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Acute obstetric coagulopathy is associated with excess plasmin generation and proteolysis of fibrinogen and factor V

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Abstract:
Haemostatic impairment may exacerbate postpartum haemorrhage (PPH). Previously, we described a distinct coagulopathy, associated with multiple causes of PPH including placental abruption and amniotic fluid embolus, termed acute obstetric coagulopathy (AOC). AOC is characterised by very high plasmin/antiplasmin complexes and rapid depletion of functional fibrinogen and factor (F)V. To determine mechanisms underlying AOC we investigated plasma from 12 women with AOC (here defined as plasmin/antiplasmin >25,000 ng/mL) and 21 severe PPH (measured blood loss >2000 mL or placental abruption) without AOC (plasmin/antiplasmin <25,000 ng/mL). Plasma from patients with AOC had a 4-fold increased ability to generate plasmin compared to severe PPH without AOC ($p<0.0002$). AOC was strongly associated with fibrinogen cleavage in the circulation, demonstrated by fragment D and other breakdown products on Western blot, ($p<0.0001$). D-dimer were increased 36-fold in AOC compared to severe PPH without AOC but thrombin/antithrombin complexes were not raised. FV was reduced on Western blot in AOC but not severe PPH without AOC ($p<0.001$) suggesting FV cleavage. Confocal microscopy showed similar clot structure between AOC and non-AOC samples but both groups differed from non-bleeding pregnant controls. These data suggest that in AOC an excess of plasmin cleaves fibrinogen and FV in the circulation causing a specific, pathognomonic depletion of coagulation factors. Fibrin(ogen) breakdown products have cofactor function for tissue plasminogen activator and these data are consistent with these breakdown products enhancing plasmin generation and potentially driving aberrant plasmin generation in AOC. These results have implications for the clinical management of coagulopathy during PPH.

Conflict of interest: COI declared - see note

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Plasmin drives acute obstetric coagulopathy

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Key points

Acute obstetric coagulopathy in postpartum bleeding is caused by an excess of dysregulated plasmin generation leading to hyperfibrinolysis

Excessive plasmin causes fibrinogenolysis and factor V cleavage resulting in depletion of these factors

Abstract

Haemostatic impairment may exacerbate postpartum haemorrhage (PPH). Previously, we described a distinct coagulopathy, associated with multiple causes of PPH including placental abruption and amniotic fluid embolus, termed acute obstetric coagulopathy (AOC). AOC is characterised by very high plasmin/antiplasmin complexes and rapid depletion of functional fibrinogen and factor (F)V. To determine mechanisms underlying AOC we investigated plasma from 12 women with AOC (here defined as plasmin/antiplasmin >25,000 ng/mL) and 21 severe PPH (measured blood loss >2000 mL or placental abruption) without AOC (plasmin/antiplasmin <25,000 ng/mL). Plasma from patients with AOC had a 4-fold increased ability to generate plasmin compared to severe PPH without AOC (p<0.0002). AOC was strongly associated with fibrinogen cleavage in the circulation, demonstrated by fragment D and other breakdown products on Western blot, (p<0.0001). D-dimer were increased 36-fold in AOC compared to severe PPH without AOC but thrombin/antithrombin complexes were not raised. FV was reduced on Western blot in AOC but not severe PPH without AOC (p<0.001) suggesting FV cleavage. Confocal microscopy showed similar clot structure between AOC and non-AOC samples but both groups differed from non-bleeding pregnant controls. These data suggest that in AOC an excess of plasmin cleaves fibrinogen and FV in the circulation causing a specific, pathognomonic depletion of coagulation factors. Fibrin(ogen) breakdown products have cofactor function for tissue plasminogen activator and these data are consistent with these breakdown products enhancing plasmin generation and potentially driving aberrant plasmin generation in AOC. These results have implications for the clinical management of coagulopathy during PPH.

Introduction

Bleeding during childbirth (postpartum haemorrhage, PPH) is a potentially life-threatening complication leading to approximately 70,000 maternal deaths per year globally.[1] Bleeding is caused by obstetric complications and exacerbated by haemostatic impairment which is found in about 5% of PPH with blood loss >1000 mL and 17% >2500 mL.[2] Despite fibrinogen levels being raised to 4-6 g/L at term, fibrinogen falls rapidly in some cases of PPH and the mechanisms underpinning this are not fully understood. In contrast, procoagulant factors do not fall below the non-pregnant normal until bleeds of more than 3-4L have occurred.[3 4] Coagulopathy, defined as Clauss fibrinogen less than 2 g/L, is strongly associated with bleed progression and worse outcomes for women and their babies.[5-8] Our recent study of 518 cases of severe PPH found that approximately half the cases of haemostatic impairment showed evidence of a unique coagulopathy defined by raised plasmin/antiplasmin (PAP) complexes which we termed acute obstetric coagulopathy (AOC) whilst the remaining cases arose predominantly due to haemodilution.[4 9]

AOC was primarily associated with placental abruption and amniotic fluid embolism (AFE), although it also occurred with other causes of PPH. The underlying clinical causes for AOC remain unclear and require further investigation. It is characterised by early, severe hypofibrinogenemia, acute acquired dysfibrinogenaemia, hyperfibrinolysis, reduced factor (F)V and decreased FVIII:VWF ratio. AOC was associated with very high rates of fetal and neonatal mortality.[4] The coagulopathy appears to be caused by excessive plasmin generation, evidenced by very high PAP levels. A similar coagulopathy associated with excessive plasmin, fibrinogen depletion and hyperfibrinolysis has also been described in a recent study of 39 women with AFE or placental abruption.[10] Mechanisms underlying AOC, and its relationship with obstetric disseminated intravascular coagulation (DIC), as defined by the International Society on Thrombosis and Haemostasis (ISTH) Obstetric DIC score of 26 or above, are unclear.[11] The ISTH Obstetric DIC score is based on Clauss fibrinogen, prothrombin time and platelet count but, unlike the non-pregnant DIC score,[12] does not take into account D-dimer levels or fibrin degradation products.

Current guidelines recommend that haemostatic impairment associated with PPH should be treated with early tranexamic acid to reduced fibrinolysis and empirical fixed-ratios of red blood cells and fresh frozen plasma (FFP) to replace coagulation factors.[13-15] These recommendations are based on data extrapolated from major trauma, which predominantly affects young males.[16] These treatment strategies may not be applicable to PPH because the baseline haemostatic systems of pregnant women and trauma cases differ significantly. The increased procoagulant factor such as FVIII, FII and FX and decreased protein S seen in pregnancy[17] are not reduced in PPH until very large bleeds have occurred whilst they are seen early in trauma.[18]. In addition, the observed coagulopathies in PPH and trauma are distinct and FFP is a relatively poor source of fibrinogen.[19]

In normal haemostasis, thrombin cleaves fibrinogen to fibrin monomers which polymerise into protofibrils. Fibrin αC regions mediate lateral aggregation of the protofibrils. The fibrin clot is stabilised by activated FXIII which cross-links fibrin-fibrin strands and incorporates $\alpha_2$antiplasmin (α_2AP) into the clot.[20 21] The zymogen plasminogen is incorporated into the developing clot and is converted to its active form plasmin via the action of tissue plasminogen activator (tPA) or urokinase (uPA).[22] Fibrin acts as a cofactor for its own destruction by augmenting tPA-mediated plasminogen activation >1000-fold.[23] Plasmin proteolysis of the fibrin clot yields multiple degradation products including cross-linked D domains (D-dimers).

