

Preclinical evaluation of point-of-care prothrombin time as a biomarker test to guide prothrombin replacement therapy in coagulopathic bleeding

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Summary

Background: Hemorrhage is still a common cause of death in trauma. Central lab measured prothrombin time (lab PT) is predictive of low prothrombin concentration and clinical outcome in trauma patients, however, treatment guidance is limited by slow turnaround times. Here, we have preclinically evaluated the potential of a point-of-care prothrombin time test (POC PT) as a faster alternative to identify patients with low prothrombin concentration.

Methods: Human whole blood was serially diluted and prothrombin time measured by POC PT (CoaguChek XS Pro, Roche) and lab PT. Recombinant human prothrombin (MEDI8111) was added to human whole blood with or without depletion of prothrombin by pretreatment with prothrombin neutralizing antibodies.

Results: There was no observable difference in the sensitivity of either test to dilution at blood volumes of 60–100%. At blood volumes of ≤55% (equivalent to 47 mg/L prothrombin), PT sharply increased, with greater dilutional sensitivity observed in the POC test. Both tests were insensitive to prothrombin up to 194 mg/L added MEDI8111 (equivalent to 328 mg/L prothrombin versus endogenous concentration of 129 mg/L). Depletion of endogenous prothrombin inversely correlated with an increase in PT which returned to baseline following addition of 97 mg/L MEDI8111 or above. Both assays correlated well above 48.5 mg/L added MEDI8111 (65.9 mg/L prothrombin).

Conclusions: Our data supports that POC PT tests, such as the CoaguChek XS Pro, are fit for purpose to confirm a coagulopathic threshold for prothrombin and provide a fast, simple, and mobile method to guide MEDI8111 therapy in bleeding trauma patients.

KEYWORDS

biomarker, coagulopathy, point-of-care, prothrombin, PT, severe bleeding

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Essentials

- Prediction of bleeding risk in trauma patients is difficult.
- We evaluated a test which measures prothrombin time/ability of blood to clot.
- We showed the prothrombin time test reflected prothrombin concentration in blood.
- The test could be used in trauma patients to identify those in need of prothrombin replacement.

1 | INTRODUCTION

Uncontrolled bleeding in severely injured trauma patients is still a leading cause of preventable death in modern society.^{1,2} In trauma hemorrhage, coagulation factors are lost through blood loss itself but also through dilution with replacement fluids and/or consumption. Fibrinogen and prothrombin (Factor II) are considered rate limiting for coagulation.^{3,4} A critical role for fibrinogen and prothrombin are supported by *in vivo* studies⁵ and fibrinogen is included in current guidelines for early replacement in the form of blood products, eg, fresh frozen plasma (FFP) and blood-derived factors such as fibrinogen concentrate and cryoprecipitate.⁶ Prothrombin complex concentrates (PCC) in which prothrombin is an important component has also been suggested for treatment of trauma-induced bleeding.⁶⁻⁸ Recombinant human prothrombin (MEDI8111) is being evaluated as a replacement therapy on top of standard-of-care for the indication of coagulopathic bleeding in trauma.

Treatment today is guided through the clinical assessment of hemorrhage using a combination of the patient's physiology, the mechanism and anatomical pattern of injury, and the patient's response to initial resuscitation.⁹ In order to assess the coagulation status of a bleeding patient, viscoelastic methods including rotation thromboelastometry (ROTEM) and thromboelastography (TEG) are increasingly utilized in trauma centers and the number of publications describing viscoelastic methodology has risen over recent years. However, viscoelastic analysis is not available in all trauma centers, the methods employed by investigators differ significantly and questions remain regarding the utility of these tests for the detection of post-traumatic coagulopathy.¹⁰

In the majority of hospitals standard central lab coagulation monitoring includes measurement of prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen, and platelets. However, the value of these measurements in guiding acute treatment is hampered by the delay in obtaining central lab results.¹¹ A number of studies have evaluated point-of-care (POC) devices such as the CoaguChek XS (Roche, Bromma, Sweden) for the rapid determination of PT in trauma patients. Whilst limitations in the accuracy of POC tests have been reported for coagulopathic patients particularly at low haematocrit levels^{11,12} other studies have indicated that POC tests could have an important role in trauma to rule in or out a clinically significant coagulopathy.^{13,14}

