D-dimer corrected for thrombin and plasmin generation is a strong predictor of mortality in patients with sepsis

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Background - D-dimer (DD) is the most used fibrin-related marker and has been proposed, either alone or in combination with other variables, as prognostic factor in patients with sepsis. However, DD generation depends on both coagulation and fibrinolysis, meaning that it may give false negative results in conditions associated with marked fibrinolytic inhibition such as sepsis. In this study, we tested whether correction of DD for thrombin and plasmin generation could improve its prognostic significance in septic patients.

Material and methods - We performed a nested study in 269 septic patients from the ALBIOS trial. DD, prothrombin fragment 1+2 (F1+2) and plasmin-antiplasmin complex (PAP) were assayed at day 1. Corrected DD (DD_{corr}) was calculated by the formula DD×PAP/F1+2, such that the lower the DD_{corr} the greater the imbalance in favour of fibrin formation over fibrin lysis, and *vice-versa.* Primary outcome was 90-day mortality.

Results - DD_{corr} showed a J-shaped relationship with mortality, which was highest in the first DD_{corr} tertile (low fibrinolysis), intermediate in the 3rd (high fibrinolysis), and lowest in the 2nd (balanced fibrinolysis), suggesting an increased risk whenever the coagulation-fibrinolysis balance is tilted ($p<0.0001$). Neither DD, nor PAP or F1+2 showed a comparable association with mortality. DD_{corr} was an independent prognostic factor in multivariable Cox models and significantly improved risk stratification (cNRI≥0.28). Finally, by combining DD_{corr} and SOFA tertiles, we developed a score with high discriminatory power.

Discussion - DD_{corr} is a good marker of the *in vivo* coagulation-fibrinolysis balance and displays a prognostic value in sepsis much higher than DD.

Keywords: *D-dimer, mortality, plasmin generation, sepsis, thrombin generation.*

INTRODUCTION

Circulating D-dimer (DD) is a degradation product of cross-linked fibrin and is widely used as a fibrin-related marker for diagnostic and prognostic purposes in different clinical settings. In venous thromboembolism, the DD assay has been validated by numerous studies as a reliable test to exclude thrombosis[1], meaning that low DD levels denote absence of fibrin deposition, either intra- or extravascularly. In critically ill patients, who are at risk of disseminated intravascular coagulation (DIC), DD measurement is included in the scores proposed by the Japanese Ministry of Health and Welfare

Arrived: 31 July 2019
Revision accepted: 16 October 2019
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Blood Transfus 2020; 18: 304-11 DOI 10.2450/2019.0175-19

(JMHW), by the International Society on Thrombosis and Haemostasis (ISTH), and by the Japanese Association for Acute Medicine (JAAM) to facilitate the diagnosis of overt DIC[2-5]. Moreover, DD elevation *per se* has been reported to be associated with increased mortality in critically ill patients[6-9], even in the absence of overt DIC[7,8]. In patients with newly diagnosed bacteraemia, elevation of DD was a frequent finding and predicted in-hospital mortality[10]. Similarly, early evaluation of DD in patient with sepsis displayed a significant prognostic performance[11], particularly when associated with an inflammatory marker[12]. These findings suggest that DD elevation may represent a potentially useful early sign of poor prognosis in critically ill patients. It should be considered, however, that DD is not a true marker of intravascular fibrin deposition because its circulating levels depend on both the amount of fibrin formed and the extent of fibrin breakdown. This implies that conditions of hypo- or hyper-fibrinolysis will have a great impact on DD levels, which in turn may lead to a misinterpretation of DD results. Sepsis, in particular, is known to be associated with strong inhibition of fibrinolysis resulting from a dramatic increase in plasminogen activator inhibitor 1 (PAI-1) level, enhanced activation of thrombin-activatable fibrinolysis inhibitor (TAFI, CPB-2) and release of nuclear products[13-15]. It is conceivable, therefore, that DD level might not accurately mirror intravascular fibrin formation during sepsis because the marked inhibition of fibrinolysis is likely to offset fibrin breakdown and a rise in DD. This view is supported by two observations: 1) combining DD with soluble fibrin monomer (FM, a genuine marker of fibrin formation), in order to correct DD by the amount of fibrin formed, improves risk stratification in patients with septic shock[16]; 2) among patients with severe sepsis and septic shock, those few with 'normal' DD levels had the highest mortality and the strongest inhibition of fibrinolysis[17]. In the light of these observations, and in view of the specific pathogenetic mechanisms behind diffuse microvascular thrombosis in sepsis, which involve both clotting activation and fibrinolysis shut down[18,19], we hypothesised that the adjustment of DD level for both thrombin generation (prothrombin fragment 1+2, F1+2) and plasmin formation (plasmin-antiplasmin complex, PAP) might provide a better picture of the balance between fibrin formation and fibrinolysis, with a potential impact on prognosis. Therefore, we evaluated the prognostic significance of corrected DD (DD_{corr}) in a group of 269 patients with sepsis, selected from the Albumin Italian Outcome Sepsis trial (ALBIOS) population[20]. We found that DD_{corr} was significantly and independently associated with mortality and improved the stratification of death risk when combined with illness severity score.