Here we investigated mechanisms underpinning the early and potentially catastrophic fall in functional fibrinogen in AOC.

Methods

Patient samples

The Obstetric Bleeding Study+ (REC16/WA/0282) recruited 518 women with PPH after written consent. Twelve cases were designated as having AOC based on PAP levels >25,000 ng/mL during PPH[4] Platelet poor plasma samples from women with AOC were compared to those from women with PPH without AOC, selected by a measured blood loss >2,000 mL (n=16) or placental abruption (n=5) and PAP <25,000 ng/mL, and 20 healthy women at term pregnancy before elective caesarean section. Samples at the time of the highest PAP levels were analysed for fibrinolytic parameters. The first sample after onset of bleeding was analysed for clot structure. Clot strength was measured using the Intem mean clot firmness on a Rotem Sigma device.

The ISTH Obstetric DIC score was calculated based on the lowest Clauss fibrinogen and platelet count and the highest prothrombin time measured during the bleed.[11 24] The highest D-dimer was used to compare groups.

Ethics committee approval was granted with reference REC16/WA/0282.

Plasmin generation assay

Plasmin generation capacity in plasma (10%) was measured using 0.5 mM S-2251 (Chromogenix) in the presence of 10 nM tPa (alteplase) and cyanogen bromide fibrinogen fragments (10 µg/mL; Technoclone).[25] Absorbance readings at 405 nm were taken every min for 8 h at 37^{o}C on a Multiskan microphotometer (Thermo Scientific). Previous work had shown that the saturating concentration of cyanogen bromide fragments in the assay is 50 µg/mL (data not shown) thus the assay could detect both increased and decreased plasmin generation.

Determination of plasma protein concentrations

PAI-1 and uPA and were measured using Simple Plex™ assays on Ella™ system. PAI-1 activity, PAI-2 and tPA were measured by ELISA Innovative Research and TAT levels quantified by ELISA from Abcam. Thrombin/antithrombin (TAT) complexes were quantified by ELISA from Abcam. D-dimer, coagulation factors and PAP were measured as described.[4]

Western blotting

PPH samples with and without AOC, pregnant control samples and pooled normal plasma (PNP) were separated on 4‑12% NuPAGE Bis-Tris gels under non-reducing conditions, then transferred to PVDF membrane, as described[26]. Fibrinogen was detected with a sheep anti-human polyclonal antibody (Enzyme Research Laboratories) and FV with a monoclonal antibody recognising a specific epitope in the heavy chain (FV; AHV-5146 Prolytix). The positive controls in the FV blots are PNP (dilute 1/40 in in a Hepes (20 mM), NaCl (150 mM), $CaCl_2$ (5 mM) Polyethylene glycol-8000 (0.1 % v/v)) in the presence of phospholipids (20 µM) incubated with activated protein C (1.5 nM) for 15 min or plasmin (2.25 nM) for ten mins.

Confocal microscopy of fibrin structure

Plasma (30%) with 0.25 μM AlexaFluor 488 (AF488) fibrinogen in TBST, CaCl2 (10 mM) and thrombin (0.125 U/ml) was added to Ibidi VI 0.4 slides and the clots formed as described[25]. Images were recorded on Zeiss 710 laser scanning confocal microscope with a 63 × 1.40 oil immersion objective using Zeiss Zen 2012 software. Images were analysed using ImageJ 1.51w and the Diameter J plugin.

Statistical analysis

Data are presented as number and percentage and median, interquartile range and range for categorical and continuous variables, respectively. AOC and PPH without AOC groups are compared by Fisher exact test and Kruskal-Wallis and Mann Whitney U tests with Bonferonni correction. Spearman correlations were used to investigate the relationship between parameters.

Results

Demographics and haemostatic parameters and management

Baseline clinical characteristics and blood component therapy of the women included in this study are shown in Table 1. Clinical data from the whole cohort of 518 women have been previously reported.[4] Patients with AOC had higher gravida (3 [2-4] vs 2 [1-3], *p*=0.03) and earlier gestation (36.5 weeks [29.5-39] vs 40 [37-41], *p*=0.01) than those with PPH without AOC. Fibrinogen concentrate was infused in 10/12 women with AOC and 3/21 with PPH without AOC based Rotem Fibtem A5.[27] There was no difference in red blood cell transfusion. All women with PPH received intravenous tranexamic acid. One woman with AOC and one with PPH without AOC received FFP.

Haemostatic parameters relevant to AOC are shown in Figures 1a to 1e and Table 2. Of the 12 cases of AOC, 11 had PAP >40,000 and one case had a maximum PAP 25,393 ng/mL. Alongside the extremely elevated PAP, women with AOC exhibit significantly reduced fibrinogen and have dysfunctional fibrinogen, as evidenced by the discrepancy between the antigenic and Clauss fibrinogen assays. Whilst FV was reduced in AOC, prothrombin, FX and thrombin generation were similar in the two groups, as previously reported (Table 2).[4] TAT complexes were similar in all groups (Fig 1e).

In the whole cohort of 518 women with PPH reported previously,[4] there were sufficient data to calculate the ISTH Obstetric DIC score in 448 cases. Of these 448 women, 45 scored 26 or above and hence fulfilled the ISTH criteria for obstetric DIC. All 12 cases of AOC had a positive score, median (IQR) 35 (27-39). There were 33 women with severe PPH but without AOC who had a positive score, median (IQR) 28 (26-37). Amongst the obstetric DIC positive scores, D-dimer were 18-fold higher in the AOC cases compared to the AOC negative cases (*p*<0.00001).

The D-dimer levels in the 33 women with non-AOC PPH, that met the ISTH criteria for obstetric DIC, were similar to those in the 403 women with PPH who scored negative for obstetric DIC *p*=0.17 (Fig 1f).

The fibrinolytic phenotype of AOC is accompanied by evidence of systemic fibrinogenolysis

We investigated whether the AOC and PPH without AOC groups had distinctive fibrin and fibrinogen degradation products. Western blot analysis identified bands corresponding to the molecular weight of fragments D and Y in 10/12 (83%) cases of AOC whilst these fragments were only detected in 1/21 cases without AOC (p<0.0001) (Fig 2a-c and Table 3). Fibrinogen breakdown products were seen in 12/12 (100%) cases of AOC compared to 3/21 (14 %) without AOC (p<0.0001) (Fig 2b and Table 3).

AOC was associated with 36-fold higher D-dimer level compared to PPH without AOC (Fig 2d), indicating massive breakdown of cross-linked fibrin. There were no significant differences in tPA, uPA, PAI-1 or PAI-2 levels between groups (Table 2).

Confocal microscopy showed no significant difference in plasma clot structure between AOC and severe PPH without AOC, although both groups exhibited fewer and smaller fibres, larger pores and less intersections than pregnant controls (all P<0.05) (Table 3 and figure 2e). Clots from patients with AOC showed thicker fibres than pregnant controls (P<0.05) (Table 3). Maximum clot firmness, dependent on Clauss fibrinogen, decreased more rapidly at lower fibrinogen levels but with no differences between AOC and PPH without AOC cases (Fig 2f).

