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ORIGINAL ARTICLE

Study of in vitro thrombin generation after neutralization of heparin

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Abstract

Introduction: Thrombin generation (TG) documents hypercoagulability. TG in platelet-poor plasma is exquisitely sensitive to heparins, which thus must be neutralized before testing. Heparinase and hexadimethrine bromide (polybrene) have been used for that purpose, but their effects per se on TG have been poorly studied so far.

Methods: (i) TG was studied in commercial normal pooled plasma (NPP; CryoCheck®, Cryopep) in absence or presence of neutralizing agents. (ii) NPP was spiked with increasing concentrations of unfractionated heparin (UFH; up to 1.0 IU/mL) or low-molecular-weight heparin (LMWH; enoxaparin up to 1.2 IU/mL) and TG studied after incubation of heparinase (Hepzyme®; 15 minutes) or polybrene (0.025 mg/mL; 10 minutes).

Results: (i) With ThromboScreen reagent to initiate TG, addition of heparinase was associated with increased peak, whereas polybrene caused lengthening of lag time and time to peak, compared with nonsupplemented NPP. (ii) With polybrene, TG was completely restored over the whole range of UFH and LMWH studied. By contrast, heparinase failed to fully restore TG in presence of UFH concentrations ≥0.8 IU/mL or LMWH concentrations ≥1.0 IU/mL. Those effects were matched with detectable tiny residual amounts of non-neutralized heparin (as assessed with an anti-Xa assay) and were less pronounced with a higher picomolar concentration of tissue factor (DrugScreen reagent).

Conclusion: Polybrene fully restored TG of heparinized plasma at the expense of an alteration of TG, pointing to the need to use adapted reference ranges. Heparinase failed to do so in presence of high concentrations of both heparins.

KEYWORDS

heparin, heparin lyase, hexadimethrine bromide, thrombin generation

1 | INTRODUCTION

The laboratory assessment of individual thrombotic risks remains challenging. Thrombin generation (TG) assays are integrative coagulation tests that are used to assess potential hypercoagulable states for patients such as those with venous thromboembolism, cirrhosis, or more recently COVID-19.[1-4] However, they frequently receive anticoagulants to manage the thrombotic risk associated with the condition prior to any laboratory investigation. Heparin is the most used anticoagulant in the hospital setting. Nevertheless, TG when performed with platelet-poor plasma is exquisitely sensitive to heparin, which precludes the evaluation of the underlying coagulation

phenotype, at least when intermediate or therapeutic doses are used.[2,5-7] Moreover, different heparin molecules may be used, at different dosages and plasma samples may be collected more or less close to the actual peak effect, which leads to huge variability in TG. Therefore, heparin neutralization is mandatory to have a reliable quantitative overview of the hypercoagulability, its inter-patient variability, and its changes over time.

Three molecules have been mainly used for heparin neutralization in the hematology laboratory: heparinase, hexadimethrine bromide (a.k.a polybrene), and protamine sulfate. Heparinases are enzymes produced by *Flavobacterium heparinum* that degrade heparin and heparan sulfate into small oligosaccharides.[8] Polybrene and protamine sulfate are polycations that interact with negatively charged heparin chains thereby inhibiting their anticoagulant effect. Heparinase and polybrene have been successfully used in clinical practice for heparin neutralization for decades, protamine sulfate being a second choice because of its pronounced interaction with clotting in the absence of heparin.[9,10] However, TG study after neutralization with heparinase and polybrene remains poorly validated so far.[10,11]

The aim of this study was thus to assess the ability of heparinase and polybrene to neutralize unfractionated heparin (UFH) and enoxaparin, a low-molecular-weight heparin (LMWH), added in vitro before TG measurement and to evaluate their potential side effects on TG.

2 | MATERIAL AND METHODS

This in vitro study was conducted at the hematology laboratory of the CHU UCL Namur. Tests were performed using either commercial normal pooled plasma (NPP; Cryocheck®, Cryopep, Montpellier, France, batches A1278 & A1291; citrate levels equivalent to 109 mM (due to the margin of tolerance during manufacturing by mixing numerous bags from healthy donors)) or home-made NPP prepared from healthy volunteers at the University of Namur, as previously described.[12] Plasma aliquots (1 mL aliquots for commercial NPP and 4mL aliquots for home-made NPP) were stored at −70°C and thawed for five minutes at 37°C in a thermostatic bath before use.