MATERIALS AND METHODS

Patients

Details on the design and main results of the ALBIOS trial[20], biomarker substudy[21], and selection of patients[14] have been previously reported. Briefly, we selected 280 patients with severe sepsis and septic shock according to the following criteria: patients with baseline severe thrombocytopenia (platelet count ≤50×10^9/L, n=85); patients with baseline platelet count >100×10^9/L, who developed severe thrombocytopenia during hospital stay (n=100); patients without thrombocytopenia throughout the study (platelet count ≥100×10^9/L, n=95). Patients who had D-dimer, PAP and F1+2 assays performed at day 1 were included in the present study (n=269). The primary outcome was 90-day mortality. The study complied with the 1975 Declaration of Helsinki as revised in 2008 and was approved by the Institutional Review Board of each centre. Written informed consent or deferred consent was obtained from each participant, according to Italian legislation.

Clinical and physiological variables

Organ dysfunction was assessed daily with the Sequential Organ Failure Assessment (SOFA) score[22], which ranges from 0 to 4 for each of five organ sub-components (respiratory, coagulation, liver, cardiovascular and renal systems), higher scores indicating higher degrees of dysfunction. The severity of systemic illness was assessed using the Simplified Acute Physiology Score (SAPS II, ranging from 0 to 163)[23], with higher scores indicating more severe systemic illness.

Blood collection and assay of circulating biomarkers

Venous blood was collected on 3.2% sodium citrate (9 volumes of blood in 1 volume citrate) before 9:00 am on the morning after enrolment (day 1). Plasma was prepared by centrifugation and stored in a biobank at –70 °C until tested. The following biomarkers were assayed with commercially available methods by trained personnel who

were blinded to the patients' characteristics: PAP complex by Technozym PAP Complex Elisa (Technoclone, Vienna, Austria); DD by a 2-step immunoassay (HemosIL AcuStar D-dimer; Werfen, Milan, Italy); F1+2 by Enzygnost F1+2 ELISA (Dade Behring, Marburg, Germany). All assays were performed within 5 years of blood collection, which is an interval known not to influence the results of DD, PAP and F1+2 assay in samples stored below −60 °C[24-26]. In order to reflect the balance between fibrin formation and lysis, DD was corrected for thrombin and plasmin formation by the following formula: DD_{corr} = DD×PAP/F1+2. In this way, the lower the DD_{corr}, the greater the imbalance in favour of fibrin formation over fibrin lysis, and *vice-versa*. Hence, low DD_{corr} values denote a condition of insufficient fibrinolysis whereas high values denote a condition of excessive fibrinolysis. All other assays were performed by standard laboratory methods.

Statistical analysis

Results are presented as proportion, median with interquartile range or mean±standard deviation, as appropriate. Groups were compared by the χ^2 test for categorical variables, and the non-parametric Kruskal-Wallis test or analysis of variance (ANOVA) for continuous ones. Bonferroni's method was used for multiple comparisons. Differences between survivors and non-survivors were assessed by the Kruskal-Wallis test or *t*-test in the entire study population. Survival estimates were calculated according to the Kaplan-Meier method and compared with the log-rank test.

Linearity of risk between DD_{corr} or sub-components of DD_{corr} and 90-day mortality was evaluated by restricted cubic splines (RCS), testing whether the non-linear component was statistically significant[27].