In a retrospective study of trauma patient data we have previously investigated the relationship of coagulation factor concentrations at emergency department admission with coagulation biomarkers and

clinical outcome. We have shown that median prothrombin plasma concentration is a good predictor of 24-hour mortality and transfusion. Furthermore, PT is predictive of prothrombin concentration of less than 60 IU/dL (60% of normal) and of transfusion demand and clinical outcome.¹⁵ The correlation between prolonged PT and increased patient mortality has also been shown by others^{16,17} supporting its utility as a predictive biomarker of outcome.

In order to demonstrate the potential of PT as a biomarker to acutely guide initial and repeat dosing of prothrombin-containing therapies, such as MEDI8111, we have evaluated a point-of-care PT test as a faster alternative to central lab analysis. In this study we have compared PT measured by CoaguChek XS Pro, now referred to as "POC PT" to a lab PT test, Thromborel S measured by Amelung coagulometer in this study, now referred to as "lab PT." In order to mimic all potential clinical situations that could affect assay accuracy we have evaluated POC vs lab PT in diluted whole blood, under conditions of neutralized prothrombin and by addition of MEDI8111 to compensate for neutralized prothrombin.

2 | METHODS

2.1 | *In vitro* studies of POC PT sensitivity to prothrombin concentration

Whole blood was collected from healthy volunteers, free of any medication for 7 days prior to blood donation, after approval from the local Gothenburg ethical committee, Sweden (ethical permit number: 033-10). Blood was collected by free flow from a 17-gauge Venflon needle (Becton Dickinson, Helsingborg, Sweden) after venipuncture into 4.5 mL Vacutainer tubes containing 0.105 mol/L citrate (Becton Dickinson, Helsingborg, Sweden). The first 2 mL was discarded to avoid unwanted coagulation activation. Plasma was prepared by centrifugation 10 000 × g at room temperature for 15 minute.

Prothrombin time (PT) was measured in citrated plasma by Thromborel S (Siemens, Marburg, Germany) in a coagulometer (Amelung, KC 10A micro coagulometer, Lemgo, Germany) or in venous blood with the CoaguChek XS Pro system and test strips (Roche, Bromma, Sweden). POC PT native whole blood assessments were performed on uncoagulated blood within 4 to 15 minutes of phlebotomy. For each read, 35 µL blood was loaded onto the application area of the test strip and a reproducible volume of blood drawn into the reading chamber of the test strip through capillary action. The POC PT range is 0-96 seconds with values >96 seconds reported as 96 seconds. Citrated plasma samples for lab PT assessment were prepared within

30 minutes of phlebotomy, and assessed for PT immediately or frozen at -80°C prior to assessment.

2.1.1 | Dilution of human blood

To generate a dilution series for POC PT analysis 500 μL native whole blood was added to 0–1165 μL (*or pro rata*) hydroxyethyl-starch (HES, 60 mg/mL, Voluven, 130/0.4, Fresenius Kabi AB, Uppsala, Sweden) to generate samples of 100%, 75%, 70%, 65%, 60%, 55%, 50%, 40%, and 30% whole blood. PT was then measured using the POC PT assay as described earlier. A dilution series for lab PT analysis was generated in the same way with the exception that whole blood was collected into citrated tubes prior to dilution. Following dilution, plasma was prepared from the citrated samples by centrifugation as previously described. Plasma concentrations of prothrombin functional activity were measured using a chromogenic prothrombinase end-point method (PI 200040 Rox prothrombin, Rossix AB, Mölndal, Sweden).