2.1 | Laboratory assays

Thrombin generation was studied with the ST Genesia device (Diagnostica Stago, Asnières-sur-Seine, France) using either STG-DrugScreen (tissue factor (TF) concentration in the high picomolar range; generally used to assess TG in anticoagulated patients) or STG-ThromboScreen (lower picomolar TF concentration than STG-DrugScreen; generally used to assess thrombotic states) reagent (Stago) as previously described.[13-15] Briefly, calibration (using the dedicated calibrator STG-ThrombiCal) and internal quality controls (provided with STG-DrugScreen and STG-ThromboScreen kits) were performed before each test series. Plasma samples were first incubated on board with the initiating reagent (containing TF and phospholipids; STG-DrugScreen or STG-ThromboScreen; 80 µL plasma plus 20 µL initiating reagent per mixture) for 10 minutes at 37°C. Then, 20 µL of the triggering reagent (STG-FluoStart), containing calcium chloride and the fluorogenic substrate, were automatically added to the mixture. The conversion of the fluorogenic substrate by thrombin produced in plasma samples generates a fluorescent signal that is measured over time and converted into thrombin concentration using the calibration curve. Four parameters were derived: the lag time (LT) corresponding to the time-lapse between the addition of calcium chloride and the beginning of TG, the peak height (PH) corresponding to the maximal thrombin concentration measured, the time to peak (ttP) corresponding to the time-lapse between the addition of calcium chloride, and the peak and the endogenous thrombin potential (ETP) corresponding to the area under the TG curve. Chromogenic anti-Xa assays were performed with a STA-R Max 2 analyzer using STA-Liquid anti-Xa reagent (Stago) calibrated with STA-Multi Hep Calibrator (Stago) to measure heparin levels. The reagent contains neither exogenous antithrombin nor dextran sulfate.

2.2 | Heparin neutralization

We evaluated two means of heparin neutralization. First, we used lyophilized heparinase type I (Dade Hepzyme®, Siemens Healthcare Diagnostics, Deerfield, United States; batches 557 514 and 557 516). According to manufacturer's recommendations, 1 mL of plasma was added to heparinase vials, the content of which was then homogenized by five gentle inversions and kept at room temperature (20°C) for 15 minutes before analysis. Second, we used hexadimethrine bromide (a.k.a polybrene; Sigma Aldrich, Saint-Louis, United States), supplied as powder, and dissolved with water at the desired concentration. To limit sample manipulation, we mixed polybrene with the initiating reagent of TG (ie, STG-DrugScreen or STG-ThromboScreen; polybrene solution used as reconstitution solution) to achieve the previously proposed final concentration of 0.025 mg/mL in test mixtures.[10,11,15] TG was then studied as described above; incubation of polybrene with plasma samples was 10 minutes due to the usual processing of the sample in the ST Genesia.

2.3 | Effect of heparinase and polybrene on TG

To evaluate the effect of heparinase and polybrene on TG, we studied TG in commercial NPP supplemented or not with heparinase as mentioned above and performed another TG run using initiating reagents reconstituted with a polybrene solution (Figure 1). Three series were performed in duplicate on separate days for each condition. As the commercial NPP used was prepared from plasmapheresis and therefore might differ from clinical plasma sample prepared by direct venipuncture and double centrifugation, we confirmed the results by performing the same study with

