Platelet drop and fibrinolytic shutdown in patients with sepsis

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ABSTRACT

Objective: Thrombocytopenia is the most common hemostatic disorder during sepsis and is associated with high mortality. We examined whether fibrinolytic changes precede incident thrombocytopenia and predict outcome in patients with severe sepsis.

Design: Nested study from the multicenter, randomized, controlled trial on the efficacy of albumin replacement in severe sepsis or septic shock (the Albumin Italian Outcome Sepsis trial).

Setting: Forty ICUs in Italy.

Patients: Three groups of patients were selected: 1) patients with platelet count ≤50x10⁹/L at study entry (n=85); 2) patients with baseline platelet count ≥100x10⁹/L who developed thrombocytopenia (≤50x10⁹/L) within 28 days (n=100); 3) patients with platelet count always ≥100x10⁹/L (n=95).

Interventions: Fibrinolytic variables, including fibrinolysis inhibitors and in vivo markers of plasmin generation, were measured on day 1.

Measurements and Main Results: Patients with early thrombocytopenia (group 1) and those who developed it later (group 2) had similar illness severity and 90-day mortality, while patients without thrombocytopenia (group 3) had milder disease and lower mortality. Fibrinolysis was markedly (and similarly) depressed in groups 1 and 2 as compared to group 3. Major fibrinolytic changes included increased levels of plasminogen activator inhibitor 1 (PAI-1) and extensive activation/consumption of thrombin activatable fibrinolysis inhibitor (TAFI). Most fibrinolytic variables were significantly associated with mortality in univariate models. However, only TAFI level and in vivo markers of fibrinolysis activation, namely plasmin-antiplasmin complex (PAP), and D-dimer, were independently associated with mortality after adjustment for SAPSII score, sex and platelet count. Moreover, the coexistence of impaired fibrinolysis and low platelets was associated with an even greater mortality.

Conclusions: Impaired fibrinolysis, mainly driven by PAI-1 increase and TAFI activation, is an early manifestation of sepsis and may precede the development of thrombocytopenia. TAFI level, in particular, proved to be an independent predictor of mortality, which may improve risk stratification of patients with severe sepsis.

Key words: severe sepsis – septic shock – thrombocytopenia – fibrinolysis – thrombin activatable fibrinolysis inhibitor
INTRODUCTION

During sepsis, there may be a wide spectrum of hemostatic alterations, ranging from subclinical to uncontrolled clotting activation with massive thrombin formation and fibrin deposition in the microcirculation. Thrombocytopenia is one of the most common hemostatic disorders, with a prevalence as high as 50% (1). Not only its early manifestation (2-5) but also its development during the course of sepsis (4, 6, 7) is associated with increased mortality, implying that the identification of prognostic markers that precede thrombocytopenia might improve risk stratification.

The current interpretation of sepsis is that an infectious agent triggers an overwhelming host inflammatory response, generating multiple inflammatory mediators. These, with the micro-organism and its components, activate leucocytes, endothelial cells and platelets, which then cooperate to promote microvascular thrombosis by inducing excessive activation of coagulation and impairment of physiologic anticoagulant pathways (8-11).

Besides massive clotting activation, impaired fibrinolysis is a major player in the pathophysiology of sepsis, as it contributes to widespread microvascular thrombosis by preventing fibrin removal (10-12). This is traditionally attributed to up-regulation of plasminogen activator inhibitor-1 (PAI-1) (12, 13-15), whose plasma levels show good prognostic performance in septic patients (15-18). However, other factors may be implicated in the down-regulation of fibrinolysis during sepsis. Thrombin-activatable fibrinolysis inhibitor (TAFI) is a plasma procarboxypeptidase that, once activated by thrombin or plasmin (TAFIa), removes the plasminogen binding sites from partially degraded fibrin, reducing plasmin generation and fibrin degradation. It also inactivates bradykinin, anaphylatoxins C3a and C5a, and osteopontin, functioning as an anti-inflammatory agent (19, 20). In human sepsis, most studies have shown a decrease in TAFI levels, due to extensive activation/consumption (21-24). However, information on the clinical utility of TAFI as a prognostic marker is still limited (23).

An additional factor that may influence fibrinolysis in severe sepsis is cell-free DNA (cfDNA), which is released upon cell activation or death, or by neutrophils as part of neutrophil extracellular traps (NETs) (25). cfDNA inhibits plasmin-mediated fibrin degradation (25, 26) and the increased levels of cfDNA in sepsis were shown to predict mortality (27-29). The main mechanisms of fibrinolysis inhibition in sepsis are summarized in Supplemental Digital Content, Fig. S1.

