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# Studies on hemostasis in COVID-19 deserve careful reporting of the laboratory methods, their significance, and their limitations

We read with much interest the recent observational study of Nougier et al, which aimed at studying thrombin generation (TG) and fibrinolysis profiles of coronavirus disease 2019 (COVID-19) pa-tients admitted to an intensive care unit or to an internal medicine ward and receiving various schemes of prophylactic heparin.[1] They reported that thrombin potential remained within normal range de-spite heparin and that fibrinolysis was decreased in relation with increased plasminogen activator inhibitor 1 and thrombin-activat-able fibrinolysis inhibitor antigen plasma levels. Using the rotational thromboelastometry delta device with EXTEM reagents and the ad-dition of 0.625 µg/mL tPA (referred to as "TEM-tPA"), they reported decreased clot lysis in COVID-19 patients, which was more pro-nounced in patients who presented a thrombotic event, compared with event-free patients.

This important report provides us the opportunity to raise some crucial methodological issues.

First, a high variability in heparin plasma levels measurements between the available anti-Xa kits has been reported, especially for unfractionated heparin (UFH) and for low anti-Xa levels.[2] The au-thors did not specify the kit they used. If they have used a reagent that contains dextran sulfate (as most currently available reagents do), this could have led to an overestimation of the heparin levels.

Indeed, dextran is reported to displace heparin from its binding to plasma proteins other than antithrombin (AT), including acute phase reactants, which are increased in COVID-19 patients, and from platelet factor 4, which can be released by activated platelets.[3] Furthermore, the authors did not differentiate patients according to the heparin they received (UFH or low molecular weight heparin [LMWH]) although they have different effects on laboratory tests (anti-Xa activity, TG, and rotational thromboelastometry). Of note, some anti-Xa kits containing exogenous AT can also lead to an over-estimation of heparin levels in case of AT deficiency, which was in-frequent in this series however.[3]

Second, the authors found in vitro TG (calibrated automated thrombogram, reagent with the high tissue factor [TF] concentration [PPP High Reagent]) within normal range despite prophylactic hepa-rin administration. Based on this finding, they conclude that the pa-tients presented a major hypercoagulability that was not controlled by heparin administration. However, they could measure heparin levels in a limited number of samples only while two different dos-ages were used (standard and intensified prophylaxis). Mean anti-Xa levels measured were low ($0.35 \pm 0.20$ IU/mL) and could have been overestimated in the presence of dextran in the reagent, as dis-cussed previously. It would thus not be unexpected to observe nor-mal TG profiles. Of note, the reagent they used for TG (ie, the PPP High Reagent) has been designed to measure thrombin potentials in plasma samples containing anticoagulant drugs and therefore uses

high TF concentrations to initiate TG (ie, 20 pmol/L TF). The ability of such analytical conditions to show evidence of a prothrombotic profile is therefore likely to be poor. The utilization of less intense activation seems more appropriate for this objective, but would require neutralization of heparin.

Third, the authors also suggested that what they refer to as "heparin resistance" could be an explanation to the normal TG profiles observed, but they thought it not likely because AT plasma levels were normal, in most patients at least. However, laboratory "resistance" to UFH (ie, failure to achieve the therapeutic target (activated partial thromboplastin time or anti-Xa levels) despite the administration of recommended UFH doses (ie, 400-600 IU/kg/d) cannot be asserted based on TG because the corresponding inhibition of TG under current, commercially available conditions (calibrated automated thrombogram or ST-Genesia), has not been determined, and definitely not without linking the assessment of heparin activity to the doses administered.[3] Furthermore, other factors besides low AT levels have been identified as potential causes of laboratory "resistance" to heparin, such as elevated platelet factor 4 or heparanases. Elevated factor VIII or fibrinogen levels can also shorten the activated partial thromboplastin time, but without any effect on the anti-Xa assays. Altogether, in our opinion, the observation of normal TG profiles despite heparin administration instead reflects the low heparin levels measured with regard to the hyperinflammatory state observed in most COVID-19 patients.

To illustrate differences among anti-Xa reagents and among types of heparin (UFH vs LMWH), we measured in parallel anti-Xa levels with kits containing or not dextran sulfate (Biophen Heparin LRT, calibrated with Biophen Heparin Calibrator, Hyphen biomed, Neuville-sur-Oise, France, and STA-Liquid anti-Xa, calibrated with STA-Multi Hep Calibrator, Stago Diagnostica, respectively) with a STA-R Max analyzer (Stago Diagnostica) in 28 COVID-19 plasma samples prospectively prepared from six COVID-19 patients admitted to the intensive care unit (11 samples from patients treated with weight-adjusted UFH [Leo Pharma, Lier] and 27 samples from patients treated with weight-adjusted enoxaparin [Sanofi]). We included both peak and trough samples to cover a wide range of anti-Xa levels. Blood was drawn into 109 mmol/L sodium citrate tubes, underwent a double centrifugation (at 1500*g* for 15 minutes at room temperature) within 1 hour after blood collection, and plasma samples were frozen at −80°C. We studied TG with the ST-Genesia analyzer using STG-DrugScreen reagent (ie, the reagent with the highest TF concentration available for the ST Genesia) according to manufacturer's recommendations (Stago Diagnostica).[4] Results were normalized using a reference plasma provided with STG-DrugScreen kit of reagents and expressed in percentage.