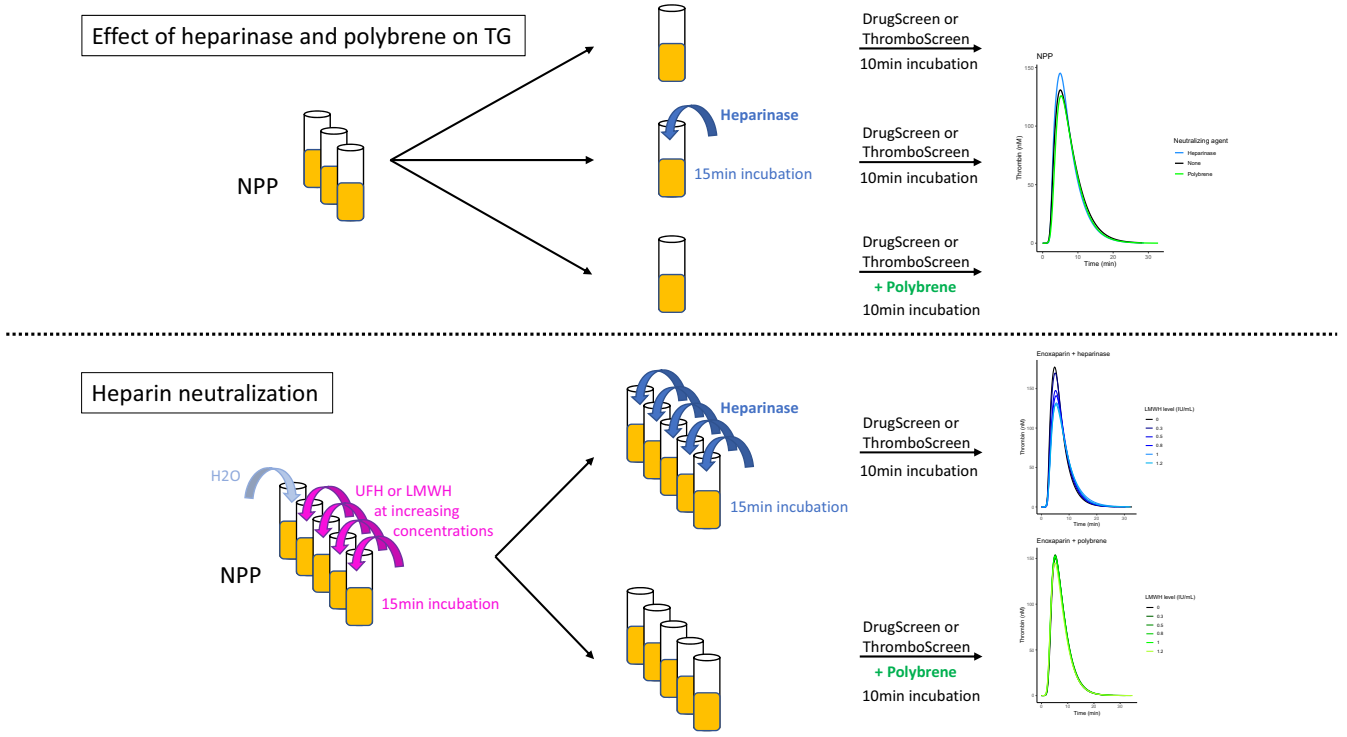

FIGURE 1 Study protocol. The first part (upper panel) aimed at assessing the effect of heparinase and polybrene per se on thrombin generation (TG) studied in normal pooled plasma (NPP). NPP was either tested alone, or after incubation with heparinase or after addition of polybrene to the initiating reagent (STG-DrugScreen or STG-ThromboScreen). The second part (lower panel) aimed at assessing the ability of heparinase and polybrene to neutralize heparin effect on TG. NPP was spiked with increasing concentrations of heparin (unfractionated heparin or enoxaparin). Then, samples were incubated with heparinase before TG or polybrene was added to the initiating reagent (10 min interval before triggering with recalcification). NPP, normal pooled plasma; UFH, unfractionated heparin; LMWH, low-molecular-weight heparin (enoxaparin)

our home-made NPP. We also assessed the effect of polybrene on the reference plasma provided with STG-ThromboScreen kit as this plasma would also be exposed to polybrene in the standard test sequence on ST Genesia.

2.4 | Neutralization of unfractionated and low-molecular-weight heparins

Commercial NPP was spiked with unfractionated heparin (Héparine Leo®, Leo Pharma, Lier, Belgium) at 0.0, 0.3, 0.5, 0.8, and 1.0 IU/mL or with enoxaparin (Clexane®, Sanofi Belgium, Machelen, Belgium) at 0.0, 0.3, 0.5, 0.8, 1.0, and 1.2 IU/mL (final concentrations in plasma; dilution 1/20 vol/vol in water). Adequacy of spiking was checked by measuring anti-Xa levels. TG was then studied with ST Genesia after heparin neutralization with heparinase (for 15 minutes) or polybrene (for 10 minutes) as described above, within 1 hour after spiking (Figure 1). Two series were tested in duplicate for each heparin concentration and neutralizing agent. Anti-Xa activity was also measured after TG on residual plasma treated with heparinase or spiked with polybrene (to achieve the same final concentration of 0.025 mg/mL) to assess heparin neutralization.

2.5 | Statistics

Results were expressed as percentages of the reference condition (NPP without neutralization agent for the first set; or NPP without heparin for the second set). Mean percentages (±standard deviation) were then calculated.

Kruskal-Wallis test was used to compare TG in presence or absence of neutralizing agents (heparinase or polybrene). Post hoc analysis was performed using Dunn's tests with a control group (without neutralization agent) with Bonferroni's correction for multiplicity. The same statistical approach was used to compare TG in the presence or absence of heparin at increasing concentrations with the addition of heparinase or polybrene.

Differences were deemed acceptable if not significantly higher than the analytical variability of the method, which was calculated using the coefficients of variation (CVa, analytical coefficient of variation) of internal quality controls and STG-RefPlasma processed locally (with the same batch; 180 tests for STG-DrugScreen and 32 tests for STG-ThromboScreen), as previously described[16-18]:

$$Acceptable\ difference = z_{0.975} \times \sqrt{2} \times CVa = 2.77 \times CVa$$

Based on those criteria, differences in LT, ttP, PH, and ETP were judged acceptable if lower than 10%, 7%, 10%, and 13%, respectively, for TG studied with STG-DrugScreen reagent, and if lower than 8%, 7%, 14%, and 11%, respectively, for TG studied with STG-ThromboScreen reagent.

Analyses were performed in R, version 4.1.0 using *PMCMR* and *ggplot2* packages.[19]

We also analyzed the effect of polybrene on the reference plasma provided with the STG-ThromboScreen reagent kit. When polybrene was added in the initiating reagent, we observed prolonged LT and ttP and reduced PH and ETP (Figure S1). The effect of polybrene on this reference plasma was more pronounced than its effect on commercial NPP (mean relative differences: LT +21%, ttP +11%, PH −20%, and ETP −16%).

3 | RESULTS

3.1 | Effect of heparinase and polybrene on TG

Using STG-ThromboScreen reagent in absence of heparin, both heparinase and polybrene per se modified TG studied in commercial NPP ($P < .01$ for LT, ttP, PH, and ETP, Kruskal-Wallis tests; Figure 2). The addition of heparinase to commercial NPP was associated with an increased PH (relative mean difference 14% ± 6%, $P = .006$, Dunn's test). The addition of polybrene was associated with a prolongation of LT and ttP (mean relative difference 17% ± 7% ($P = .002$, Dunn's test) and 8% ± 4% ($P = .02$, Dunn's test) respectively). When the same experiments were performed using home-made NPP, we also identified the increased PH observed with heparinase and the prolonged LT and ttP with polybrene (data not shown). When using the reagent with higher picomolar tissue factor concentration (STG-DrugScreen), differences between commercial NPP exposed or not to heparinase and polybrene were no longer statistically significant (Figure 3).

