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BRIEF REPORT

Is lupus anticoagulant testing with dilute Russell's viper venom clotting times reliable in the presence of inflammation?

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Abstract

Background: Testing for lupus anticoagulant (LA) is not recommended in case of inflammation as C-reactive protein (CRP) can interfere *in vitro* with the phospholipids present in the activated partial thromboplastin time test used to detect an LA. However, the potential interference of an acute phase protein (ie, CRP) in LA testing using the dilute Russell's viper venom (DRVV) test is poorly studied.

Objectives: To study the effect of inflammation, as evidenced by increased CRP levels, on DRVV tests.

Methods: First, a retrospective analysis (2013-2023) was performed: data on all LA workups were retrieved, and the association between CRP levels and DRVV screen, mix, and confirm clotting times was studied. Second, data on DRVV panels and CRP levels were extracted from 2 prospective studies involving intensive care unit patients to study the association between both variables. Third, CRP was added to normal pooled plasma at 6 relevant concentrations (up to 416 mg/L) to study the association between CRP itself and DRVV coagulation times.

Results: In the retrospective analysis, DRVV screen and confirm clotting times significantly increased as CRP increased (increase of 0.11 seconds and 0.03 seconds per 1 mg/L increase of CRP level, respectively). In the prospective analysis, only DRVV screen was prolonged with high CRP levels (increase of 0.06 seconds for a 1 mg/L increase in CRP level); DRVV screen/confirm ratio was also increased with high CRP levels. *In vitro*, the addition of CRP did not significantly increase any DRVV clotting times.

Conclusion: LA testing should be performed with much caution in the presence of inflammation as it may be associated with prolongation of both activated partial thromboplastin time and DRVV clotting times.

KEYWORDS
antiphospholipid antibodies, C-reactive protein, dilute Russell's viper venom, inflammation, lupus anticoagulant

Essentials

- The aPTT can be increased by a high CRP level, but the effect of inflammation on DRVVT is poorly studied.
- We assessed the association between DRVVT and CRP levels retrospectively, prospectively and *in vitro*.
- DRVVT is increased in the presence of inflammation, but this is not the result of direct CRP interference.
- Lupus anticoagulant testing should be performed with much caution in the presence of inflammation.

1 | INTRODUCTION

Antiphospholipid antibodies have been suggested by some authors as a potential contributor to the high incidence of thrombotic complications observed in COVID-19 patients [1–3]. However, antiphospholipid antibodies were mostly detected during the first wave of COVID-19 before the widespread use of dexamethasone [4]. In addition, most patients were single positive for lupus anticoagulant (LA), which did not persist beyond the acute phase, and the association of antiphospholipid antibodies with increased thrombotic risk has been debated [5–8].

The usual first step in laboratory diagnosis of these antibodies consists of the search for anticardiolipin, anti–beta-2-glycoprotein I, and LA. The first 2 are identified with solid-phase immunoassays (eg, enzyme-linked immunosorbent assay), while the latter is with clotting tests (sensitive activated partial thromboplastin time [aPTT]– and dilute Russell's viper venom [DRVV]–based clotting times). However, LA tests are subject to several interferences, which are commonly encountered in COVID-19 patients: aPTT-based tests are sensitive to the presence of anticoagulant drugs [9–12] and the interference of C-reactive protein (CRP) per se, ascribed to binding to phospholipids, part of clotting reagents [9,13,14]. DRVV tests (DRVVTs) can also be sensitive to residual oral anticoagulant drugs levels (vitamin K antagonists and direct oral anticoagulants [DOACs]) [15] but are only sensitive to high levels of unfractionated heparin (UFH) since a heparin inhibitor is present in the reagent [9] and are generally considered unaffected during inflammation, even if few data support this affirmation [14,16].

