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Hemostasis in patients with acute kidney injury secondary to acute liver failure

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Acute kidney injury (AKI) occurs in over half of patients with acute liver failure. Despite prolonged prothrombin times and thrombocytopenia, continuous renal replacement therapy circuits frequently develop clots during patient treatment. Here we assessed factors contributing to this by measuring coagulation parameters (standard coagulation tests, pro- and anticoagulant factors, thromboelastography, and thrombin generation) in 20 consecutive patients with acute liver failure; mean age 42 years. Within 48 h, 10 had developed stage 3 AKI and 9 required continuous renal replacement therapy, of whom 2 had frequent circuit clots. The patients with stage 3 AKI were found to have significantly lower platelet counts and levels of factor V and the natural anticoagulants antithrombin, Protein C and Protein S, but increased extrinsic pathway activation and von Willebrand factor levels. Tissue factor levels were greater in those with stage 3 AKI, as was microparticle activity. Although patients with acute liver failure and advanced AKI requiring continuous renal replacement therapy have an even more marked thrombocytopenia and more prolonged extrinsic pathway activation, this was not associated with increased bleeding. Thus, more frequent circuit clots during continuous renal replacement therapy appear to be due to a combination of increased tissue factor and microparticle release, endothelial activation, and reduction in natural anticoagulants.

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Patients with acute liver failure (ALF) typically have prolonged prothrombin times (PT) and international normalized ratios (INR) because of a reduction in hepatic synthesis of clotting factors.[1] As such, patients with ALF are considered at risk of bleeding, with PT and INR frequently used in clinical practice to guide prophylactic blood product support before invasive procedures.

Acute kidney injury (AKI)[2] is a well-recognized complication of ALF,[3] and is associated with increased mortality. Continuous forms of renal replacement therapy are the preferred renal replacement modality for these patients in view of their cardiovascular instability and increased risk of intracranial hypertension.[4] Despite prolonged standard laboratory coagulation times and reduced peripheral platelet counts in ALF, patients who develop AKI paradoxically often display frequent clotting of the renal replacement therapy extracorporeal circuits,[5] requiring formal anticoagulation.[6] AKI is associated with a systemic inflammatory syndrome,[7] and the link between systemic inflammation and activation of the coagulation cascades[8] may explain the predisposition to thrombosis in this group of patients.

Our aim in this study was to determine whether development of acute kidney injury in patients with ALF changes the hemostatic balance from risk of bleeding to a prothrombotic state by studying hemostatic pathways and global coagulation potential in these patients.

RESULTS

Clinical characteristics

A total of 20 patients with ALF: 9 (45%) following acetaminophen self-poisoning, 4 (20%) secondary to ischemic hepatitis, 4 due to acute hepatitis (non-A non-B), and 3 due to drug toxicity (65% male and median age 42.5 years (28.5–50)), were studied. Within 24 h of admission to the intensive care unit, despite resuscitation, five patients were started on continuous renal replacement therapy (CRRT), all of whom met serum creatinine for stage 3 AKI,[2] and by 48 h, 1 patient had died of multiorgan failure, and 5 more had developed AKI stage 3, 3 meeting serum creatinine criteria, 1 with severe oliguria, and 1 due to rising

creatinine and metabolic acidosis, with 9 patients in total supported by CRRT, with 2 patients receiving unfractionated heparin anticoagulation, following repeated CCRT circuit clotting.

Baseline hematological investigations from the day of admission to the intensive care for the 10 patients who developed AKI grade 3 as well as those who did not are set out in Table 1. Apart from serum creatinine being greater on admission for those who developed AKI stage 3 (201 μmol/l (110–306) vs. 73 μmol/l (57–98), $P = 0.065$), there were no significant differences in standard laboratory tests, although the AKI stage 3 group generally had more prolonged clotting times, lower platelet counts, and higher Model for End-Stage Liver Disease (MELD) scores,[9] but C-reactive protein and ammonia levels were similar.

At 48 h, 6 patients remained at AKI stage 0, 1 patient at AKI stage 1, and 2 patients at stage 2. Sequential Organ Failure Assessment (SOFA) scores were significantly higher for AKI stage 3 (median 12.5 (7–14)) compared with stage 0 (4 (1.5–5.5), $P = 0.01$), as were MELD scores (Table 2). C-reactive protein was greater in those with higher stages of AKI (median 67 mg/l (11.5–98.5)) compared with those with stages 0/1 (12.5 mg/l (10–17); Table 2).

