Universiteit
Antwerpen

This item is the archived peer-reviewed author-version of:

Inhibition of the procarboxypeptidase U (proCPU, TAFI, proCPB2) system due to hemolysis

Reference:
Mertens Joachim, Claesen Karen, Leenaerts Dorien, Sim Yani, Lambeir Anne-Marie, Hendriks Dirk.- Inhibition of the procarboxypeptidase U (proCPU, TAFI, proCPB2) system due to hemolysis
Journal of thrombosis and haemostasis - ISSN 1538-7933 - 17:6(2019), p. 878-884
Full text (Publisher's DOI): https://doi.org/10.1111/JTH.14432
To cite this reference: https://hdl.handle.net/10067/1598100151162165141

uantwerpen.be
Institutional repository IRUA

Inhibition of the procarboxypeptidase U (proCPU, TAFI, proCPB2) system due to hemolysis.

Joachim C. Mertens, Karen Claesen, Dorien Leenaerts, Yani Sim, Anne-Marie Lambeir, Dirk Hendriks

Laboratory of Medical Biochemistry, Department of Pharmaceutical Sciences, University of Antwerp, Antwerp, Belgium.

Running head: Inhibition of carboxypeptidase U due to hemolysis

Correspondence: Dirk Hendriks, Laboratory of Medical Biochemistry, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium.

Tel.: +32 3 265 27 27; E-mail: dirk.hendriks@uantwerpen.be

Abstract

Introduction: Spurious hemolysis of samples is the leading cause of interference in coagulation testing and was described to interfere in fibrinolysis assays. The influence of hemolysis on the procarboxypeptidase U (proCPU) system is not known.

Methods: By means of spiking of hemolysate in pooled normal plasma, the effect of hemolysis on CPU, proCPU and functional clot lysis assays was assessed. The influence of hemolysis on CPU generation during *in vitro* clot lysis was also evaluated. Cut-offs corresponding to maximal acceptable bias were determined.

Results and discussion: When active CPU was added to pooled plasma, a severe decrease in activity – up to 97.2 % inhibition – was seen with increasing plasma concentrations of oxyhemoglobin. The 10% cut-off value was 0.3 g/L oxyhemoglobin. Using an activity based assay, proCPU levels appeared to decrease gradually with increased hemolysis (maximal reduction of 19.5%) with a 10% cut-off value of 4.2 g/L oxyhemoglobin. The relative clot lysis time (%CLT) showed a maximal negative bias of 68.5%. The reduction in CLT paralleled a significant reduction of the first CPU activity peak. The cut-off value for the CLT was 0.4 g/L oxyhemoglobin. In presence of thrombomodulin (TM), the CLT+TM was not affected up to 8.0 g/L oxyhemoglobin.

Conclusion: These data indicate a clear inhibition of the CPU system due to hemolysis resulting in an increase of lysis in functional fibrinolysis assays. We were able to quantify the effect and to propose cut-off values for every parameter.

Essentials

- Hemolytic influence on the (pro)carboxypeptidase U ((pro)CPU) system is not known.
- In the current manuscript this was assessed by spiking pooled normal plasma with hemolysate.
- CPU activity, proCPU levels and clot lysis times showed a dose dependent hemolytic bias.
- The observed bias in the several CPU related parameters is due to inhibition of CPU activity.

Keywords

- Carboxypeptidase B2
- Carboxypeptidase U
- Fibrinolysis
- Hemolysis
- Thrombin-Activatable Fibrinolysis Inhibitor

Body Text

Introduction

Procarboxypeptidase U (proCPU, TAFI, proCPB2) – after activation into CPU – is a potent antifibrinolytic enzyme that was discovered three decades ago [1]. CPU can be measured with activity based, functional or immunological assays [2–5]. Preliminary experiments showed a bias in CPU activity in hemolytic plasma samples (unpublished data). Spurious hemolysis of samples is the leading cause of interference in hemostasis testing [6]. It can cause spectral interference (maximal absorbance of free hemoglobin at 410 nm). Furthermore, release of cytoplasmic and membrane molecules (tissue factor, proteases, fragmented phospholipid membrane…) can result in spurious activation of the coagulation cascade and platelets, causing biological interference [6]. Hemolysis significantly enhances fibrinolysis in thromboelastography experiments, suggesting a biological interference in functional fibrinolysis assays [7]. In this study, the influence of hemolysis on the CPU system was investigated.

Methods

Pooled plasma

Blood from 25 healthy volunteers was collected into vacutainer tubes containing 0.109 M trisodium citrate (9:1 v/v; Greiner Bio-One, Austria) and immediately placed on ice. The study was approved by the Medical Ethics Committee UZA-UAntwerp (B300201214328) and all volunteers provided informed consent. Blood samples were centrifuged (2000 *x* g; 15 min; 4°C) after which plasma was pooled, aliquoted and stored at -80°C.

