mL. Fibrinogen was added to increase the total fibrinogen level by 6 g/L, corresponding with final plasma concentrations of >7.5 g/L for all samples.

2.2. Thrombin generation

Thrombin generation in platelet-poor plasma was performed using the Calibrated Automated Thrombogram (CAT) method (Thrombinoscope BV, Maastricht, The Netherlands). Our previously described standardized protocol was used [15]. In brief, thrombin generation was triggered by either high tissue factor (in-house reagent, comparable to PPP Reagent HIGH) or 10 μg/mL ellagic acid (Sigma-Aldrich), both in the presence of 4 μM phospholipids. Thrombin generation was assessed after addition of a low-affinity fluorescent substrate (*Z*-Gly-Gly-Arg 7-amino-4-methylcoumarin) for thrombin using Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland) utilizing a 390/460 filter, and recorded using the Thrombinoscope software (Thrombinoscope BV).

2.3. CAT validation of thrombin generation

Validation of the CAT method for within- and between run variation was performed using the Clinical and Laboratory Standards Institute (CLSI) EP5 protocol in normal pool plasma [16].

Repeated measurements of normal and heparin spiked plasmas were performed within a 10-day period, with two runs per day and two replicates per run. All experiments were performed using the same batch of reagent to avoid batch to batch differences. Coefficient of variation (CV %) for within-run and between-run variation was calculated (Table 1).

2.4. tPA-ROTEM

Rotational thromboelastometry using the ROTEM delta (Werfen; Barcelona, Spain), to assess viscoelastic properties of a whole blood sample during clot formation and/or dissolution, was performed in the presence of recombinant tissue plasminogen activator (r-tPA) to induce