2.1.2 | Spiking of MEDI8111 in prothrombin depleted and non-depleted human blood

Recombinant human prothrombin (MEDI8111) at 9.9 mg/L was obtained from MedImmune (Gaithersburg, MD, USA). The human prothrombin neutralizing monoclonal antibody (mAb) used in this study was a mouse IgG2b mAb produced at 10.2 mg/mL from a hybridoma clone at MedImmune (Cambridge, UK). Hybridoma clones were obtained after repeated immunisation of mice with human prothrombin. Clones were first screened for binding to human prothrombin, and then screened for prothrombin neutralizing activity in a prothrombinase assay. The screened mAbs were further characterized for binding to prothrombin fragments F1+2, F1 and thrombin, and, for prothrombin neutralizing activity in FII clot, PT and APTT assays, in addition to the prothrombinase assay. A mouse IgG2b mAb that binds to but does not neutralize prothrombin was also identified for control experiments.

500 μL native whole blood was spiked with 0–10 μL MEDI8111 or 10 mol/L sodium citrate vehicle buffer to give a final concentration range of 0–194 mg/L recombinant human prothrombin in a final volume of 510 μL prior to POC PT analysis as described earlier.

To demonstrate the specific role of prothrombin, 11 mL native whole blood was incubated \pm 89.5 μL prothrombin neutralizing antibody at room temperature for 4 minutes giving a final antibody concentration of 83 mg/L. The amount of antibody was chosen to be sufficient for neutralizing the prothrombin present and had been verified experimentally. Following incubation, 470 μL samples were removed and spiked with 0–40 μL MEDI8111 at 2.475 mg/L or 9.9 mg/L or 10 mol/L sodium citrate vehicle buffer to give a final concentration range of 0–297 mg/L recombinant human prothrombin in a final volume of 510 μL before POC PT analysis.

In both prothrombin-depleted and non-depleted spiking experiments, a citrated whole blood sample was also generated and treated as for the native whole blood sample. Citrated whole blood was

treated \pm human prothrombin neutralizing antibody prior to spiking with MEDI8111 or vehicle buffer. Samples were then centrifuged to obtain plasma for PT analysis with the lab PT assay. Plasma concentrations of prothrombin were measured as described for the dilution experiments.

2.2 | Statistical analyses

In the in vitro dilution experiment, the mean difference in PT (POC PT–Lab PT) was estimated with two sided 95% confidence intervals using the *t*-statistic. In order to check for any systematic bias between the two methods Bland-Altman graphs were constructed.¹⁸

3 | RESULTS

3.1 | Sensitivity of PT to dilution

Bleeding trauma patients are given replacement fluids and FFP transfusions as one element of emergency care. A potential undesired consequence of this is dilution of the blood and a decrease in the concentration of all coagulation factors. A decrease in all coagulation factors could also occur due to consumption in severe bleedings. Therefore it was important to determine the sensitivity of the POC and lab PT assays to dilution and a consequential decrease in all coagulation factors. Dilutions of human whole blood were performed using HES, a fluid still used to avoid circulatory shock due to blood loss in trauma bleeding, although it is recognized to have effects on coagulation itself.¹⁹ Prothrombin concentration was determined at each dilution step together with measurement of PT by both the POC and lab assays. The results show that POC PT is more sensitive to dilution (Figure 1A). There is no difference in mean PT at blood volumes of 75%, 70%, 65%, and 60%, with point estimates of <2 seconds. At blood volumes of 55% and below the mean difference in PT increases sharply (Table 1). Plasma prothrombin concentration, together with other coagulation factors, are decreased with dilution, with a mean of 111 mg/L in undiluted blood and 47 mg/L at 55% remaining blood volume. In Figure 1B, Bland-Altman plots are shown to illustrate that there seems not to be any systematic bias in the differences between POC PT and lab PT.

3.2 | Super-physiological levels of prothrombin

In addition to triggering initial MEDI8111 dosing, there is an anticipated requirement for an analytical test to determine the need for further MEDI8111 dosing in patients with continued bleeding. In order to assess the POC PT test for this purpose, the sensitivity to prothrombin concentrations above the normal physiological range was evaluated. Figure 2 shows that following addition of increasing concentrations of MEDI8111 to whole blood or plasma there is no evident impact of super-physiological prothrombin concentrations on PT in either of the assays. Mean prothrombin concentrations ranged from 129 mg/L, in the absence of added MEDI8111, up to 328 mg/L following addition of 194 mg/L MEDI8111 (data not shown).