Proteolysis of FV is evident in AOC

FV and FVIII:VWF ratio were reduced in AOC compared to PPH without AOC, in contrast, other procoagulant factors and thrombin generation are similar. Western blot analysis demonstrated a reduction in FV, suggesting FV cleavage, in 10/12 cases of AOC and 0/21 cases of severe PPH without AOC (Table 3 and Fig 3a and 3b).

AOC promotes enhanced plasmin generation capacity

tPA-initiated plasmin generation was increased 4-fold in AOC plasma compared to PPH without AOC (p<0.0002) (Figure 3c and Table 2). There was a strong correlation between D-dimer and plasmin generation capacity (rho=0.83) in patients with PPH (Fig 3d).

Illustrative case study of dynamic changes in coagulation parameters in AOC

A 57 kg woman in premature labour delivered vaginally at 20 weeks gestation. She developed a coagulopathy of unknown cause and the Clauss fibrinogen rapidly fell from 4.2 to 0.4 g/L (Fig 4a) in association with extremely high D-dimer and PAP (Fig 4b). She was given intravenous tranexamic acid as soon as abnormal bleeding was recognised and plasmin generation capacity fell after this time. Obstetric interventions to control bleeding included uterotonics and a Bakri balloon and the total measured blood loss was 2500 mL. She was infused 6 gm of fibrinogen concentrate when her Clauss fibrinogen was 0.4 g/L. This resulted in an increment of 1.6 g/L for Clauss fibrinogen whilst the increment for antigenic fibrinogen was almost double at 3.1 g/L (Fig 4a). After the fibrinogen concentrate had been infused the amount of fragment D increased in the patient's plasma (time course shown in Fig 4b). There was a strong correlation between the amount of fragment D and plasmin generation capacity (Figure 3e).

Discussion

In this paper we demonstrate that AOC is associated with plasmin-mediated proteolysis of circulating fibrinogen in addition to excessive fibrinolysis of cross-linked fibrin. Plasma from patients categorised as having AOC on the basis that they have previously made excess plasmin, as indicated by high PAP, had a 4-fold increased capacity to generate more plasmin compared to other cases of severe PPH. This suggests the potential for a positive feedback loop leading to dysregulated plasmin generation. In contrast, there was no increase in TAT complexes in AOC or severe PPH meaning that there was limited evidence for systemic activation of coagulation in these cases. These findings provide insights into the rapid and potentially catastrophic fall in functional fibrinogen that is associated with some cases of obstetric bleeding.

Fragment D is a specific marker of plasmin-mediated proteolysis of fibrinogen within the circulation, and was found in 10/12 cases of AOC demonstrating that this process contributes to the early, rapid fall in fibrinogen. Reduced Clauss fibrinogen is the critical, clinically quantifiable, haemostatic change during PPH and levels <2 g/L are associated with poor outcomes for mother and baby.[4 8] Our previous work has shown that, in addition to reduced amounts of fibrinogen, AOC is associated with inhibition of fibrinogen function. Understanding the mechanism underlying these processes may help to devise treatment strategies in these life-threatening cases.

Fibrinogen breakdown products,[28] and specifically fragment D,[29] interfere with fibrin polymerisation, possibly contributing to the acute dysfibrinogenaemia seen in AOC. Hypodysfibrinogenaemia may have clinically important consequences for treatment of PPH. Trials show that Clauss fibrinogen >2 g/L is adequate for controlling bleeding during PPH[30] and this is reflected in current guidelines.[14 15] It is not known whether infused fibrinogen is susceptible to plasmin-mediated cleavage or inhibition of fibrin polymerisation in AOC. If so, higher than expected doses of fibrinogen or repeat infusions, might be required to achieve the therapeutic target of Clauss fibrinogen >2 g/L.

In the case study shown, 6 g of fibrinogen concentrate were infused into a woman with a Clauss fibrinogen of 0.4 g/L. This resulted in increments of 1.6 g/L for Clauss and 3.1 g/L for antigen fibrinogen demonstrating rapid functional inhibition of about half the infused fibrinogen. Studies in congenital fibrinogen deficiency showed no different in incremental recovery between Clauss and antigenic fibrinogen[31] suggesting that in AOC the difference in recovery is due to functional inhibition. Interference by high levels of circulating fragment D and D-dimer[29] is a possible mechanism for the observed rapid inhibition of fibrinogen function observed. The pattern on the Western blots, including an increase in fragment D, would indicate that some of the infused fibrinogen has undergone proteolysis. There were insufficient data to assess *in vivo* increments in other patients because samples had not been taken at appropriate times as clinicians dealt with the acute emergency. Further studies are required to corroborate this observation from a single patient and to this end we have established a new study to investigate fibrinogen increments during PPH.

Fibrin clots formed in the presence of plasmin-mediated fibrinogen cleavage products (such as fragment X which lacks the αC regions) show increased susceptibility to lysis.[32] This mechanism may contribute to the rapid clot breakdown and very high D-dimer levels in AOC. The αC domains of fibrinogen are most vulnerable to plasmin cleavage;[33] in addition to the crucial role they play in lateral aggregation of fibrin, they are the site for cross-linking to the inhibitor α_2AP.[20] It is feasible that clots formed from fibrinogen cleavage products have less α_2AP incorporated, enhancing susceptibility of the clot to fibrinolysis, although this requires further study. There was insufficient sample to assay α_2AP in this study however this is an important parameter to consider in future work

because α_2AP depletion may contribute to the excess plasmin activity and instability of fibrin clots seen in AOC. We have previously shown that approximately 60% of normal plasma levels (0.7 ± 0.6 mg/L) of α_2AP are necessary to stabilize clot against tPA-mediated fibrinolysis and that the rate of lysis inversely correlates with total cross-linked α_2AP.[34] Importantly, α_2AP harbours a C-terminal tail that binds plasminogen and competes against its incorporation into the forming fibrin clot. Therefore, a reduction in circulating levels of α_2AP, via consumption or proteolysis, has the capacity to accelerate plasmin generation at various levels.

Plasma from AOC cases had a markedly increased ability to generate tPA-initiated plasminogen activation despite the extremely high amounts of plasmin that had already been made, as demonstrated by the PAP levels. As fibrin(ogen) breakdown products have cofactor activity for tPA-mediated plasmin generation in the absence of fibrin,[35] it is plausible that these breakdown products increased plasmin generation capacity in AOC. This hypothesis is indirectly supported by the finding that plasmin generation capacity correlated strongly with D-dimer and, in the one AOC case where analysis was possible, with the amount of fragment D. Although fragment D and D-dimer are not strong co-factors for tPA-mediated plasminogen activation their presence is likely to be a good surrogate for fragment E and (DD)E complexes which are potent cofactors for tPA-mediated activation of plasmin.[36] Although the mechanism of the increased plasmin generation in AOC cannot be establish with certainty from the data presented, enhanced co-factor activity of fibrin(ogen) breakdown products is a plausible suggestion. Further work is need to investigate which breakdown products could be implicated. These findings have the potential to create a positive feedback loop for dysregulation of plasmin generation thereby exacerbating the coagulopathy.