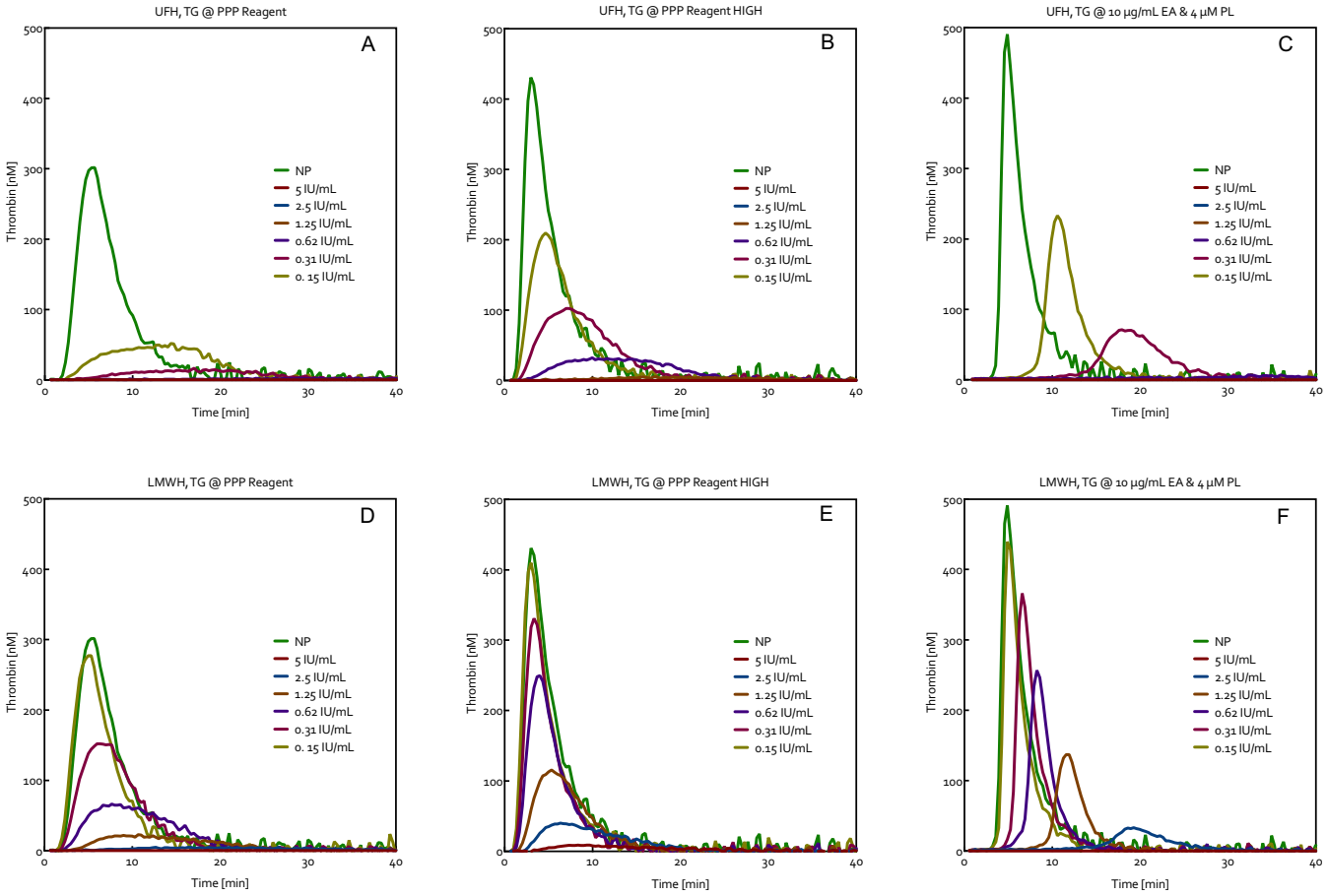

Fig. 1. Titration of UFH (upper panels) and LMWH (lower panels) in several thrombin generation triggers. Panels A and D show PPP Reagent triggered thrombin generation, panels B and E the PPP Reagent HIGH assay and panels C and F the ellagic acid triggered thrombin generation. (Heparin LEO: Unfractionated heparin; LMWH: Nadroparin; NP: Normal pool; PL: Phospholipids; TF: Tissue factor; TG: Thrombin generation).

fibrinolysis, as described previously, according to the in-house standardized and validated protocol [17]. In brief, 125 ng/mL r-tPA (actilyse; Boehringer Ingelheim BV) was added simultaneously with 10 mM $CaCl_2$ and 35 pM tissue factor (Innovin, Siemens) to initiate both fibrinolysis and clot formation at the start of the measurement. The following standard tPA-ROTEM parameters were analyzed: CT (clotting time in seconds), MCF (maximum clot firmness in mm), LOT (lysis onset time in seconds; time from CT until a 15% drop in MCF) and LT (lysis time in seconds; time from CT until a 90% drop in MCF).

2.5. Fibrinogen and anti-Xa

For validation of fibrinogen and heparin spiking in plasma or whole blood from donors, the fibrinogen levels were assessed by the Clauss method (Dade Thrombin Reagent; Siemens, Marburg, Germany) and Anti-Xa levels by the Hyphen method (Biophen Heparin LRT; Hyphen Biomed, Neuville-Sur-Oise, France) on a Sysmex CS2100i (Sysmex Corporation, Kobe, Hyogo, Japan) hemostasis analyzer. Anti-Xa (aXa) test samples were diluted (1 unit sample: 2 units pooled reference plasma) and determined using a LMWH calibration line (Biophen LMWH calibrator; Hyphen Biomed). For UFH a previously determined correction factor was applied: aXa-UFH = 1.55 * aXa-LMWH [18].

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.4 (GraphPad, San Diego USA). Descriptive statistics included calculation of median for each of the thrombin generation or tPA-ROTEM conditions. Multiple measurement comparison between the conditions was performed using the Friedman's test. Post hoc analysis using Dunn's multiple comparisons test was performed if a statistically significant difference was found using Friedman's test. A *P*-value cutoff of $P < 0.05$ was used for significance.

3. Results and discussion

3.1. Thrombin generation

The presence of heparin (both LMWH and UFH) anticoagulants in plasma causes diminished thrombin generation, which was more pronounced at lower tissue factor triggers (eg. TG triggered with PPP Reagent). Therefore, higher tissue factor levels (PPP Reagent HIGH) were compared to PPP Reagent LOW, PPP Reagent, and to intrinsic activation of thrombin generation through addition of ellagic acid. To this purpose, thrombin generation was assessed in plasmas spiked with a various concentration (0–5 IU/mL) of nadroparin or unfractionated heparin using the two tissue factor triggers or ellagic acid (Fig. 1).

