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Platelet Enhanced Plasma: Characterisation of a novel candidate resuscitation fluid's extracellular vesicle content, clotting parameters and thrombin generation capacity

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Conflict of Interest

The authors declare no conflicts of interest.

For Transfusion

Abstract

BACKGROUND:

Platelet transfusion is challenging in emergency medicine because of short platelet shelf-life and stringent storage conditions. Platelet-derived extracellular vesicles (PEV) exhibit platelet-like properties. A plasma generated from expired platelet units rich in procoagulant PEV may be able to combine the benefits of plasma and platelets for resuscitation whilst increasing shelf life and utilising an otherwise wasted resource.

STUDY DESIGN AND METHODS:

Freeze-thaw cycling of PRP followed by centrifugation to remove platelet remnants was utilised to generate platelet enhanced plasma (PEP). An *in vitro* model of dilutional coagulopathy was also designed and used to test PEP. Rotational Thromboelastometry and Calibrated Automated Thrombography were used to assess clotting and EV procoagulant activity. Capture arrays were used to specifically measure EV sub-populations of interest (ExoView™, NanoView Biosciences). Captured vesicles were quantified and labelled with: Annexin-V-FITC, CD41-PE and CD63-AF647. Platelet alpha granule content (PDGF-AB, soluble P-Selectin, VEGF-A and NAP2-CXCL7) was measured. Commercially available platelet lysates were also characterised.

RESULTS:

PEP is highly procoagulant, rich in growth factors, exhibits enhanced thrombin generation and restores haemostasis within an *in vitro* model of dilutional coagulopathy. The predominant vesicle population were PEV with 7.0×10^9 CD41+PS+ EV/ml compared to 4.7×10^7 CD41+PS+ EV/ml in platelet-free plasma ($p = 0.0079$). Commercial lysates show impaired but rescuable clotting.

DISCUSSION:
PEP is a unique candidate resuscitation fluid containing high PEV concentration with preliminary evidence indicating a potential for upscaling the approach using platelet concentrates. Commercial lysate manufacturer workflows may be suitable for this, but further optimisation and characterisation of PEP is required.

Introduction

In 1967, Wolf coined the term “platelet dust” to describe the material underpinning the procoagulant properties observed in platelet poor plasma (PPP) and serum [1-3]. This material is now recognised to be a subpopulation of extracellular vesicles (EV) derived from platelets [1,4]. EV are released by all cells, identified in virtually all biological fluids and act as key mediators of intracellular communication, performing roles in homeostasis and disease[5,6]. They are small cell-derived vesicles that cannot replicate, are delimited by a lipid bilayer and can be released constitutively or induced in response to activation from injury, inflammation and stress (e.g. oxidative, shear). [7,8]. Current classification divides EV into 3 major sub-types: endosomal origin (e.g. exosomes (30-150nm) and plasma membrane origin (e.g. microvesicles (100-1000 nm) and apoptotic bodies (50-5000 nm)) [8-11]. EV are highly heterogeneous, varying and overlapping in: size, biological function and cargo (which can include various RNA species, DNA species, protein and lipids) [12-14]. Procoagulant platelet-derived EV (PEV) are characterised by externalised phosphatidylserine (PS). Externalised PS provides a negatively charged surface that promotes assembly of the tenase and prothrombinase complexes facilitating thrombin generation and mediating conversion of fibrinogen into fibrin. PEV contribute to the procoagulant properties of fresh frozen plasma (FFP), with differences in PEV content potentially affecting clinical outcomes[21-25]. Physiologically, PEV are released upon traumatic injury and are positively associated with survival [26,27].

Platelets are stored at room temperature under gentle agitation and have a shelf life of 5-7 days. Stringent storage conditions leads to high wastage and demand for constant acquisition [35]. Platelets have been described as essentially unavailable in remote and austere environments [36-39]. The PROMMT study, a multicentre prospective observational trial of civilian trauma, highlighted that increased use of plasma or platelets relative to packed red blood cells (PRBC)s was associated with a reduction in 24-hour mortality [40].

Human platelet lysates (HPL) are generated via freeze-thaw cycling of expired platelet units and contains high levels of growth factors and form an alternative to foetal bovine serum (FBS) for the expansion of a diverse range of cell types[41,42]. Here we provide evidence for the *in vitro* potency of Platelet Enhanced Plasma (PEP) generated through freeze-thaw cycling of platelet rich plasma (PRP) and some preliminary evidence on utilising the process on platelet units.

Methods

Volunteers

This study received ethical approval from the University of Birmingham's Science, Technology, Engineering, and Mathematics Ethical Review Committee (ERN-18-1017). Healthy volunteers were recruited from the University of Birmingham Research Laboratories, Queen Elizabeth Hospital. Exclusion criteria included a history of bleeding symptoms and use of aspirin/NSAIDs and other antiplatelet drugs within 1 week. Informed written consent was received from all participants. Blood was withdrawn from each participant via antecubital venepuncture using a sterile 21-gauge needle and aseptic technique. The first 4ml of blood were withdrawn into EDTA anticoagulant BD vacutainers for full blood counts using the Sysmex XN1000 (Sysmex, UK, Milton Keynes). Up to 42ml of blood was then drawn into tri-sodium citrate anticoagulant BD vacutainers for experiments (9:1 vol/vol).

PEP generation

PRP was prepared from whole blood, following the recommendations of the Platelet Physiology Subcommittee of SCC/ISTH by 200 x g centrifugation for 10 minutes with no brake [43]. PRP was aspirated without disturbing the buffy coat. Residual blood underwent a second centrifugation at 1500 x g for 20 minutes. PPP was aspirated without disturbing the buffy coat. PEP was generated by 3x freeze-thaw cycling of PRP at -80°C and 37°C. After the final thaw sample underwent 3000 x g centrifugation to remove residual platelet remnants and aggregates and was stored frozen until required for experiments. For some experiments platelet free plasma (PFP) was generated by spinning whole blood once at 2000 x g for 20 minutes, followed by a second spin of the aspirated plasma at 13,000 x g for 2 minutes.

Commercial Lysates

HPL were also obtained from 3 different companies: MultiPL'100 (MacoPharma, Mouvaux, France) $PL_{SOLUTION}$ Research Grade (PL Bioscience GmbH, Dennewartstr, Germany), CRUX RUFA Research Trinova Biochem GmbH, Rathenaustr, Germany). All HPL preparations were thawed at 37°C, aliquoted and stored at -80°C. HPLs were thawed at 37^0C for 10 minutes prior to experimental analysis.

Thrombin Generation

Thrombin generation was assessed by using calibrated automated thrombography (CAT) as described by Hemker et al [44]. Briefly, 96 well round bottomed plates were prepared with 4 wells containing 80 μl of sample and 20 μl of either PRP reagent (5 pM tissue factor) or thrombin calibrator. Plates were then incubated for 10 minutes at 37°C, after which 20 μl of FLUCA reagent (fluorescent thrombin substrate Z-Gly-Gly-Arg-aminomethylcoumarine (ZGGR-AMC) and $CaCl_2$ (CAT# 86197, Stago) was automatically added to initiate thrombin generation. After sample activation samples were monitored using a fluorescent plate reader (Fluoroskan Ascent, Thermo Scientific™, UK). Thrombin generation parameters (lag time, peak, time to peak, ETP (endogenous thrombin potential/area under the curve, start tail) were calculated by the Hemker software (Thrombinoscope Software Version V5.0.0.742, Stago).