Univariate and multivariable Cox proportional hazard models were used to analyse the association between DD_{corr} and 90-day mortality. Two different multivariable models were tested: model 1 included SOFA score, age, sex, body mass index (BMI), and serum lactate concentration; model 2 included SAPS II score, sex, BMI, presence of shock, serum lactate concentration, and blood platelet count. The co-variates included in the models, besides age and sex, were selected because they showed a statistical difference between 90-day survivors and non-survivors (*data not shown*). Hazard ratios were calculated between tertiles of DD_{corr}, considering the mid-tertile as the reference category. Continuous net reclassification improvement (cNRI) was calculated to assess the improvement in reclassification of 90-day mortality risk by adding DD_{corr} to each of the two multivariable models. In all analyses, we considered the clinical and laboratory variables at day 1. Two-sided p=0.05 was considered statistically significant. Statistical analyses were performed with SAS software, version 9.4 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Clinical characteristics

The characteristics of the whole cohort and of patients grouped according to DD_{corr} tertiles are summarised in **Table I**. Patients in the 3rd tertile of DD_{corr} had higher SOFA score and serum creatinine levels than patients of the other two tertiles. Moreover, the former patients showed a higher percentage of positive blood cultures, a higher heart rate and a lower platelet count than patients in the 2nd tertile but not than patients in the 1st tertile, indicating that intermediate values of DD_{corr} were associated with less pronounced modifications of such variables. Concerning the DD_{corr}-related variables, DD and PAP increased significantly moving from the lowest to the highest tertile of DD_{corr}, whereas F1+2 did not show significant changes.

DD_{corr} and mortality

Cubic spline model analysis showed a non-linear, J-shaped relationship between DD_{corr} and mortality (**Figure 1A**). DD, instead, showed a linear relationship with mortality (**Figure 1B**) as did PAP and F1+2, the other two sub-components of DD_{corr} (*data not shown*).

By Kaplan-Meier plots, the highest 90-day mortality was recorded in the 1st tertile of DD_{corr}, which corresponds to insufficient fibrinolysis, while the lowest in the 2nd tertile, which corresponds to intermediary fibrinolysis (**Figure 1C**). Patients with excessive fibrinolysis (3rd tertile) had a mortality rate that lays between the other two tertiles, suggesting that either extreme condition, i.e. insufficient (1st tertile) or excessive (3rd tertile) fibrinolysis, is associated with a greater risk of death. Even though there was no appreciable difference in F1+2 levels among DD_{corr} categories (**Table I**), removal of F1+2 from the DD_{corr} formula decreased the difference in mortality among tertiles of DD corrected for plasmin only (Logrank test p=0.002) (*data not shown*). Contrary to DD_{corr}, tertiles of DD had a poor discriminatory capacity (**Figure 1D**). As to the

Blood Transfus 2020; 18: 304-11 DOI 10.2450/2019.0175-19

Table I - *Characteristics of the whole cohort and of patients grouped according to corrected D-dimer tertiles*