All heparin concentrations in plasma were based on clinically expected concentrations in COVID patients. PPP Reagent LOW was unable to provide reliable thrombin generation curves at all heparin levels (data not shown), PPP Reagent resulted in moderate curves, whereas both PPP Reagent HIGH and 10 µg/mL ellagic acid provided reliable thrombin generation curves within the full heparin ranges. Combined with the intermediate results obtained for PPP Reagent, the PPP Reagent HIGH and ellagic acid were selected as the two triggers for analytical validation. Repeated analysis of control plasma samples revealed coefficients

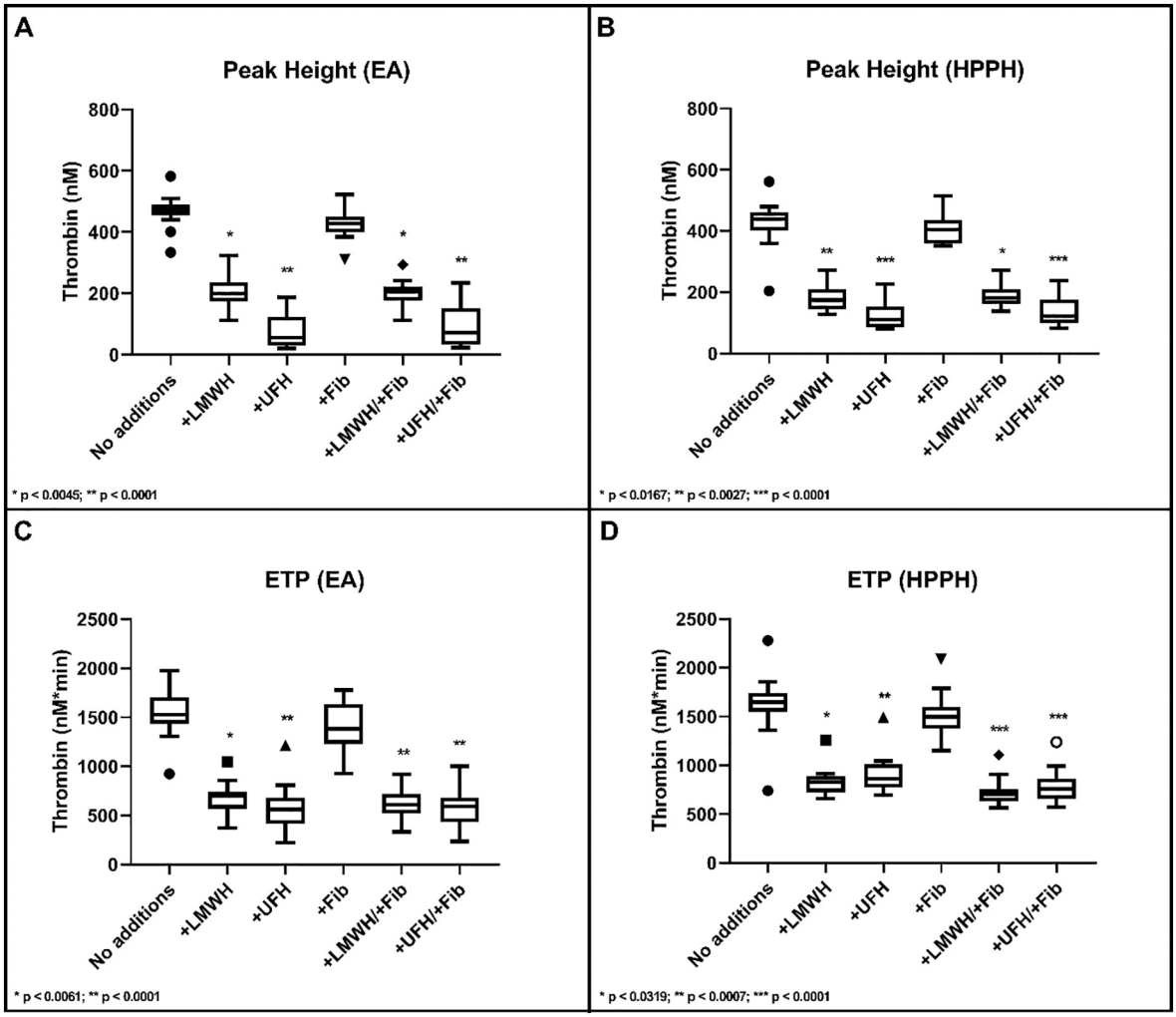

Fig. 2. Medians and ranges of peak height and ETP for control, fibrinogen, UFH and LMWH spiked samples from healthy volunteers. Panel A and C show ellagic acid (10 μg/mL, 4 μM PL) triggered thrombin generation baseline and in several spiking conditions. Panel B and D show the same conditions in PPP Reagent HIGH triggered thrombin generation. Final concentrations: 0.6 IU/mL LMWH, 0.3 IU/mL UFH, and 7.5 g/L fibrinogen. (EA: Ellagic Acid; HPPH: High tissue factor mix; LMWH: low-molecular weight heparin; UFH: Unfractionated heparin; Fib: Fibrinogen).

of variation (CV) for lag time, ETP and peak height below 10%CV for both the within-run and between-run reproducibility (Table 1), suggesting that both the PPP Reagent HIGH and ellagic acid conditions for thrombin generation were suitable for routine clinical laboratory analysis.

Following validation, the two triggers for thrombin generation were applied to evaluate the effects of various heparins and elevated fibrinogen levels on thrombin generation in plasma from healthy participants. Spiking of LMWH and UFH (0.45 IU/mL and 0.23 IU/mL UFH, respectively) retained the expected decrease in thrombin generation, comparable to the previously observed dose-dependent effects. Overall, LMWH and UFH reduced the peak height by 58% to 85%, respectively and up to 60% reduction of ETP was observed for both in the EA-triggered