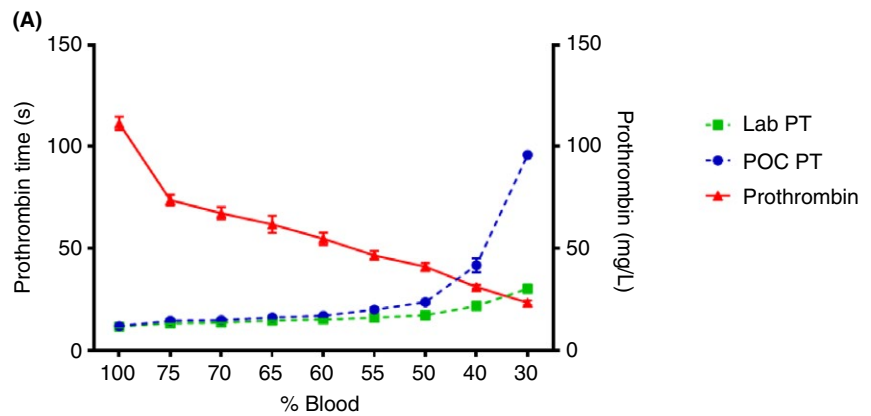


FIGURE 1 (A) Effect of dilution on PT measured by POC or lab PT assays in whole blood or citrated plasma respectively. Each data point represents mean \pm SEM per dilution per volunteer ($n=7$). The concentration of prothrombin (mg/L) in the citrated plasma was determined using a prothrombinase activity assay ($n=5$). At 30% remaining blood volume, PT measured by the POC assay exceeded the instruments upper limit of >96 seconds and data is therefore reported as a maximum value of 96 second. (B) Bland-Altman plots showing the difference in PT (POC PT-lab PT) vs the mean PT of the two methods for each individual at decreasing blood volumes. The solid lines indicate the mean difference and the hashed lines indicate 95% limits of agreement. POC, point of care; PT, prothrombin time

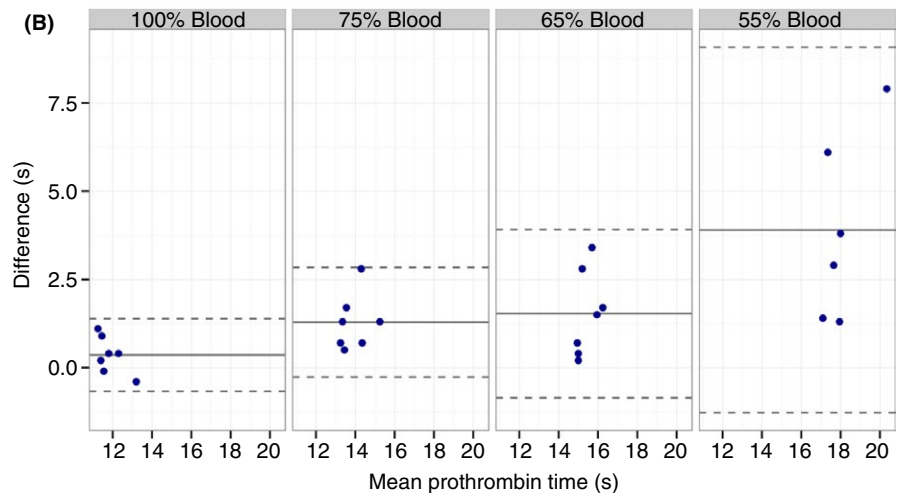


TABLE 1 Summary statistics of prothrombin time (PT) measurements ($n=7$) using POC PT and Lab PT at decreasing blood volume

