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DR MICHAËL HARDY (Orcid ID : 0000-0001-6701-9417)

PROF. JONATHAN DOUXFILS (Orcid ID : 0000-0002-7644-5298)

PROF. PIERRE FONTANA (Orcid ID : 0000-0003-1546-0774)

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

Michael Hardy*,†, Jonathan Douxfils‡,§, Marion Bareille*, Sarah Lessire†, Isabelle Gouin-Thibault¶, Pierre Fontana**, Thomas Lecompte**, François Mullier*

*Université catholique de Louvain, CHU UCL Namur, Namur Thrombosis and Hemostasis Center (NTHC), NARILIS, Hematology Laboratory, Yvoir, Belgium.

†Université catholique de Louvain, CHU UCL Namur, Namur Thrombosis and Hemostasis Center (NTHC), NARILIS, Anesthesiology Department, Yvoir, Belgium.

‡Université de Namur, Département Pharmacie, Namur Thrombosis and Hemostasis Center (NTHC), NARILIS, Namur, Belgium.

§ Qualiblood s.a., Namur, Belgium.

¶INSERM, CIC 1414 (Centre d'Investigation Clinique de Rennes), Université de Rennes, CHU de Rennes, Département d'Hématologie Biologique, Rennes, France.

**Département de Médecine, Hôpitaux Universitaires de Genève, service d'angiologie et d'hémostase et Faculté de Médecine, Geneva Platelet Group (GpG), Université de Genève, Geneva, Switzerland.

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Corresponding author:
Prof François Mullier
CHU UCL Namur
Hematology laboratory
Avenue G. Thérasse, 1
B-5530 Yvoir – Belgium
Tel + 32 (0)81 42 49 86
Francois.mullier@uclouvain.be

Dear Editor,

We read with much interest the recent observational study of Nougier et al., which aimed at studying thrombin generation (TG) and fibrinolysis profiles of COVID-19 patients admitted to an intensive care unit (ICU) or to an internal medicine ward and receiving various schemes of prophylactic heparin.[1] They reported that thrombin potential remained within normal range despite heparin and that fibrinolysis was decreased in relation with increased plasminogen activator inhibitor 1 (PAI-1) and thrombin-activatable fibrinolysis inhibitor (TAFI) antigen plasma levels. Using the rotational thromboelastometry (ROTEM) delta device with EXTEM reagents and the addition of 0.625µg/mL tPA (referred to as 'TEM-tPA'), they reported decreased clot lysis in COVID-19 patients, which was more pronounced in patients who presented a thrombotic event, compared to 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 authors 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 (PF4), which can be released by activated platelets.[3] Furthermore, the authors did not differentiate in the analysis patients according to the heparin they received (UFH or low molecular weight heparins (LMWH)) while they have different effects on laboratory tests (anti-Xa activity, TG and ROTEM). Of note, some anti-Xa kits containing exogenous AT can also lead to an overestimation of heparin levels in case of AT deficiency, which was infrequent in this series though.[3]

Second, the authors found *in vitro* TG (calibrated automated thrombogram (CAT), reagent with the high tissue factor (TF) concentration (PPP-High)) within normal range despite prophylactic heparin

administration. Based on this finding, they conclude that the patients 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 dosages were used (standard and intensified prophylaxis). Mean anti-Xa levels measured were low (0.35 +/- 0.20 IU/mL) and those levels could have been overestimated in the presence of dextran in the reagents as discussed above. It would thus not be unexpected to observe normal TG profiles. Of note, the reagent they used for TG (i.e. 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 (i.e. 20 pM TF). The ability of such analytical conditions to evidence 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 (i.e. failure to achieve the therapeutic target (activated partial thromboplastin time (aPTT) or anti-Xa levels) despite the administration of recommended UFH doses (i.e. 400 to 600 IU/kg/day)) cannot be asserted based on TG, since the corresponding inhibition of TG under current, commercially available conditions (CAT 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 PF4 or heparanases. Elevated factor VIII or fibrinogen levels can also shorten the aPTT but without any effect on the anti-Xa assays. Altogether, to our opinion, the observation of normal TG profiles despite heparin administration rather reflects the low heparin levels measured with regards 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, Asnières-sur-Seine, France, 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 ICU (11 samples from patients treated with weight-adjusted UFH (Leo Pharma, Lier, Germany) and 27 samples from patients treated with weight-adjusted enoxaparin (Sanofi, Paris, France)). We included both peak and trough samples to cover a wide range of anti-Xa levels. Blood was drawn into 109mM sodium citrate tubes, underwent a double centrifugation (at 1500g for 15 minutes at room temperature) within one hour after blood collection and plasma samples were frozen at -80°C. We studied TG with the ST-Genesia analyzer using STG-DrugScreen reagent (i.e. 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 the anti-Xa levels compared to 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; **Fig. 1A**); by contrast, anti-Xa levels were not different between both 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 differently influence laboratory tests.