thrombin generation. Comparable anticoagulant effects for LMWH and UFH were seen in the HPPH-triggered assay, leading to slightly less pronounced decrease in peak height (56%; 71%) and ETP (46%; 42%). Thrombin generation was not significantly altered in presence of high concentrations of fibrinogen, suggesting that the anticoagulant effects of both LMWH and UFH were not influenced by high fibrinogen levels, which are commonly seen in the COVID-19 population. Data of the spiking experiments suggest that the EA triggered thrombin generation might be more sensitive in detecting the difference in the inhibitory effect of UFH compared to LMWH on coagulation, however additional data would be needed to confirm this further. The lag time of the thrombin generation assay (data not shown) was unaffected by any of the study conditions. Although our data using spiked healthy controls are consistent, further clinical validation with plasma of COVID-19 patients is needed. The assay shows sensitivity in differentiation between LMWH and UFH inhibition as suggested by the difference of anticoagulant effects in thrombin generation (Fig. 2).

In contrast to our data, an inhibitory effect of high fibrinogen concentrations on thrombin generation was reported previously [13,19]. However, the effect of fibrinogen might only be evident in thrombin generation activated by lower concentrations of TF or EA where small offsets have a relatively large effect on the total measurement. The concentration of both EA and TF in our assay is high to compensate in part for the presence of LMWH and UFH in order to obtain measurable TG in hospitalized COVID-19 patients, while on LMWH or UFH therapy.

3.2. tPA-ROTEM

Similarly, tPA-ROTEM was performed to assess the effect of hyperfibrinogenemia and heparins on clot formation and fibrinolysis in whole blood of 10 healthy volunteers. Compared to control samples (median [IQR], 62.5 s [59.5–71.75]), CT was prolonged in both UFH and LMWH spiked samples (101.5 s [91.0–106.8] and 90.5 s [81.25–91.25],