We observed an overall good correlation between anti-Xa levels measured with both reagents (Pearson's correlation coefficient = 0.98 for UFH samples and = 0.98 for LMWH samples). However, for UFH samples, the reagent containing dextran showed an overestimation of anti-Xa levels compared with reagents that did not contain dextran (proportionally to anti-Xa levels; slope of the regression line: 1.47; 95% confidence interval, 1.24-1.71;
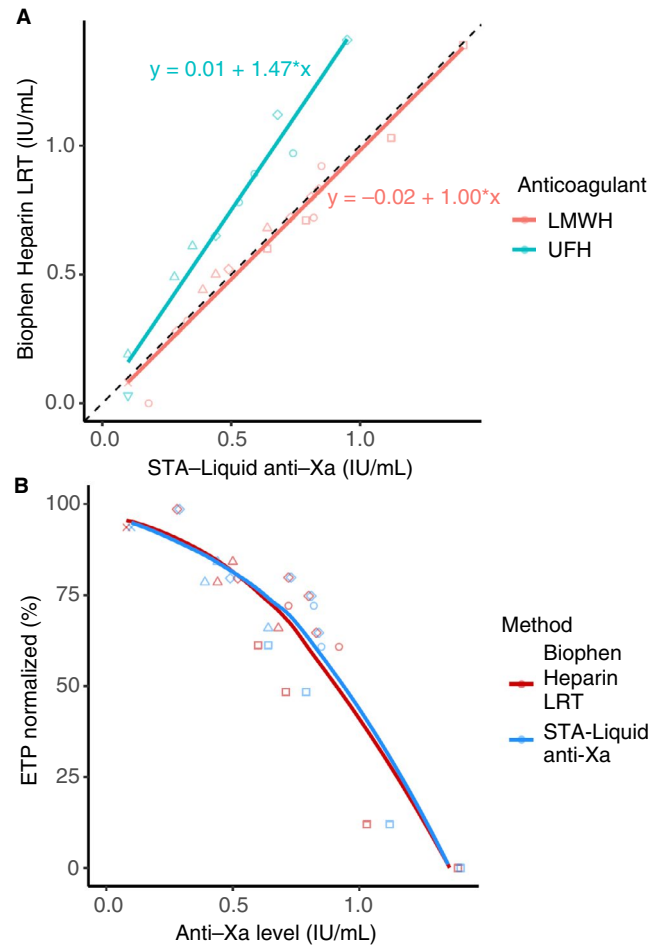


**FIGURE 1** Correlation between anti-Xa levels as measured with two different chromogenic anti-Xa assays and the endogenous thrombin potential (ETP) in plasma samples from patients treated with heparin. A, Correlation between the two chromogenic anti-Xa assays depending on the type of heparin in the sample. The Biophen Heparin LRT overestimates the anti-Xa level of UFH samples compared to the STA-Liquid anti-Xa. B, Correlation between the anti-Xa levels in LMWH samples and the ETP. A progressive inhibition of TG is observed as measured anti-Xa levels increase. TG was studied with the ST-Genesia device using the STG-DrugScreen reagent and results were normalized using a reference plasma provided with STG-DrugScreen reagent. Each subject is represented by a different symbol

Figure 1A); by contrast, anti-Xa levels were not different between the two methods for samples from patients treated with enoxaparin. These results further support the importance of a careful and thorough report on the methods used and of a clear differentiation of the anticoagulants, which frequently influences laboratory tests differently.

A decreased thrombin potential was evidenced in relation with enoxaparin levels as assessed with anti-Xa assays (Figure 1B). With more effective LMWH levels, TG was proportionally reduced. This suggests that that the normal TG profiles identified by the authors could first be the consequence of the low heparin levels measured, either as a consequence of the uncontrolled timing of blood collection with regards to LMWH administration (trough samples) or

because heparin doses were too low with regard to the hyperinflammatory state described in severe COVID-19 patients.

