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Letter to the Editors

Influence of C-reactive protein on thrombin generation assay

Marie Didembourg[1], Jonathan Douxfils[1,2], François Mullier[3], Michael Hardy[4], Julien Favresse[1,5], Laure Morimont[1,2]

[1] *University of Namur, Faculty of Medicine, Department of Pharmacy, Namur Research Institute for Life Sciences (NARILIS), Namur Thrombosis and Hemostasis Center (NTHC), Namur, Belgium,*

[2] *Qualiblood sa, Namur, Belgium*

[3] *Université catholique de Louvain, CHU UCL Namur, Laboratory Hematology, Namur Research Institute for Life Sciences (NARILIS), Namur Thrombosis and Hemostasis Center (NTHC), Yvoir, Belgium*

[4] *Université catholique de Louvain, CHU UCL Namur, Department of Anesthesiology, Namur Research Institute for Life Sciences (NARILIS), Namur Thrombosis and Hemostasis Center (NTHC), Yvoir, Belgium*

[5] *Clinique Saint-Luc Bouge A.S.B.L. "Santé & Prévoyance"*

Corresponding author:

Laure Morimont

[1]University of Namur, Faculty of Medicine, Department of Pharmacy, Namur Research Institute for Life Sciences (NARILIS), Namur Thrombosis and Hemostasis Center (NTHC), Namur, Belgium

[2]Qualiblood sa, Namur, Belgium;

Rue du Séminaire 20a, 5000 Namur, Belgium

Mail: Laure.morimont@qualiblood.eu

Phone: +32 81 444 992

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To the editors,

A close association between inflammatory state, C-reactive protein (CRP) and thromboembolic events has been described at least a decade ago [1] but has resurfaced recently with the outbreak of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[2] CRP is an acute phase reactant plasma protein considered as a systemic biomarker representative of the total burden of inflammation but also linked to the development of pro-thrombotic states.[1, 2] ~~Through direct interaction with Fcγ receptors (FcγRs) on smooth muscles cells or vascular endothelial cells, CRP promotes discharge of tissue factor (TF), production of pro inflammatory cytokines and release of plasminogen activator inhibitor.[2] The CRP-FcγR interaction also inhibits the liberation of tissue plasminogen activator, modifying the fibrinolytic balance and reducing intravascular fibrin clearance.[1]~~ Under normal circumstances, plasma CRP concentrations stand between 0,2 and 8,0mg/L, but patients being affected by diseases or conditions characterized by a strong inflammation express CRP levels up to 1000 times higher.[3] Persistent elevated CRP levels, observed in moderate to severe COVID-19 patients, in sepsis or in other chronic inflammatory diseases (e.g. inflammatory bowel~~s~~ disease) [4, 5], are reflected by higher risk of cardiovascular complications, especially venous thromboembolism and pulmonary thrombosis.[6] The assessment of the pro-thrombotic state of these patients with global coagulation tests like thrombin generation assays (TGA) may be relevant to assess the evolution of the disease or identify its severity, although their clinical utility remained to be confirmed. Nevertheless, CRP impacts coagulation assays, especially the activated partial thromboplastin time (aPTT) which has been reported to be dose-dependently prolonged in presence of CRP concentrations encountered in inflammatory states.[7, 8] It has also been demonstrated that phospholipids act as catalytic surfaces and, depending on their component, form complex with CRP leading to potential disturbance of TGA on the Calibrated Automated Thrombogram (CAT).[7] This study aims to assess how CRP impacts TGA on the ST-Genesia system. A comparison with the CAT system was performed, using the same triggering reagent, as differences between both platforms have been reported.[9]