ROTEM

Rotational thromboelastometry (ROTEM) was used to measure clotting time (CT), clot formation time (CFT) (time to reach clot amplitude of 20 mm), maximum clot firmness (MCF) and the alpha (^{0}), which is the angle of the tangent between 0 and 20 mm clot amplitude. A 4-channel instrument was used for all analysis (ROTEM® Delta, Werfen, Spain). Following the automated pipetting programme, 20 μl of recalcification reagent (TEM Innovations GmbH, Munich, Germany), 20 μl of activation reagent and 300ul of sample were mixed and instrument recording initiated. The activation reagent was either INTEM (TEM Innovations GmbH, Munich, Germany) or EXTEM reagent (TEM Innovations GmbH, Munich, Germany); for investigation of the intrinsic and extrinsic pathways, respectively. Instrument and sample temperature were maintained at 37°C, except for investigation of the coagulopathy model of whole blood and PEP treatment.

In Vitro model of coagulopathy

A model of *in vitro* coagulopathy was designed based on the independent protocols of Caballo et al and Shenkman et al [45,46]. Citrated whole blood was collected and diluted to 40% with 0.9% saline solution. Whole blood, diluted blood and diluted blood treated 1:1 with PEP were then assessed by

ROTEM. To better reproduce the coagulopathy associated with trauma hypothermia was also simulated in diluted blood, with or without PEP treatment, by measurement at 32°C[47]. Whole blood was measured at 37°C.

APTT, PT and fibrinogen

Activated partial Thromboplastin (APTT), Prothrombin time (PT) and Clauss fibrinogen assays were performed on a Sysmex CS-5100 coagulometer (Sysmex, Kobe, Japan) using Actin FS, Thromborel S and Dade® Thrombin Reagent (Siemens Healthineers, Marburg, Germany) on PEP, PPP and HPL.

EV phenotyping with ExoView

EV were detected by the ExoView R100 reader (NanoView Biosciences, Boston, MA, USA) [48]. Tetraspanin Plasma chips (NanoView Biosciences, Boston, MA, USA) were used for all samples. Chips were arrayed with capture antibodies against human CD63, CD81, CD9, CD41a and Mouse IgG. Chips were placed in separate wells of a 24 well plate. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline (HBS) was used to dilute all samples. PFP samples were diluted 1:10 and PEP samples were diluted 1:250 in HBS. 35 µl of diluted sample was applied to each chip. Distilled water was added to the void space between wells, the plate was sealed and incubated for 16 hours at room temperature in the dark. Chips were washed three times with HBS supplemented with 2.5mM Ca^{2+} and 15uM GPRP (Gly-Pro-Arg-Pro) amide (Sigma Aldrich). After each wash the plate was shaken at 500 rpm (LSE Digital Microplate Shaker, Corning) for three minutes. Following the final wash, a fluorescent antibody cocktail of annexin-V-FITC (1:2000, Biolegend), CD41-PE (1:2000, Biolegend) and CD63-Alexa-647 (1:1200, Nanoview Biosciences) was added and incubated for one hour at room temperature in the dark. Chips were washed three times as previously. A final wash was performed with distilled water. The chips were imaged with the ExoView R100 reader (nScan2 Version 2.76 software). The data was analysed using NanoViewer Version 2.82 with fluorescence gating based on Mouse IgG capture background and sizing thresholds set at 50 nm to 200 nm.

Growth Factor Characterisation by multiplex assay

Magnetic bead-based multiplex assays (Luminex) were used to assess the growth factor content (PDGF-AB, P-selectin, VEGF-alpha and CXCL7/NAP2) of PEP, PPP and HPL (R&D Systems, Minneapolis, USA). Briefly, 50 µl of diluted samples or standards were added to 96 well plates. 50 µl of analyte bead cocktail was added to each well and the plate was incubated for 2 hours at room temperature on a plate shaker at 800 rpm (LSE Digital Microplate Shaker, Corning). Following an automated programme, plates were washed three times with a magnetic plate washer. The antibody-biotin reporter was added and incubated for 1 hour at room temperature on a plate shaker at 800 rpm. Following three washes, 50 µl of streptavidin-phycoerythrin was added to each well and plates were incubated at room temperature in the dark on a plate shaker for 30 minutes. Following a final three washes the plates were read on the Bioplex 100 (Bio-Rad Laboratories, Hercules, CA). 5-parameter logistic curves were generated from the standards.

Statistical Analysis

Statistical analysis and figures were generated using RStudio [49]. For all datasets normality was not assumed. For all datasets where more than two groups are being compared Kruskall-Wallis tests were performed followed by Wicoxon signed rank tests where sample groups were related and Wilcoxon rank sum tests where samples groups were unrelated. The Holm-Bonferroni method was applied to correct for multiple comparisons. Where datasets contained two samples the Wilcoxon signed rank test was used to determine significance. Values reported in text are median values. The predefined level of significance was set at 5% (alpha level = 0.05).

Results:

Blood Cell Count:

A summary of cell counts in whole blood, PPP, PRP, PEP and PFP (N = 6) are shown in Figure 1. Expired platelet concentrate cells counts are shown in supplementary materials Table 4. The platelet count of whole blood (252 x 10^9/L, p = 0.031) was less than PRP (376 x 10^9/L), greater than PPP (9.5 x 10^9/L, p = 0.031), PEP (6 x10^9/L, p = 0.031) and PFP (<1 x 10^9/L, p = 0.031). The erythrocyte counts in whole blood (4.94 x 10^{12}/L) were greater than PRP (0.03 x 10^{12}/L, p = 0.035), PPP (0.00 x 10^{12}/L, p = 0.036), PEP (0.00 x 10^{12}/L, p = 0.031) and PFP (0.01 x 10^{12}/L, p = 0.036). As expected, the number of leukocytes was lower in PRP (0.01 x 10^9/L, p = 0.031), PPP (0.00 x 10^9/L, p = 0.031), PEP (0.00 x 10^9/L, p = 0.031) and PFP (0.00 x 10^9/L, p = 0.031) compared to whole blood (6.63 x 10^9/L). PRP is enriched in platelets with minimal leukocyte and erythrocyte contamination. Freeze-thaw cycling of PRP, generates PEP which is characterised by reduced platelet count (p = 0.031) and is free of leukocyte and erythrocyte contamination.

PEP shows Enhanced Thrombin Generation Characteristics

Figure 2 shows a comparison of PRP and PEP with PPP and freeze-thawed PPP controls. Figure 2 a) shows an enhanced leftward shift in thrombin generation dynamics for PEP compared to PPP, PRP, freeze-thawed PPP and HPL. Figure 2 b) shows PEP (255.8 nM) has the highest peak thrombin generation and is improved compared to PPP (46.13 nm, p = 0.0079), PRP (114.0 nm, p = 0.0079) and HPL (72.6 nm, p = 0.036). Interestingly, Figure 2 c) shows PEP (1351.1 nmol/min) ETP does not increase compared to fresh PRP ETP (1268.7 nmol/min, p = 0.22); indicating that the total amount of thrombin generated is not altered. PEP ETP (1351.1 nmol/min) was greater than PPP (802.0 nmol/min, p = 0.0079) and HPL ETP (1104 nmol/min, p = 0.036). As shown in Figure 2 d), PEP (3.6 mins) lag time is reduced compared to PPP (6.63 mins, p = 0.0079) and PRP (6.13 mins, p =0.0079), but was not significantly different to HPL lag time (4 mins, p = 0.14). The time to reach peak thrombin generation, (Figure 2 e)) is reduced in PEP (6.94 mins) compared to PPP (13.32 mins, p = 0.079) and PRP (14.67

mins, p = 0.0079) but not HPL (10.92 mins, p = 0.39). Figure 2 f) shows that the start tail of PEP (26.49 mins) is earlier compared to PPP (52.27 mins, p = 0.0079), PRP (37.67 mins, p = 0.0079) and HPL (41.75 mins, p = 0.036). A full breakdown of HPL thrombin generation parameters is shown in supplementary material Table 1. Expired NHSBT platelet unit's thrombin generation parameters are shown in supplementary Figure 1 and Table 5.