We suspected that inflammation might also interfere with DRVVTs during LA testing. In retrospective and prospective cohorts, we investigated the effect of inflammation, as evidenced by elevated CRP levels, on DRVVTs and sought to determine whether this interference was mediated by CRP per se.
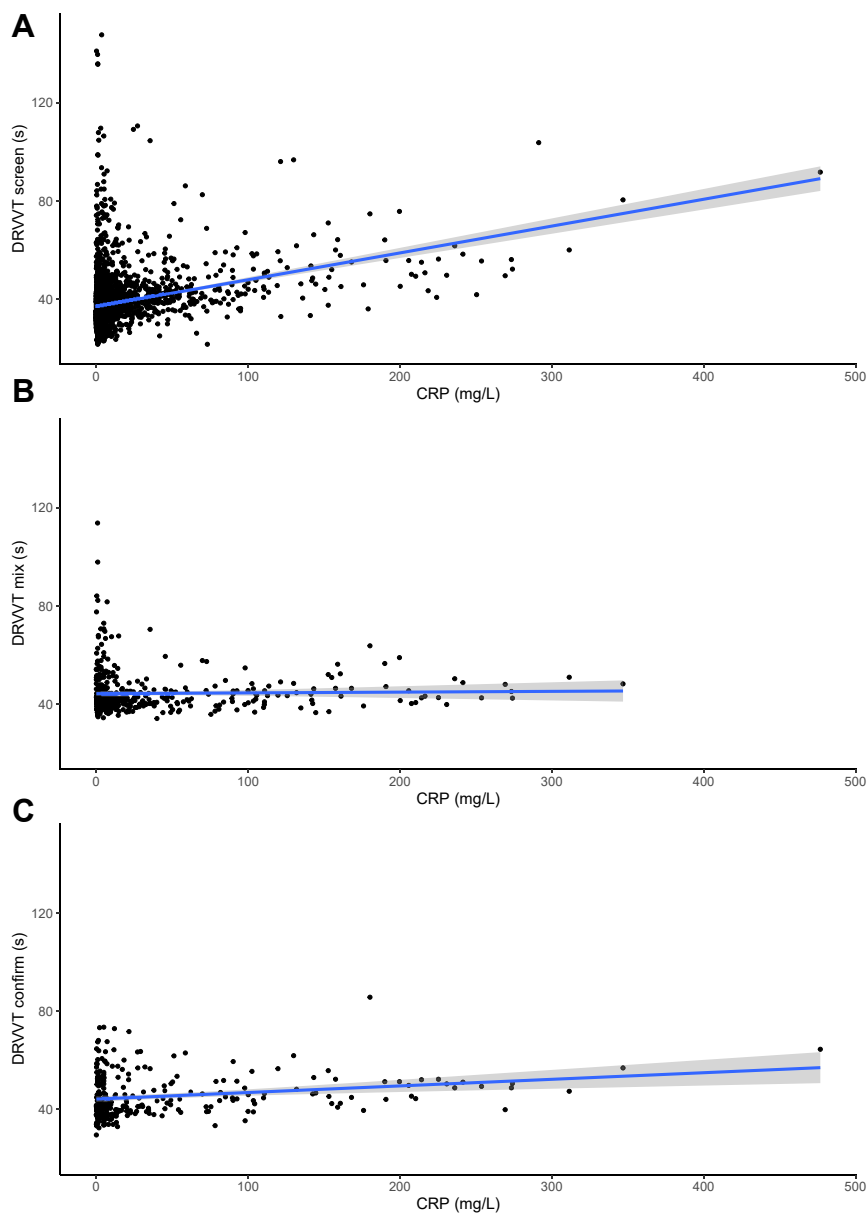
2 | METHODS

First, we reviewed all LA workups performed in our laboratory over the last 10 years (from November 1, 2013, to October 31, 2023), including at least a DRVV screen test. For 3702 out of 6699 such workups, a CRP measurement was available on the same sampling. The detection of LA was performed with a 3-step sequential procedure with double centrifuged (1500 × g for 15 minutes at 20 °C) plasma [17]: an initial screening test, then a mixing test (mixing with a commercial normal pooled plasma [CRYOcheck, Cryopep, Precision Biology] to rule out coagulation factors

defects), and a final confirmatory test, which consists of adding a higher concentration of phospholipids to overcome the LA effect. We used STA-Staclot DRVV Screen and Confirm reagents, PTT-LA and Staclot LA, and a STA R Max coagulometer (Diagnostica Stago). The LA reagents we used did not change over the study period. Every patient presenting for LA workup was asked about her or his medication intake thanks to gatekeeping, physician's computerized order entry. We excluded tests performed for patients receiving UFH (anti-Xa, ≥0.8 U/mL; as a heparin inhibitor is present in the DRVV reagent used), DOACs (anti-Xa, >30 ng/mL; measured using dedicated chromogenic tests performed for each sample from patients on DOAC), or vitamin K antagonists (international normalized ratio, ≥1.5). Patients in whom persistence of LA after at least 12 weeks could be confirmed were considered true LA and excluded from the analysis (n = 34); furthermore, for this subset of patients, no association between CRP levels and DRVV clotting times was identified (not shown). We used linear regression to examine the association of CRP levels with coagulation times after activation with DRVV (Figure 1).