In a large randomized trial, in which we tested the effectiveness of albumin in patients with severe sepsis and septic shock (ALBIOS) (30), we collected blood samples for subsequent investigations. One of the aims was
to assess whether fibrinolytic alterations could serve as early markers of sepsis outcome. Therefore, in 280
selected patients we measured several fibrinolysis-related variables with the following aims: 1) to investigate
whether, in patients with normal baseline platelets, the fibrinolytic changes precede a subsequent platelet drop;
2) to evaluate the association between fibrinolytic markers and 90-day mortality.

METHODS

Patients

The ALBIOS trial is described in the Supplemental Digital Content, Study design. From patients recruited in the
40 centers participating in the biomarker substudy (31), we selected 280 patients according to the following
criteria. Group 1 (n=85) included all patients with baseline severe thrombocytopenia (platelet count ≤50x10^9/L).
Group 2 (n=100) included all patients with baseline platelet count >100x10^9/L, who developed severe
thrombocytopenia (i.e. platelet count ≤50x10^9/L by day 28). Group 3 (n=95) included patients (matched for sex
and age with Group 2) randomly extracted from the subgroup of patients without thrombocytopenia throughout
the study (platelet count ≤100x10^9/L). The study was approved by the institutional review board of each
participating center.

To investigate the relationships between fibrinolytic variables and outcome, the three groups were pooled.

Clinical and physiological variables

Organ dysfunction was assessed with the Sequential Organ Failure Assessment (SOFA) score (32). The severity
of systemic illness was assessed using the Simplified Acute Physiology Score (SAPS II) (33). Platelets were
counted at study entry, then from day 1 to day 28 (30).

Blood collection and assay of circulating biomarkers

Venous blood was collected on day 1, centrifuged, and plasma was shipped on dry ice to a central repository and
stored at −70°C. Biomarkers were assayed in a centralized laboratory by trained personnel unaware of patients’
characteristics. Commercially available ELISA kits were used for the assay of PAI-1, TAFIa/ai (a combination
of TAFIa and its inactive derivative, TAFIai), plasmin-α₂-antiplasmin complex (PAP, a marker of in vivo plasmin generation), and prothrombin fragment 1+2 (F1+2, a marker of thrombin generation). D-dimer, a specific fragment derived from the lysis of fibrin, was measured by a two-step immunoassay. TAFI zymogen was assayed by a functional assay, plasma cf-DNA by a fluorometric assay and plasma clot lysis, which reflects the overall fibrinolytic capacity, by a turbidimetric assay (see Supplemental Digital Content, Assays, for details).

Outcome measures

Primary outcome measure was 90-day all-cause mortality.

Statistical methods

Results are presented as proportion, median with interquartile range or mean ± SD, as appropriate. Groups were compared by the Chi-Square test for categorical variables and Kruskal-Wallis test or ANOVA for continuous ones. Bonferroni’s method was used for multiple comparisons. The association between 90-day mortality and each fibrinolytic marker was assessed by the Kruskal-Wallis test or t-test in the entire study population. Survival estimates were calculated according to Kaplan-Meier and compared with the log-rank test between (i) study groups, (ii) groups divided according to median levels of relevant biomarkers, (iii) combinations of high/low platelets (median split) and high/low fibrinolysis markers.

Unadjusted and adjusted Cox proportional hazard models were used to analyze the association between fibrinolysis markers and 90-day mortality. The hazard ratios were calculated for 1 SD increase in marker levels.

Finally, we calculated the continuous net reclassification improvement (cNRI) to assess the improvement in reclassification of death risk by adding to the model each of the fibrinolytic markers that showed an association with mortality at multivariate analysis. A two-sided p-value of 0.05 was considered significant. Statistical analyses were done with SAS software, version 9.4 (SAS Institute, Inc., Cary, NC).

RESULTS

Clinical characteristics
Table 1 summarizes the main clinical and physiological characteristics of the three groups at study entry. Patients with baseline thrombocytopenia (group 1) and those who developed thrombocytopenia during the study (group 2) had similar clinical characteristics, physiological variables and high mortality. In contrast, patients without thrombocytopenia (group 3) were markedly different, with less impairment of physiological variables and lower mortality. The survival curves of the three groups are reported in Fig. 1. No appreciable differences were observed between patients admitted for medical reasons or for emergency surgery (not shown).

**Fibrinolytic markers in relation to thrombocytopenia**

Patients with early thrombocytopenia (group 1) and those who developed it later (group 2) had higher levels of PAI-1, TAFIa/ai, and cfDNA, lower levels of TAFI zymogen and longer clot lysis time than patients without thrombocytopenia (group 3), indicating stronger inhibition of fibrinolysis (Table 2). As regards the markers of clotting and fibrinolysis activation, only D-dimer was higher in groups 1-2 than in group 3. Group 2 patients, who had a median platelet count of 104x10^9/L on day 1, presented the same fibrinolytic changes as group 1, whose median platelet count was four times lower (Table 1), suggesting that the changes behind the fibrinolytic shutdown in sepsis are independent of and may precede thrombocytopenia.