Reagents

Purified proCPU was obtained as previously described [8] and was activated to purified CPU (see below). Purified human thrombin, potato tuber carboxypeptidase inhibitor (PTCI), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and $CaCl_2$ were from Merck (Germany). Rabbit-lung thrombomodulin (TM) was from Seikisui Diagnostics (USA). Bz-*o*-cyano-Phe-Arg was synthesized at the Laboratory of Medicinal Chemistry (University of Antwerp, Belgium) [3]. Purified porcine CPB was from Worthington Biochemical Corporation (USA).

Hemolysate preparation

Hemolysates were obtained by lysis of red blood cells (RBC) from five healthy volunteers. Citrated whole blood was centrifuged (2000 *x* g; 15 min) and the RBC fraction was washed three times with phosphate-buffered saline and centrifuged (2000 x g, 10 min) after which the washed RBC fraction was lysed with distilled water. A last centrifugation (2000 x g, 30 min) was performed to remove the debris and the lysates were pooled.

Measurement of cell-free hemoglobin in plasma

The extent of hemolysis was quantified by means of the cell-free oxyhemoglobin concentration in plasma. Oxyhemoglobin was measured spectrophotometrically at 562, 578 and 598 nm without interference of bilirubin and methemoglobin [9].

CPU activity measurement

CPU activity was measured with the substrate Bz-*o*-cyano-Phe-Arg based on a previously described method [2] with the modification that the background activity was not measured in heat-inactivated samples but after addition of PTCI (75 µg/mL). Pooled plasma was thawed on ice after which purified CPU was spiked in two concentrations (1:9 v/v) resulting in final CPU activities of 1.5 and 4 U/L respectively. Afterwards, samples were placed on ice for 10 min before analysis.

ProCPU measurement

Plasma was diluted 40 times in HEPES (20 mM; pH 7.4) and proCPU was activated with human thrombin (4 nM), thrombomodulin (16 nM) and $CaCl_2$ (50 mM) after which the generated active CPU was measured with the substrate Bz-*o*-cyano-Phe-Arg and the formed product quantified by HPLC [3].

Clot lysis experiments

In vitro clot lysis experiments were performed as described by Leenaerts *et al.* in the presence and absence of PTCI (20 µg/mL) and thrombomodulin (0.5 nM) [4]. To overcome analytical interference due to hemolysis, the turbidity was measured at 450 nm instead of 405 nm in a spectrophotometer (Spectramax; Molecular devices). The clot lysis time (CLT; the time between half-maximal turbidity during coagulation and fibrinolysis respectively) was calculated automatically [9]. The ΔCLT is the absolute reduction in CLT after addition of PTCI [10]. The %CLT was defined as the reduction in CLT (ΔCLT) relative to its respective CLT. The %CLT reflects the intrinsic potential of the CPU system of an individual. In the presence of 0.5

nM TM, the same parameters were determined and denoted CLT+TM, ΔCLT+TM and %CLT+TM.

CPU generation during *in vitro* clot lysis was determined as previously described [4,10] and was assessed after spiking of equal volumes of HEPES buffer (20 mM; pH 7.4) or hemolysate (5.3 g/L oxyHb) to pooled plasma.

Influence of hemolysis

The influence of hemolysis on CPU activity, proCPU levels and clot lysis determinations was assessed on pooled plasma. Hemolytic samples were prepared by spiking of pooled plasma with hemolysate. Pooled plasma diluted with the same volumes of HEPES buffer (20 mM, pH 7.4) served as comparator to correct for dilution. The range in oxyhemoglobin concentrations was selected based on the CLSI guidelines and covered all laboratory classifications of hemolysis [6,11]. The CPU activity, proCPU levels and %CLT were determined in the reference sample (no lysate or buffer added) and the observed bias due to hemolysis was expressed relative to these baseline values.

Oxyhemoglobin cut-offs

The cut-off oxyhemoglobin levels for the CPU activity and proCPU levels correspond to a mean bias of -10% compared to the result obtained in the non-hemolytic reference sample. For the clot lysis experiments, a bias of -10% on the %CLT was accepted which corresponds to a bias of approximately -5% in the absolute CLT. Both non-linear and linear relations were graphed as recommended by von Meyer *et al., c*ut-offs were determined by linear regression analysis on the data that showed a linear bias [12].