	All n=269	DD_{corr} T1 n=89	DD_{corr} T2 n=91	DD_{corr} T3 n=89	p
Clinical variables					
DD_{corr} range (ng/mL×0³)	0.11-7,491	0.11-8.86	8.88-40.28	41.33-7,491	-
Age (year)	71 (62-77)	71 (63-77)	72 (61-78)	70 (62-77)	0.80
Female sex - n (%)	119 (44.2)	38 (42.7)	41 (45.1)	40 (44.9)	0.94
Body mass index (kg/m²)	25.8±4.5	25.7±4.7	26.5±5.2	25.2±3.5	0.19
Reason for admission to ICU - n (%)					
Medical	162 (60.2)	51 (57.3)	51 (56.0)	60 (67.4)	0.33
Elective surgery	22 (8.2)	10 (11.2)	6 (6.7)	6 (6.7)	
Emergency surgery	85 (31.6)	28 (31.5)	34 (37.3)	23 (25.8)	
SOFA score	8 (6-10)	8 (5-10)	8 (5-10)	9 (7-12)[a,b]	0.007
SAPS II score	51.1±15.8	52.0±16.8	49.2±14.3	52.1±16.2	0.37
Shock - n (%)	167 (62.1)	60 (67.4)	51 (56.0)	56 (62.9)	0.28
Mechanical ventilation - n (%)	210 (78.1)	74 (83.2)	70 (76.9)	66 (74.2)	0.33
Randomised to albumin arm - n (%)	141 (52.4)	43 (48.3)	47 (51.7)	51 (57.3)	0.48
Positive blood culture - n (%)	92/242 (38.0)	31/82 (37.8)	19/81 (23.5)	42/79 (53.2)[a]	0.0006
Physiological/laboratory variables					
Heart rate (beats/min)	104.4±20.0	105.4±19.8	100.1±20.0	107.9±19.6[a]	0.03
Mean arterial pressure (mmHg)	73.3±14.5	72.1±13.7	74.8±14.1	71.9±15.6	0.34
Central venous pressure (mmHg)	10.0±4.9	9.4±4.3	9.4±4.9	10.7±5.1	0.19
PaO_2 /FiO_2	187 (129-270)	178 (126-242)	183 (120-267)	201 (134-284)	0.43
Urine output (mL/h)	50 (24-100)	50 (30-100)	50 (30-100)	50 (10-100)	0.36
Lactate (mmol/L)	2.8 (1.8-5.3)	2.9 (2.0-6.1)	2.6 (1.6-3.9)	2.9 (1.9-5.4)	0.12
Serum albumin (g/L)	23.8±6.2	24.5±7.0	22.8±5.5	24.3±6.0	0.18
Haemoglobin (g/dL)	10.8±1.9	10.8±2.0	10.7±1.8	10.8±1.8	0.94
Serum creatinine (mg/dL)	1.6 (1.0-2.7)	1.4 (0.95-2.05)	1.5 (0.9-2.7)	2.1 (1.3-3.0)[a,b]	0.001
Serum bilirubin (mg/dL)	0.9 (0.6-1.8)	0.8 (0.5-1.8)	0.9 (0.6-1.7)	1.2 (0.6-2.1)	0.40
White blood cells (10^3/mm³)	11.5 (4.5-19.2)	9.3 (2.8-18.1)	13.3 (5.4-21.8)	11.2 (5.3-17.6)	0.067
Platelet count (10^9/L)	142 (44-219)	146 (49-207)	191 (107-263)	122 (35-190)[a]	0.001
DD_{corr} related variables					
D-dimer (ng/mL)	4,201 (2,102-9,763)	1,883 (1,123-3,477)	4,061 (2,773-6,006)	13,959 (7,874-25,287)[a,b]	<0.0001
PAP (ng/mL)	1,659 (951-3,316)	672 (406-1,260)	1,662 (1,215-2,929)	3,690 (2,070-7,399)[a,b]	<0.0001
F1+2 (pM)	382 (243-669)	387 (250-800)	349 (231-609)	377 (241-687)	0.54

Continuous variables are shown as mean ± SD or as median (Q1-Q3); categorical variables as number (%). [a]: statistically different from tertile 2; [b]: statistically different from tertile 1. DD_{corr}: corrected D-dimer; ICU: intensive care unit; PAP: plasmin-antiplasmin complex; SD: standard deviation; T: tertile.

Table II - *Cox proportional models for 90-day mortality*

Independent variable	No. events (%)	Univariate	Multivariate model 1	Multivariate model 2
		HR (95% CI) / p-value	HR (95% CI) / p-value	HR (95% CI) / p-value
DD_{corr} ×10³: tertile 1	57/88 (64.8%)	2.6 (1.7-4.1) / <.0001	2.1 (1.3-3.3) / 0.0019	2.1 (1.4-3.4) / 0.0012
DD_{corr} ×10³: tertile 2	30/89 (33.7%)	reference	reference	reference
DD_{corr} ×10³: tertile 3	41/87 (47.1%)	1.6 (1.0-2.6) / 0.047	1.1 (0.68-1.8) / 0.65	1.4 (0.85-2.3) / 0.20

Multivariate model 1: adjusted for SOFA score, age, sex, BMI and lactate. Multivariate model 2: adjusted for SAPSII score, sex, BMI, shock, lactate and platelets. BMI: body mass index; DD_{corr}: corrected D-dimer; HR: hazard ratio.