Fourth, to what extent viscoelastometric tests are truly "global" can be challenged. Viscoelastometric tests are attractive because they are performed with whole blood and address not only the platelet-dependent coagulation process, but also clot mechanical properties and fibrinolysis. In our opinion, such tests have major intrinsic limitations though, among which is the initiation of clotting with massive TF concentrations (EXTEM reagents), the weak association with platelet function assays, and that fibrinolysis is initiated by endogenous, bloodborne uninhibited plasminogen activators, which are most often so low that fibrinolysis is negligible.[5,6] To account for the latter issue, the authors added exogenous tPA (of note, at very high concentrations [ie, 625 ng/mL]).[7] However, such modifications of commercial reagents still lack of clinical validation, and could lack sensitivity to the effect of the transient increase of tPA that could be present during initial stages of the disease.[8]

In addition, regarding D-dimer assays, the performance (ie, concordance with other reagents, analytical precision) in high values such as those observed in COVID-19 patients is highly variable, making comparisons of results from studies using different assays hazardous.[9] Moreover, to the best of our knowledge, the performance in high values of the kit used by the authors (HemosIL D-Dimer HS 500) has not been evaluated yet.

Finally, little information was provided regarding the preanalytical step of laboratory tests. For example, the timing of blood collection and centrifugation conditions were not specified, although these variables may have important influence on the tests.[10]

To cope efficiently with hemostatic disturbances related to COVID-19, authors of such studies should be urged to fully report the laboratory methods used and to acknowledge and comprehensively discuss their potential drawbacks. This is essential to enhancing the interpretability and applicability of the results. Studies should also be appropriately designed with regard to their objectives; otherwise, they are at the risk of not being able to make robust conclusions. Therefore, it is of upmost importance to provide the reader all relevant information needed to integrate the ever-growing data accumulating on this topic with the ultimate aim of an elaboration of well-grounded clinical guidance.

## CONFLICT OF INTEREST

The authors declare no competing interest.

## AUTHOR CONTRIBUTIONS

Michael Hardy, Thomas Lecompte, and François Mullier designed the study. Michael Hardy performed the experiments and analyzed the results. Michael Hardy, Thomas Lecompte, and François Mullier drafted the manuscript. All authors reviewed the manuscript for critical content and approved the final version.

## ETHICAL APPROVAL

The observational study was approved by the Ethics Committee of the CHU UCL Namur (NUB B0392020000031).

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# Response to "Studies on hemostasis in COVID-19 deserve careful reporting of the laboratory methods, their significance and their limitation": Don't throw the baby out with the bathwater

Dear Editor,

We appreciate the opportunity to respond to the letter from Dr Hardy and colleagues.[1] The authors stress the large variability observed between different anti-Xa kits available on the market, they contest our results of thrombin generation assay (TGA) in patients with COVID-19 receiving standard- or high-dose prophylaxis and question the relevance of thromboelastography as a global hemostasis assay. We would like to respond to the methodological concerns raised by the authors.

The key concern they raised is the choice of the anti-Xa kit. We used HemosIL Liquid anti-Xa kit (Werfen, Le Pré-Saint-Gervais, France) containing dextran sulfate but no exogenous antithrombin. It is indeed true that different anti-Xa methods can give different results.[2] The anti-Xa methods that are the least influenced by plasma proteins, ie, with dextrans and appropriate dilution, approach the plasma concentration best. In a single plasma sample there is only one heparin concentration, determined essentially by the number of high-affinity pentasaccharide sequences per unit volume. The effect of that concentration differs significantly in normal and even more in patients' plasmas. An anti-Xa test that is sensitive to binding of heparin by acute phase plasma proteins is sensitive to the same plasma variables as the in vivo thrombin generation process and thus foreshadows part of the heparin resistance in patients with COVID-19

infection. It therefore is a conceptual mistake that some tests "*over*estimate" anti-Xa values, but we must admit that inappropriate tests may *under*estimate them. As an example, in the last survey of the External Quality Control for Assays and Tests with Focus on Thrombosis and Haemostasis (ECAT survey 2019-M4), among 142 participant centers, 55 hospital laboratories using Stago Liquid anti-Xa kit without dextran, also used by Hardy et al in their study, underestimated the effect of heparin (0.10 IU/mL with a CV 35.8%) in a plasma sample loaded with unfractionated heparin (UFH) 0.30 IU/mL, compared to HemosIL Liquid anti-Xa kit with dextran used in our study or Hyphen Biomed Biophen LRT with dextran used by Hardy et al (0.29 IU/mL; CV 8.9% and 0.33 IU/mL; 10.2%, respectively). In the same survey, no difference was observed between kits when low molecular weight heparin (LMWH) was present in the plasma sample (LMWH 0.65 IU/mL). Observation reported here by Hardy et al was earlier reported by previous ECAT surveys, which pointed out that Stago Liquid anti-Xa kit underestimates the effect of UFH compared to other kits.

Hardy et al report their own results obtained in COVID-19 patients with two anti-Xa reagents containing (or not) dextran sulfate with different types of heparin and they show, as the ECAT survey did, no significant difference is observed between two methods for plasmas from patients treated with enoxaparin. In our study, the large majority (91%) of patients received prophylaxis with enoxaparin and according to the findings reported by Hardy et al our results should not be influenced by the presence of dextran contained in the