Results and discussion

CPU activity

To mimic hemolysis, different dilutions of hemolysate were added to pooled plasma resulting in cell-free oxyhemoglobin concentrations ranging from 0.03 g/L to 12.4 g/L. After addition of two different concentrations of purified CPU (1.5 U/L and 4 U/L), the remaining CPU activity was measured (Fig. 1A). There was a clear negative bias that paralleled increasing oxyhemoglobin levels. In samples with 2 g/L cell-free oxyhemoglobin, a mean reduction of 50.2% in CPU activity was observed and a further decline up to 97.2% was seen in plasma samples that contained 12.4 g/L cell-free oxyhemoglobin. The relative decrease in CPU activity was similar in samples that contained either 1.5 U/L CPU or 4 U/L CPU. The cut-off oxyhemoglobin level that resulted in a 10% decrease of the CPU activity was 0.3 g/L (Fig. 1B). Noteworthy, using the same assay, addition of hemolysate (2.4 g/L and 4.8 g/L oxyHb) also caused >90% inhibition of purified pancreatic CPB (data not shown).

ProCPU in plasma

The mean (±SEM; N=3) amount of proCPU measured in pooled plasma was 1025 (±23) U/L. As shown in Fig. 1C, spiking of the hemolysate resulted in a moderate gradual decrease of the measured proCPU level. An oxyhemoglobin concentration of 10.3 g/L cell-free oxyhemoglobin resulted in a decrease of 19.5%. The cut-off oxyhemoglobin level that resulted in a 10% reduction of the proCPU level was 4.2 g/L (Fig. 1D). The influence of hemolysis on proCPU levels was less pronounced in comparison to the bias seen in CPU activity measurements. One should consider that proCPU levels in plasma (approximately 1000 U/L) are 250-600 times higher than the spiked active CPU concentrations (1.5 – 4 U/L). Furthermore, in order to avoid substrate exhaustion, the plasma was diluted 40 times before activation of proCPU which

results in proportionally lower oxyhemoglobin concentrations during the incubation with the substrate.

Functional assays

The functionality of the CPU system was assessed in *in vitro* clot lysis experiments. The increase in turbidity during clot lysis was spectrophotometrically assessed at 450 nm instead of 405 nm to overcome spectral interference by hemoglobin. The CLT was not affected by the wavelength modification (N=10; p>0.05; Wilcoxon signed-rank test). The mean (±SEM; N=4) CLT, ΔCLT and %CLT of the pooled plasma were 49.5 (±0.6) min, 19.5 (±1.7) min and 39.3 (±1.0) % respectively. The hemolytic bias of the %CLT was expressed relative to the corresponding reference sample (Fig. 2A). The %CLT showed a dose-dependent decrease with increasing oxyhemoglobin concentrations; 2.0 g/L oxyhemoglobin resulted in a mean inhibition of 45% of the %CLT and a maximal inhibition of 68.5% was observed at 6.0 g/L. CLTs were measurable up to concentrations of 6.5 g/L oxyhemoglobin; interfering light absorbance at higher oxyhemoglobin concentrations resulted in a significant distortion of the clot lysis profile. Fig. 2B illustrates that the oxyhemoglobin cut-off in the regular clot lysis assay was 0.4 g/L.

In order to assess the effect of hemolysis on the CPU generation during *in vitro* clot lysis, hemolysate was added to pooled plasma resulting in 5.3 g/L oxyhemoglobin. This increased the optical density (absorbance) during the clot lysis experiments (range 0.2 - 1 vs. 1.75 - 2.45) and a reduced lag time in the presence of hemolysate (Fig 3A-B) was observed which is in concordance with previous observations of an accelerated clotting initiation in hemolytic samples [7].

The expected biphasic CPU generation pattern was seen in the absence of hemolysate (Fig. 3A). A clear inhibition of the generated CPU activity (mean (±SEM), N=2) was observed in the

presence of 5.3 g/L oxyhemoglobin (Fig. 3B). The first CPU peak which is formed upon the activation of proCPU by thrombin(-thrombomodulin), decreased from 9.2 (±0.1) U/L to 3.9 (±0.1) U/L, a reduction of 57.6%. As a result the CPU activity drops faster below the CPU threshold value resulting in a reduction of the CLT as described by Leurs *et al.* [13,14]. The mean (±SEM) CLT decreased from 48.5 (±1.5) min to 35.9 (±0.2) min in the presence of the hemolysate. This implied a reduction in the ΔCLT and %CLT of 73.1%. After lysis of the clot, the plasmin-dependent CPU activity peak was generated, but was similarly reduced with maximal CPU activities declining from 7.9 (±0.1) U/L to 2.7 (±0.1) U/L, which corresponds to a reduction of 65.6%.