To further examine the association between DRVV clotting times and CRP levels, we retrieved plasma samples from patients with severe inflammation (high CRP levels) prospectively collected and frozen at −80 °C as part of 2 studies performed in patients admitted to the intensive care unit (ICU): one was a longitudinal study performed on COVID-19 patients admitted to the ICU, and the other focused on the monitoring of UFH in a general ICU population (Ethics Committee NUBs: B0392020000031 and B039201940886) [18–20]. Only patients on UFH therapy or without anticoagulant treatment were considered for inclusion. Briefly, 109 mM citrated plasma collected as part of daily care was double centrifuged at 1500 × g for 15 minutes at room temperature and frozen at −80 °C. Plasma samples were then thawed at 37 °C for 5 minutes, and DRVV screen and confirm tests were performed twice during the ICU stay for each patient as part of both study protocols; mixing tests were not performed due to a limited volume of plasma frozen. Later, additional blood samples were retrieved to cover CRP levels up to 400 mg/L (maximum of 2 additional samples per patient). CRP levels (CRP Gold Latex, DiAgam) and UFH anti-Xa levels (Liquid anti-Xa, Diagnostica Stago) were measured as part of daily clinical management. Associations between CRP levels and DRVV clotting times were analyzed using mixed-effects linear models to account for possible correlations between measurements performed in the same patient; anti-Xa levels were included in all models as a potential confounder by modeling a node at 0.8 U/mL anti-Xa level to account for the presence of a heparin inhibitor in the reagent capable of neutralizing heparin up to this level, according to the manufacturer.

FIGURE 1 Retrospective analysis of the association of C-reactive protein (CRP) levels with dilute Russell's viper venom (A) screen (n = 3702; P < .0001), (B) mix (n = 401; P = .05), and (C) confirm (n = 243; P = .0006) clotting times. Linear regression was used to model the association between both variables. Dots represent individual data, and the line represents the linear regression line (with its 95% CI). Patients with a residual effect of heparin (anti-Xa level, ≥0.8 U/mL), direct oral anticoagulants (plasma level, >30 ng/mL), or vitamin K antagonists (international normalized ratio, ≥1.5) and patients for whom lupus anticoagulant persistence was confirmed at 12 weeks were excluded. Note that for dilute Russell's viper venom test (DRVVT) mix, the abscissa represents the CRP level before dilution with normal pooled plasma.

RESULTS AND DISCUSSION

In the retrospective study, CRP levels were statistically associated with an increase in DRVV screen clotting time (increase of 0.11 seconds per 1 mg/L increase in CRP level; P < .0001; n = 3702), but not after mixing with normal plasma (DRVV mix; P = .05; n = 401).

Finally, a spiking experiment was performed with CRP to assess whether the interference in the DRVVT was due to CRP itself, as demonstrated for aPTT-based tests reagent [13], or not. Commercial normal pooled plasma (CRYOcheck) was spiked with human CRP (from human pleural fluid, purity >98%; Sigma Aldrich) at 6 final concentrations (0, 26, 52, 104, 208, and 416 mg/L) in triplicate. DRVV screen, mix, and confirm tests were performed as previously described.

DRVV clotting time after the addition of a higher concentration of phospholipids (DRVVT confirm) also slightly increased with increasing CRP levels (increase of 0.03 seconds per 1 mg/L increase in CRP level; P = .0006; n = 243). We also identified an association between PTT-LA and Staclot LA and CRP levels (P < .0001 and P = .01, respectively).

In the prospective cohorts, we analyzed 89 plasma samples from 66 patients (46 tests from the COVID cohort and 43 tests from the general ICU cohort). We identified an association between increasing CRP levels and prolonged DRVV screen clotting times (increase of 0.06 seconds for a 1 mg/L increase of CRP level; P = .0002), but not with DRVV confirm test (P = .60; Figure 2). DRVV screen/confirm ratio also increased with increasing CRP levels (P < .001; Figure 3). Both tests were influenced by increased UFH anti-Xa levels above 0.8 U/mL (P = .0006 and P = .005, respectively).