Subsequently, nine of the patients who did not develop AKI stage 3 were successfully discharged from the intensive care unit, 1 after liver transplant, whereas five deaths occurred in those with AKI stage 3, including 1 after acute liver transplant and 1 after second acute liver transplant.

Table 1 | Patient demographics and results of standard laboratory investigations on admission to the ICU for patients with ALF who developed AKI stage 3 (AKI-3) within 48 h, and those who did not (AKI 0–2)

	AKI stages 0–2	AKI stage 3
Etiology of ALF		
Acetaminophen	4	5
Drug-induced ALF[a]	2	1
Ischemic ALF	2	2
Seronegative hepatitis	2	2
Encephalopathy grade	1 (1–2)	2.5 (1–3)
Hemoglobin, g/l	123 ± 8.6	104 ± 9.4
Total white cell count, $\times 10^9$/l	7.84 ± 1.5	12.4 ± 1.9
Peripheral platelet count, $\times 10^{12}$/l	129 (107–164)	74 (65–166)
CRP, mg/l	14.5 (8–49)	16.5 (7.8–49)
Serum urea, mmol/l	4.1 (3.2–9.1)	10.6 (7.0–16.4)
Serum creatinine, μmol/l	73 (57–98)	201 (110–306)*
Serum phosphate, mmol/l	0.77 (0.49–1.34)	1.03 (0.85–1.13)
Serum albumin, g/l	35 (29–39)	31 (21.3–35.5)
Arterial ammonia, μmol/l	162 (112–212)	110 (81–131)
Serum aspartate transaminase, U/l	2407 (995–7592)	4230 (2583–9489)
Serum alanine transaminase, U/l	2968 (1025–6620)	4352 (2395–8337)
Serum bilirubin, μmol/l	61 (38–163)	91 (61–180)
INR	2.35 (1.8–6.1)	4.2 (2.6–6.3)
Prothrombin time, s	28.5 (21.9–67.3)	44.2 (31–82.5)
APTT, s	32.7 (29.4–38)	36.6 (34.7–42.9)
Fibrinogen, g/l	1.75 ± 0.19	1.77 ± 0.23
MELD score	26 (19–32)	34 (15–38)
MELD sodium score	26.5 (21.5–32)	34.5 (15–38.5)
Outcome		
Alive at ICU discharge	9	5
Liver transplant	1	2
Death without transplant	1	3
Death after liver transplant	0	2

Abbreviations: AKI, acute kidney injury; ALF, acute liver failure; APTT, activated partial thromboplastin time; CRP, C-reactive protein; ICU, intensive care unit; INR, international normalized ratio; MELD score, Model for End-Stage Liver Disease score; MELD sodium, MELD score including serum sodium.
Seronegative hepatitis (biopsy-proven acute hepatisis with negative tests for hepatitis A, B, C, and autoimmune serology), and final patient outcomes.
Results are expressed as mean ± s.e.m. or median (interquartile range).
*$P < 0.05$ versus groups.
[a]Drugs other than acetaminophem (paracetamol).

Clotting factors

As expected, patients with ALF had deranged clotting factors compared with normal healthy subjects (Table 2). Patients in stage 3 AKI had lower platelet counts, and more deranged (elevated) INR and activated partial thromboplastin time (APTT), than those with stage 0 AKI (Table 2). FV and antithrombin levels were significantly lower in those with AKI stage 3, and there was a trend for both lower pro- and anticoagulant (Protein S (PS) and Protein C (PC)) factors for AKI stage 3 patients. Similarly, patients with stage 3 AKI had significantly higher levels of von Willebrand factor antigen (VWF:Ag), and greater levels of tissue factor (TF), and factor VIII (FVIII). Endogenous heparinoids (> 0.05 U/ml) were detected in 75% of patients with stage 3 AKI compared with

Table 2 | Laboratory clotting tests after 48 h of admission according to AKI staging