PEP is Enriched in Procoagulant CD41+ EV

Figure 3 shows secondary labelling of captured PEP and PFP EV. As expected, there were higher concentrations of PS+ vesicles captured on CD41 (7.0 x 10^9 particles/ml) (Figure 3 a) and CD9 (3.7 x 10^9 particles/ml) (Figure 3 c) spots in PEP compared to PFP (4.7 x 10^7 particles/ml, 1.8 x 10^7 particles/ml, p = 0.0079). Figure 3 b) shows greater PS+ and CD41+ EV (5.1 x 10^8 particles/ml, 1.6 x 10^9 particles/ml) compared to PFP (3.3. x 10^6 particles/ml, 4.4 x 10^6 particles/ml, p = 0.0079) captured on CD63. The increased EV count in PEP on CD63 capture is less than that recorded on other spots. CD81 captured EV, shown in Figure 3 d), increase modestly in PEP compared to PFP and indicate that platelets have low capacity to generate CD81+ EV. Supplementary table 3 shows EV size. The size of PEP EV captured on CD63 (65 nm) and CD9 (80 nm) were not significantly different to PFP EV captured on CD63 (65nm, p = 0.91) and CD9 (80 nm, p = 0.75). CD41 captured (100 nm) PEP EV were larger than CD41 captured PFP EV (75 nm, p = 0.012). CD81 PEP EV (55 nm) were smaller than CD81 PFP EV (70 nm, p = 0.043).

PEP Clotting Characteristics

Figure 4 a) shows PEP (40.5 s) exhibits an enhanced CT with EXTEM stimulation compared to PPP (51 s, p = 0.210) No differences were observed in INTEM CT between groups (Figure 4 e). With EXTEM and INTEM testing there were no significant differences in CFT between groups (Figure 4 b and f). MCF (Figure 4 c)) is significantly greater in PRP (79 mm) with EXTEM testing compared to PPP (24 mm, p = 0.0079),) and PEP (26.8mm, p = 0.0079). MCF is also greater in PRP (79mm) compared to PPP (23.0mm, p = 0.012) and PEP (26.0mm p =0.0079) with INTEM testing. With EXTEM testing alpha

angle was similar in PRP (84.0), PPP (81.0, p = 0.25) and PEP (83, p = 0.53). Alpha angle was also similar, with INTEM testing, between PRP (84.0) and PPP (79.5, p = 0.071) and between PRP and PEP (81, p = 0.071). Figure 4 i), j) and k) shows no significant differences in APTT, PT or fibrinogen between PEP and PPP.

PEP Treatment of an *In Vitro* Model of Coagulopathy: Clotting Characteristics

With EXTEM testing the CT of the dilutional model of coagulopathy (122 s) was increased compared to whole blood (82 s, p = 0.012) and PEP treatment of the coagulopathic blood (56 s) resulted in a shorter CT compared to both whole blood (p = 0.012) and the model (p = 0.012). CT of the dilutional model (302 s) was increased with INTEM testing compared to whole blood (159 s, p = 0.012) and restored upon PEP treatment (151s, p = 0.016), with no significant difference to whole blood CT (p = 0.53). The MCF of whole blood (67 mm) with EXTEM testing was reduced in the model conditions (43 mm, p = 0.0079) and did not significantly increase with PEP treatment (50 mm), remaining unchanged compared to the model (p= 0.25). INTEM testing shows that MCF is reduced in the model (45 mm) compared to whole blood (67 mm, p = 0.012) remaining unchanged upon PEP treatment (43.5 mm, p = 0.75) and lower than whole blood MCF (p = 0.012). With EXTEM testing the alpha fell in the model conditions (49) compared to whole blood (75, p = 0.0079). Upon PEP treatment (78, p = 0.012) EXTEM alpha was significantly improved and similar to whole blood alpha (p = 0.75). With INTEM testing model alpha (47) was lower than whole blood alpha (78, p = 0.012), PEP treatment improved alpha (79, p = 0.012), restoring to a similar value to whole blood (p = 0.67).

PEP and Commercial Lysates are Enriched in Platelet-Derived Growth Factors

PEP shows significant enhancements in PDGF-AB (4138.99 pg/ml), P-selectin (137.94 pg/ml), VEGF-A (57820.77 pg/ml) and NAP-2/CXCL7 (63794500 pg/ml) compared to PPP (PDGF-AB = 336.31 pg/ml, p = 0.0022; P-selectin = 40.77 pg/ml, p = 0.0022; VEGF-A = 28027.42 pg/ml, p = 0.005; NAP-2/CXCL7 = 3073850 pg/ml, p = 0.0022). Similar enhancements were observed with HPL (PDGF-AB = 3244.14

pg/ml, p = 0.024; P-selectin = 308.75 pg/ml, p = 0.024; VEGF-A = 84262.8 pg/ml, p = 0.0028; NAP-2/CXCL7 = 102080000 pg/ml, p = 0.024) compared to PPP. Interestingly, HPL show similar concentrations of PDGF-AB (p = 1) to PEP but have higher concentrations of P-selectin (p = 0.024), VEGF-A (p = 0.048) and NAP2/CXCL7 (p = 0.024). Individual breakdown of growth factor content of HPL is shown in Supplementary materials Table 1.

Commercial Lysate Clotting is Impaired but Rescued with Fibrinogen Supplementation

Figure 7 shows characterisation of HPL clotting parameters. Supplementary materials Table 2 shows HPL exhibit impaired clotting, if any clotting at all. However, thrombin generation characteristics of the lysates (Figure 2), show good capacity to generate thrombin. Upon exogenous addition of fibrinogen to 2mg/ml, 3mg/ml and 4mg/ml there is restoration and improvement of clotting parameters as measured by ROTEM (Figure 7).

Discussion

PEP is a platelet enhanced plasma that contains high levels of procoagulant PEV with significantly enhanced thrombin generation dynamics compared to donor matched PRP and PPP. PEP can be generated from platelets from fresh whole blood (PRP), with preliminary evidence indicating an optimised procedure could be utilised on platelet concentrates (preliminary data shown in supplementary material). PEP also contains high amounts of platelet derived growth factors which could aid in wound healing and immune cell recruitment [50]. PEP successfully restores clotting time in an *in vitro* model of dilutional coagulopathy and forms a novel candidate resuscitation fluid. The MCF of PEP is not as high as PRP and is more analogous to PPP. Platelets are incorporated into the clot and in their absence clot strength declines. PEP treatment of the *in vitro* dilutional coagulopathy model did not restore MCF but did successfully restore clotting time. The clotting and thrombin generation data together suggest PEP would be useful in accelerating the formation of a stable clot. PEV have been shown to have positive effects on platelet function as assessed by aggregometry[51]. PEV have also been shown to exhibit non-haemostatic effects on the vessel wall, inflammation and vascular tone which may be beneficial for the treatment of haemorrhage and trauma [20].