Blood volume (%)	Mean POC PT (min, max) (seconds)	Mean Lab PT (min, max) (seconds)	Mean difference POC PT-Lab PT (95% CI) (seconds)
100	12.0 (11.5, 13.0)	11.7 (10.7, 13.4)	0.36 (-0.13, 0.84)
75	14.6 (13.6, 15.9)	13.3 (12.7, 14.6)	1.29 (0.55, 2.02)
70	14.9 (13.8, 16.2)	13.6 (12.1, 15.2)	1.30 (0.05, 2.55)
65	16.2 (15.1, 17.4)	14.7 (13.8, 15.4)	1.53 (0.40, 2.65)
60	17.1 (15.5, 18.9)	15.3 (14.1, 16.5)	1.84 (0.50, 3.18)
55	20.1 (17.8, 24.3)	16.1 (14.3, 17.3)	3.90 (1.13, 6.67)
50	23.7 (19.9, 26.4)	17.4 (15.5, 19.0)	6.36 (4.03, 8.68)
40	41.8 (35.6, 60.6)	21.8 (17.7, 23.4)	20.0 (11.5, 28.5)

POC, point of care.

3.3 | Dependency of PT on prothrombin

The specific dependency of PT on prothrombin concentration was assessed for both the POC and lab assays by treatment of whole blood with prothrombin neutralizing antibodies to block the activity of thrombin. Neutralizing of endogenous prothrombin inversely correlated with an increase in PT by both POC and Lab assays (Figure 3A baseline and 0). Addition of MEDI8111 at 97 mg/L or above returned prothrombin to baseline concentrations or higher and a subsequent

decrease in PT close to baseline was observed for both POC and Lab measurements (Figure 3A). Furthermore, Figure 3A shows that above 48.5 mg/L added MEDI8111 (65.9 mg/L prothrombin measured by prothrombinase assay) POC PT and lab PT correlated well. Below this cut-off both assays reflect prothrombin concentration but lab PT shows a higher sensitivity compared to POC PT. At least for the extremely low prothrombin concentrations this is a consequence of the POC but not the lab PT test having an upper limit for PT detection set at 96 seconds. Parallel control experiments were performed using a

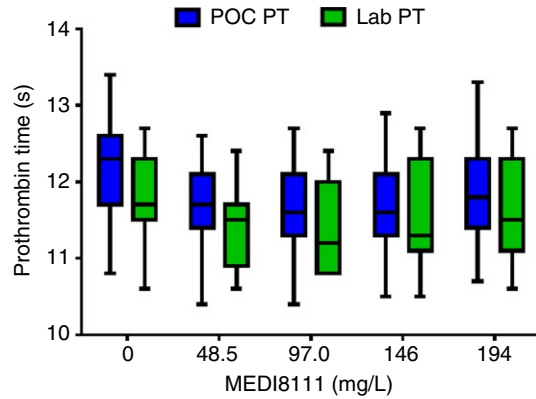


FIGURE 2 Effect of super-physiological concentrations of prothrombin on the ability of POC or lab assays to measure PT. 0-194 mg/L MEDI8111 was added in vitro to baseline blood containing normal concentrations of endogenous prothrombin (Box plots represent Min to Max. 1 data point per sample treatment per volunteer [n=4]). POC, point of care

mouse IgG2b mAb that binds to but does not neutralize prothrombin. The control mAb at a final concentration of 0-170 mg/L had no effect on lab PT compared to the prothrombin neutralizing mAb which increased PT at concentrations of 124 mg/L or above (Figure 3B).

4 | DISCUSSION

As replacement coagulation factor(s), such as MEDI8111, become available to treat coagulopathy, there is an increased need for simple, cheap, and readily available portable devices for diagnosing low coagulation factor concentrations in bleeding trauma patients. MEDI8111, produced by MedImmune, is being evaluated for the indication of coagulopathy due to severe bleeding in trauma. We have previously confirmed a link between critically low prothrombin concentration at admission and clinical outcome in trauma patients and demonstrated that lab measured PT is a good predictor of prothrombin concentration.¹⁵ In addition, we have provided prior evidence that PT is a more sensitive and specific biomarker than the commonly used ROTEM EXTEM CT and MCF for predicting massive blood transfusion and mortality.¹⁵ In this paper we have evaluated CoaguChek XS Pro in preclinical models of dilutional coagulopathy and the potential utility of POC PT tests to guide replacement of prothrombin in bleeding trauma patients.