In the presence of 0.5 nM TM – resulting in an increased CPU generation during coagulation – the mean (±SEM; N=4) CLT+TM was 69.9 (±0.7) min and the ΔCLT+TM and %CLT+TM were 33.2 (±0.4) min and 47.6 (±0.8) % respectively. The clot lysis time in the presence of thrombomodulin was not affected by hemolysis as no significant variations in the %CLT+TM were observed compared to non-hemolytic pooled plasma (Fig. 2C), although substantial distortion of the clot lysis profiles was observed at cell-free oxyhemoglobin levels >8.0 g/L.

Fig. 3B shows that severe hemolysis (5.3 g/L oxyHb) did not significantly influence the mean CLT+TM (77.3 *vs.* 78.7 min), ΔCLT+TM (42.9 *vs.* 44.8 min) or %CLT+TM (55.5% *vs.* 56.9%). Although the first CPU peak (mean (±SEM); N=2) did show a decrease of 35.7% (99.0 (±0.5) U/L *vs*. 63.7 (±2.5) U/L) which - based on the threshold hypothesis - should theoretically correspond to a reduction in the CLT+TM of approximately 2.5 min. Most likely, this difference is too small to be observed in the current experimental setup. Furthermore, the second CPU peak showed no inhibition (5.1 (±0.1) U/L *vs.* 6.4 (±0.2) U/L) (Fig. 2C), probably due to the high amount of CPU generated during the first peak, saturating the inhibitory effect.

Moore *et al*. demonstrated that RBC lysis exacerbated fibrinolysis in thromboelastography experiments and proposed a plasminogen-driven mechanism [7]. CPU generation assessment during regular CLT demonstrated that the inhibition of the first CPU peak (generated after proCPU activation by thrombin-thrombomodulin) is the driving force behind the increased fibrinolytic capacity and the observed bias in hemolytic samples.

In conclusion, our findings suggest that the results of CPU activity, proCPU levels and CLT determinations should not be reported when oxyhemoglobin levels exceed the cut-offs summarized in Table 1. These cut-offs were determined using hemolysate in order to mimic hemolysis and our data indicate that RBC lysis is a major cause of inhibition of CPU activity. Consistent application of the cut-off values is therefore crucial for the correct interpretation of data from observational studies and clinical trials. Additionally, sampling for CPU determinations is already challenging with blood collection and centrifugation on ice in tubes containing PPACK and aprotinin to avoid thermal inactivation of CPU and *ex vivo* activation of proCPU respectively [15]. Therefore it is of utmost importance that sample collection for CPU determinations is well-standardized and performed by well-trained and well-equipped teams, especially when samples are collected in acute settings or emergency departments (e.g. acute ischemic stroke, acute myocardial infarction, pulmonary embolism…) where spurious hemolysis is frequently seen and resampling is mostly impossible [16].

References

1 Hendriks D, Scharpé S, van Sande M, Lommaert MP. Characterisation of a carboxypeptidase in human serum distinct from carboxypeptidase N. *J Clin Chem Clin Biochem* 1989; **27**: 277–85.

2 Heylen E, Van Goethem S, Augustyns K, Hendriks D. Measurement of carboxypeptidase U (active thrombin-activatable fibrinolysis inhibitor) in plasma: Challenges overcome by a novel selective assay. *Anal Biochem* 2010; **403**: 114–6.

3 Heylen E, Van Goethem S, Willemse J, Olsson T, Augustyns K, Hendriks D. Development of a sensitive and selective assay for the determination of procarboxypeptidase U (thrombin-activatable fibrinolysis inhibitor) in plasma. *Anal Biochem* 2010; **396**: 152–4.

4 Leenaerts D, Loyau S, Mertens JC, Boisseau W, Michel JB, Lambeir AM, Jandrot-Perrus M, Hendriks D. Carboxypeptidase U (CPU, carboxypeptidase B2, activated thrombin-activatable fibrinolysis inhibitor) inhibition stimulates the fibrinolytic rate in different in vitro models. *J Thromb Haemost* 2018; **16**: 2057–69.

5 Hulme JP, An SSA. Detecting activated thrombin activatable fibrinolysis inhibitor (TAFIa) and inactivated TAFIa (TAFIai) in normal and hemophilia a plasmas. *Bull Korean Chem Soc* 2009; **30**: 77–82.

6 Lippi G, Plebani M, Favaloro EJ. Interference in coagulation testing: Focus on spurious hemolysis, icterus, and lipemia. *Semin Thromb Hemost* 2013; **39**: 258–66.

7 Moore HB, Moore EE, Gonzalez E, Hansen KC, Dzieciatkowska M, Chapman MP, Sauaia A, West B, Banerjee A, Silliman CC. Hemolysis exacerbates hyperfibrinolysis, whereas platelolysis shuts down fibrinolysis: evolving concepts of the spectrum of fibrinolysis in response to severe injury. *Shock* 2015; **43**: 39–46.