There were no differences in clot structure when comparing those formed in plasma from AOC and cases of PPH without AOC although both were different from non-bleeding pregnant controls. Clots with thicker fibres and a more porous network are reported to be more susceptible to fibrinolysis.[37] Indeed, fibrin lacking αC domains due to congenital dysfibrinogenemia or cleavage of circulating fibrinogen significantly impacts on the mechanical properties of the clot and susceptibility to lysis.[21 32]

The demonstration of reduced FV on Western blot in the AOC cases provides a mechanism for the reduced FV activity observed in AOC and reported in cases of AFE.[38-40] FV may be proteolysed by plasmin or aPC, which are both increased in AOC[4] and further investigation is required to unravel the contributions of these enzymes. The high levels of plasmin combined with the presence of fragment D, a product of plasmin-mediated proteolysis of fibrinogen, propel plasmin into the limelight as a potential candidate.

The fall in FVIII was masked by a rise in VWF and is more evident when the FVIII/VWF ratio is calculated. Despite reduced levels of FV and FVIII in AOC, thrombin generation remained above the non-pregnant normal range.[4] Although thrombin generation cannot be used to predict the risk of bleeding, the increased ability to generate thrombin in PPH compared to healthy non-pregnant controls suggests that deficiencies of procoagulant clotting factors are unlikely to be the main cause of haemostatic impairment in AOC. We have suggested that tranexamic acid and targeted fibrinogen replacement should be prioritised above infusion of fresh frozen plasma (FFP) in PPH. This approach has been investigated in a trial of more than 600 cases of severe PPH where viscoelastic haemostatic assays were used to withhold FFP if results were normal. In that study no cases of clinically significant haemostatic impairment were seen.[41] This treatment strategy has been associated with a reduction in the rate of massive PPH and the need for red blood cell transfusion[27 42] and is currently being investigated in a randomised control trial (ISRCTN17679951). If bleeding continues dilutional deficiency of clotting factors may also develop and in these cases FFP is required.[9]

The findings described herein are compatible with a specific rather than generalised consumption of coagulation factors in AOC. The phenotype of the coagulopathy previously reported in cases of AFE and placental abruption are very similar to those identified here as AOC, featuring low Clauss fibrinogen, FV, and very high PAP and D-dimers.[10 39] There have been no previous reports of fibrinogenolysis or enhanced plasmin generation capacity in AFE and placental abruption but it is interesting to speculate that similar mechanisms may come into play.

The definition of AOC, based on a specific very high PAP level as described here, is arbitrary and unlikely be optimal. Ide et al reported 39 cases of AFE and placental abruption with PAP levels ranging from about 2000 to 200,000 ng/mL, medians roughly 50,000 and 20,000 ng/mL in AFE and placental abruption, respectively. The median D-dimer reported by Ide et al was 107,000 ng/mL in abruption and 300,000 ng/mL in AFE.[10] As can be seen from the case reported here, the PAP levels change rapidly with time and the highest level in each case is partly dependent on when samples were taken during an acute emergency. A definition of AOC based on PAP is not relevant in clinical practice because PAP is not available as a routine test. Further research is required to develop a pragmatic definition relevant to clinicians and it is likely that D-dimer will be important for a clinically applicable definition. It is possible that AOC will be better defined by the combination of low fibrinogen, high PAP and high D-dimer. We have established a prospective study to investigate this further.

All cases of AOC in the original OBS+ study of 518 patients with PPH fulfilled the ISTH criteria for obstetric DIC.[11] The finding that TAT complexes were not raised in either AOC or severe PPH without AOC does not support generalised activation of coagulation as would be expected in DIC. TAT is elevated in pregnant women (>20 ng/L) in the third trimester compared to non-pregnant healthy controls (1-5 ng/L)[43] and the levels reported here are consistent with this previous report. Although the very high PAP in AOC suggest hyperfibrinolytic DIC, this diagnosis usually requires evidence of activation of coagulation and the absence of raised TAT complexes suggests that AOC may be a distinct coagulopathy.

In addition to the 12 cases of AOC, 33 women without AOC were also classified as obstetric DIC based on the ISTH scoring system.[11] The ISTH obstetric DIC score positive/AOC negative cases did not have raised D-dimer compared to women with PPH who scored negative for obstetric DIC. The lack of raised D-dimer means that evidence for DIC is limited in these cases suggesting that in PPH the ISTH score may reflect haemostatic impairment rather than DIC in the non-AOC cases. It is possible that the ISTH obstetric DIC score might be more specific for DIC in PPH if D-dimer was included in the algorithm although pregnancy relevant cut offs would need to be derived. It is important to note that the ISTH Obstetric DIC score is not designed specifically for PPH and can be used in all obstetric scenarios. Further research is required to better define the coagulopathies associated with PPH and other obstetric complications.

The main limitations of this study are that it has been performed on a relatively small group of patients with a limited number of samples which were taken during acute emergency situations. This has limited the number of assays that could be performed to unravel the various mechanisms driving plasmin generation in AOC. The underlying mechanism that caused the initial very high levels of PAP in AOC is not known and will be investigated in future studies. Assays such as tPA/PAI-1 and uPA/PAI-1 complexes would be instructive and will be included in future work. A possible underlying trigger for excess plasmin generation in our cases of AOC could be sub-clinical AFE because AFE is commonly associated with AOC[10] but this suggestion will require further investigation. The findings need to be confirmed in a larger cohort with further investigation of potential mechanisms and pathology.

In conclusion, we demonstrate for the first time that AOC is characterised by excessive and dysregulated plasmin generation leading to systemic proteolysis of fibrinogen and FV in the circulation thereby promoting acute acquired dysfibrinogenaemia and massive hyperfibrinolysis. These findings strongly support a role for tranexamic acid[44] and early fibrinogen replacement in AOC and will help inform future prospective clinical trials and guidelines on optimal treatment strategies.

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Eric West performed the plasmin generation assays and contributed to the Western blot analyses.

Authorship Contributions

PWC contributed to study design, data collection, data analysis and interpretation and manuscript preparation.

CSW contributed to experimental design, experimental analysis, data interpretation and manuscript preparation.

LdeL was study PI and contributed to study design, data collection, data analysis and interpretation and manuscript preparation.

AN contributed to experimental analysis, data interpretation and manuscript preparation.

PVJ contributed to experimental design, sample analysis, data interpretation and manuscript preparation.

SFB contributed to study design, data collection, data analysis and interpretation and manuscript preparation.

NG contributed to data analysis and interpretation and manuscript preparation.

REC contributed to study design, data collection, data analysis and interpretation and manuscript preparation.

NJM contributed to experimental design, experimental analysis, data interpretation and manuscript preparation.

Disclosure of Conflicts of Interest

PWC has received research support from CSL Behring, Haemonetics Corporation and Werfen. He has acted as a paid consultant to CSL Behring, Haemonetics Corporation and Werfen.

LdeL has received research support from Haemonetics Corporation. She has acted as a paid consultant to Octapharma.

SFB has received research support from CSL Behring, Haemonetics Corporation and Werfen. She has acted as a paid consultant to CSL Behring and Werfen.

RC has received research support from CSL Behring, Haemonetics Corporation and Werfen.

CSW, AN, NG, PVJ and NJM have no conflicts of interest relevant to the current study to declare.