Since currently there are no POC devices approved for analysis of PT in the trauma population we wanted to evaluate the suitability of existing devices, with a specific focus on dilution and sensitivity to prothrombin. Under conditions of in vitro human whole blood dilution, and an equal reduction in all coagulation factors, the estimated mean difference between lab and POC PT is similar at blood volumes 60-100%. However, at 55% blood volume (47 mg/L prothrombin) the estimated mean difference doubles and, based on the 95% confidence intervals, continues to increase as the blood volume decreases (Table 1). We therefore propose a blood volume of 60% or above as a cut-off where

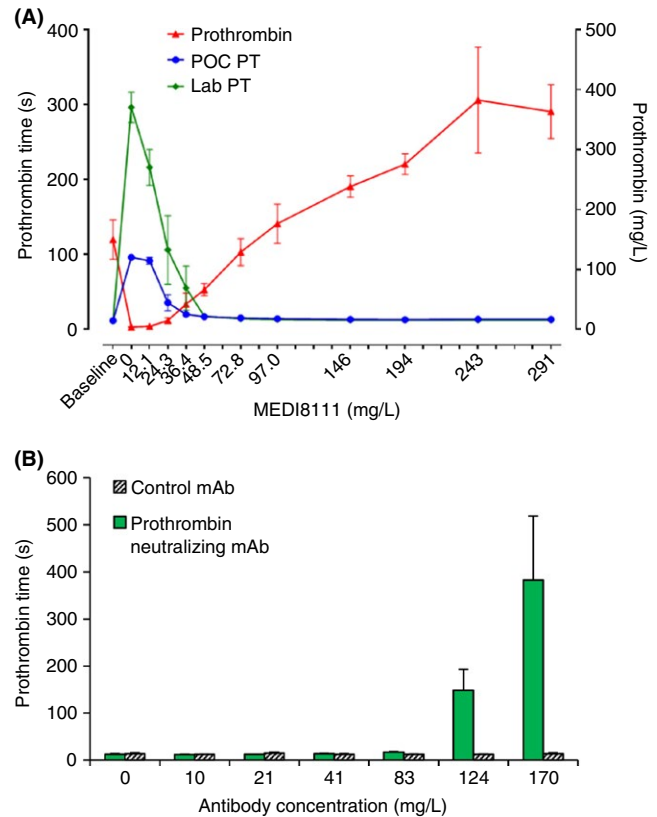


FIGURE 3 (A) Analysis of PT by POC (whole blood) or lab (citrated plasma) assays in prothrombin neutralized blood with in vitro added MEDI8111. Data was obtained prior to neutralization of prothrombin (baseline) and after incubation with increasing concentrations of added MEDI8111. The concentration of prothrombin (mg/L) in the citrated plasma samples was determined using a prothrombinase activity assay (Mean±SEM, n=4). POC PT data points with 0 and 12.1 mg/L added MEDI8111 exceeded the instruments upper limit of 96 seconds are reported in the graph as a value of 96 second. At added concentrations of ≥194 mg/L MEDI8111 5 data-points were outside of the linear range of the prothrombinase assay. (B). Analysis of PT in citrated plasma by the Thromborel lab assay following pretreatment with increasing concentrations (0-170 mg/L) of control or prothrombin neutralizing mAb (Mean±SD, n=5). POC, point of care; PT, prothrombin time

POC PT can reliably and accurately reflect PT as measured by standard lab-based methodology. MEDI8111 is intended to be given as a fixed rather than titrated dose to all patients below a coagulopathic threshold. We have previously observed that prothrombin below 60 IU/dL (60% of normal) was associated with coagulopathy and an increased mortality and transfusion demand in trauma patients.¹⁵ Therefore taken together the data supports that CoaguChek XS Pro could be used to trigger prothrombin therapy in coagulopathic patients since all patients with prothrombin below 60% would benefit from replenishment and would be treated with MEDI8111. Furthermore, since the POC test would be used to set a PT threshold for treatment rather than an absolute prothrombin concentration this addresses previously reported concerns around the accuracy of POC assays particularly in the most severely bleeding coagulopathic trauma patients.^{10,11}