**Fibrinolytic markers and survival**

The levels of fibrinolytic markers in 90-day survivors and non-survivors are reported in Supplemental Digital Content, Table S1. Non-survivors had higher PAI-1 and TAFIa/ai, and lower TAFI. They also had lower PAP and D-dimer, suggesting greater impairment of *in vivo* fibrinolysis. Neither F1+2 nor cfDNA differed between survivors and non-survivors. Platelet count on day 1 was significantly lower in non-survivors.

Kaplan-Meier curves for 90 day-mortality by median levels of fibrinolytic markers and platelet count are shown in Supplemental Digital Content, Fig. S2.

In univariate Cox models, PAI-1, TAFIa/ai, TAFI zymogen, D-dimer and platelets significantly predicted mortality (Fig. 2a). However, after adjustment for SAPSII score and sex, only TAFI, D-dimer and platelet count were associated with mortality (Fig. 2b). To see whether the fibrinolysis-related biomarkers contributed to risk prediction independently of platelets, we tested them in a model that included platelet count on day 1, besides
SAPS II and sex. In this model not only TAFI and D-dimer, but also PAP was significantly associated with 90-day mortality (Fig. 2c). Moreover, cNRI tests showed a 30% net improvement in reclassification of the risk of death when TAFI was added to the model (cNRI [95% CI] = 0.29 [0.05-0.52], p=0.02) or D-dimer (cNRI = 0.30 [0.06-0.53], p=0.01). Qualitatively similar results were obtained when SOFA score was tested as covariate in the Cox model instead of SAPS II and platelets or when the models included also BMI and lactate, i.e. the variables outside SOFA and SAPS II scores that showed a statistical difference between survivors and non survivors (not shown).

Finally, we tested whether the combination of low platelets and impaired fibrinolysis was associated with a worse outcome. As markers of altered fibrinolysis we used the levels of TAFI, PAP and D-dimer, i.e. the fibrinolytic variables that predicted mortality independently of platelets in multivariate Cox analysis (Fig. 2c). After dividing the patients according to median levels of biomarkers (referred to as low and high, respectively), we evaluated the survival curves of patients with different combinations of platelets and each of the three fibrinolytic markers. Patients in whom low platelet count was associated with low TAFI (Fig. 3a) or low PAP (Fig. 3b) displayed a markedly higher mortality than patients with low platelets and high level of either fibrinolytic marker (HR=1.69 [95% CI, 1.05 – 2.73], p=0.03 for low TAFI, and HR=2.01 [95% CI, 1.27 – 3.19], p=0.003 for low PAP). On the contrary, in patients with high platelets, the level of TAFI or PAP had no appreciable influence on mortality (data not shown). Moreover, the survival of the two high-platelets groups was not different from that of patients with low platelets and high TAFI or PAP. The combination of D-dimer and platelets showed a progressive increase in mortality moving from high platelets-high D-dimer to low platelets-low D-dimer (Fig. 3c). However, the difference in mortality between low and high D-dimer was not significant in neither low or high platelets groups (data not shown).

**DISCUSSION**

In this study we show that (i) in patients with sepsis, most of the changes underlying the fibrinolytic shutdown precede the onset of clinically relevant thrombocytopenia which, once established, is associated with a marked increase in mortality; (ii) some fibrinolytic markers, i.e. TAFI, D-dimer and PAP, are independent predictor of death; (iii) the combination of low platelets and strong fibrinolysis impairment greatly enhances the mortality risk.
We first examined the effect of the timing of thrombocytopenia on sepsis severity and outcome and the relationship between fibrinolytic changes and thrombocytopenia. Patients who presented with thrombocytopenia (group 1), and those who developed it later (group 2), had similar illness severity (SAPS II score at baseline). The fibrinolytic alterations on day 1 were also similar in these two groups, suggesting that the fibrinolytic shutdown may occur independently of or earlier than thrombocytopenia. In contrast, patients without thrombocytopenia had milder clinical abnormalities and less marked fibrinolytic alterations. Accordingly, 90-day mortality was much higher in groups 1 and 2 than in group 3. These data suggest that patients with early or late thrombocytopenia belong to the same cohort, the only possible difference being the time when they were observed. A platelet count higher than 100x10^9/L does not mean that the process of platelet consumption with microthrombi formation has not already started. However, the finding that patients with normal platelet count do present marked fibrinolysis inhibition could indicate that platelets will drop in the subsequent days and that the outcome is likely to deteriorate.