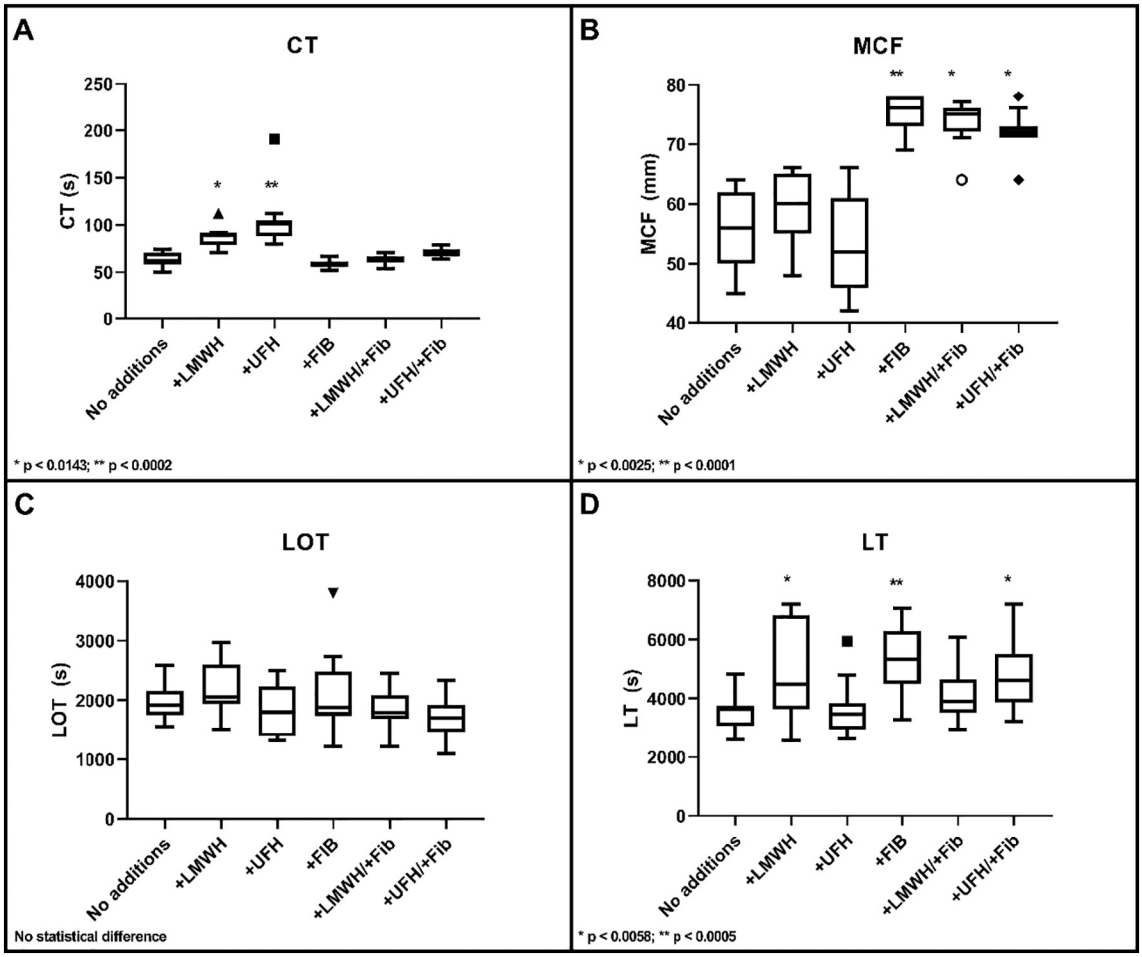

Fig. 3. Median and range of tissue plasminogen activator thromboelastometry (tPA-ROTEM) clotting time (Panel A), maximum clot firmness (Panel B), lysis onset time (Panel C) and lysis time (Panel D) for control, fibrinogen, UFH and LMWH spiked samples from healthy volunteers. (CT: clotting time; MCF: maximum clot firmness; LOT: lysis onset time; LT: lysis time; LMWH: low-molecular weight heparin; UFH: unfractionated heparin; Fib: Fibrinogen).

respectively). However, this parameter was unable to differentiate between the inhibitory effect of the two different heparins, since no significant difference between medians was observed. Addition of fibrinogen to the heparin spiked whole blood resulted in normalization of CT (71.5 s [66.75–75.0] and 62.5 s [60.0–67.75] for UFH and LMWH, respectively). MCF was consistently increased in fibrinogen spiked samples, irrespective of heparin presence, as illustrated in Fig. 3.

Fibrinolytic tPA-ROTEM parameters LOT and LT illustrate the time to initiation and dissolution of clot breakdown, respectively. The initiation of fibrinolysis, LOT, did not differ between groups. However, LT was significantly higher in fibrinogen spiked samples (5328 s [4364–6107]) compared to the control (3642 s [3224–3777]). Addition of UFH to fibrinogen spiked samples slightly diminished the effect of hyperfibrinogenemia as median LT significantly decreased to 4784 s [4233–5667] ($p = 0.0048$).

Note that LT was immeasurable within 2 h in three (2 LMWH and 1 UFH + Fibrinogen) samples, which were fixed at 7200 s for statistical analysis. Though the exact value is unknown, these results can be interpreted as >120 min, thus suggesting fibrinolysis in this subset is more diminished than presented in our results.