3.2 | Neutralization of unfractionated heparin and low-molecular-weight heparin

3.2.1 | Heparinase

When TG was studied with STG-ThromboScreen reagent, significant residual heparin effect was measurable despite the use of heparinase (Figure 4): for UFH, LT and ttP were significantly prolonged and PH was significantly reduced from UFH levels of 0.8 IU/mL (mean differences 3.9%, 6.2%, and 12.7%, p-values (Dunn's tests) 0.047, 0.04, and 0.04, respectively; Table S1). For enoxaparin, ttP was significantly prolonged, and PH and ETP were significantly decreased from enoxaparin levels of 1.0 IU/mL (mean differences 9.7%, 30.2%, and 14.8%, p-values (Dunn's tests) 0.03, 0.01, and 0.01, respectively). Of note, tiny residual amounts of non-neutralized heparin (anti-Xa levels <0.1 IU/mL) could be detected in those samples with high concentration of UFH and enoxaparin (Table S2), showing that such a marked but incomplete neutralization of heparins had an impact on TG when studied with STG-ThromboScreen reagent.

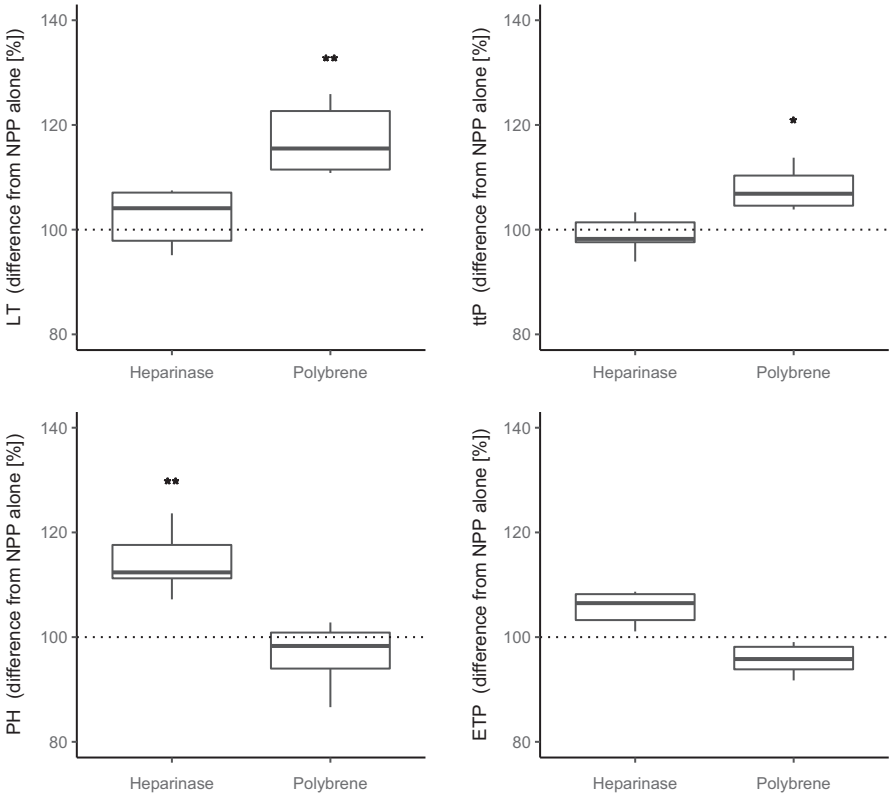

FIGURE 2 Effect of heparinase and polybrene on thrombin generation (TG) studied in commercial normal pooled plasma (NPP) with ST Genesia and STG-ThromboScreen reagent. NPP was incubated with heparinase or polybrene before TG. Three series were tested in duplicate for each condition. TG parameters are expressed as percentages of the condition without neutralizing agent. Horizontal bars represent medians, boxes represent interquartile ranges, and whiskers represent the minimum-maximum range. Comparisons were performed with Kruskal-Wallis test and post hoc analysis by Dunn's test (with Bonferroni adjustment for multiplicity). *, $P < .05$; **, $P < .01$. NPP, normal pooled plasma; LT, thrombin generation lag time; ttP, thrombin generation time to peak; PH, thrombin generation peak height; ETP, endogenous thrombin potential