AKI stage	Normal	0	3
No. of patients		6	10
MELD score		20.1 ± 3.0	30.3 ± 3.1*
CRP, mg/l	< 5	14 (10.5–178)	67 (11–110)
Hemoglobin	g/l	111.5 ± 27.1	109.2 ± 23.2
Platelets, $\times 10^9$/l	150–450	129 (112–214)	80 (63–122)*
WBC, $\times 10^{12}$/l	4.0–9.0	5.1 ± 3.2	13.6 ± 6.45*
PT (s)	9–13.5 s	20.8 (15.2–24.9)	27.7 (20.5–60.1)
INR	0.9–1.2	1.6 (1.2–2.1)	2.6 (2.0–5.6)*
APTT (s)	26–36 s	31.4 ± 1.5	42.8 ± 3.0*
Fibrinogen-C	1.5–4.0 g/l	2.25 ± 0.3	1.67 ± 0.2
Factor II	50–150 IU/dl	45.6 ± 11.5	35.5 ± 8.4
Factor V	50–150 U/dl	93.0 ± 15.8	47.6 ± 9.4*
Factor VII	70–175 IU/dl	45.8 ± 10.8	27.6 ± 5.0
Factor VIII	50–150 IU/dl	228 ± 13	241 ± 36
Factor IX	50–150 IU/dl	56.8 ± 8.9	55.1 ± 8.2
Factor X	50–150 U/dl	54.8 ± 9.8	39.3 ± 9.0
Factor XI	70–150 U/dl	55.0 ± 6.9	50.9 ± 3.9
Factor XII	50–150 U/dl	59.4 ± 8.5	45.9 ± 7.6
VWF:Ag	45–175 IU/dl	233 ± 30	389 ± 41*
Tissue factor	< 2 pmol/l	5.0 ± 2.2	18.6 ± 5.1
AT	80–140 IU/dl	50.3 ± 7.7	31.4 ± 2.7*
Protein C	66–126 IU/dl	49.0 ± 18.1	21.3 ± 5.7
Protein S	50–150 IU/dl	86.5 ± 14.1	63.2 ± 10.2
Anti-Xa(ch)	< 0.05 U/ml	0.02 (0–0.06)	0.09 (0.02–0.11)
MP activity	4.0 ± 0.3 n mol/l	15.0 ± 3.8	27.7 ± 9.4
MP clotting time	65.2 ± 2.0 s	52.3 ± 4.2	56.9 ± 6.7

Abbreviations: AKI, acute kidney injury; Anti-Xa(ch), Anti-factor Xa activity-chromogenic; APTT, activated partial thromboplastin time; AT, antithrombin; CRP, C-reactive protein; ICU, intensive care unit; INR, international normalized ratio; MELD score, Model for End-Stage Liver Disease score; MP, microparticle; PT, prothrombin time; VWF:Ag, von Willebrand factor antigen; WBC, white blood cell.
Results are displayed as mean ± s.d. or median (interquartile range).
*$P < 0.05$ versus AKI stage 0.

25% for those without AKI stage 0. Anti-Xa activity however did not differ between AKI stages, despite two patients with AKI stage 3 being given unfractionated heparin for CRRT.

Thromboelastography (TEG)

TEG analysis showed a procoagulant pattern for those with normal renal function, with shortened R values in 86%, coupled with normal K values (86%), increased angle (54%), and normal maximal amplitude in 57% (Table 3). Those with AKI stage 3 had more heterogenous TEG appearances; R and K values were shortened in 30% and 10%, normal in 30% and 50%, and prolonged in 40% and 40% patients, respectively. The α-angle was narrowed in 30% and normal in 60%, with normal or reduced maximum amplitude in 30 and 60% of patients. As two patients with stage 3 AKI had received heparin, the TEG was repeated with heparinase to neutralize any heparin effect, but there were no significant changes between the native and heparinase TEG results (Table 3).

Thrombin generation

The endogenous thrombin potential (ETP)[10] at 48 h was generally reduced for all stages of AKI. With the addition of Protac, those with stage 3 AKI showed smaller reduction in ETP (Figure 1), and higher % ETP resistance compared with healthy controls, and with stage 0 AKI (Table 4). Similarly, the velocity index or the slope of endogenous thrombin generation was greater for those in AKI stage 3 when normalized to controls (162 ± 46%), and also following addition of Protac (149 ± 42%). Microparticle activity was significantly increased in patients with ALF, both those with and without AKI, but greater for those with AKI ((15.3 (10.5–26.4) vs. 12 (7.2 (23.8) nmol/l).