8 Schatteman K a., Goossens FJ, Scharpé SS, Hendriks DF. Proteolytic activation of purified human procarboxypeptidase U. *Clin Chim Acta* 2000; **292**: 25–40.

9 Kahn SE, Watkins BF, Bermes EW. An evaluation of a spectrophotometric scanning technique for measurement of plasma hemoglobin. *Ann Clin Lab Sci* 1981; **11**: 126–31.

10 Leenaerts D, Aernouts J, Van Der Veken P, Sim Y, Lambeir A, Hendriks D. Plasma carboxypeptidase U (CPU, CPB2, TAFIa) generation during in vitro clot lysis and its interplay between coagulation and fibrinolysis. *Thromb Haemost* 2017; **117**: 1498–508.

11 CLSI. Hemolysis, Icterus, and Lipemia/Turbidity Indices as Indicators of Interference in Clinical Laboratory Analysis; Approved Guideline. CLSI document C56-A. Wayne, PA; 2012.

12 von Meyer A, Cadamuro J, Lippi G, Simundic AM. Call for more transparency in manufacturers declarations on serum indices: On behalf of the Working Group for Preanalytical Phase (WG-PRE), European Federation of Clinical Chemistry and Laboratory Medicine (EFLM). *Clin Chim Acta* 2018; **484**: 328–32.

13 Leurs J, Nerme V, Sim Y, Hendriks D. Carboxypeptidase U (TAFIa) prevents lysis from

proceeding into the propagation phase through a threshold-dependent mechanism. *J Thromb Haemost* 2004; **2**: 416–23.

14 Leurs J, Wissing BM, Nerme V, Schatteman K, Björquist P, Hendriks D. Different mechanisms contribute to the biphasic pattern of carboxypeptidase U (TAFIa) generation during in vitro clot lysis in human plasma. *Thromb Haemost* 2003; **89**: 264–71.

15 Willemse JL, Brouns R, Heylen E, De Deyn PP, Hendriks DF. Carboxypeptidase U (TAFIa) activity is induced in vivo in ischemic stroke patients receiving thrombolytic therapy. *J Thromb Haemost* 2008; **6**: 200–2.

16 Söderberg J, Jonsson PA, Wallin O, Grankvist K, Hultdin J. Haemolysis index - An estimate of preanalytical quality in primary health care. *Clin Chem Lab Med* 2009; **47**: 940–4.

Addendum

J.C. Mertens and D. Hendriks: Study concept and design. J.C. Mertens, K Claesen and Y. Sim: recruitment of control population, sample processing and measurements. J.C. Mertens: processing and interpretation of data. J.C. Mertens: drafting of the manuscript. J.C. Mertens, K. Claesen and Y. Sim, D. Leenaerts, A.M. Lambeir, D. Hendriks: critical revision of the manuscript.

Acknowledgements

J.C. Mertens is a research assistant of the Research Foundation - Flanders (FWO Vlaanderen).

Conflict of Interest disclosures

J.C. Mertens reports a grant from the Research Foundation - Flanders (FWO Vlaanderen), during the conduct of the study. D. Hendriks reports grants from the Research Foundation - Flanders (FWO Vlaanderen), during the conduct of the study and consultancy and service fees from the Institut de Recherches Internationales Servier (I.R.I.S.) - France, outside the submitted work. K. Claesen, D. Leenaerts, Y. Sim and A.-M. Lambeir have nothing to disclose.