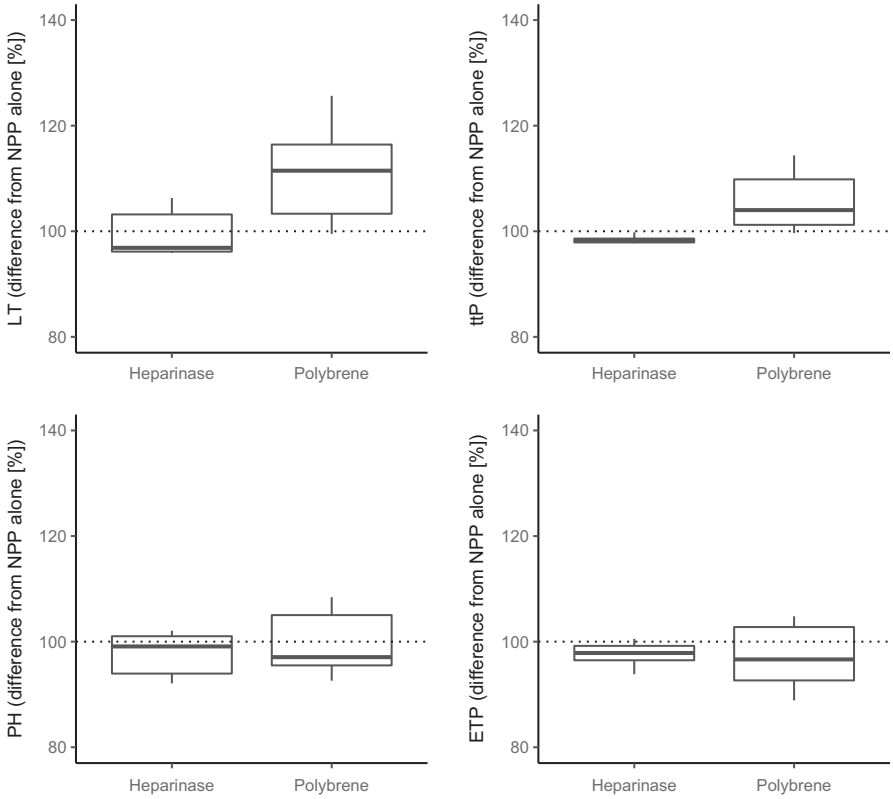

FIGURE 3 Effect of heparinase and polybrene on thrombin generation (TG) studied in commercial normal pooled plasma (NPP) with ST Genesia and STG-DrugScreen reagent. NPP was incubated with heparinase or polybrene before TG. Three series were tested in duplicate for each condition. TG parameters are expressed as percentages of the condition without neutralizing agent. Horizontal bars represent medians, boxes represent interquartile ranges, and whiskers represent the minimum-maximum range. None of the comparisons reached statistical significance according to Kruskal-Wallis testing. NPP, normal pooled plasma; LT, thrombin generation lag time; ttP, thrombin generation time to peak; PH, thrombin generation peak height; ETP, endogenous thrombin potential

When TG was studied upon stronger TF stimulus for its initiation (STG-DrugScreen reagent), no relevant effect of residual heparin could be detected (Figure 5, Table S3).

3.2.2 | Polybrene

Regarding polybrene, a slight PH decrease could be measured at initial UFH concentrations of 0.5 and 0.8 IU/mL (mean differences 6.6% and 7.1%, p-values (Dunn's tests) 0.03 and 0.009, respectively; Table S1, Figure 4). These differences were however acceptable as per the criteria defined previously (lower than 14% for PH). When higher picomolar concentration of tissue factor was used (STG-DrugScreen reagent), no relevant effect of both UFH and LMWH could be measured after neutralization with polybrene (Figure 5, Table S3). Contrary to what had been observed with heparinase, tiny residual amounts of non-neutralized heparin could not be detected by chromogenic anti-Xa assays when polybrene was used to neutralize UFH (up to 1.0 IU/mL) or enoxaparin (up to 1.2 IU/mL).

4 | DISCUSSION

Heparin neutralization was more efficacious using polybrene, especially in presence of higher heparin concentrations and weaker TF stimulus for TG initiation (STG-ThromboScreen reagent). Indeed, with polybrene, no relevant effect of residual heparin was observed in the presence of both UFH and enoxaparin in concentrations covering the range of observed levels in clinical practice. Conversely, heparinase seemed unable to fully neutralize concentrations of heparin corresponding to the higher part of the therapeutic range, leaving tiny amounts of heparin able to impact TG. This was especially true for the LMWH enoxaparin and more pronounced when using the lower picomolar concentration of TF of the two reagents (ie, STG-ThromboScreen reagent).

This was at the expense of an impact of the neutralizing agents per se (ie, in absence of heparin) on TG. The addition of polybrene was associated with a significant prolongation of LT and a slight prolongation of ttP. Regarding heparinase, its effect was less pronounced and mainly consisted of an increased PH. Those effects of neutralizing agents per se should be considered when interpreting TG results after heparin neutralization using these agents. Specific reference ranges should be determined in presence of heparinase or polybrene to better assess TG patterns in this setting.