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Table 1. Clinical characteristics and haematological management

	Non-bleeding term pregnant controls (N=20)	Acute obstetric coagulopathy (AOC) (N=12)	Severe postpartum haemorrhage without acute obstetric coagulopathy (N=21)	AOC vs non AOC P value
Age (Years) Med (IQR) range	ND	30.5 (26.5-35.5) 21-45	32.0 (27.0-35.0) 21-44	0.85
Gravida N Med (IQR) range	ND	3 (2-4) 2-6	2 (1-3) 1-6	0.03
Parity N Med (IQR) range	ND	2 (1-2) 0-5	1 (0-1.5) 0-3	0.09
Gestation (months) Med (IQR) range	ND	36.5 (29.5-39) 20-40	40 (37-41) 25-42	0.01
Mode of birth				
Unassisted vaginal N (%)	0	5 (41.7)	7 (33.3)	0.34
Instrumental vaginal N (%)	0	1 (8.3)	7 (33.1)	
Elective CS N (%)	20	2 (16.7)	1 (4.8)	
Non-elective CS N (%)	0	4 (33.3)	6 (28.6)	
Primary cause of bleeding				
Atony N (%)	NA	1 (8.3)	1 (4.8)	0.12
Surgical/trauma N (%)	NA	1 (8.3)	11 (52.4)	
Placental abruption N (%)	NA	5 (41.7)	5 (23.8)	
Placenta accretia/praevia N (%)	NA	2 (16.7)	3 (14.3)	
Retained products of conception N (%)	NA	1 (8.3)	1 (4.8)	
Amniotic fluid embolus or coagulopathy of unknown cause N (%)	NA	2 (16.7)	0 (0)	
Total measured blood loss (mL) Med (IQR) range	ND	1525 (1050-2650) 200-5500	2300 (2000-2800) 1200-8500	0.06

Blood component and haemostatic management				
Red blood cells (units) Med (IQR) range	0	1.5 (1-2.5) 0-9 10/12 (83%)	2 (0-3.5) 0-16 12/21 (57%)	0.7
Fresh frozen plasma (units) Med (IQR) range	0	0 (0-0) 0-4 1/12 (8%)	0 (0-0) 0-2 1/21 (4.8%)	1.0
Fibrinogen concentrate (g) Med (IQR) range N (%)	0	4 (5-7) 0-10 10/12 (83%) 10/12 (83)	4,4,8 3/21 (15%)	 0.002
Tranexamic acid infused N (%)	0	11/11 (100%)*	21/21 (100%)	NA

ND is no data. Med (IQR) is median (interquartile range). * no data was available for one patient.

Table 2. Haematological parameters relevant to acute obstetric coagulopathy

	Non-bleeding term pregnant controls (N=20)	Acute obstetric coagulopathy (AOC) (N=12)	Severe postpartum haemorrhage without acute obstetric coagulopathy (N=21)	AOC vs non AOC P value
Prohaemostatic factors				
Factor V (IU/dL) Med (IQR) range	109.4 (97.9-121.0) 74.4-141.0	50.1 (32.2-77.1) 15.5-101.6	81.7 (63.5-115.1) 15.7-174	0.009
Factor VIII (IU/dL)	181.8 (147.1-244.6) 108.4-398.2	159.4 (58.6-314.5) 49.8-439.5	296.1 (198.9-378.7) 97.2-908.4	0.06
Factor VIII/VWF ratio Med (IQR) range	0.6 (0.5-1.0) 0.4-1.4	0.28 (0.19-0.44) 0.12-0.74	0.81 (0.60-1.07) 0.28-1.73	0.0005
Factor II (IU/dL) Med (IQR) range	145.3 (131.7-150.2) 123.6-172.2	96 (71-119) 61-161	109.3 (89.4-123.9) 32.4-155.3	0.23
Factor X (IU/dL) Med (IQR) range	159.0 (139.7-177.3) 121.6-238.2	98.0 (78.6-116.5) 61.5-182.8	106.9 (93.7-153.1) 31.2-203.2	0.32
Endogenous thrombin potential nM/min Med (IQR) range	2067 (1944-2367) 1273-3008	2089 (1886-2379) 1223-3808	2040 (1815-2423) 1015-3006	0.86
Fibrinolytic parameters				
Plasmin generation (pM/s) Med (IQR) range	0.35 (0.32-0.52) 0.07-0.66	1.95 (1.09-2.49) 0.51-3.0	0.48 (0.39-0.86) 0.02-1.28	0.0002
PAI-1 antigen (ng/mL) Med (IQR) range	52.9 (41.9-52.9) 21.6-88.9	30.0 (16.0-77.6) 2.1-326	23.9 (17.1-46.9) 4.9-128.3	0.89
PAI-1 activity (ng/mL) Med (IQR) range	164.6 (126.5-227.6) 54.3-327.6	73.6 (18.9-456.7) 4.9-914.6	86.7 (21.6-220.4) 1.5-630.8	0.87
PAI-2 antigen (ng/mL) Med (IQR) range	133.9 (83.6-198.1) 57.4-329.2	92.8 (67.5-179.0) 46.9-245.5	124.0 (88.2-300.1) 18.1-441.8	0.23
uPA antigen (ng/mL) Med (IQR) range	1.9 (1.6-2.1) 1-2.8	1.0 (0.7-1.3) 0.53-1.66	1.2 (0.92-1.38) 0.41-2.94	0.13
tPA antigen (ng/mL) Med (IQR) range	1.4 (1.0-1.9) 0.5-6.0	1.8 (1.3-3.0) 0.4-12.5	1.17 (0.92-1.38) 0.41-2.94	0.53

ND is no data. Med (IQR) is median (interquartile range). If more than one sample was available from a patient the samples at the time of the highest PAP was analysed except for haemoglobin and platelet counts which were taken before the bleed started.

Table 3. Fibrinolytic parameters and markers of fibrinogen and factor V breakdown

	Non-bleeding term pregnant controls (N=20)	Acute obstetric coagulopathy (N=12)	Severe postpartum haemorrhage without acute obstetric coagulopathy (N=21)	AOC vs non AOC p value
Fibrinogen and Factor V breakdown products				
Fragment D and Y present N (%)	0	10 (83)	1 (5)	<0.0001
Breakdown products on reducing gel N (%)	1 (5)	12 (100)	3 (14)	<0.0001
Decreased factor V heavy chain N (%)	1 (5)	10 (83)	0 (0)	<0.0001
Fibrin clot structure				
Fibre length (μM) Med (IQR) range	3206 (3076-3422) 2977-3530	2881 (2678-3142) 1984-3235	2972 (2776-3118) 2603-3584	0.62
Pore area (μM^2) Med (IQR) range	19.8 (18.2-23.0) (17.1-27.9)	25.0 (20.7-28.3) 4.2-31.0	25.7 (22.9-28.7) 15.6-34.5	0.79
Number of pores (N) Med (IQR) range	346.4 (301.7-401.5) 273.3-434.0	259.4 (230.7-292.4) 141.7-370.0	257.6 (234.0-313.5) 183.0-476	0.89
Number of intersections (N) Med (IQR) range	1135.0 (1023.5-1254.0) 986.0-1384.0	956.5 (878.2-1117.5) 665.0-1231.0	951.5 (867.9-1052.0) 764.7-1399.0	0.90
Characterised length (μM) Med (IQR) range	3.03 (2.91-3.15) 2.73-3.24	3.22 (2.94-3.36) 2.77-3.63	3.26 (3.13-3.38) 2.80-3.62	0.60
Fibre thickness (μM) Med (IQR) range	1.90 (1.87-2.02) 1.72-2.27	2.19 (1.0-2.37) 1.89-2.82	2.08 (1.97-2.36) 1.38-2.59	0.51
Intersection density ($ints/\mu M^2$) Med (IQR) range	0.064 (0.058-0.071) 0.056-0.078	0.054 (0.040-0.063) 0.038-0.070	0.054 (0.049-0.060) 0.043-0.079	0.93

NA is not applicable. Med (IQR) is median (interquartile range).