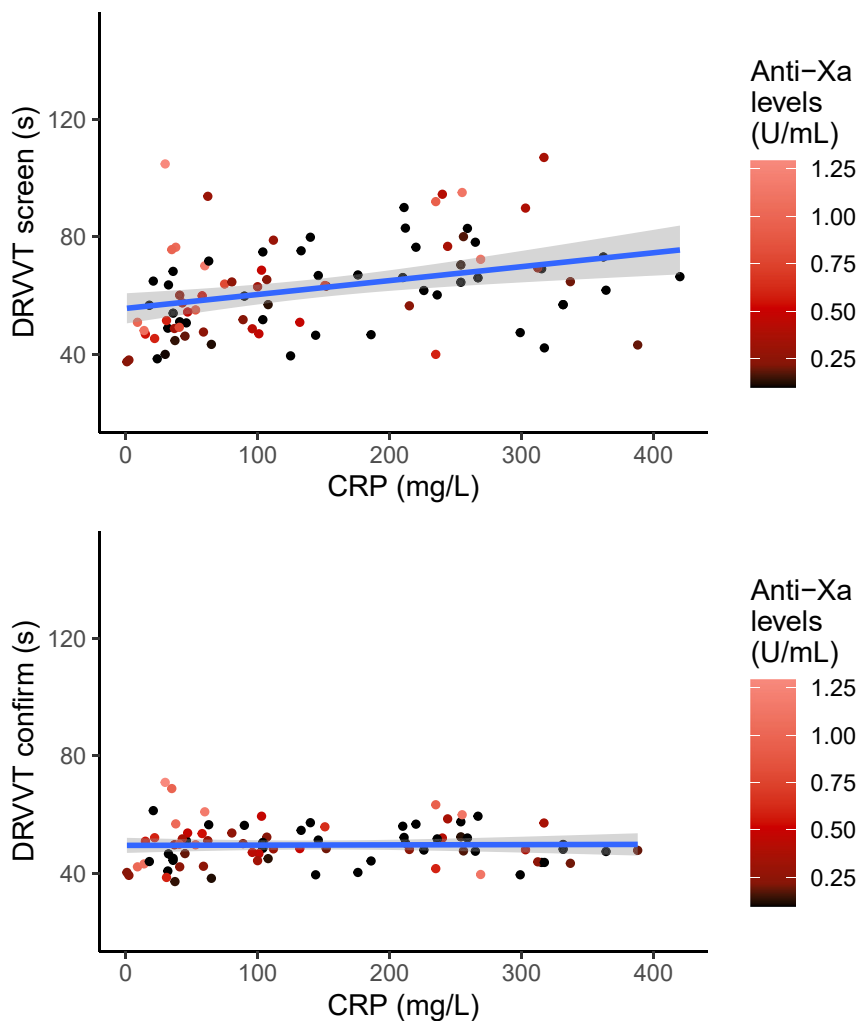**FIGURE 2** Prospective analysis (*n* = 89) of the association of C-reactive protein (CRP) with dilute Russell's viper venom (DRVV) screen and confirm clotting times (mixing tests were not performed due to a limited volume of plasma frozen). Intensive care patients from 2 prospective cohorts, receiving or not receiving unfractionated heparin, were included in the analysis. Linear regression was used to model the association between both variables. Dots represent individual measurements, and the line represents the linear regression line (with its 95% CI). DRVV screen clotting time increased with increasing levels of CRP (*P* = .0002) but not the DRVV confirm clotting time (*P* = .60). Both tests were also influenced by unfractionated heparin anti-Xa levels above 0.8 U/mL (*P* = .0006 and *P* = .005, respectively). DRVVT, dilute Russell's viper venom test.

In the spiking experiment, clotting times increased by only a few seconds at the highest added CRP levels for DRVV screen and confirm tests (+1.75 and +3.25 seconds at 400 mg/L added CRP, respectively); for DRVV mix test, the increase was even smaller, possibly due to the lower CRP levels reached due to the 1:1 dilution with normal pooled plasma. This suggests that the increases in DRVV screen clotting times observed with increasing CRP levels are not due to CRP itself, as described with aPTT-based reagents [13].