The potency of PEV has recently been demonstrated in mouse models of severe haemorrhage, where PEV alone restored mean arterial blood pressure, plasma protein concentration, lactate levels and the base deficit compared to vehicle or fresh platelet administration [28]. A recent publication suggests a PEV rich platelet lysate generated via sonication and suspension in a balanced salt solution could be used as a topical treatment to treat bleeding in trauma patients [34]. Saline has repeatedly been shown to be less efficacious than plasma for fluid replacement in trauma patients, where it can promote dilutional coagulopathy and contribute to metabolic acidosis[53-55]

In contrast to other PEV rich solutions[28,30-32,34] PEP is suspended in plasma. EV are historically challenging to measure, direct measurement in complex biofluids such as plasma is difficult. We utilised a surface-molecule affinity EV capture and secondary labelling of captured EV with minimal

sample manipulation. This vesicle phenotyping shows PEP EV are largely platelet derived and procoagulant (CD41+PS+).

Using the PEP procedure on NHSBT platelet units could form an avenue for extending clinical utility of platelets and aid in reducing wastage[56]. As HPL manufacturers already exist, it may be possible to generate an off-the-shelf resuscitation fluid using an existing workflow. However, despite the strong capacity for thrombin generation in HPL, clotting was impaired. However, we have shown fibrinogen supplementation restores clotting. Other processes are likely involved in commercial HPL generation that impede clotting as the same impairment is not observed in 8-day old NHSBT platelet units. Certainly, HPL have been optimised for cell culture and this is reflected in the significantly greater concentrations of P-Selectin, VEGF-alpha and NAP2/CXCL7 present in HPL compared to PRP-derived PEP. It is feasible that the PEP generation procedure may be more beneficial if started earlier. A time course following platelet ageing and subsequent PEP composition and functional activity will be necessary to determine the optimal timepoint for PEP generation. A future PEP-like product would undergo full proteomic analysis, measurement of serotonin, plasminogen activator 1 (PAI-1), polyphosphate[59]. PEV interaction with leukocytes, the endothelium and platelet function will also be important parameters [60].

An additional challenge with EV based therapeutics is establishing dosage and ensuring a standardised final product as covered in in this cited position paper [61]. A final PEP preparation derived from platelet units could be investigated through *ex vivo* treatment of blood from trauma patients with coagulopathy, followed by investigation in an *in vivo* small animal model of trauma. Limitations to this work include the scaled-down approach, the model of dilutional coagulopathy used, which could be improved by simulating metabolic acidosis and strengthened with an additional control of diluted blood measured at 37°C to assess the relative contribution of hypothermia to the model.