Figure legends

Figure 1: Acute obstetric coagulopathy is characterised by increased plasmin and loss of functional fibrinogen

Fibrinogen levels in plasma samples from patients with AOC, severe PPH without AOC and non-bleeding pregnant controls were measured by (A) Clauss assay, (B) as total antigen and (C) the ratio of Clauss:antigenic fibrinogen was then calculated. (D) plasmin/antiplasmin complexes and (E) thrombin/antithrombin complexes are shown. (F) shows D-dimer levels in cases of PPH with and without AOC grouped based on those that scored positive (score ≥26) or negative (score ≤25) on the ISTH criteria for obstetric DIC. Data are median (IQR). Analysis was by Kruskal-Wallis one-way ANOVA and Mann Whitney U test with Bonferroni correction.

Figure 2: Evidence of fibrinogenolysis and fibrinolysis in acute obstetric coagulopathy

(A) shows a representative Western blot run under non-reducing conditions using an anti-human fibrinogen detection antibody of samples from patients with AOC (red) and severe postpartum haemorrhage without AOC (blue); the non-bleeding pregnant control sample (green) was taken before an elective caesarean section. Fragment D indicated is a specific marker of fibrinogen proteolysis, fragment Y is approximately 160 kD. (B) is a reducing Western blot with samples as in (A), breakdown products less than about 30 kD are detected in AOC but not in postpartum haemorrhage without AOC. (C) is a representative non-reducing gel of sequential samples over time from a single patient with AOC, showing fibrinogen in the earliest sample on the left and the latest on the right. A confocal microscopy image of the plasma fibrin clot at the earliest time point (lane 3) is shown. (D) shows D-dimer levels in non-bleeding pregnant controls, AOC and severe PPH without AOC. (E) shows representative confocal microscopy images of plasma fibrin clots for pooled normal plasma (PNP), non-bleeding pregnant control taken before an elective caesarean section (control) PPH without AOC (PPH non-AOC), and a case of AOC (AOC). (F) shows the relationship between Clauss fibrinogen and Intem A5, as a measure of clot strength, performed on a Rotem Sigma device. Blue dots are cases of AOC and grey dots postpartum haemorrhage without AOC, individual cases might contribute more than one data point. There was no difference in clot firmness, as measured by Rotem, between the AOC and postpartum haemorrhage without AOC groups at similar Clauss fibrinogen levels. Dashed line is the linear correlation for all non-AOC cases (R^2=0,24) and the solid line is the linear correlation for all AOC cases (r^2=0.63).

Figure 3 Depletion and proteolysis of factor V and enhanced plasmin generation in acute obstetric coagulopathy

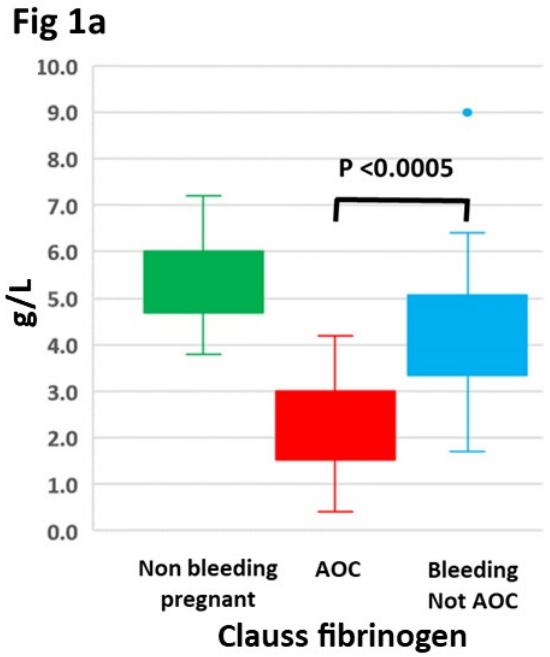
(A) shows FV activity in non-bleeding pregnant controls, AOC and severe PPH without AOC. (B) FV degradation was analysed by Western blotting. Samples were run under reducing conditions and FV detected by a monoclonal antibody directed to the heavy chain. Two patients with AOC are shown to have reduced or undetectable FV compared to pooled normal plasma (PNP) and a non-bleeding pregnant control at the time of elective caesarean section (shown in green). The positive controls are PNP + activated protein C (aPC) for 15 minutes or PNP + plasmin for 10 minutes, in both cases the FV is reduced. (C) Plasmin generation was analysed in plasma from the non-bleeding pregnant controls, AOC and severe PPH without AOC. (D) shows the relationship between D-dimer and tPA-initiated plasmin generation capacity in all cases of PPH including AOC and non-AOC cases. (E) shows

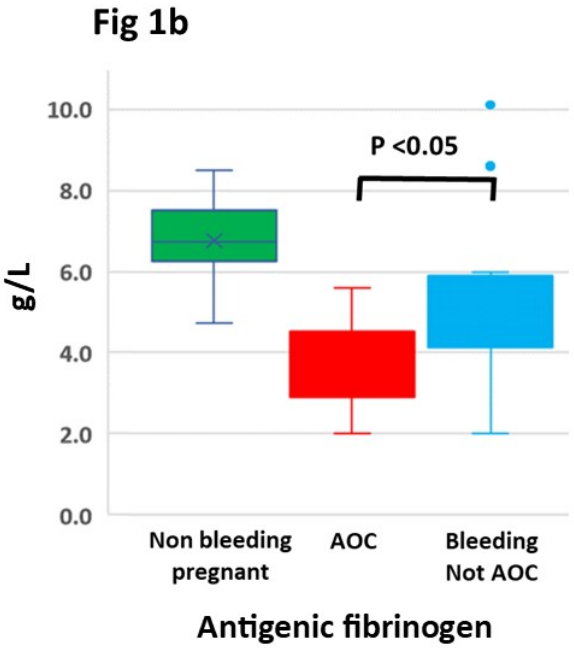
the relationship between plasmin generation capacity and the amount of fragment D in the blood of a single patient with AOC at different time points who is described in the case report. The fibrinogen degradation in these patient samples are shown in Fig 2C.