To avoid the need of spiking each plasma sample individually and to increase the reproducibility of the addition of polybrene, we added it directly in the initiating reagent. Therefore, the STG-RefPlasma used to normalize the results and increase the reproducibility of the measurement,[20] was also exposed to polybrene. Under this condition, STG-RefPlasma had longer LT and ttP and lower PH and ETP than when it was analyzed in absence of polybrene (Figure S1). Changes in PH and ETP were more pronounced than those observed with the commercial NPP in the presence or absence of polybrene. This could be partly due to the fact that STG-RefPlasma is a reconstituted lyophilized preparation while the CryoCheck NPP is supplied as frozen plasma. Therefore, PH and ETP could be artificially

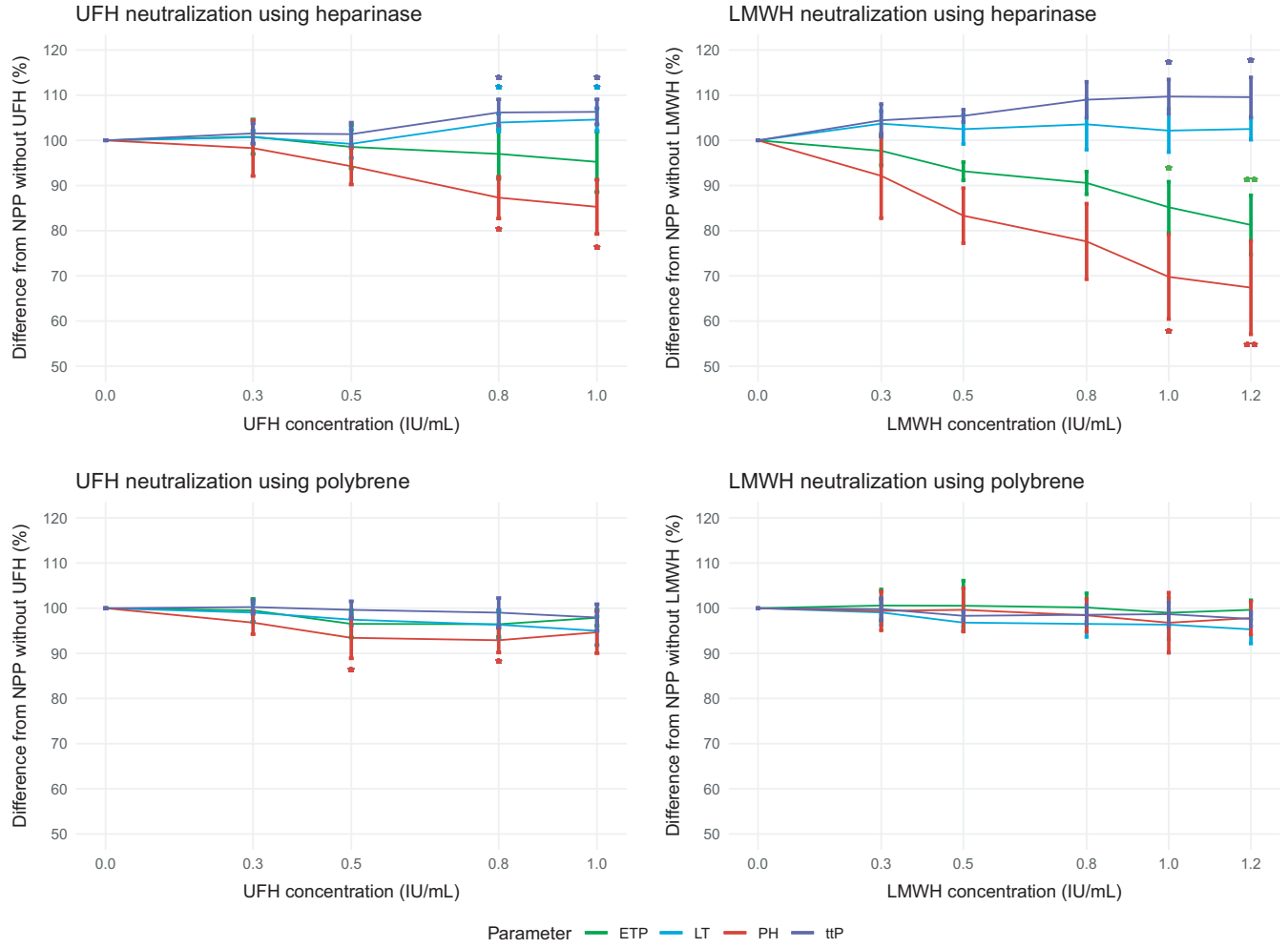

FIGURE 4 Unfractionated and low-molecular-weight heparins (enoxaparin) neutralization using heparinase and polybrene before thrombin generation analyses with the ST Genesia using the STG-ThromboScreen reagent. The ordinate represents the ratio of TG between heparinized samples and the reference condition (without heparin, dilution with distilled water only), expressed in percentage. Vertical bars represent standard deviations. Two series were performed in duplicate for each condition (the same effect was observed in the two series despite a different lot of heparinase). Comparisons were performed by Kruskal-Wallis tests and post hoc analysis by Dunn's tests (with Bonferroni adjustment for multiplicity). *, $P < .05$; **, $P < .01$. UFH, unfractionated heparin, LMWH, low-molecular-weight heparin (enoxaparin); ETP, endogenous thrombin potential; LT, lag time; PH, peak height; ttP, time to peak

increased when results are normalized using this reference plasma exposed to polybrene, leading to overly hypercoagulable patterns. This reinforces the need for reference ranges established under those specific testing conditions.