Tables and Figures

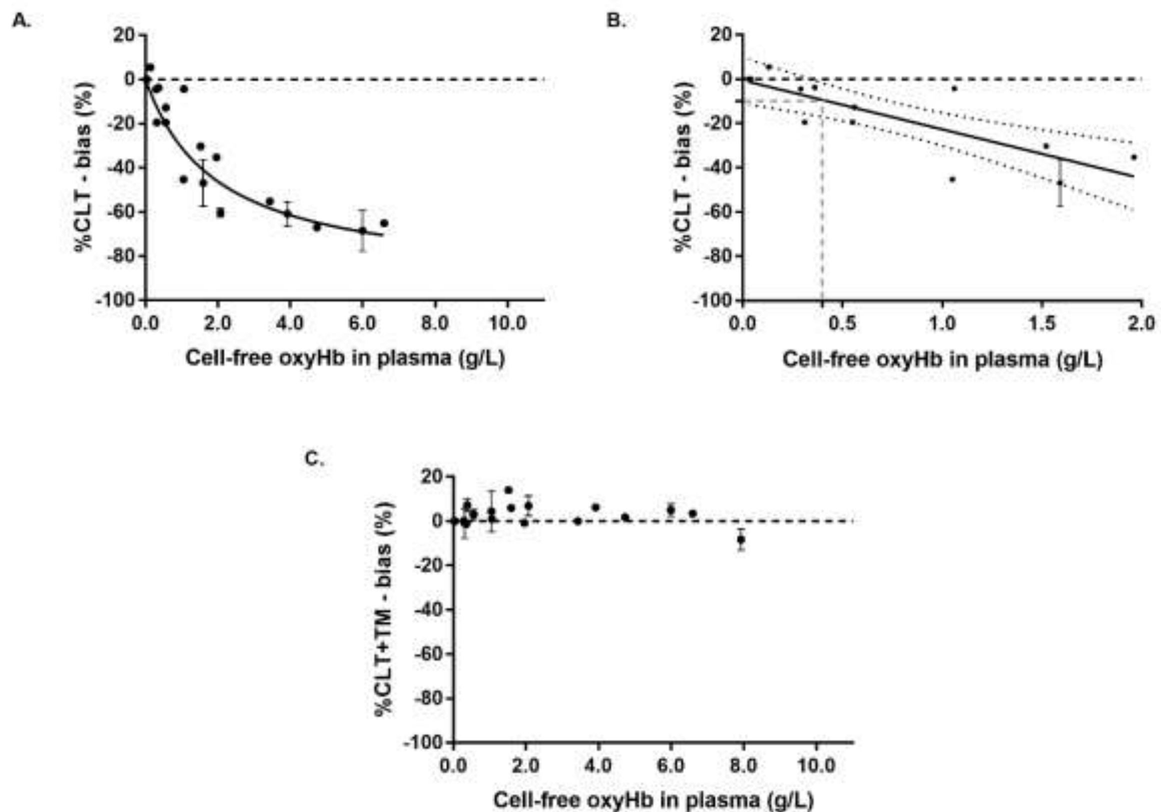

Fig. 1: Influence of hemolysis on activity based CPU assays.

CPU activity (A-B) and proCPU levels (C-D) were measured in the presence of increasing concentrations of oxyhemoglobin (hemolysate). The bias in (pro)CPU compared to the non-hemolytic reference level (black dashed line) was determined for every sample. All data were presented as mean, whiskers indicate SEM. The presented cell-free oxyhemoglobin (oxyHb) levels were measured in plasma. For measurement of proCPU, samples were diluted 1:40 to avoid substrate exhaustion, final in-assay oxyhemoglobin levels were equally lower. **Panel A: CPU activity.** Non-hemolytic baseline levels of 1.5 U/L (grey squares) and 4 U/L (black dots) of purified CPU showed a dose-dependent negative bias with increasing levels of cell-free oxyhemoglobin. The non-linear correlations are respectively displayed by the grey and black hyperbolas. **Panel B: Oxyhemoglobin cut-off for CPU activity determinations.** The linear

regression curve and its 95% CI (dotted lines) are displayed in black. The grey dashed lines indicate a reduction of 10 % in the CPU activity that coincided with 0.3 g/L cell-free oxyhemoglobin. **Panel C: ProCPU levels as measured with the in-house activity based proCPU assay.** Mean (±SEM; N=3) non-hemolytic baseline proCPU levels in the normal pooled plasma were 1025 (±23) U/L. Increasing oxyhemoglobin levels in the plasma resulted in a limited dose-dependent decrease of the proCPU levels. A mean negative bias of 19.8% was observed in the presence of 10.3 g/L oxyhemoglobin in plasma. The non-linear correlation is displayed by the black hyperbola. **Panel D: Oxyhemoglobin cut-off for proCPU determinations with the activity based assay.** The linear regression curve and its 95% CI (dotted lines) are displayed in black. The grey dashed lines indicate a reduction of 10% from the baseline proCPU that coincided with 4.2 g/L of cell-free oxyhemoglobin.