The study protocol was in accordance with the Declaration of Helsinki. Recruitment of healthy volunteers for the constitution of a normal pooled plasma (NPP) has been approved by the Ethical Committee of the CHU-UCL Namur, Yvoir, Belgium (approval number: B03920096633). Trisodium citrate tubes (3.2% i.e., 0.109M) were used for blood collection. Platelet-poor-plasma was obtained from the supernatant fraction after a double centrifugation for 15 minutes at 2500g . NPP was constituted of 50 healthy individuals (median age= 20 years,

from 18 to 56 years; mean BMI= 23 kg.m^{-2}) not carrier of a factor V Leiden or G20210A mutation. Human C-reactive protein (Merk KGaA, Darmstadt,Germany) was spiked in NPP at five ~~plasma~~ concentrations (0 [phosphate buffer saline], 50, 100, 200, and 350 mg/L). These concentrations were confirmed by an immunoturbidimetric assay on the Cobas® 8000 (Roche Diagnostics, Meylan, France). The selected concentrations correlated with CRP levels observed in patients suffering from inflammatory diseases such as COVID-19 or rheumatoid arthritis.[3, 6] Thrombin generation (TG) was first assessed on the new automated system, the ST-Genesia (Diagnostica Stago, Asnières-sur-Seine, France). Secondly, TG was assessed on the CAT using the Thrombinoscope software version 5,0 (Thrombinoscope bv, Maastricht, the Netherlands). The triggering reagent on both platforms was the STG-ThromboScreen-TM (Diagnostica Stago) which contains phospholipids and TF (exact concentrations not provided by the manufacturer). Both TGA methods were performed in duplicate and assessed by 3 independent runs on each platform. The aPTT was measured at the highest CRP concentration (350 mg/L) and compared to the NPP buffer concentration ~~(0 mg/L)~~ in order to confirm that our model was able to replicate previous observations and to validate our experiments.[7] The aPTT was performed on a STA R Max system (Diagnostica Stago) using Dade®Actin®FS (Siemens Healthcare, Munich, Germany) as activator reagent, which has been previously proven to be sensitive to the presence of CRP.[7] All data and statistical analysis were~~-~~ processed and performed using GraphPad Prism 8,0 for macOS (GraphPad Software, San Diego, California USA, www.graphpad.com). Mean velocity rate index (mVRI) was calculated for TGA performed on the ST-Genesia system as the algorithm does not determine~~d~~ it automatically. The ratio for the lag time (LT), time-to-peak (Ttpeak), endogenous thrombin potential (ETP), peak height (PH), and mVRI were calculated relative to the NPP buffer concentration ~~(i.e. 0 mg/L).~~ Descriptive statistics were used to describe the data. An ordinary one-way ANOVA was performed to assess the differences on TGA parameters of the 5 CRP concentrations tested. In case of significance, a Tukey's multiple comparison test was performed. Difference between both analyzers was assessed using a paired t-test with individual variance computed for each comparison followed by a Holm-Sidak's multiple comparison test. The threshold for significance was set at 0,05.

Mean values and mean ratios ± standard deviation (SD) and coefficient of variation (CV%) were calculated for each TGA parameter at each plasma CRP concentrations. Results are reported in **Table 1**. Based on mean values, no statistically significant difference was observed between the five tested concentrations (ANOVA p-value>0,05), regardless of the platform used. However, on the CAT system, a decrease of 7% of the ETP and an

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increase of 10% of the mVRI were observed at the highest CRP concentration (350 mg/L) compared to NPP buffer concentration.

Comparison between both platforms, represented in Figure 1, showed significant difference for LT and Ttpeak (Holm-Sidak p-value<0,05). LT was significantly higher at CRP levels of 50, 100 and 150 mg/L as well as Ttpeak at CRP levels of 50 and 150 mg/L when TGA was assessed on the CAT compared to the ST-Genesia System. Significant differences observed between LT and Ttpeak are possibly associated with the specific algorithm ~~signal acquisition sequence~~ ~~specific of~~to ~~to~~ each platform. The ETP was not significantly different between both equipment (p-value>0,05).

mg/L of CRP. This corresponds to a prolongation of 4,2 s and a ratio of 1,15 when compared to the baseline. ~~This~~ A similar increase was ~~previously~~ also observed by Devreese *et al.* [7].