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Figures

Figure 1

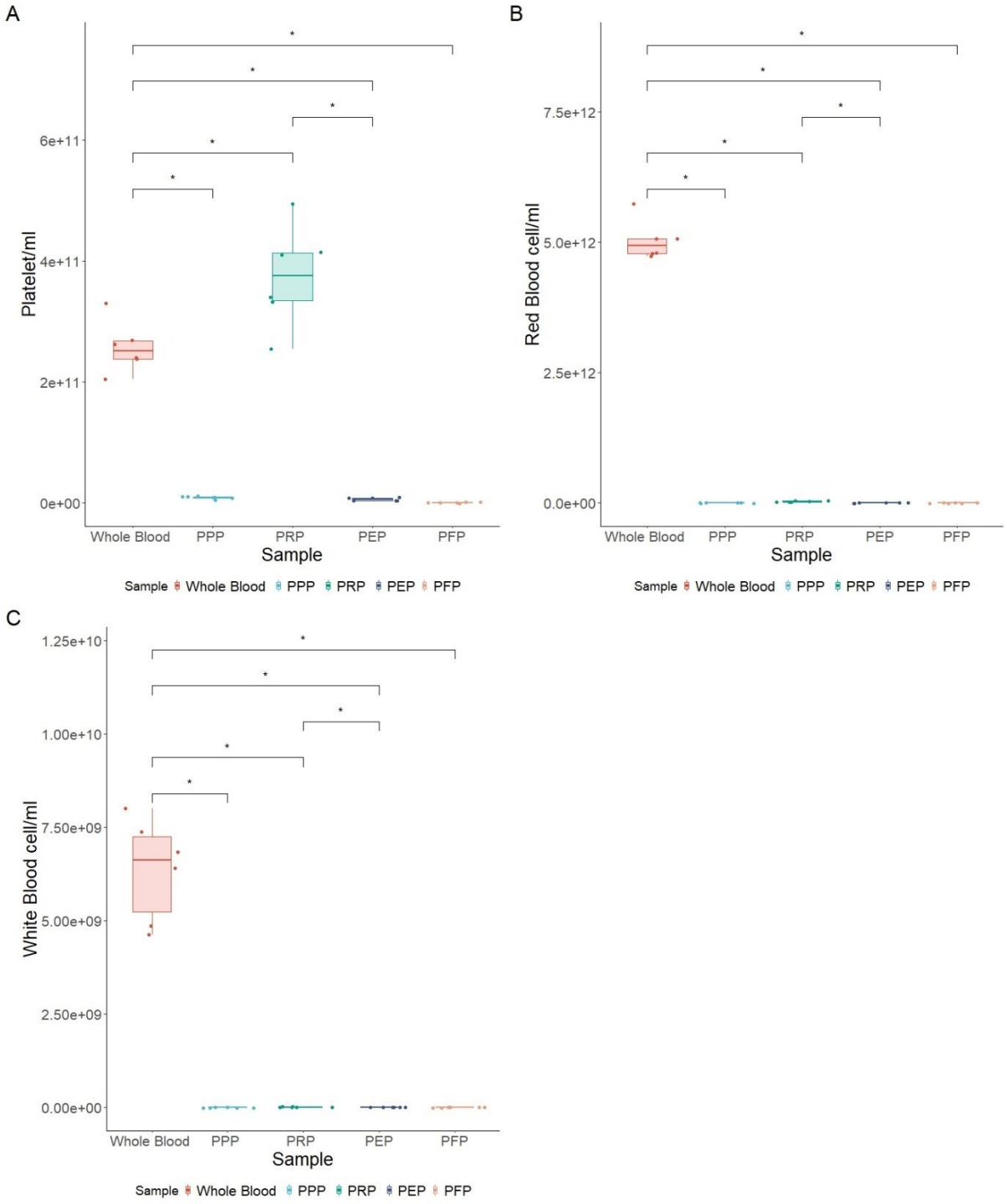


Figure 2

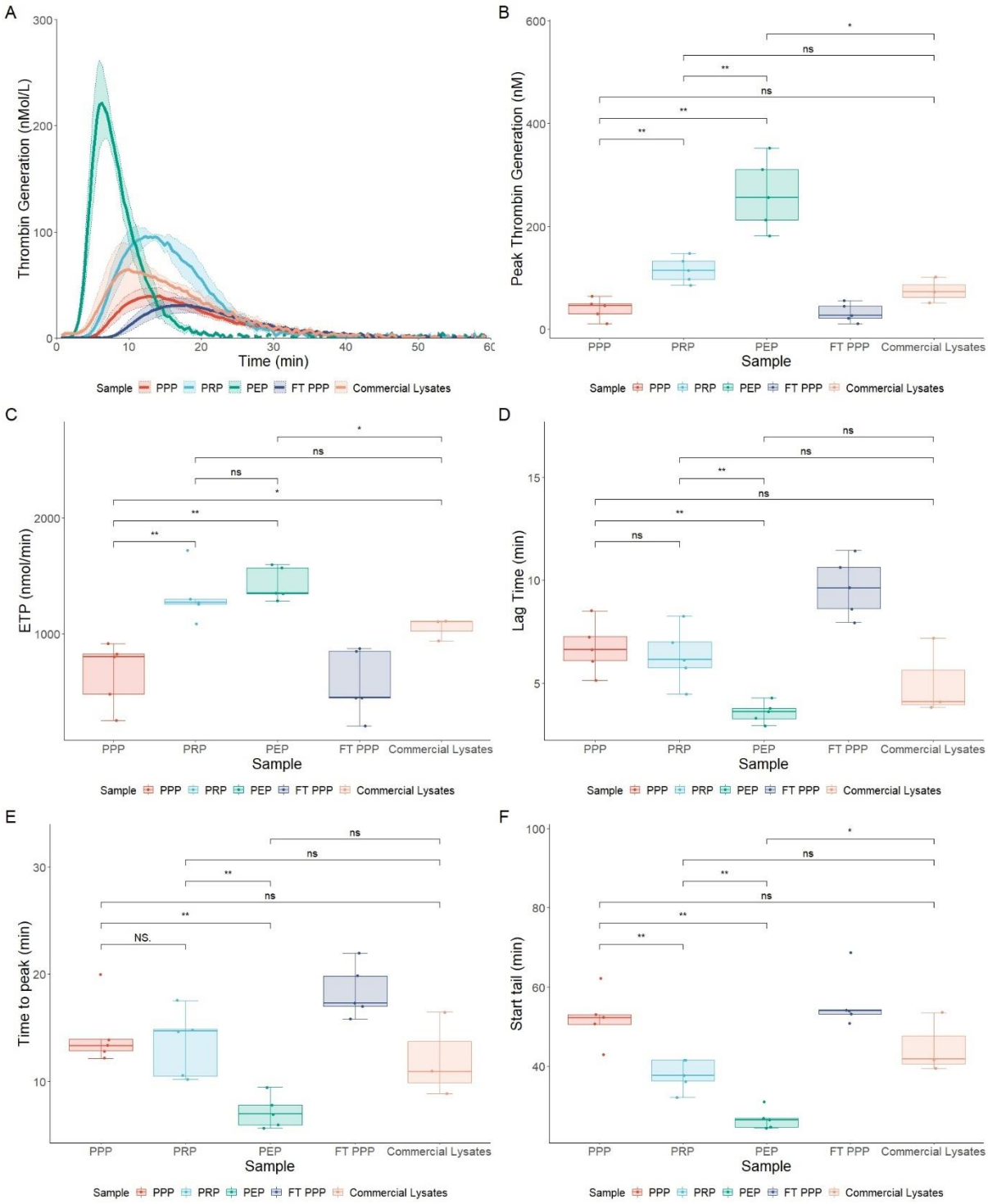
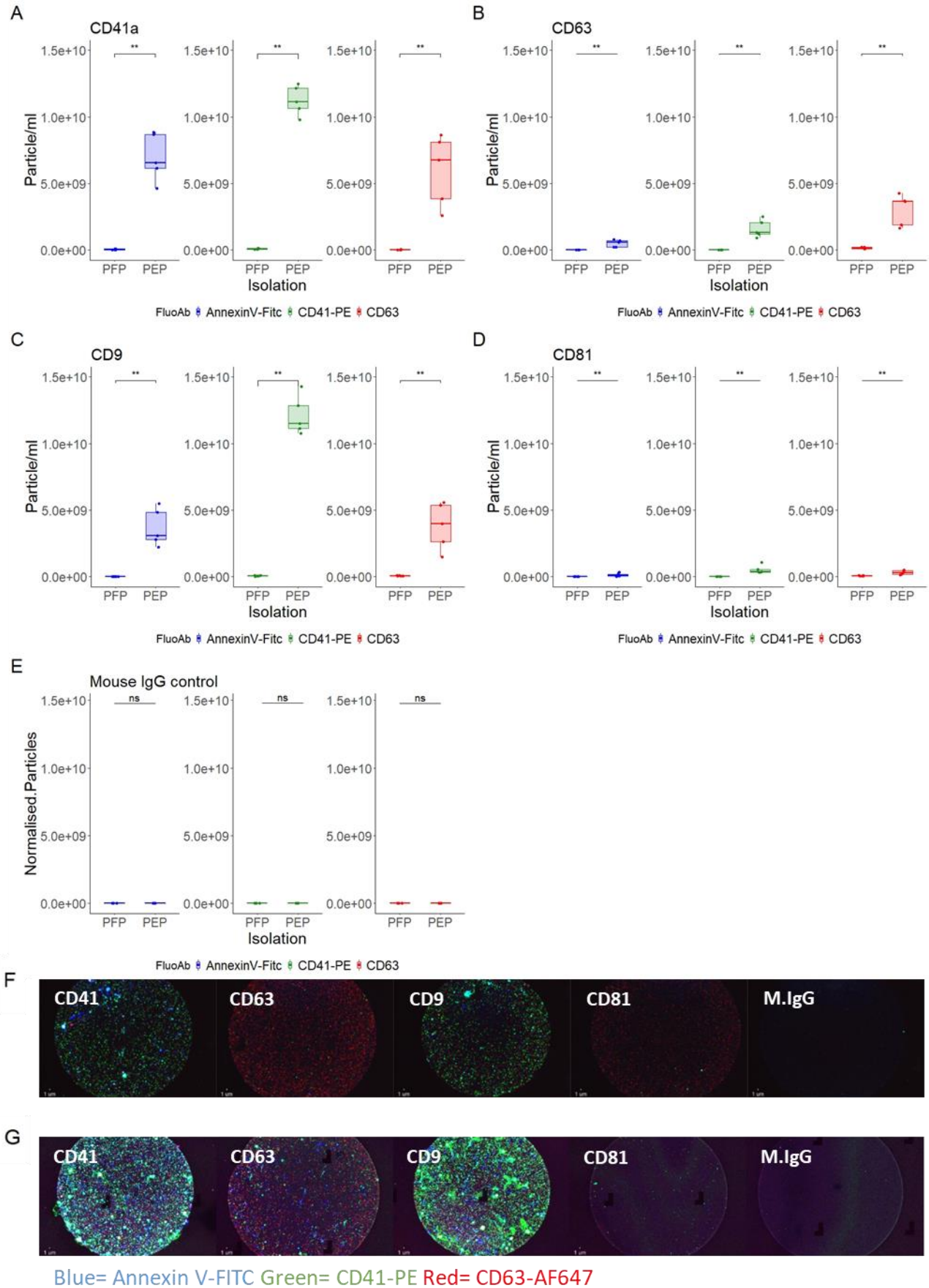
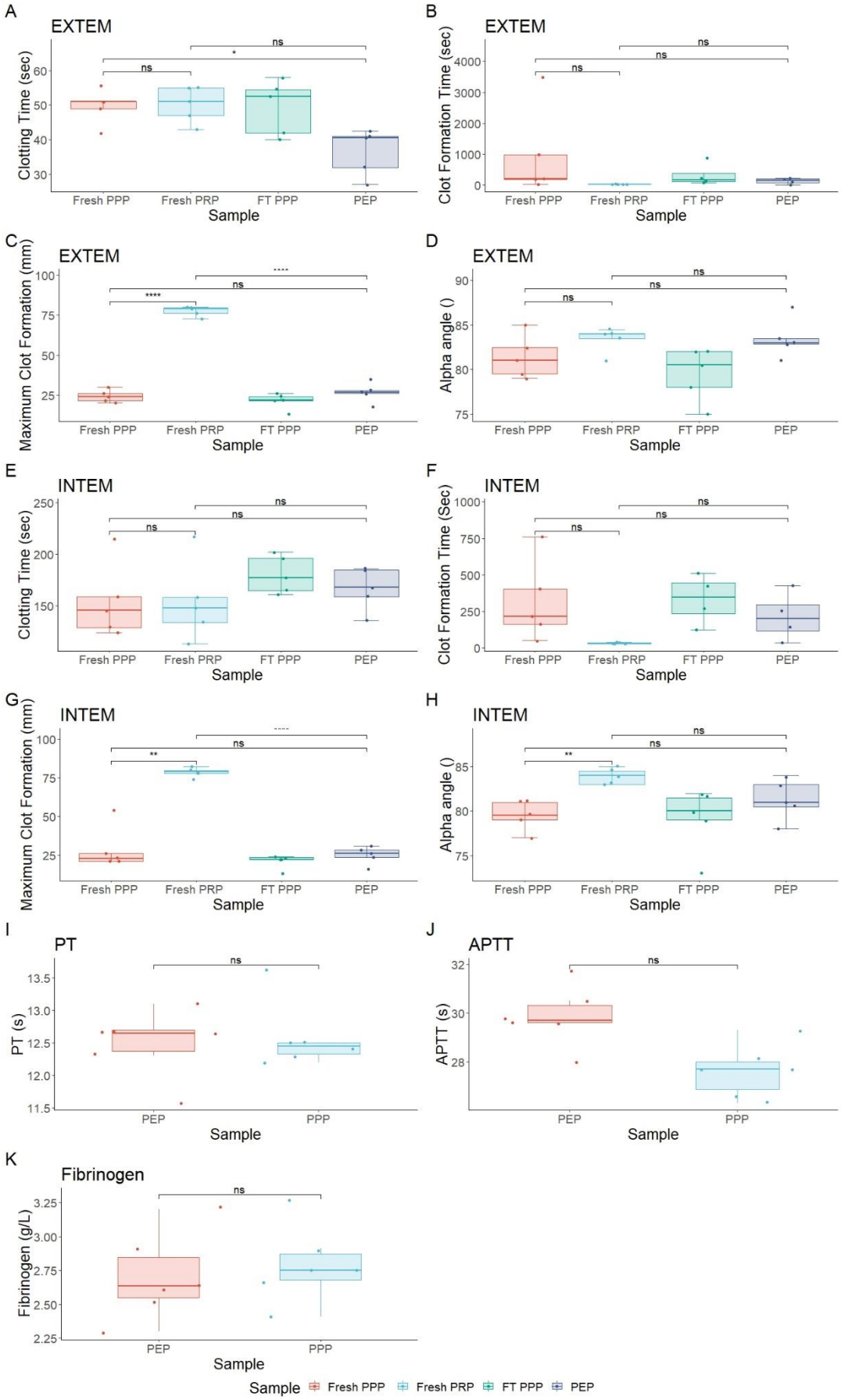