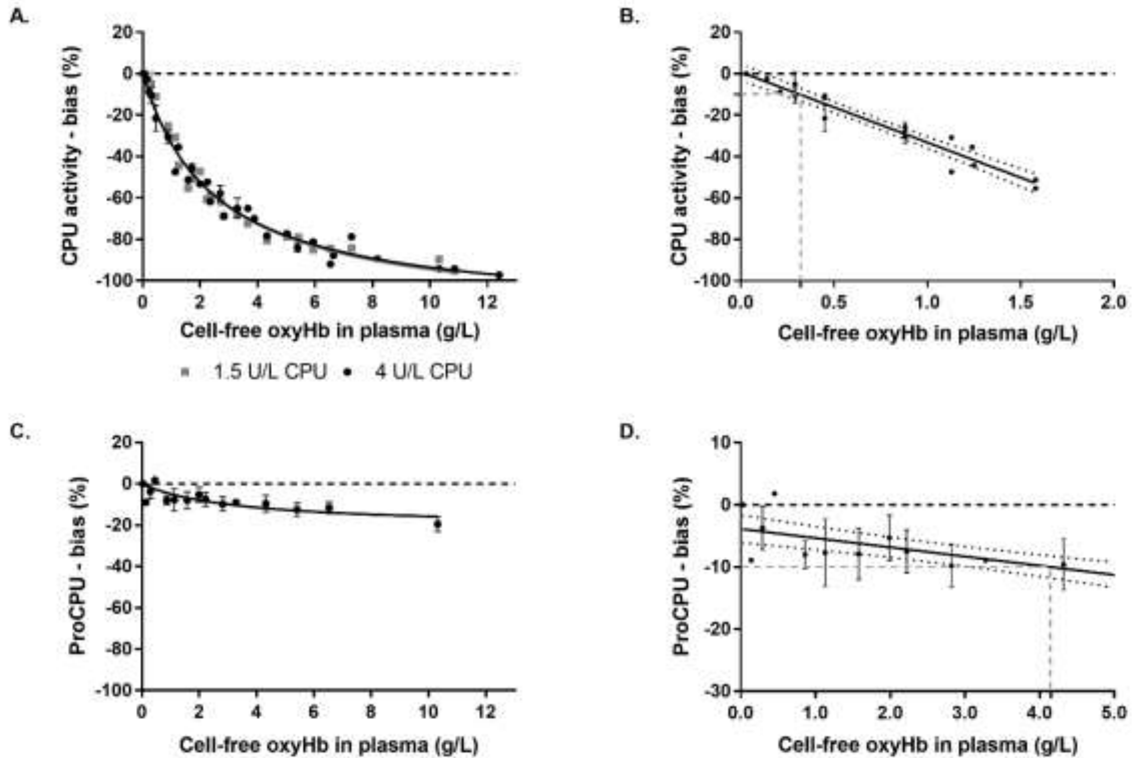

Fig. 2: Influence of hemolysis on functional CPU assays (*in vitro* clot lysis experiments).

The %CLT (A-B) and %CLT+TM (C) were measured in the presence of increasing concentrations of cell-free oxyhemoglobin (hemolysate). The bias in %CLT(-TM) compared to the non-hemolytic reference level (black dashed line) was determined for every sample. All data were presented as mean, whiskers indicate SEM. The presented cell-free oxyhemoglobin (oxyHb) levels were measured in plasma. In the clot lysis assays, plasma was 1:2 diluted, final in-assay oxyhemoglobin levels were proportionally lower. **Panel A: %CLT.** Mean (±SEM) non-hemolytic baseline levels of the %CLT were 39.3 (±1.0) %. Clot lysis times were measurable in the presence of oxyhemoglobin levels up to 6.5 g/L. A negative bias in the %CLT was observed with increasing levels of cell-free oxyhemoglobin with a maximal negative bias of 68.5% at 6.0 g/L oxyhemoglobin. The non-linear correlation is displayed by the black hyperbola. **Panel B: Oxyhemoglobin cut-off value for %CLT determinations.** The linear regression curve and its

95% CI (dotted lines) are displayed in black. The grey dashed lines indicate a reduction of 10% in the %CLT that coincided with 0.43 g/L of cell-free oxyhemoglobin. **Panel C: %CLT+TM.** Mean (±SEM) baseline levels of the %CLT+TM were 69.9 (±0.7) %. None of the tested oxyhemoglobin concentrations up to 8.0 g/L affected the %CLT+TM.