Previous reports identified concentration-dependent inhibition of TG in presence of polybrene, which completely neutralized heparins.[10,11] Rotteveel et al identified a prolongation of the time to peak with polybrene concentrations of 3.0 heparin equivalent units/mL and above (which corresponds approximatively to 0.03 mg/mL) and a reduction in ETP only at the highest polybrene concentration tested (ie, 12 heparin equivalent units).[10] At low polybrene concentrations (3.0 heparin equivalent units), such as those used in our study, polybrene was able to fully restore TG even with the presence of UFH at 3 IU/mL.[10] In another study, polybrene displayed a concentration-dependent inhibition of TG studied with the Calibrated Automated Thrombography, even at intermediate polybrene concentration (ie, 0.05 mg/mL).[11] With lower polybrene concentrations (ie, 0.025 mg/mL), the effect of UFH and dalteparin (spiked up to 2.0 IU/mL) on TG peak height was fully neutralized.[11] The mechanism of action of polybrene on the initiation of TG has been previously studied. Among the proposed mechanisms, Chu et al identified that polybrene could inhibit TF-dependent FVII activation, which would be consistent with the prolongation of LT and ttP we observed.[21]

Regarding heparinase, a previous study did not identify an effect of heparinase on TG of normal plasma,[10] an observation that we did not confirm. This is possibly due to differences in the initiating reagent used in the two studies (the exact composition of the initiating reagent is not available in both cases). The effect is not easy to explain; heparinase could increase TG by degrading endogenous heparin-like substances, yet inhibition by polybrene should have the