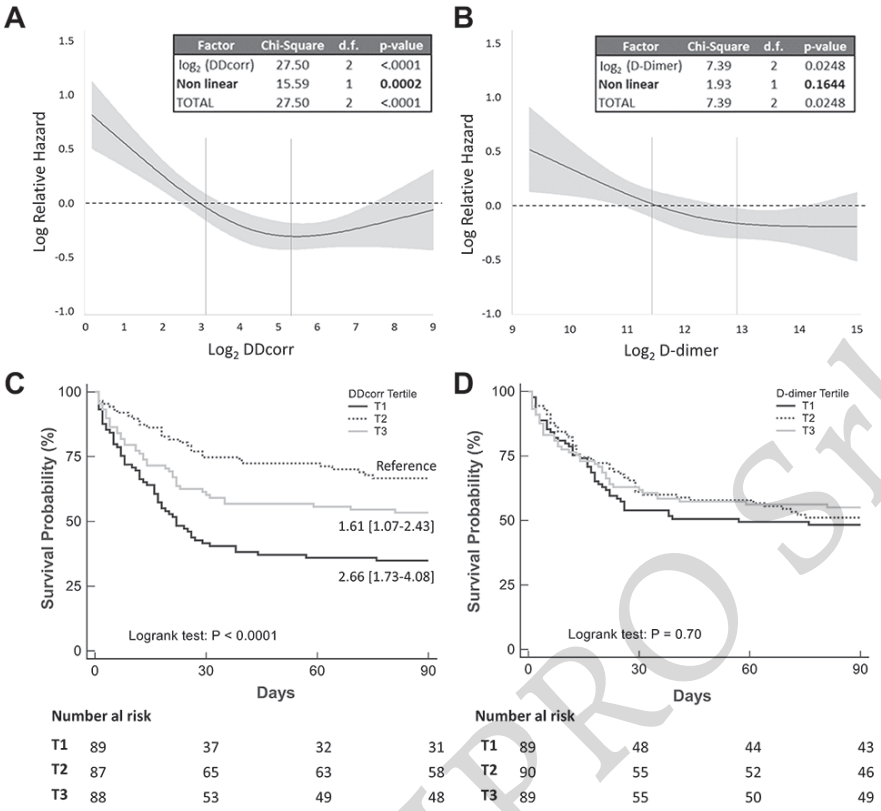

Figure 1 - Restricted cubic spline (knots=3) for 90-day mortality (Cox PH models) by levels of corrected D-dimer (DD_{corr}) (A) or DD (B)
Kaplan-Meier survival curves by tertiles of DD_{corr} (C) or DD (D). Hazard ratio (95% CI) is reported for each DD_{corr} tertile. (A and B). Vertical lines represent cut-offs for tertiles. CI: confidence interval: df: degree of freedom.