In sum, we found that STA-Staclot DRVV screen test used for LA testing could be prolonged in the presence of inflammation (evidenced by elevated CRP levels), contrary to what was previously reported with another reagent (LA Screen reagent, Gradipore Ltd) [14]. More
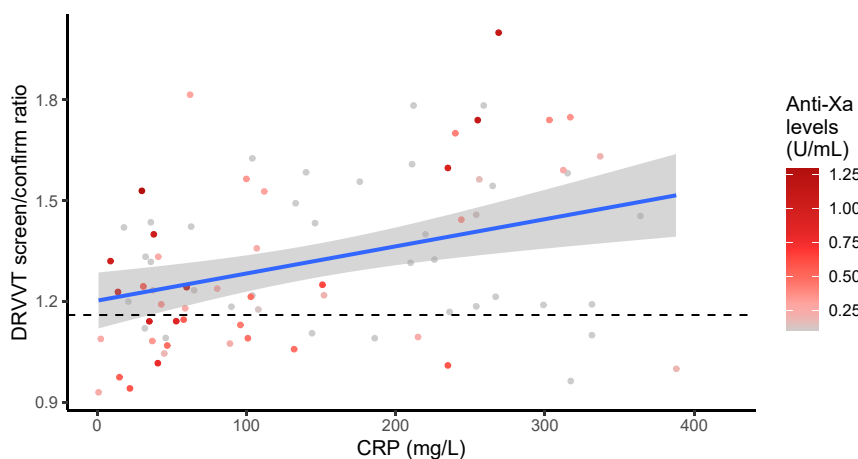

FIGURE 3 Association between C-reactive protein (CRP) levels and dilute Russell's viper venom (DRVV) screen/confirm ratios in the prospective cohort. Each point represents DRVV screen/confirm ratio performed for the same patient with a given plasma sample. The color represents the unfractionated heparin anti-Xa level measured in the same plasma sample. The dashed line represents the local positivity cutoff value for DRVV test (DRVVT) screen/confirm ratio.

recently, Reyes Gil et al. [8] identified in a small cohort of COVID-19 patients an association between high CRP levels and DRVVT positivity (tested with STA-Staclot DRVV reagents). However, this was not explained by a direct effect of CRP on the phospholipids present in the test, contrary to what has been described for aPTT [13], as we found that the addition of CRP *in vitro* to plasma samples does not reproduce the prolongation of the DRVVT. Therefore, this could correspond either to a transient LA profile [21], as reported in various infectious diseases [22,23], or to the interference of another acute phase reactant. It also appears that the DRVV clotting time is less prolonged after the addition of excess phospholipids (DRVV confirm). A strength of our study is that it assessed the association between inflammation and increased DRVV clotting times both retrospectively in a large cohort and prospectively. However, the 2 cohorts included different patients (patients with thrombotic or obstetrical complications in the retrospective part [24] and critically ill intensive care patients in the prospective part), which may have contributed to the differences observed in the results of the 2 parts. Its limitations are that it was a monocenter study, and only 1 reagent kit was studied (but a few different ones are available on the market). Future work should confirm this association and may attempt to identify its mechanism.

CONCLUSION

Overall, LA testing should be performed with much caution in the presence of inflammation due to the risk of false-positive DRVV and aPTT tests, at least with the reagents used in this study (STA-Staclot DRVV and PTT-LA reagents). This may have contributed to the high incidence of (isolated, single positive) LA reported in first-wave COVID-19 patients. Retesting at least 12 weeks later under optimal conditions is therefore mandatory [25]. Manufacturers should mention this interference in the DRVVT package insert information.

AUTHOR CONTRIBUTIONS
Conception of the study: M.H., F.M. Data collection: M.H. Data analysis: M.H. Writing of the first draft: M.H., T.L., F.M. Critical revision of the manuscript: M.H., E.C., M.P., T.L., F.M.

RELATIONSHIP DISCLOSURE
M.H., E.C., M.P., and T.L. have no relevant disclosure related to this work. F.M. reports institutional fees from Stago, Werfen, Nodia, Roche Sysmex, and Bayer. He also reports speaker fees from Boehringer-Ingelheim, Bayer Healthcare, Bristol-Myers Squibb-Pfizer, Diagnostica Stago, Sysmex, and Aspen, all outside the submitted work.

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