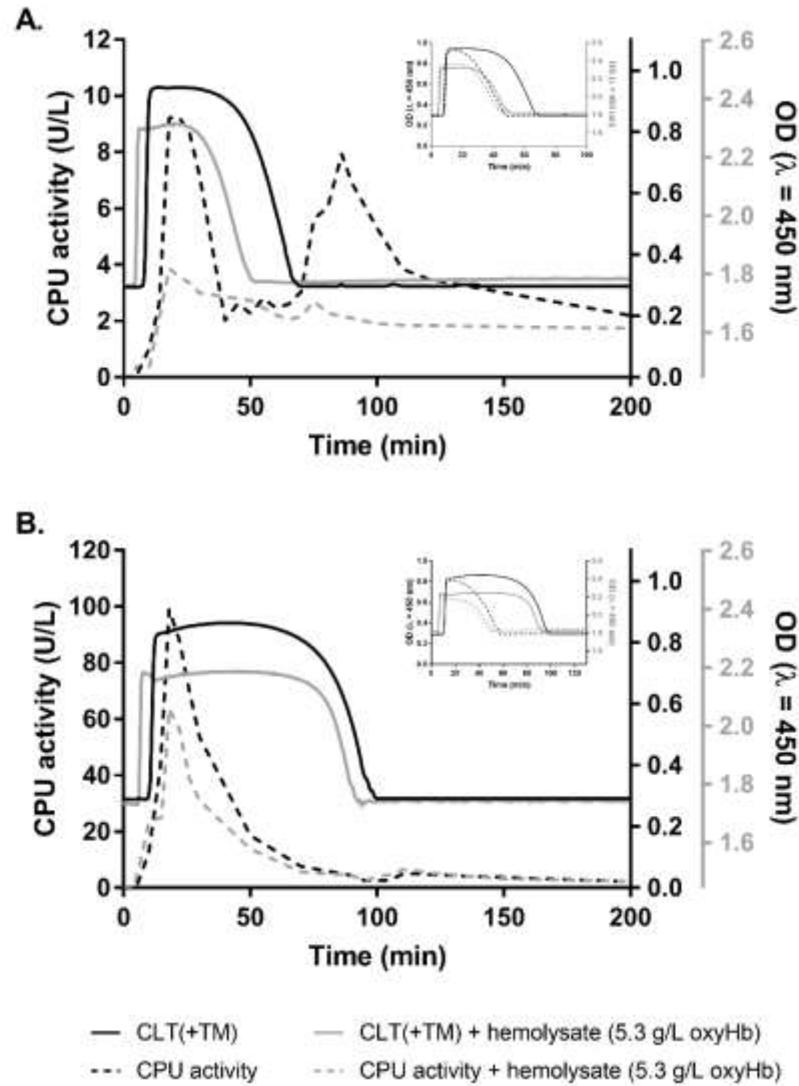

Fig. 3: Influence of hemolysis (hemolysate – 5.3 g/L oxyhemoglobin) on the CPU generation during *in vitro* clot lysis.

The clot lysis profiles in the absence of hemolysate are displayed as the black uninterrupted curve on the right y-axis. Addition of hemolysate (grey uninterrupted curve) increased the optical density (range 0.2 - 1 vs. 1.75 - 2.45). Profiles in the presence of hemolysate are displayed on the grey right y-axis. CPU generation profiles in the absence and presence of hemolysate are displayed as black and grey dashed curves respectively on the left y-axis. Data are presented as mean only (N=2). **Panel A: CPU generation during regular CLA.** Addition of

hemolysate reduced the CLT and ΔCLT (from 48.5(±1.5) to 35.9 (±0.2) and from 20.5 min to 5.5 min respectively) corresponding to a reduction of the %CLT of 73.1 %. The first and second CPU peak were both reduced in the presence of the hemolysate. The first peak showed a 57.6 % reduction from 9.2(±0.1) U/L to 3.9 (±0.1) U/L, the second peak a 65.6 % reduction from 7.9 (±0.1) to 2.7 (±0.1) U/L. The lag time which corresponds to the clot initiation was reduced from 6.5 min to 4.5 min. **Panel B: CPU generation during CLA in the presence of thrombomodulin (TM).** The CLT+TM, ΔCLT+TM and % CLT were not affected by the addition of hemolysate (77.3 *vs.* 78.7 min; 42.9 *vs.* 44.8 min and 55.5% *vs.* 56.9% respectively). The first CPU generation peak showed a 35.7 % reduction from 99.0 (±0.5) U/L to 63.7 (±2.5) U/L, the second peak was slightly increased from 5.1 (±0.1) U/L to 6.4 (±0.1) U/L. The lag time was reduced from 7.0 min to 4.5 min. **Insets:** The insets in both panels show the clot lysis profiles with and without PTCI (dashed and uninterrupted curves respectively). Non-hemolytic conditions are displayed in black and plotted on the left y-axis, hemolytic conditions are displayed in grey and plotted on the modified right y-axis.

Table 1: Overview of recommended oxyhemoglobin cut-off values for CPU related parameters.

Parameter	Bias	oxyHb cut-off (g/L)	Hemolysis category*	Visual assessment*
CPU activity	-10 %	0.3	Slightly hemolyzed	Yellow to slightly pink
ProCPU†	-10 %	4.2	Grossly hemolyzed	Red to brown
Clot lysis assay (CLA)				
CLT ‡	-5 %	0.4	Mildly hemolyzed	Pink to slightly red
ΔCLT	-10 %	0.4		
%CLT	-10 %	0.4		
CLA with TM	N/A	8.0	Grossly hemolyzed	Red to brown

*Categories according to: Lippi *et al*. *Semin Thromb Hemost* 2013; **39**: 258–66. †ProCPU levels as measured with our in-house activity based proCPU assay. ‡Based on the bias observed in the %CLT. (pro)CPU: (pro)carboxypeptidase U, CLT: clot lysis time, N/A: not applicable, oxyHb: Oxyhemoglobin, TM: thrombomodulin.