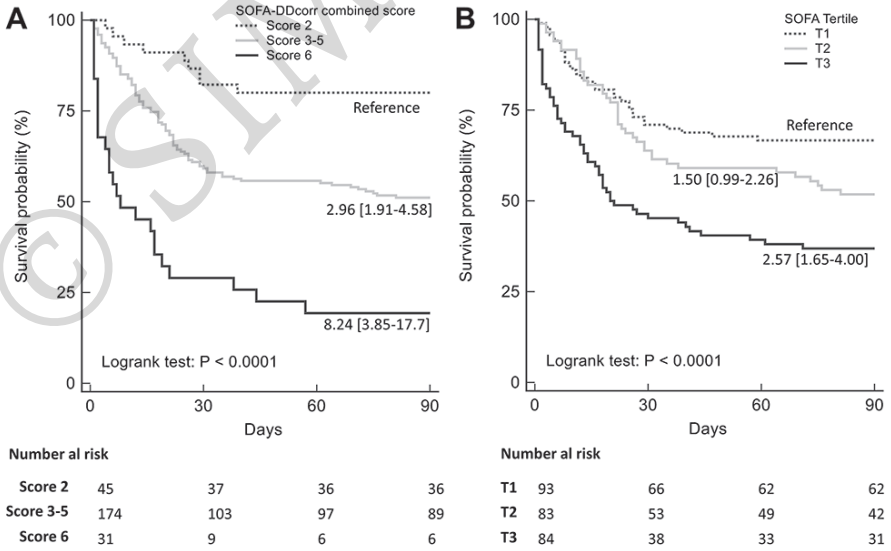

Figure 2 - (A) 90-day survival curves of groups defined by the combination of corrected D-dimer (DD_{corr}) and Sequential Organ Failure Assessment (SOFA) score categories (see text for additional information)**. (B) 90-day survival curves by tertiles of SOFA are illustrated for comparative purposes**
Hazard ratio (95% CI) is reported for each group.CI: confidence interval.

other sub-components of DD_{corr}, only PAP tertiles showed a significant association with mortality, which was highest in the lowest tertile (Logrank test p=0.016 (*data not shown*). Univariate and multivariate Cox models were used to evaluate the prognostic significance of DD_{corr}, which was categorised into tertiles, using tertile 2 as reference. Two different multivariate models were tested, one including SOFA score, age, sex, BMI, and lactate, the other including SAPSII score, sex, BMI, shock, lactate, and platelets. As reported in **Table II**, DD_{corr} was significantly associated with mortality in all 3 models, even though the difference between T2 and T3 was no longer significant in the multivariable models.

By continuous net reclassification (cNRI) test, we found that the addition of DD_{corr} to multivariable models significantly improved the assessment of death risk, overall cNRI amounting to 0.35 (0.12-0.59) for model 1 and to 0.28 (0.04-0.52) for model 2.

Based on these findings, we tested if the combination of SOFA and DD_{corr} would improve the discriminatory power of each single variable. To that purpose, we developed a simple score, as follows. We assigned a score of 1, 2 and 3 to the first, second and third tertile of SOFA. For DD_{corr} score, we assigned 1 point to the 2nd tertile, 2 points to the 3rd tertile, and 3 points to the 1st tertile, such that the higher the DD_{corr} score the higher the mortality risk. By the sum of the two scores we identified 3 groups: group 1, whose combined score equalled 2 (low risk); group 2, whose score ranged between 3 and 5 (intermediate risk); group 3, whose score equalled 6 (high risk). As shown in **Figure 2A**, there was a dramatic increase in mortality rate moving from the lowest to the highest combined score; the hazard ratio rose by >8 times in the high risk group, which consisted of patients with high SOFA score and low DD_{corr} levels. Even though the two extreme groups (scores 2 and 6, respectively) were much smaller than the intermediate one, meaning that the survival confidence intervals of the former were obviously greater, there was no overlap among the 95% confidence intervals of the three groups (*data not shown*).

DISCUSSION

We show that DD corrected for thrombin and plasmin generation has a high prognostic value in septic patients. Based on the formula we used, low levels of DD_{corr} are expected when plasmin generation, as measured by PAP level, is low relative to thrombin generation (i.e., F1+2), meaning that fibrin deposition overwhelms fibrin removal by the plasminogen/plasmin system. At the opposite extreme, high levels of DD_{corr} suggest an imbalance towards fibrin degradation, indicative of an active fibrinolytic state. In between these two conditions, DD_{corr} appears to reflect a more or less balanced coagulation-fibrinolysis equilibrium. Thus, patients with a given DD level may fall in the low, intermediate or high DD_{corr} category depending on the balance between thrombin and plasmin generation. In our patients with severe sepsis and septic shock, we found a non-linear, J-shaped relationship between DD_{corr} levels and 90-day survival. Mortality rate was high in patients with elevated DD_{corr} (tertile 3) and even more so in those with low DD_{corr} (tertile 1), suggesting that the imbalance between fibrin formation and fibrin removal increases the mortality risk, regardless of which way the balance tips. The highest mortality in patients with low DD_{corr} harmonises with the well-known fibrinolytic shutdown associated with sepsis. The mechanisms behind fibrinolysis suppression are multiple and include PAI-1 elevation, TAFI activation, and release of nuclear products such as histones and DNA[13-15]. Each of these factors has been shown to be associated with the severity of illness and mortality[13,14,28-33], even though not consistently[13,14,34-36]. As a matter of fact, in the very same patients, we showed that both PAI-1 increase and TAFI activation/consumption were significantly associated with mortality[14], but neither of the two showed a discriminatory power as high as that of DD_{corr}. This is not surprising because the level of single factors, albeit pathophysiologically important, cannot provide a full picture of the ongoing coagulation-fibrinolysis processes, which are influenced by a multitude of players directly or indirectly involved in fibrin formation and lysis. In this respect, DD_{corr} is likely to better reflect the *in vivo* situation because it takes into account reliable markers of thrombin and plasmin formation as well as fibrin degradation. Mortality rate was also increased in patients with high DD_{corr}, i.e., in patients displaying enhanced fibrinolysis. This finding is in line with the evidence that DIC may present in different forms depending on the degree of fibrinolysis activation: i) DIC with suppressed fibrinolysis; ii) DIC with balanced fibrinolysis; and iii) DIC with