~~As expected, the aPTT performed in our study was prolonged by 1,15 at CRP concentration of 350 mg/L. This correlates with results previously obtained in the study of Devreese et al.~~

Different hypotheses may explain the absence of the impact of CRP on TGA. Firstly, it has been shown that the impact on aPTT was reagent-dependent, suggesting that the composition of the phospholipids content of the reagents influences the impact of CRP on aPTT clotting times.[7] It is therefore possible that the phospholipid~~s~~ content of the STG-ThromboScreen is not or weakly sensitive to the presence of CRP. Secondly, aPTT and TGA induce coagulation through different pathways. Indeed, aPTT induces coagulation through the intrinsic pathway whereas in TGA, the TF contained in the reagent activates the extrinsic pathway. A third hypothesis which completes the second one is the way TG is dependent to phospholipids. Namely, it has been shown that TG reaches a plateau at phospholipids concentrations above ± 3µM meaning that if the residual non-complexed phospholipids is above this threshold, the impact on TG would be negligible.[10] However, as no formal information is provided by the manufacturer regarding the final phospholipids concentration in the STG-ThromboScreen reagent, this is only an assumption.

~~Thus, the~~ ~~The~~ The weak affinity of CRP for the phospholipids molecules contained in the STG-ThromboScreen, the difference in the triggering coagulation pathway and the weaker dependence for phospholipids of the TF-induced TG are hypotheses that may explain the robustness of TG towards CRP. In conclusion, this study demonstrated that CRP levels up to 350 mg/L did not impact significantly TG performed either on a CAT or on a

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ST-Genesia system. Thrombin generation assay is therefore an efficient test to assess the hemostatic function of patients with elevated CRP, like those in sepsis and suffering from chronic or acute inflammatory conditions.

Conflict of Interest

Among the authors. J. Douxfils is CEO and founder of QUALIblood s.a.. a contract research organization manufacturing the DP-Filter. is co-inventor of the DP-Filter (patent application number: PCT/ET2019/052903) and reports personal fees from Daiichi-Sankyo. Mithra Pharmaceuticals. Stago. Roche and Roche Diagnostics outside the submitted work. F. Mullier reports institutional fees from Stago, Werfen, Nodia, Roche Sysmex and Bayer. He also reports speaker fees from Boehringer Ingelheim, Bayer Healthcare, Bristol-MyersSquibb-Pfizer, Stago, Sysmex and Aspen all outside the submitted work. The other authors have no conflicts of interest to disclose.

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Figure 1: ***Mean values of each TGA parameter; (A) ETP, (B) mVRI, (C) Lag time, (D) Time-to-peak and (E) Peak height; at each CRP concentration (0 – 50 – 100 – 200 – 350 mg/L) for both equipments****. Data from CAT system are shown in blue. Data from ST Genesia system are shown in orange.*