A
Thrombin Generation (nMol/L)
Time (min)
B
Peak Thrombin Generation (nM)
C
ETP (nmol/min)
D
Lag Time (min)
E
Time to peak (min)
F
Start tail (min)
Sample
PPP
PRP
PEP
FT PPP
Commercial Lysates

Figure 3



Blue= Annexin V-FITC Green= CD41-PE Red= CD63-AF647

Figure 4


A
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Fresh PPP
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FT PPP
PEP
Sample
B
EXTEM
Clot Formation Time (sec)
C
EXTEM
Maximum Clot Formation (mm)
D
EXTEM
Alpha angle ()
E
INTEM
Clotting Time (sec)
F
INTEM
Clot Formation Time (Sec)
G
INTEM
Maximum Clot Formation (mm)
H
INTEM
Alpha angle ()
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PT
PT (s)
PEP
PPP
J
APTT
APTT (s)
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Fibrinogen
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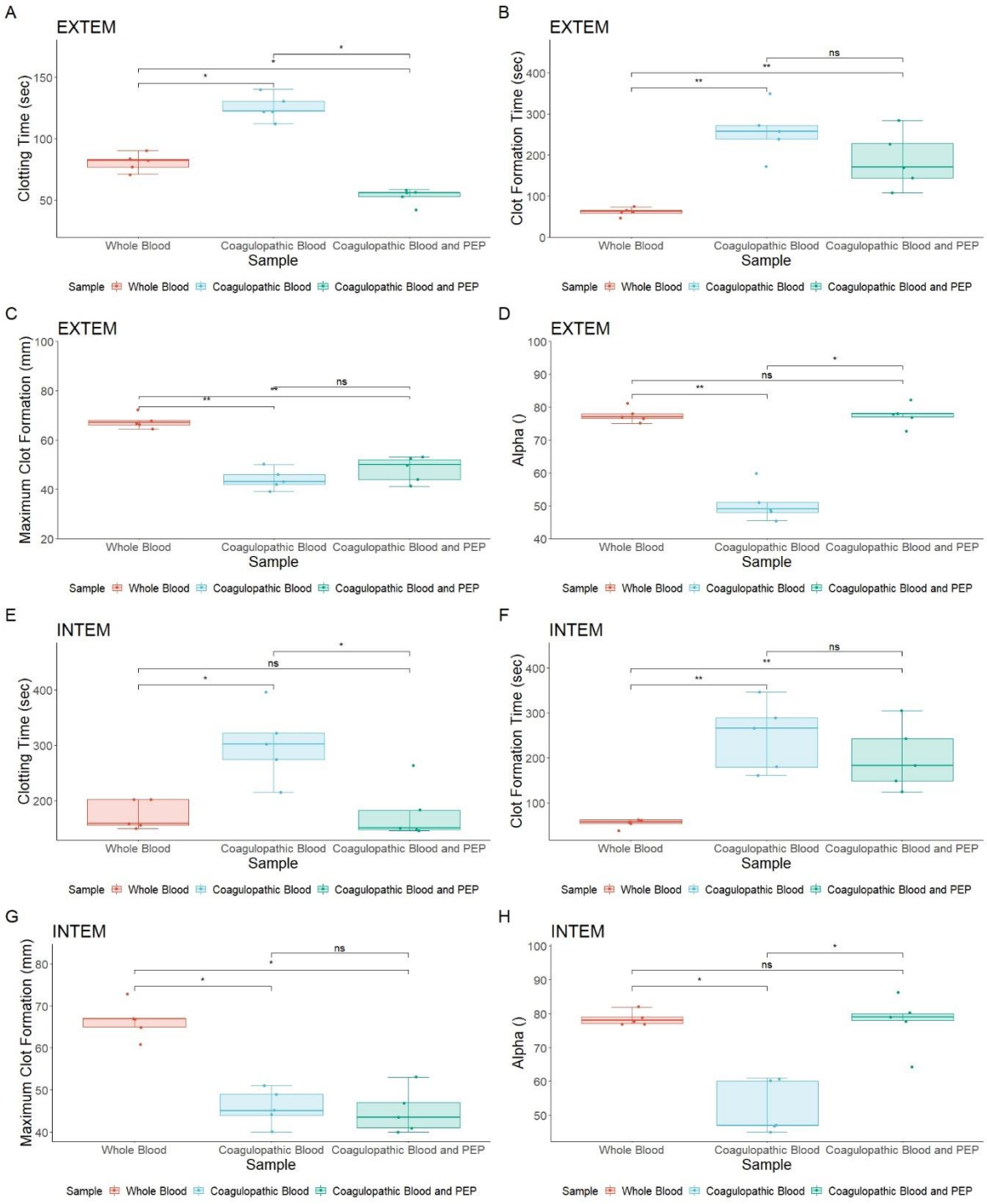
ns
Sample
Fresh PPP
Fresh PRP
FT PPP
PEP