During this study, no patient suffered major hemorrhage or received a blood transfusion. Some patients received fresh frozen plasma (2 units) on the day of admission before central venous and arterial line insertions. These patients included one patient of AKI stage 0, one patient of stage 2, and two patients of AKI stage 3.

Table 3 | Standard TEG with and without heparinase to neutralize any heparin effect (TEG heparinase) results after 48 h from patients with ALF divided according to AKI stage

AKI stage	0	3
TEG native		
R time	10.2 (6.1–12.6)	26.2 (12.2–48.6)
K time	3.0 (2.4–9.2)	21.4 (4.8–33.5)
α-Angle	47.6 (24.8–50.0)	11.5 (4.6–40.9)
MA	49.0 (39.0–54.1)	31.1 (19.9–48.9)
Lysis %	<10	<10
TEG heparinase		
R time	9.2 (6.2–10.7)	20.4 (11.1–30.7)
K time	4.4 (3.3–6.7)	8.4 (4.9–22.0)
Angle	40.4 (36.4–50.6)	24.9 (12.6–38.4)
MA	46.2 (39.2–53.8)	39.4 (28.8–49.9)
Lysis %	<10	<10

Abbreviations: AKI, acute kidney injury; ALF, acute liver failure; MA, maximum amplitude; TEG, thromboelastography.
Normal ranges: R, 12–26 min; K, 3–13 min, α-angle: 14–46 °, MA:, 42–63 mm; and lysis at 30 min, 0–5%.

DISCUSSION

As expected, in our study, patients with ALF were found to have reduced peripheral platelet counts and hepatic-dependent coagulation factors.[11] Typically, the degree of thrombocytopenia and prolongation of laboratory PT, particularly following acetaminophen (paracetamol) self-poisoning, are much greater than that observed in patients with cirrhosis who develop AKI. However, not all liver-dependent clotting proteins were reduced, as fibrinogen levels were normal. FVIII and VWF, which are synthesized by the endothelium, and TF released on endothelial injury or from blood cells such as monocytes were all increased, suggesting that ALF has a systemic inflammatory component. Traditionally, patients with ALF were thought to be 'auto-anticoagulated,' but the balance between pro- and anticoagulant factors may not have been as distorted as once thought[11] because of a parallel reduction in the hepatically synthesized natural anticoagulants, antithrombin, PS, and PC.[12] More recently, it has been realized that similarly patients with cirrhosis may also be less at risk of bleeding than previously thought, possibly associated with increased endothelial activation.[13]

Patients with chronic kidney disease are well recognized to have platelet dysfunction, and prolonged bleeding times.[14]

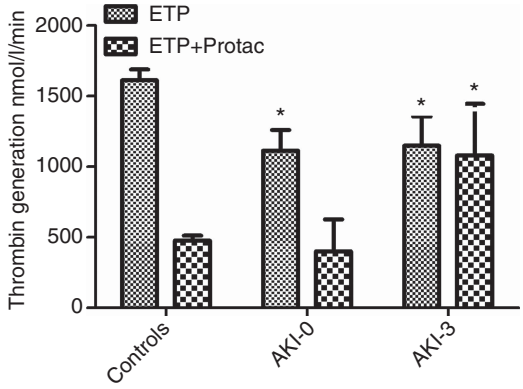

Figure 1 | Endogenous thrombin potential (ETP) measured without (native) and with Protac in healthy controls, acute liver failure patients without acute kidney injury (AKI-0), and AKI stage 3 (AKI-3). *$P < 0.05$ versus healthy controls.

Table 4 | Thrombin generation 48 h after admission, patients divided according to stage of AKI

	Normal	Stage 0	Stage 3
ETP (nmol/l)	1613 ± 76	1114 ± 147	1149 ± 206
ETP + Protac (nmol/l)	477 ± 36	539 (117–587)	807 (597–1144)
ETP %	30.0 ± 2.1	42.2 ± 14.5	66.9 ± 11.3

Abbreviations: AKI, acute kidney injury; ETP, endogenous thrombin potential.
ETP (nmol/l) in the presence of exogenous tissue factor trigger, with addition of protein C activator (Protac).
% ETP, percentage inhibition of ETP in the presence of Protac = ((ETP + Protac)/(ETP − Protac) × 100).