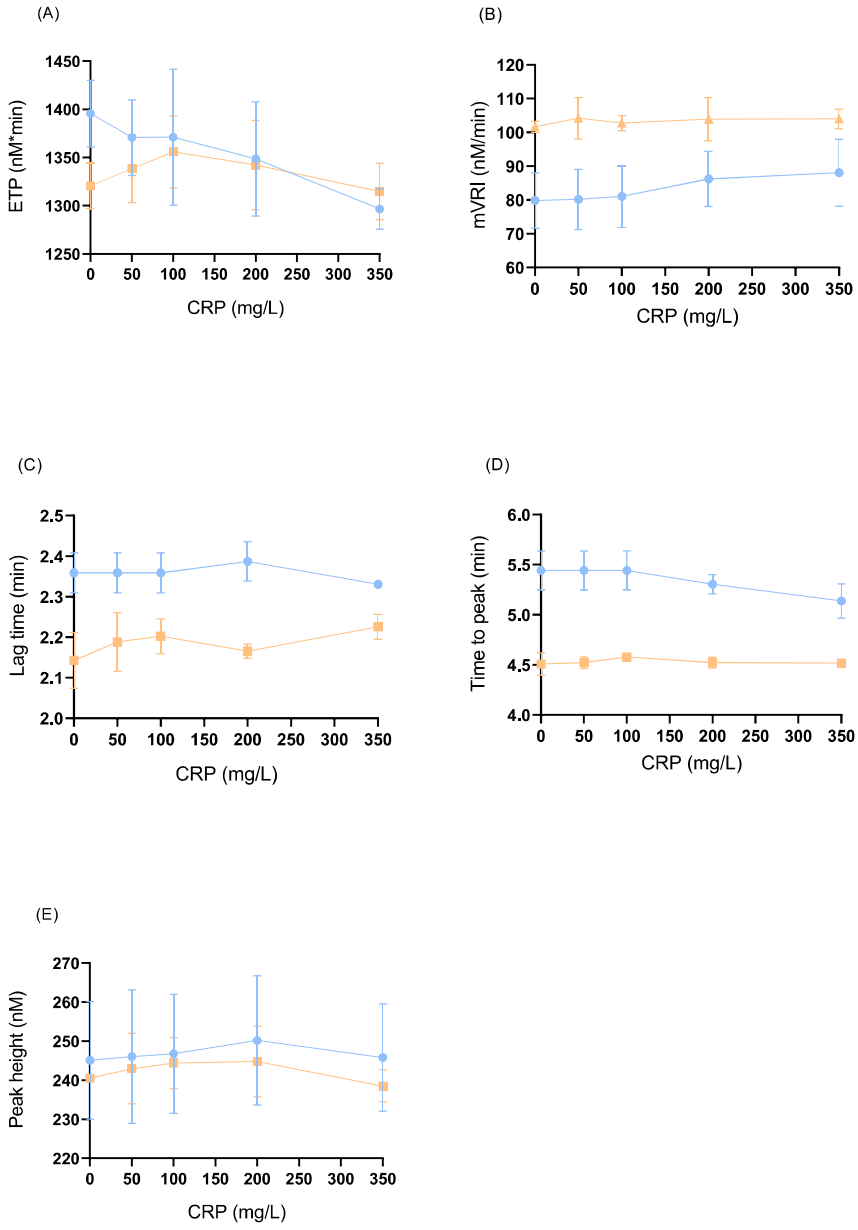

Table 1: ***Mean values ± SD , mean ratios ± SD and calculated CV (%) of TGA parameters reported for each plasma CRP concentration on the CAT and the ST GENESIA system. Reference intervals described for each TGA parameter on both equipments are extracted from the article of Talon et al.[9]*** *P-value of the one-way ANOVA expresses difference between CRP concentrations for each analyzer. P-value of the Holm-Sidak's multiple comparison test shows the difference between equipment for each CRP concentration and each parameter.*