enhanced fibrinolysis[37]. DIC with suppressed fibrinolysis is the predominant DIC type during sepsis and one of the major pathogenetic mechanisms of organ failure[18,19]. Nevertheless, heightened fibrinolysis may be observed during sepsis, even in the presence of inhibitors[38], as suggested by the high levels of PAP found in a large percentage of patients by ourselves and by others[39,40]. When it occurs, enhanced plasmin generation exacerbates consumptive coagulopathy, thereby increasing the risk of bleeding.

From a clinical standpoint, DD_{corr} turned out to be an independent predictor of mortality and to significantly increase risk stratification when combined with other relevant risk factors, including illness severity scores. More importantly, by combining DD_{corr} with SOFA, we developed a simple score with a high discriminatory power, being able to identify patients with low risk (20% mortality) and patients with very high risk (> 80% mortality). Even if we could not perform a head-to-head comparison with DIC score, it could be speculated that DD_{corr}, thanks to its independent association with mortality, might offer an advantage over DIC score (either ISTH or JAAM), which, in a large cohort of sepsis patients, was shown to predict outcome in univariate but not multivariable models including illness severity[41].

Our study has a number of limitations. First, in the current formulation, DD_{corr} has practically no clinical applicability for it requires three distinct assays, two of which (F1+2 and PAP ELISAs) are not suitable for emergency use. However, it should be feasible to develop automated assays (e.g., latex methods) that can be performed relatively quickly. Moreover, F1+2 might be replaced by soluble fibrin, another marker of fibrin formation, that can be easily measured and that has already been shown to improve risk stratification in sepsis when combined with DD[16]. Second, patients were selected from the ALBIOS population on the basis of platelet trajectories and thus patients with moderate thrombocytopenia (50-100×10^9/L) at baseline were not included[14]. Third, we did not evaluate the changes of DD_{corr} over time, which, in the case of a dynamic process like sepsis, might even have improved the prognostic capabilities of DD_{corr} measurements. Finally, the study has a retrospective design.

CONCLUSIONS

Our results suggest that a score reflecting the balance between fibrin formation and breakdown like the DD_{corr} may represent a new prognostic marker in septic patients, which may significantly increase stratification of death risk if combined with illness severity score. Moreover, DD_{corr} level may offer clues for a more targeted therapeutic strategy for the treatment of sepsis-associated coagulopathy as it has the potential to distinguish between hypo- or hyper-fibrinolysis conditions. Prospective studies in larger patient cohorts are warranted to validate our data and to define the exact prognostic significance of DD_{corr}.

ACKNOWLEDGEMENTS

The Authors are grateful to Werfen (Milan, Italy) for providing the kits and the equipment for D-dimer assay (courtesy of Dr. Alessandro Berti).

FUNDING

This trial was partially funded by grants from the Italian Ministry of Health (*Ricerca Finalizzata* 2011-2012, grant RF-2011-02348358), and by a grant from "Aldo Moro" University, Bari .

AUTHORSHIP CONTRIBUTIONS

MC designed and supervised the study and wrote the manuscript. FS and CTA performed the research. LG, PC, AP and MP supervised the clinical study management. SM and RL performed the statistical analysis and contributed to data interpretation. NS contributed to data interpretation and writing of the manuscript. LG, RL and PC critically revised the manuscript. All Authors read the final version of the manuscript and approved it prior to submission.

The Authors declare no conflicts of interest.

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