However, as AKI develops rapidly, uremic toxins do not have sufficient time to accumulate,[15,16] and the changes associated with AKI are therefore related to systemic inflammation with elevated FVIII, VWF, and TF, with increased fibrinolysis rather than uremia.[17,18]

Patients with AKI stage 3 had greater SOFA and MELD scores, C-reactive protein values, and more prolonged INR and APTT times with lower peripheral platelet counts, as well as lower (but statistically nonsignificant) levels of plasma clotting proteins than those with AKI stages 2 and 3. Only FV, which has a half-life of ~15 h, was significantly reduced in AKI 3 stage, which is in keeping with more severe liver injury and elevated MELD scores. As such, FV is widely used as a poor prognostic indicator (as in the Clichy criteria) for non-acetaminophen ALF.[19] FVII, which has the shortest half-life of the hepatically synthesized clotting factors,[20] was lower but not significantly different in those with AKI stage 3.

TEG appearances were either normal or prothrombotic for those without AKI, whereas they appeared to be normal to even hypocoagulable in the presence of AKI stage 3. This may be explained by the fact that TEG is an *ex vivo* test initiated by the contact pathway, and hence dependent on the procoagulant factors and the platelet count,[21] both of which were relatively more affected (reduced) in severe AKI (stage 3) than the milder forms of AKI. The clot formed *ex vivo* in the TEG cup depends predominantly on platelet numbers and function and fibrinogen.[21] Although the TEG pattern was either normal or hypocoagulable compared with normal controls, when taking into consideration the reduced platelet count and possible dysfibrinogenemia, then it could be argued that the TEG appearances showed quicker clot formation than would have been expected for the platelet count and fibrinogen levels. Unfortunately, we did not have sufficient blood to test all patients with a TEG with additional fibrinogen to determine whether there was a functional fibrinogen defect. Furthermore, TEG does not take into account other determinants of clotting, particularly the effects of anticoagulants such as the PC pathway, which are more likely to be detected using thrombin generation assays. More patients with AKI stage 3 had detectable circulating heparinoids, probably a reflection of endothelial dysfunction or liver injury, but there was no difference in anti-Xa activity or any significant change in TEG profiles following neutralization with heparinase.[19] There was no increased clot lysis observed in either group, in keeping with reports that plasminogen activator inhibitor 1 levels are often increased in ALF, leading to reduced fibrinolysis.[20] Indeed, the *in vitro* thrombin generation test (thrombography) confirmed that the effect with Protac was greater in those without AKI, as these patients had higher PC concentrations, suggesting that patients with AKI stage 3 have activated PC resistance and were actually more prothrombotic. This potential prothrombotic tendency was supported in that although thrombin generation was delayed, once the reaction began, the actual velocity of the reaction was increased. In addition, both TF and microparticles, which are important activators of the coagulation cascade *in vivo*, were found to be more elevated in patients with AKI stage 3, along with increased FVIII and VWF, suggesting increased endothelial activation. The prothrombotic tendency in advanced kidney disease in our study was clinically corroborated with repeated circuit clots in two of nine patients requiring renal replacement therapy. Other mechanistic possibilities for frequent filter clot in these patients can be explained by the larger reduction observed in FXII and FXI as they tend to deposit on the dialyzer capillary fibers, and this in conjunction with activation of FVII by tissue factor promotes thrombin formation, leading to dialyzer clotting.[22]

Despite these apparent changes reducing thrombin and clot formation, there was no increase in clot lysis, and clinically no patient required blood transfusion or had bleeding during the preceding 48 h of study. As such, although clotting factors were generally reduced in our cohort, this was balanced by reduction in the natural anticoagulant factors, and hence patients with the combination of acute liver and kidney failure are not at a greater risk of bleeding. Indeed, heparin may not be an effective anticoagulant for CRRT for these patients because of the low levels of antithrombin.[9,23] Thus, other anticoagulant options should be considered for patients with both chronic cirrhosis[24] and ALF who develop AKI, as anticoagulant-free circuits are prone to clot.[6] Citrate has been reported by several groups to be an effective regional anticoagulant in patients with liver failure requiring extracorporeal support.[25,26] However, as citrate is predominantly metabolized in the liver and kidney, and to a lesser extent in muscle,[27] then patients with ALF and AKI can potentially develop citrate toxicity if unable to metabolize citrate adequately.[28] However, just as lactate-containing dialysates and replacement solutions can be adequately metabolized by the majority of patients with liver disease,[29] so can be citrate.[25,26] The risks of citrate toxicity can be reduced by using less citrate, aiming for a slightly higher postfilter ionized calcium, 0.4 mmol/l rather than 0.2 mmol/l, and CRRT in dialysis mode rather than pure filtration.[27] However, patients with ALF and AKI with reduced muscle blood and raised lactate are prone to citrate toxicity, and hence other anticoagulants such as prostacyclin and nafamostat maleate may have a role.[30]