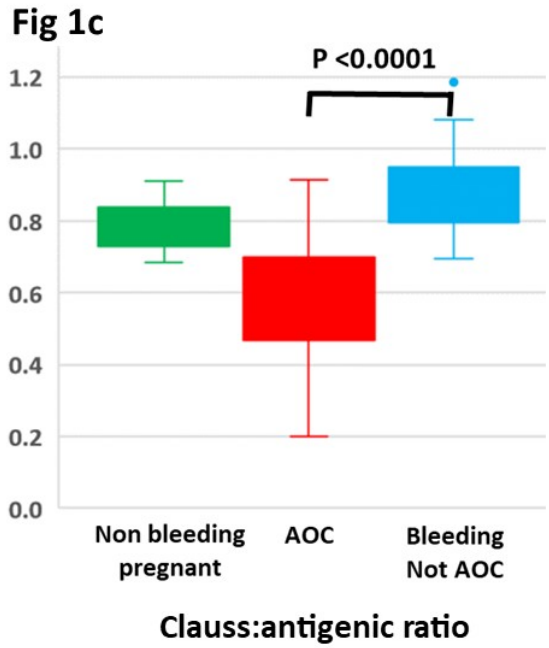
Figure 4 Dynamic changes in haemostatic parameters in case study of acute obstetric coagulopathy

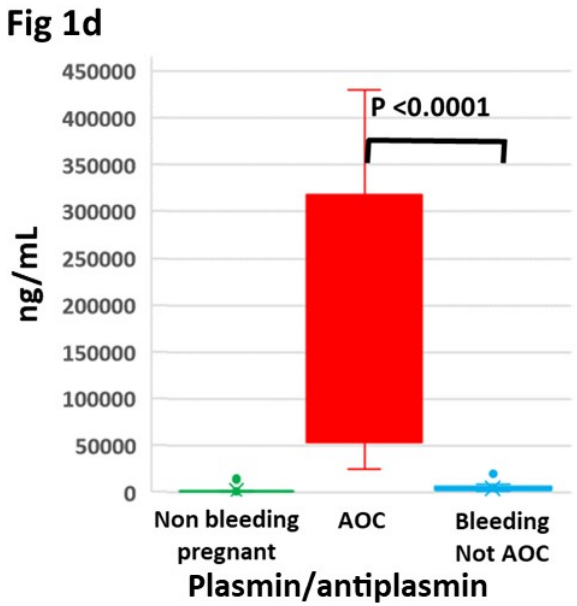
(A) shows Clauss and antigenic fibrinogen levels measured during the bleed and the response to am infusion of 6g of fibrinogen concentrate. The increment for Clauss fibrinogen (orange arrow) is about half that seen for antigenic (blue arrow). The measured blood loss at the time of each sample is shown in pink. (B) shows the levels of plasmin/antiplasmin complexes, D-dimer, fragment D and plasmin generation capacity at each time point. The time of the tranexamic acid (TXA) infusion is shown.