Next we examined the relation of fibrinolytic biomarkers with mortality, after grouping the patients in a single cohort. In univariate Cox models, most fibrinolytic markers, including PAI-1 and TAFI-related variables, predicted mortality. After correction for SAPS II score, which is a well-established score to predict mortality in critically ill patients (33), and platelet count on day 1, which was also significantly associated with mortality (Fig. 2), we found that TAFI, D-dimer and PAP were still independently associated with mortality, whereas PAI-1 and TAFIa/ai were not. The results were still consistent in a fully adjusted analysis which included also sex, serum lactate and body mass index (not shown). These findings suggest that fibrinolysis impairment, as reflected by low levels of PAP and D-dimer and extensive TAFI consumption, is a clinically relevant marker of disease severity, and this may further improve the risk stratification of patients with severe sepsis, as inferred from NRI analysis. Our data also suggest that the platelet drop and the fibrinolytic shutdown may occur independently of each other and that the combination of the two is associated with a poorer outcome. We did in fact find that patients who had both low platelets and low fibrinolysis (i.e. low PAP or low TAFI) had a much higher mortality than patients who had low platelets but milder fibrinolysis impairment. Interestingly, the latter group did not differ from patients who had high platelets, no matter what their fibrinolytic status was, suggesting that both conditions (i.e. low platelets and low fibrinolysis) must coexist to worsen the outcome.

From a pathophysiological perspective, it appears that platelet activation plays a major role in dictating the overall severity, as it contributes to the formation of thrombi in the microcirculation, while the inhibition of fibrinolysis adds a further hit by preventing or delaying the clearance of these microthrombi.
The relation between impaired fibrinolysis and unfavorable outcome in septic patients is supported by numerous studies (reviewed in ref. 12). However, while there is ample evidence of the role of high PAI-1, it is not clear whether TAFI activation/consumption is a marker of sepsis severity. Clinical studies are inconsistent (22-24, 37, 38), very likely because of heterogeneous patient populations, relatively small sample and different assay methods (12). Our patients with severe sepsis showed a significant association with 90-day mortality for both high levels of TAFIa/ai and low levels of TAFI zymogen, which suggests extensive TAFI activation (24). While on the one hand this may contribute to suppressing fibrinolysis, on the other, the sustained TAFI activation may eventually lead to its excessive consumption, with the consequent loss of a major inhibitor of inflammatory mediators, such as C3a and C5a, which have been implicated in human and experimental sepsis (39).

At variance with several reports (27-29, 40), we did not find any significant association between cfDNA levels and outcome. The reason is not clear but it might be related to the time and type of assay and/or the length of follow-up. In fact, in a study of 255 septic patients, cfDNA was found to be independently associated with ICU mortality but not with hospital mortality (40). Moreover, contrary to Rodelo et al (41), we found a greater mortality in patients displaying low rather than high levels of D-dimer. The apparent discrepancy may be explained by the type of patients and illness severity. Indeed, Rodelo’s study included patients with suspected infection or sepsis (median SOFA=3), whereas we studied patients with severe sepsis (median SOFA=8). Being circulating D-dimer influenced by both fibrin formation and fibrinolysis, it is likely that, in a sample including patients with and without (moderate) sepsis, elevated D-dimer is principally a marker of hypercoagulability, as in patients with venous thromboembolism (42). On the contrary, in patients with severe sepsis, a less marked elevation of D-dimer may reflect the inability to degrade the microthrombi.

Our study presents some limitations. First, because of the study design, patients with moderate thrombocytopenia (50-100x10^9/L) were not included. Second, the study, although planned in the original ALBIOS trial, has a retrospective design. Third, no information is available on the time course of fibrinolytic biomarkers or how their trajectories correlate with clinically relevant outcomes.

CONCLUSIONS
Our study shows that fibrinolysis suppression, mainly driven by increased PAI-1 and TAFI activation, is an early event in patients with severe sepsis and is associated with disease severity and survival. TAFI, in particular, proved to be an independent predictor of mortality that might facilitate clinical decision-making. Among the numerous treatments aiming to interfere with the pathophysiology of sepsis (anti-inflammatory, anti-cytokine, anticoagulant drugs) a specific intervention to correct the inhibition of fibrinolysis has never been attempted in man.

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REFERENCES


Legend to Figures

Figure 1. Kaplan-Meier curves for 90-day survival in the three groups. Information on 90-day mortality was not available for five patients who were excluded from this analysis (respectively 3, 1 and 1 from thrombocytopenia, developing thrombocytopenia and no thrombocytopenia).

Figure 2. Fibrinolysis-related markers and 90-day mortality. Cox proportional hazard models for the relationship between fibrinolysis-related markers and 90-day mortality. a, univariate model; b, adjusted for SAPS II score and sex; c, adjusted for SAPS II score, sex and platelet count on day 1. Data shown as hazard ratios (HR) [95%CI].

Figure 3. Kaplan-Meier curves of 90-day survival for the combinations of platelet count (PLT) with either TAFI (a), PAP (b) or D-dimer (c). Low and high denote below and above the median, respectively.