Figure 5


A
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EXTEM
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Whole Blood
Coagulopathic Blood
Coagulopathic Blood and PEP
Sample
Sample Whole Blood Coagulopathic Blood Coagulopathic Blood and PEP
G
INTEM
Maximum Clot Formation (mm)
80
70
60
50
40
ns
*
*
Whole Blood
Coagulopathic Blood
Coagulopathic Blood and PEP
Sample
Sample Whole Blood Coagulopathic Blood Coagulopathic Blood and PEP
H
INTEM
Alpha ()
100
90
80
70
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Whole Blood
Coagulopathic Blood
Coagulopathic Blood and PEP
Sample
Sample Whole Blood Coagulopathic Blood Coagulopathic Blood and PEP

Figure 6

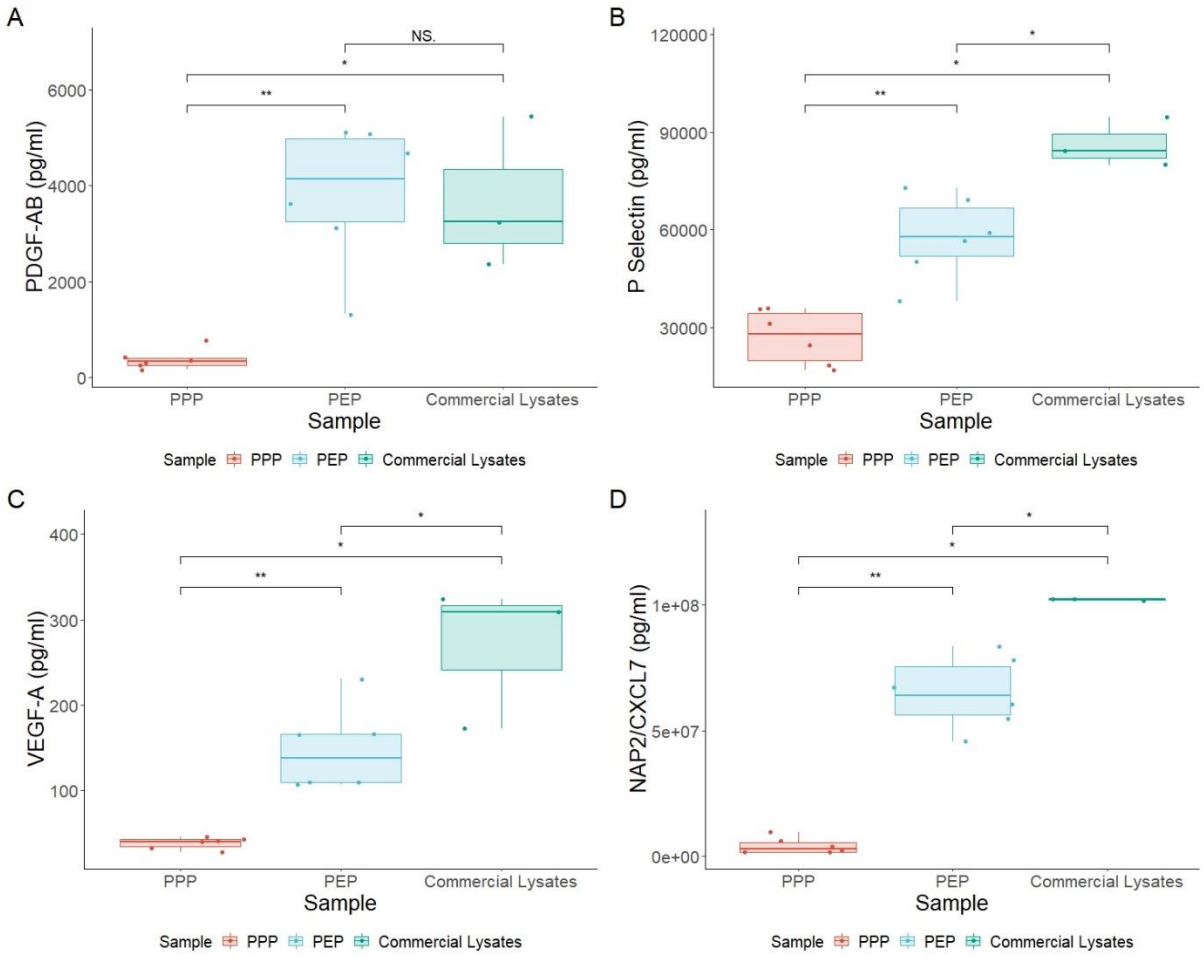
A
NS.
*
**
6000
4000
2000
0
PDGF-AB (pg/ml)
PPP
PEP
Commercial Lysates
Sample
Sample PPP PEP Commercial Lysates
B
120000
90000
60000
30000
P Selectin (pg/ml)
C
400
300
200
100
VEGF-A (pg/ml)
D
1e+08
5e+07
0e+00
NAP2/CXCL7 (pg/ml)