TGA parameters	CRP (mg/L)																			
	0 mg/mL			50 mg/mL				100 mg/mL				200 mg/mL				350 mg/mL				
	Mean values ± SD	CV (%)	Mean ratios ± SD	Mean values ± SD	CV (%)	Mean ratios ± SD	CV (%)	Mean values ± SD	CV (%)	Mean ratios ± SD	CV (%)	Mean values ± SD	CV (%)	Mean ratios ± SD	CV (%)	Mean values ± SD	CV (%)	Mean ratios ± SD	CV (%)	p-value of the one-way ANOVA
ETP (nM*min)																				
CAT system [1090-1320]	1395,53 ± 34,53	2,47%	1,00 ± 0,00	1370,78 ± 39,27	2,86%	0,98 ± 0,01	1,13%	1371,00 ± 70,63	5,15%	0,98 ± 0,01	0,75%	1348,65 ± 59,52	4,41%	0,97 ± 0,01	0,70%	1296,53 ± 21,10	1,63%	0,93 ± 0,00	0,19%	*0,207*
ST GENESIA system [1256-1505]	1320,39 ± 23,83	1,80%	1,00 ± 0,00	1338,40 ± 35,25	2,63%	1,01 ± 0,03	2,67%	1355,93 ± 37,60	2,77%	1,03 ± 0,01	0,97%	1342,30 ± 46,36	3,45%	1,02 ± 0,02	1,76%	1314,69 ± 29,31	2,23%	1,00 ± 0,01	0,83%	*0,623*
p-value of the Holm-Sidak test	*0,056*			*0,851*				*0,851*				*0,851*				*0,260*				
mVRI (nM*min-1)																				
CAT system [65-105]	79,83 ± 8,4	10,32%	1,00 ± 0,00	80,16 ± 8,90	11,10%	1,00 ± 0,01	1,33%	81,04 ± 9,05	11,17%	1,01 ± 0,01	0,86%	86,21 ± 8,20	9,52%	1,08 ± 0,01	0,59%	88,06 ± 9,95	11,30%	1,10 ± 0,03	2,50%	*0,705*
*ST GENESIA system**	101,68 ± 4,93	4,85%	1,00 ± 0,00	104,19 ± 9,18	8,81%	0,98 ± 0,03	3,05%	102,79 ± 4,99	4,85%	1,00 ± 0,04	4,41%	103,95 ± 4,15	3,99%	0,99 ± 0,02	2,06%	104,03 ± 2,82	2,71%	1,01 ± 0,02	1,66%	*0,821*
p-value of the Holm-Sidak test	*0,053*			*0,061*				*0,061*				*0,082*				*0,082*				
Lag time (min)																				
CAT system [2,5-3,0]	2,36 ± 0,05	2,08%	1,00 ± 0,00	2,36 ± 0,05	2,08%	1,00 ± 0,00	0,00%	2,36 ± 0,05	2,08%	1,00 ± 0,00	0,00%	2,39 ± 0,05	2,06%	1,01± 0,02	1,70%	2,33 ± 0,00	0,00%	0,99 ± 0,02	1,62%	*0,655*
ST GENESIA system [2,3-2,8]	2,14 ± 0,07	3,21%	1,00 ± 0,00	2,19 ± 0,07	3,30%	1,02 ± 0,05	4,77%	2,20 ± 0,04	1,97%	1,03 ± 0,03	3,34%	2,17 ± 0,02	0,82%	1,01 ± 0,02	2,36%	2,23 ± 0,03	1,38%	1,04 ± 0,02	2,18%	*0,376*
p-value of the Holm-Sidak test	*0,058*			*0,033**				*0,033**				*0,033**				*0,055*				
Time to peak (min)																				
CAT system [5,2-6,3]	5,44 ± 0,20	3,61%	1,00 ± 0,00	5,44 ± 0,20	3,62%	1,00 ± 0,00	0,00%	5,44 ± 0,20	3,61%	1,00 ± 0,00	0,00%	5,31 ± 0,10	1,80%	0,98 ± 0,01	0,72%	5,14 ± 0,17	3,33%	0,94 ± 0,01	0,80%	*0,989*
ST GENESIA system [4,3-5,4]	4,51 ± 0,11	2,45%	1,00 ± 0,00	4,52 ± 0,06	1,26%	1,00 ± 0,04	3,56%	4,58 ± 0,02	0,52%	1,02 ± 0,02	1,91%	4,52 ± 0,05	1,20%	1,00 ± 0,03	2,95%	4,52 ± 0,03	0,60%	1,00 ± 0,02	2,08%	*0,673*
p-value of the Holm-Sidak test	*0,056*			*0,047**				*0,056*				*0,047**				*0,056*				
Peak height (nM)																				
CAT system [200-262]	245,11 ± 15,00	6,12%	1,00 ± 0,00	246,06 ± 17,04	6,93%	1,00 ± 0,01	1,31%	246,77 ± 15,27	6,19%	1,01 ± 0,02	1,93%	250,21 ± 16,61	6,64%	1,02 ± 0,01	0,50%	245, 83 ± 13,73	5,58%	1,00 ± 0,00	0,14%	*0,995*
ST GENESIA system [220-325]	240,54 ± 0,47	0,20%	1,00 ± 0,00	242,95 ± 9,04	3,72%	1,01 ± 0,04	3,53%	244,36 ± 6,60	2,70%	1,02 ± 0,03	2,53%	244,75 ± 9,02	3,68%	1,02 ± 0,04	3,51%	238,52 ± 4,07	1,71%	0,99 ± 0,01	1,51%	*0,757*
p-value of the Holm-Sidak test	*0,985*			*0,985*				*0,985*				*0,985*				*0.949*				

* Ranges are not set for the parameter mVRI for the ST-Genesia as this parameter was calculated.