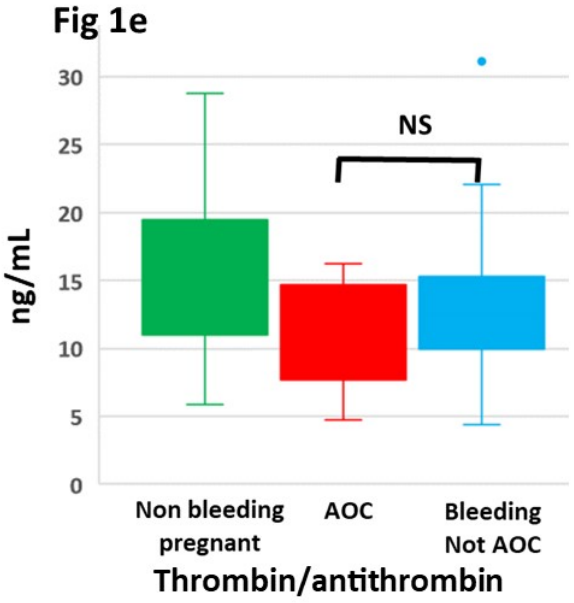
Figure 1











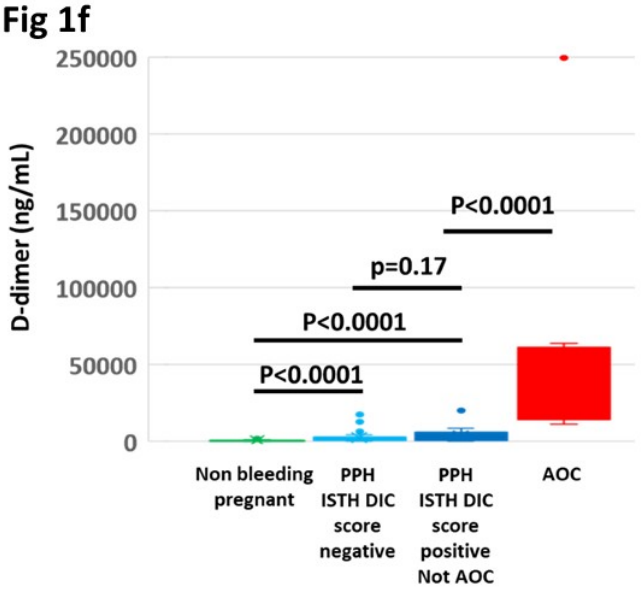


Figure 2

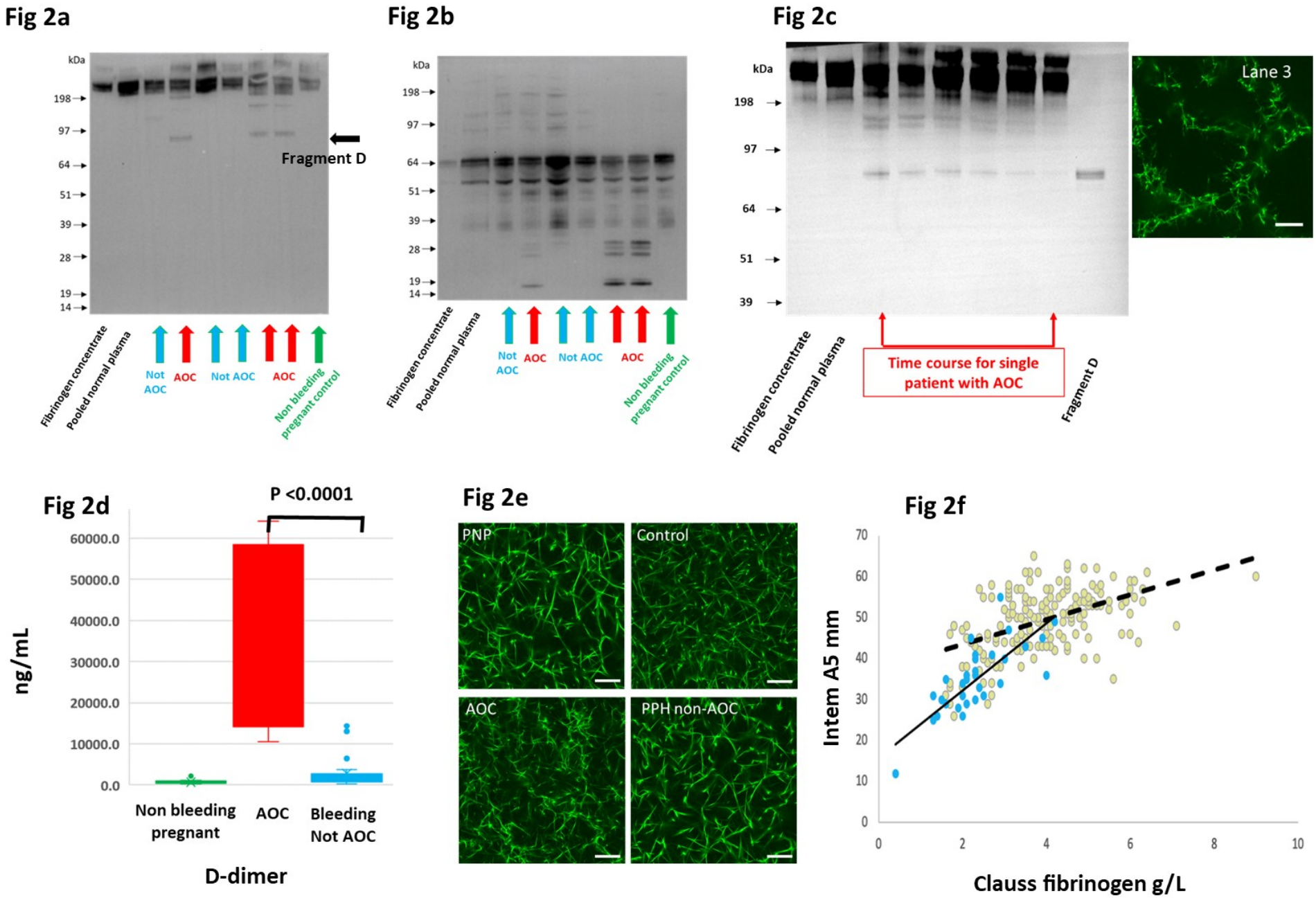


Fig 3a

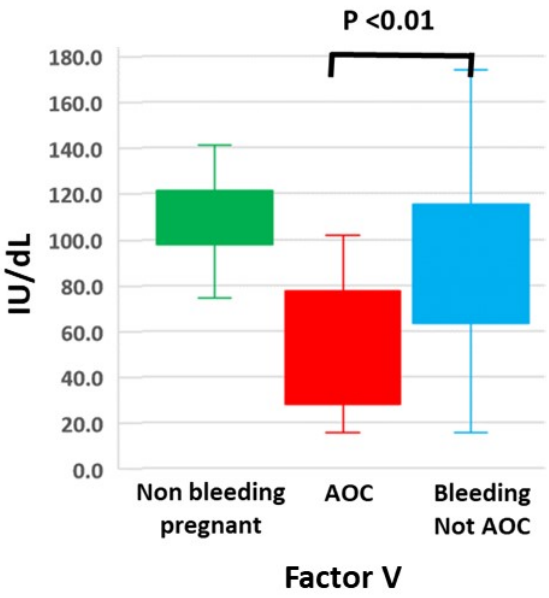


Fig 3b

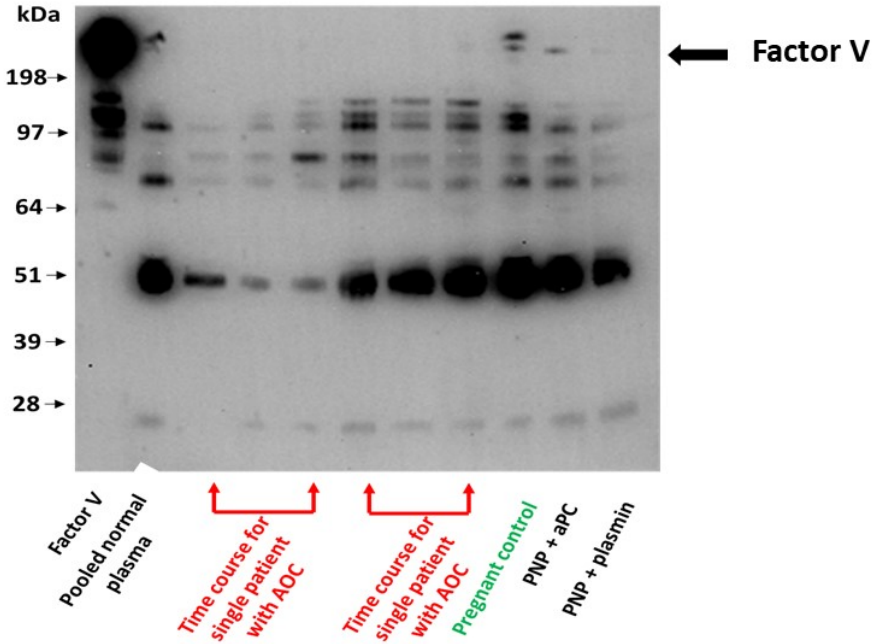


Fig 3c

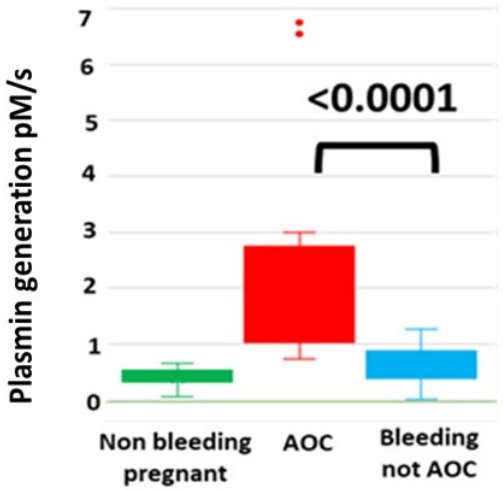


Fig 3d

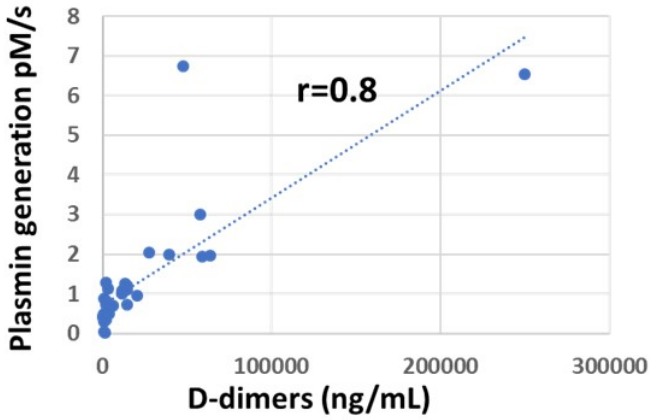


Fig 3e

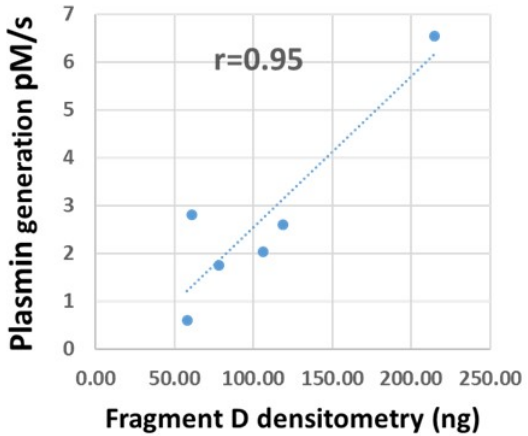


Figure 4