It has previously been reported that PT can be abnormally prolonged by prothrombin in some commercially available assays but not others.²⁰ Since this could potentially impact the clinical utility of the POC assay for re-dosing of MEDI8111 in patients with ongoing bleeds the effect of super-physiological levels of prothrombin on the POC assay was evaluated. 0-194 mg/L added MEDI8111 on PT was assessed in both POC and lab assays. Figure 2 shows that although it is unlikely that patients will ever be exposed to these high concentrations of prothrombin there is no abnormal prolongation on PT with either assay.

Since in vitro dilution of human whole blood reduces the concentration of all coagulation factors we wanted to specifically improve our understanding of the precise role of prothrombin on PT when analyzed by the POC versus lab assay. To assess this, prothrombin was neutralized by a prothrombin-neutralizing antibody and then prothrombin was added back in the form of MEDI8111. Although the software of the POC PT assay caps the maximum PT readout at 96 seconds, the POC PT values mirror the more sensitive lab PT. Both assays accurately reflected the change in prothrombin throughout the concentration curve and overlapped at or above 48.5 mg/L added MEDI8111. Based on this in vitro study approximately 66 mg/L measured prothrombin appears to be the rate-limiting threshold for coagulation. This is comparable to the prothrombin threshold observed in the in vitro dilution experiment (Figure 1) supporting the concept that PT is critically dependent on prothrombin concentration alongside sensitivity to fibrinogen, FV, and FX deficiency.²¹⁻²³ We have not examined the impact of deficiencies of these factors, which is a limitation of this study. A key consideration in using PT to guide prothrombin administration would be the possibility that a prolonged PT may have other reasons than low prothrombin concentration and that optimal treatment may require administration of additional coagulation factors.

A further limitation of the study is that HES is used as the only dilution fluid which is accepted to be controversial.¹⁹ The use of HES, however, creates a worst-case-scenario model with deranged fibrin polymerisation together with coagulation factor deficiency. We therefore believe it is relevant and valuable since we are testing the robustness of the POC PT test under most stringent conditions. A more procoagulant situation as compared to baseline has been shown at lower in vitro dilutions (<20-40%) with HES as well as other dilution fluids when coagulation were measured with ROTEM or TEG.²⁴⁻²⁶ If this phenomenon is applicable for PT then the measured value would not reflect the prothrombin concentration correctly at low dilutions. However, PT seem to be less impacted by this initial hypercoagulable state as seen with both saline²⁵ and HES dilution (Figure 1, Table 1) of whole blood. The hypercoagulability at low dilutions of blood observed with TEG has been shown to be dependent on the dilution of anti-thrombin.²⁷ We believe this might be the reason why PT is less impacted since PT has low sensitivity to changes in anti-thrombin concentrations.

5 | CONCLUSIONS

In this paper we have evaluated the suitability of a POC assay for measurement of PT in preclinical models of dilutional coagulopathy.

We have demonstrated that lab and POC measured PT is comparable when blood and prothrombin is above 60% of baseline and that both assays are insensitive to super-physiological concentrations of prothrombin. We recognize that accurate POC PT values cannot be obtained in extreme coagulopathy but for the purpose of triggering and monitoring fixed MEDI8111 dosing, CoaguChek XS Pro is a suitable assay to provide a PT threshold above which prothrombin replacement is beneficial. Our studies are limited to preclinical evaluation but provide the rationale for future clinical validation of POC PT tests to support MEDI8111 development and ultimately clinical use in trauma.

AUTHOR CONTRIBUTIONS

CAB and KMH contributed to experimental design, interpretation of results, and writing of the manuscript. NH performed and evaluated in vitro experiments and participated in writing of the manuscript. MO performed statistical analysis. AL advised on experimental design, interpretation of data, and participated in the manuscript review. All authors read and approved the final manuscript.

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RELATIONSHIP DISCLOSURES

CAB, NH, MO and KMH are employed by AstraZeneca. AL is a former employee of AstraZeneca. All authors declare no competing interests.

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