Although we extensively measured hepatically dependent pro- and anticoagulant factors, and other key factors involved in coagulation, including microparticles, tissue factor, and endothelial derived factors, in combination with *in vitro* tests of clot formation and thrombin generation, our study is limited by the number of patients studied. The definition of AKI was predominantly based on changes in serum creatinine. Creatinine generation is typically reduced in AKI and creatinine measurements may also be lowered in liver failure because of the interfering effects of bilirubin.[31] Creatinine is also one of the variables used to calculate the MELD score,[32] which is used to estimate early mortality in patients with liver failure, and hence the question arises as to

whether the changes in coagulation reported are primarily related to severity of kidney injury, or severity of ALF, as higher creatinine values also increase the MELD score and, similarly, SOFA scores contain a renal score component. Although our groups of patients who developed AKI stage 3 and those who did not were matched for underlying liver disease, further studies are required to tease out the procoagulant effects of AKI from those due to the severity of liver failure, although our results would probably support severity of ALF as the major determinant. However, as both severe AKI and ALF cause systemic inflammation, which can increase tissue factor, phospholipids, and microparticles, and AKI is more likely with increasing severity of ALF, this may prove somewhat difficult in clinical practice.

Thus, in this study, although traditionally patients with AKI have been thought to be auto-anticoagulated and at risk of bleeding, we found that these patients are actually more prothrombotic than originally thought because of the combination of increased endothelial activation with increased release of tissue factor and circulating microparticles, active phospholipids, FVIII, and VWF, and hence patients with ALF and AKI requiring extracorporeal renal support are more likely to clot CRRT circuits.[9]

MATERIALS AND METHODS

Patients

A total of 20 patients admitted with ALF, defined by *de novo* liver failure, coagulopathy—PT $\geqslant$20 s or INR $\geqslant$1.5—and with hepatic encephalopathy,[1] were studied. The severity of liver disease was assessed with the MELD score,[32] and that of acute illness with the SOFA score.[33] Bleeding and thrombotic episodes were recorded according to the criteria set out by The International Society for Thrombosis and Hemostasis.[34] AKI classification was based on the RIFLE (*R*isk, *I*njury, and *F*ailure) criteria, modified recently by the Kidney Disease Improving Global Outcomes (KDIGO) group[2] to incorporate the significance on patient outcomes of the smaller changes in the creatinine levels, and the development of renal injury over a relatively longer period (up to 7 days). Grading of AKI in patients who did not require renal support was based on serum creatinine measurements, as urine output did not increase grading of AKI, whereas both serum creatinine and urine output were used to define grade 3 AKI. Serum creatinine was measured by a modified Jaffe method and then by enzymatic creatinine for patients with elevated bilirubin.[31]

In addition, 20 healthy volunteers were studied for thrombin generation and the circulating microparticle assays[35] after signed informed consent.

Coagulation tests

All coagulation tests were performed on citrated samples using BD Vacutainer tubes with a blood to citrate concentration of 9:1 (Becton Dickinson, Plymouth, Devon, UK). All samples were taken atraumatically 48 h after admission in the intensive care setting using an indwelling arterial catheter kept patent by continuous saline flushing. A minimum of 5 ml of blood was discarded before sample collection. All samples were double centrifuged for 12 min at 2000 *g*. The plasma was aliquoted and stored at $-85\,^{\circ}$C until testing.

Standard coagulation tests

PT was measured using HemosIL PT Fibrinogen HS Plus thromboplastin reagent (Instrumentation Laboratory (IL), Warrington, UK) with an International Sensitivity Index of 1.15, according to the manufacturer's instructions using an ACL TOP automated coagulometer (IL), and PT ratios were calculated for each patient from the geometric mean PT (GMPT) calculated from normal individuals. INRs were calculated using the geometric mean PT and the International Sensitivity Index of the manufacturer. The APTT was obtained using HemosIL SynthASIL APTT reagent (IL) on an ACL TOP coagulometer.