A decreased thrombin potential was evidenced in relation with enoxaparin levels as assessed with anti-Xa assays (**Fig. 1B**). With more effective LMWH levels, thrombin generation 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 regards to the hyperinflammatory state described in severe COVID-19 patients.

Fourth, to what extent viscoelastometric tests (VETs) 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 at fibrinolysis. In our opinion such tests have major intrinsic limitations though, among which the initiation of clotting with massive TF concentrations (EXTEM reagents), the weak association with platelet function assays and the fact that fibrinolysis is initiated by endogenous, blood-borne 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 very high concentrations, i.e. 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-dimers assays, the performance (i.e 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, while these variables may have important influence on the tests.[10]

In order to cope efficiently with hemostatic disturbances related to COVID-19, authors of such studies should be urged to fully report on the laboratory methods used and to acknowledge and to comprehensively discuss their potential drawbacks. This is essential to enhance the interpretability and the applicability of the results. Studies should also be appropriately designed with regards 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.

Declarations

Ethical Approval

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

Competing interests

The authors declare no competing interest.

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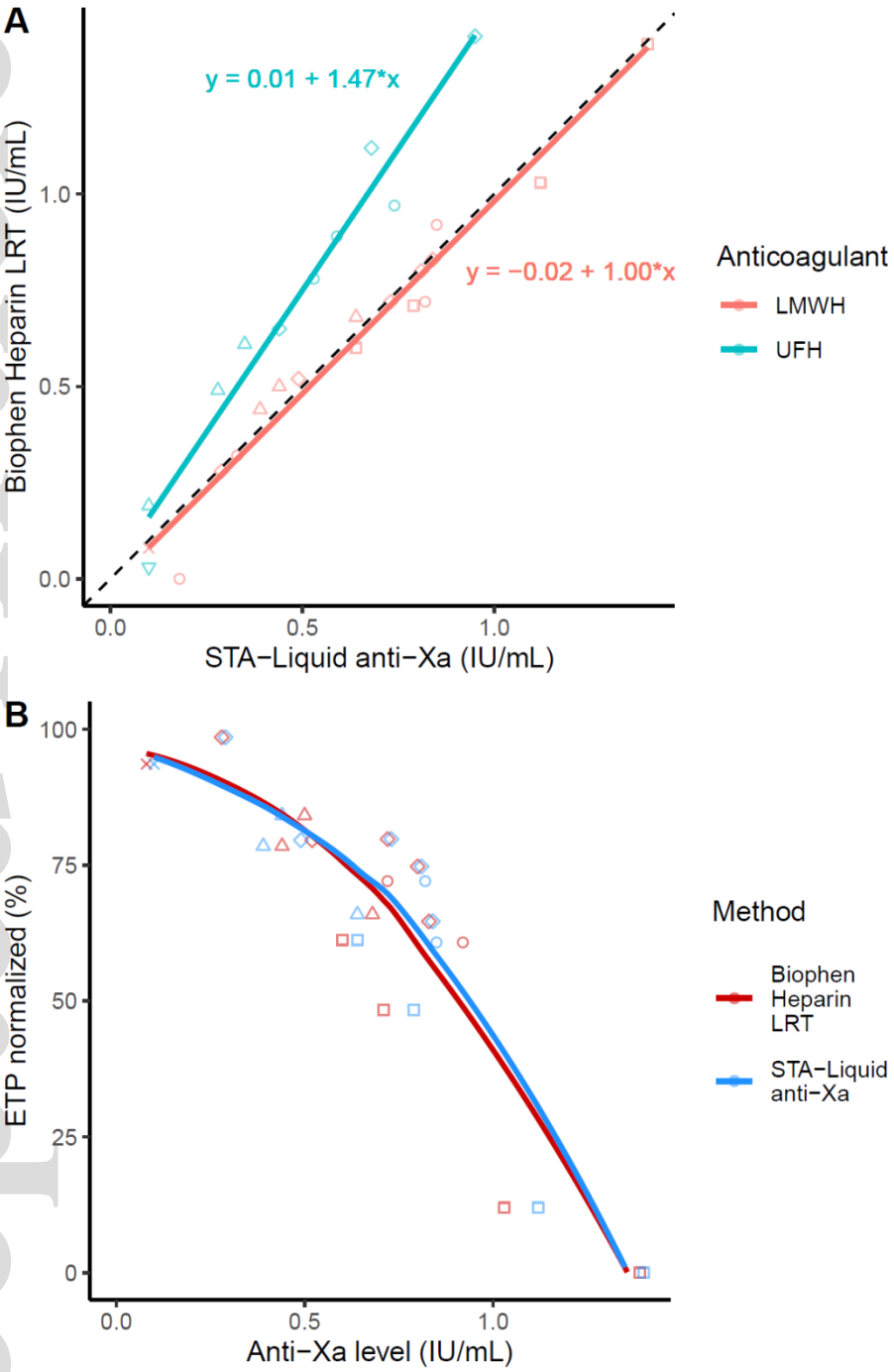
Authors' contributions

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

Figure

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.** Panel A represents the 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. Panel B represents the 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.



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