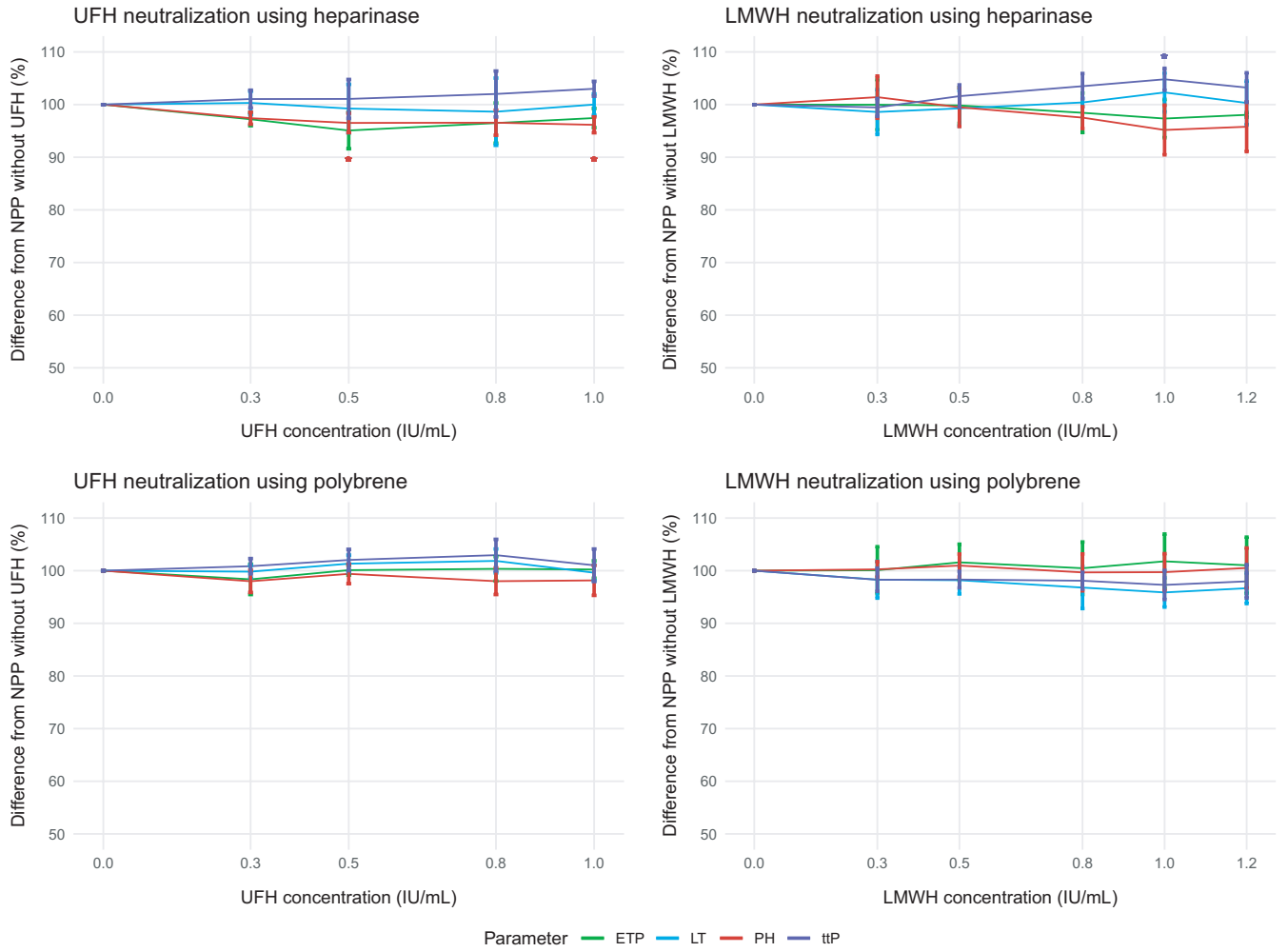

FIGURE 5 Unfractionated and low-molecular-weight heparins (enoxaparin) neutralization using heparinase and polybrene before thrombin generation study with ST Genesia using STG-DrugScreen reagent. The ordinate represents the ratio of TG between heparinized samples and the reference condition (without heparin, dilution with distilled water only), expressed in percentage of the reference condition. Vertical bars represent standard deviations. Two series were performed in duplicate for each condition (the same effect was observed in the two series despite a different lot of heparinase). Comparisons were performed by Kruskal-Wallis tests and post hoc analysis by Dunn's tests (with Bonferroni adjustment for multiplicity). *, $P < .05$. UFH, unfractionated heparin, LMWH, low-molecular-weight heparin (enoxaparin); ETP, endogenous thrombin potential; LT, lag time; PH, peak height; ttP, time to peak

same effect; this was not the case perhaps due to a concurrent anticoagulant effect discussed just above. On the other hand, heparinase was unable to completely restore the ETP after spiking plasma samples with UFH at high concentrations (3 IU/mL).[10] No further improvement was noticed after incubation of the plasma in a second heparinase vial either, which is consistent with an enzymatic process.[10] Heparinase had also been shown unable to fully neutralize both UFH and LMWH with other so-called global coagulation tests[22] and standard coagulation tests.[10] The duration of incubation should have been sufficient for the completion of heparinase effect. The residual heparin effect after incubation with heparinase could be explained by its inability to digest the smallest heparin fragments, which can still exhibit an anti-Xa activity.[23,24] This would be consistent with the fact that low anti-Xa activity is expected to impact TG mainly as a reduction in PH.[6] In our study, we also detected a weak anti-Xa activity persisting after the use of heparinase (with the two batches used). This anti-Xa activity was lower than the detection limit provided by the manufacturer for clinical use (ie, 0.1 IU/mL). However, as the residual anti-Xa levels after heparinase use were consistent between runs and increased monotonically with heparin concentration, we consider that they are relevant and could account for the observed impairment of TG.

The main limitation of this study is that it was performed with a commercial NPP spiked in vitro with heparins. Indeed, the effect of polybrene and heparinase could be different with patients' samples. For example, endogenous heparin-like substances, which could also be neutralized by heparinase and polybrene, can increase or decrease in various clinical contexts such as cardiovascular or infectious diseases.[25-27] Further work should be performed on plasma from patients on heparin therapy. Moreover, we only studied enoxaparin; results could be different with other LMWH. Nevertheless, our results are consistent with previous work performed with

dalteparin.[11] Second, we only evaluated a single concentration of polybrene and heparinase based on the results of previous studies.[10,11,14] Furthermore, the calibration was performed in the absence of the two neutralizing agents. Further work could assess whether different concentrations of the two reagents could lead to a better balance between heparin neutralization and the direct effect on TG measured on the ST Genesia system, and could ensure that the two neutralizing agents do not interfere with the fluorogenic substrate, as it is likely. Third, some in vivo effects of heparin will not be neutralized by either polybrene or heparinase. Indeed, heparin administration mobilizes tissue factor pathway inhibitor (TFPI) from the endothelial surface; TFPI has been shown to significantly contribute to the anticoagulant effect of heparin.[28] Therefore, residual TG inhibition is expected to be identified after in vitro neutralization of heparin administered in vivo. Finally, these results are not applicable for other types of TG initiation. Indeed, polybrene has been shown to interfere with contact activators (eg, celite and kaolin) by direct electrostatic interactions. The timing of polybrene administration (ie, before or after contact activation) would then significantly impact the results.[14]

5 | CONCLUSION

Contrary to heparinase, polybrene is able to fully restore TG in presence of concentrations of UFH and LMWH (enoxaparin) up to 1.0 and 1.2 IU/mL, respectively. Indeed, an effect on TG of tiny amounts of residual heparin as judged with anti-Xa assay was detected despite the addition of heparinase when UFH and enoxaparin were added at concentrations corresponding to the high therapeutic range; trough samples should then be preferred when heparinase is used to neutralize heparin administered by intermittent subcutaneous injections. However, the use of polybrene was associated with an increase in TG lag time and time to peak and the use of heparinase with an increased PH, which highlights the importance of using reference values specific to those neutralization procedures. Those effects were only observed with STG-ThromboScreen kit: they were not significant with a stronger TF stimulus for initiation of TG (ie, STG-DrugScreen kit).

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CONFLICT OF INTEREST

JD is the CEO and founder of QUALIblood s.a., a contract research organization manufacturing the DP-Filter, is a coinventor 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. AC is an employee of Diagnostica Stago Company, which markets the ST Genesia system. TL reports fees (used in the frame of matters at work) from Diagnostica Stago for educational materials. FM 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, Stago, Sysmex, and Aspen all outside the submitted work.

AUTHOR CONTRIBUTIONS

MH and FM involved in the conceptualization. MH involved in in the investigation. MH, JD, LM, MD, TL, and FM involved in formal analysis. MH, TL, and FM involved in writing the original draft. MH, JD, LM, MD, AC, EdM, TL, and FM involved in reviewing and editing the manuscript. FM involved in supervision.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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