Pro- and anticoagulant factors

Platelet-poor plasma stored at $-85\,^{\circ}$C was analyzed for FVIII, FIX, FXI, and FXII by standard one-stage APTT-based assays, and FII, FV, and FVII were analyzed by one-stage clotting PT-based assay on an ACL 3000 (IL).[27] VWF:Ag was analyzed by an in-house enzyme-linked immunosorbent assay.[36] We also analyzed anticoagulant factors PC, PS, and anti-thrombin as previously described.[36] Anti-Xa assays were used to determine the presence of endogenous heparinoids as previously described.[37] Fibrinogen was measured using the recommended Clauss method.[38]

Microparticles were measured using the Zymuphen MP-Activity assay (Huphen BioMed, Neuville-sur-oise, France) in platelet-poor plasma, and procoagulant phospholipids (Stago, Asnieres sur Seine, France) on an ACL TOP coagulometer.

Thromboelastography

Whole blood clotting was assessed by thromboelastography (TEG native) (Thromboelastograph Hemostasis Analyzer 5000 (Haemonetics, Niles, IL) using whole blood at room temperature. In addition, we measured TEG-heparinase (with heparinase embedded cup and pin) to assess the endogenous or exogenous heparin effect. Five parameters were assessed: *R-time* (min): latency of clot formation from beginning of clotting reaction to initial fibrin formation (defined as a change of 2 mm in amplitude), representing the enzymatic component of coagulation, the point at which standard tests of coagulation reach their end point, and therefore thought to reflect PT/INR; *K-time* (min): time from initial fibrin formation required to reach specific clot firmness (defined as 20 mm amplitude), representing clot dynamics; *α-angle* (degrees): a measurement of rate of fibrin formation and cross-linkage representing fibrinogen levels; *Maximum amplitude* (mm): maximal clot strength representing platelet function and aggregation as is mainly contributed to by platelets and fibrinogen; and *Lysis-30* (%): clot lysis at 30 min, representing a measure of fibrinolysis. Interpretation of TEG data was based on a global assessment of coagulation incorporating the cumulative effect of the interactions at various levels between plasma components (clotting proteins) and cellular components (platelets, red and white blood cells, and microparticles) of coagulation, thus allowing a dynamic assessment at different stages of clot formation/fibrin polymerization (clot initiation, amplification, and propagation to fibrinolysis).

Thrombin generation test

(TG was determined using the calibrated automated thrombography method.[36] TF reagent 5 pmol/l; Thrombinoscope B.V., Maastricht, The Netherlands) was added to 80 µl of platelet-poor plasma and the thrombin generation dynamics were derived using a calibrator with previously assigned thrombin activity and dedicated software.[38]

The calibrated automated thrombography TG was used to determine the ETP, peak height (PH), time to peak (TP), and lag time (LT), and the velocity index (slope) (PH/TP − LT)). We also used a modified TG assay as previously described,[35] in the presence of a snake venom extract, Protac (Pentapharm, Basel, Switzerland), which is an activator of PC. This brings in the effect of the PC pathway in the TG test. In normal plasma, the addition of Protac leads to a reduction in the ETP. We derived the ETP%, a measure of percentage inhibition of PC by Protac and calculated as ((ETP with Protac)/(ETP without Protac×100). The lower the ETP%, the better preserved the levels and the function of PC, and conversely higher ETP% means more severe PC deficiency or PC resistance and a potentially greater susceptibility for thrombosis.

Ethical approval was granted by the local ethical committee as audit and clinical service development.

Statistical analysis

Statistical analysis was by Students' *t*-test for normally distributed data and Mann–Whitney *U*-test for nonparametric data (GraphPad Prism version 5.0, San Diego, CA). In addition, χ^2 analysis with correction for small numbers and one-way analysis of variance with Tukey's or Dunn's post analysis correction were also performed using SPSS software for Windows version 15.0 (SPSS, University of Chicago, Chicago, IL) to adjust for multiple testing. Data are expressed as mean ± s.e.m., median, and interquartile range, or percentages. Statistical significance was taken at $\leqslant 5\%$ level.

DISCLOSURE

All the authors declared no competing interests.

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