Figure 7

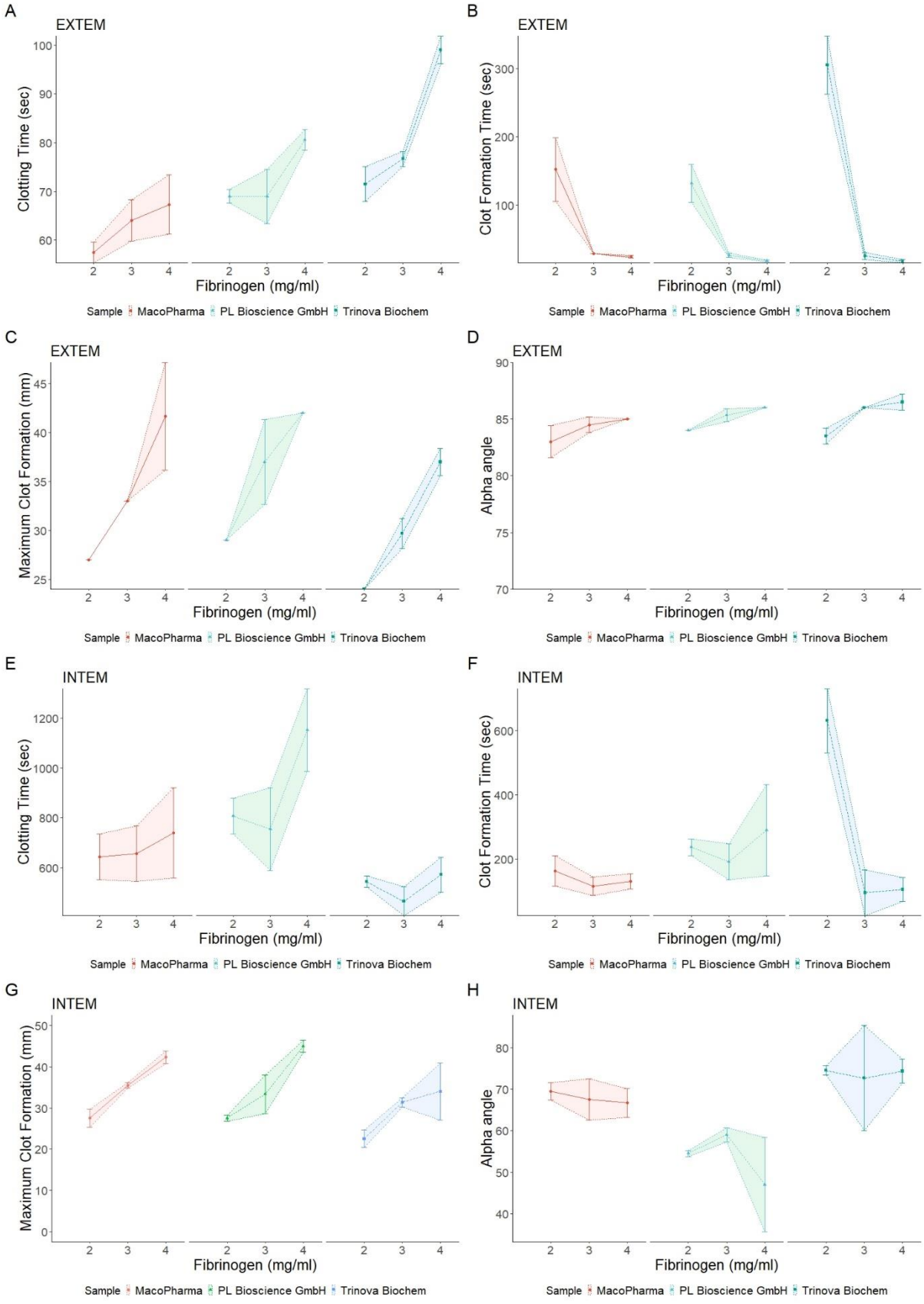
A
EXTEM
Clotting Time (sec)
Fibrinogen (mg/ml)
B
EXTEM
Clot Formation Time (sec)
Fibrinogen (mg/ml)
C
EXTEM
Maximum Clot Formation (mm)
Fibrinogen (mg/ml)
D
EXTEM
Alpha angle
Fibrinogen (mg/ml)
E
INTEM
Clotting Time (sec)
Fibrinogen (mg/ml)
F
INTEM
Clot Formation Time (sec)
Fibrinogen (mg/ml)
G
INTEM
Maximum Clot Formation (mm)
Fibrinogen (mg/ml)
H
INTEM
Alpha angle
Fibrinogen (mg/ml)
Sample MacoPharma PL Bioscience GmbH Trinova Biochem

Figure Legends

Figure 1- Cell Counts from Sysmex XN1000 in whole blood, PPP, PRP, PEP and PFP, (N = 6) a) Platelet counts b) Erythrocyte counts (c) Leukocyte counts . Kruskall-Wallis tests followed by Wilcoxon sign rank tests, select significant differences are shown; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 2- CAT Thrombin generation with PRP reagent of PEP, donor-matched PRP donor-matched PPP (fresh and freeze-thawed) (N= 5, measured in duplicate) and commercial lysates (N = 3, measured in duplicate). a) Trace (nmol/L) with shading representing the 95% confidence interval of the median, b) Peak Thrombin Generation (nM), c) ETP (nmol/min), d) Lag Time (min), e) Time to Peak (min), f) Start Tail (min). Kruskall-Wallis tests followed by Wilcoxon sign rank tests for PEP, PRP, PPP (fresh and freeze-thawed); for comparison with commercial lysates Wilcoxon ranks sum tests were used select significant differences are shown; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 3- EV Characterisation of PFP and PEP by ExoView with secondary labelling of EV captured on antibody capture spots: Annexin-FITC (blue), CD41-PE (green), CD63-AF647 (red) (N = 5, measured in triplicate). a) Labelled EV captured on anti-CD41a spots, b) Labelled EV captured on anti-CD63 spots, c) Labelled EV captured on anti-CD81 spots, d) Labelled EV captured on anti-CD9 spots, e) Labelled EV captured on control Mouse IgG spots, f) Representative fluorescent images of PFP measured at a dilution factor of 1:10, g) Representative fluorescent images of PEP measured at a dilution factor of 1:250. Wilcoxon sign rank tests, select significant differences are shown; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 4- Clotting parameters of PPP (fresh and freeze-thawed), PRP and PEP measured by ROTEM (N= 5, measured in duplicate) (a-h) and clotting parameters of freeze-thawed PPP and PEP measured with the Sysmex CS5100 coagulometer (N = 6, measured in duplicate) (i-k). a) EXTEM Clotting time, b) EXTEM Clot Formation Time, c) EXTEM Maximum Clot Formation, d) EXTEM Alpha Angle). e) INTEM Clotting time, e) INTEM Clot Formation Time, g) INTEM Maximum Clot Formation, h) INTEM Alpha Angle) (N= 5, measured in duplicate). i) Sysmex CS5100 PT, j) Sysmex CS5100 APTT, k) Sysmex CS5100 Fibrinogen (N= 6, measured in duplicate). Kruskall-Wallis tests followed by Wilcoxon sign rank tests, select significant differences are shown; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 5- ROTEM parameters of whole blood, a dilutional coagulopathy model (Whole blood diluted to 40% with saline and measured at 32°C) and PEP treatment of the coagulopathy model (N= 5, measured in duplicate). a) EXTEM Clotting time, b) EXTEM Clot Formation Time, c) EXTEM Maximum Clot Formation, d) EXTEM Alpha Angle) e) INTEM Clotting time, f) INTEM Clot Formation Time, g) INTEM Maximum Clot Formation, h) INTEM Alpha Angle) Kruskall-Wallis tests followed by Wilcoxon sign rank tests, select significant differences are shown; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 6- Luminex characterisation of PDGF-AB, P-Selectin, VEGF-A and NAP-2/CXCL7 in PEP generated via freeze-thaw cycling (N = 6, measured in duplicate). Kruskall-Wallis tests followed by Wilcoxon sign rank tests for PEP, PRP and PPP; for comparison with commercial lysates Wilcoxon ranks sum tests were used, select significant differences are shown; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 7- ROTEM parameters of commercially available human platelet lysates and effect of fibrinogen supplementation to 2 mg/ml, 3mg/ml and 4mg/ml a) EXTEM Clotting time, b) EXTEM Maximum Clot Formation, c) EXTEM Clot Formation Time, d) EXTEM Alpha Angle) e) INTEM Clotting time, f) INTEM Maximum Clot Formation, g) INTEM Clot Formation Time, h) INTEM Alpha Angle) (N= 3, measured in duplicate). Mean values with standard deviation shown.

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