Addition of heparins resulted in the expected CT prolongation in the tPA-ROTEM assay. Although no significant difference between UFH and LMWH was present, there seems to be a trend that the stronger effect of UFH can be observed (Fig. 3). MCF was consistently increased significantly in presence of fibrinogen confirming the importance of increased fibrinogen in whole blood viscoelastic clot strength measurements. No clear fibrinolysis suppressive effect of high fibrinogen could be observed in our results, since LOT did not differ between groups and LT wasn't consistently increased in fibrinogen spiked samples.

In addition to recombinant human tissue factor, Innovin (recombinant tissue factor concentrate) contains a heparin-neutralizing compound. However, Innovin was diluted prior to use and small volume was added to the test cup to obtain a final tissue factor concentration of 35 pM. The significant CT increase in heparin spiked samples suggests that the residual heparin-neutralizing compound in this dilution is no longer able to neutralize the heparin in our samples.

Both fibrinolysis parameters (LOT and LT) showed considerable variability between individuals, which was even more pronounced in spiked samples. In the control group, however, only one value was out of reference range for LT (2100–4620 s), illustrating the large interindividual differences when assessing fibrinolysis in whole blood of healthy volunteers. Nonetheless, hyperfibrinogenemia resulted in increased LT. Cushing et al. [20] showed similar t-PA induced ROTEM (100 ng/mL) results with fibrinogen spiked whole blood of 10 healthy volunteers. They demonstrated a decrease in fibrinolysis, which did not reach statistical significance due to large variability in fibrinolysis parameters.

Both UFH and LMWH have previously been shown to shorten clot lysis time and, thus, induce fibrinolysis [21–23]. Addition of UFH to fibrinogen spiked samples did result in significantly shorter LT in our experiments, possibly demonstrating the fibrinolytic properties of heparins. However, this fibrinolysis inducing effect is absent in sole UFH and LMWH spiked samples and no significant difference between fibrinogen and fibrinogen + LMWH spiked samples was observed. Thus, the hypothesis that the pro-fibrinolytic effect of heparins can be detected

using tPA-ROTEM cannot be confirmed by our data.

4. Conclusion

In this paper we describe our systematic approach for adaptation and validation of both thrombin generation and tPA-ROTEM protocols to assess hypercoagulability, hypofibrinolysis and monitoring of antithrombotic therapy for the hospitalized COVID-19 population. Although the exact pathophysiology of severe coagulopathy in COVID-19 is not known, the mechanism involves a complex interaction of inflammation, hemostasis and endothelial damage, amongst others. Modification of global haemostatic assays might include multiple components affected in COVID-19, however, the aim of the presented study was to estimate the anticoagulant potential of heparins on top of the occasionally observed hyperfibrinogenemia. To this purpose, the thrombin generation assay was modified in order to detect anticoagulant effects in plasma, in presence of variable levels of fibrinogen. Hyperfibrinogenemia did not have a significant influence on the thrombin generation. The effect of both LMWH and UFH was measurable within the assay and a clear distinction between the inhibitory activity of LMWH and UFH could be measured. The inhibitory activity of both LMWH and UFH was not altered by the hyperfibrinogenemia.

Hyperfibrinogenemia merely showed a trend towards decreased fibrinolysis in tPA-ROTEM, which appeared diminished in presence of additional heparins. As expected, spiking with UFH and LMWH resulted in prolongation of CT and fibrinogen spiking resulted in MCF increase irrespective of heparins.

Author contributions

TWvdB and AMH performed experiments, analyzed data and wrote the manuscript. MN, JWS, BvB and HtC discussed data and revised manuscript. RvO supervised experiments and revised manuscript. YH designed study and revised the manuscript. HMHS design the study and wrote the manuscript.

Declaration of competing interest

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

HtC and HMHS received funding for research from Bayer and Pfizer and are stakeholders in Coagulation Profile. HtC is a consultant for Alveron and has served on advisory boards for Bayer, Pfizer, Daiichi, Leo and Gilead. The other authors declare no conflict of interest. The Dutch Covid-19 and Thrombosis Coalition (DCTC) is supported by grants from the Netherlands Thrombosis Foundation and The Netherlands Organization for Health Research and Development (ZON-MW). This project has been made possible in part by the Van de Laar Foundation for Cardiovascular Biochemistry.

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