Early rise in circulating endothelial protein C receptor correlates with poor outcome in severe sepsis.

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Abstract

Purpose

The endothelial protein C receptor (EPCR) negatively regulates the coagulopathy and inflammatory response in sepsis. Mechanisms controlling the expression of cell-bound and circulating soluble (s)EPCR are still unclear. Moreover, the clinical impact of EPCR shedding and its potential value to predict sepsis progression and outcome remain to be established.

Methods

We investigated the time-course of plasma sEPCR upon the 5 first days (D) of severe sepsis in 40 patients.

Résults

Firstly, no significant difference was observed when comparing sEPCR at admission (D1) to healthy volunteers and to patients with vasculitis. We report that the kinetic profile of plasma sEPCR in patients was almost stable at the onset of sepsis with no change from D1 to D4 and then a significant decrease at D5. This pattern of release was consistently observed whatever the level of sEPCR at D1. Characteristics of patients or of infections (except Gram negative) had no or poor critical influence on sEPCR profile. However, we found that sEPCR kinetic was clearly influenced by patient's outcome (D28 survival). We demonstrate that a significant but moderate (< 15% of basal level) and transient increase in sEPCR level at Day2 associates with poor outcome at Day28.

Conclusion

Severe sepsis, at the onset, only triggers moderate quantitative changes in plasma sEPCR levels. Our findings suggest that in severe sepsis, an early (at D2), transient but significant increase in circulating sEPCR may be detrimental suggesting that sEPCR could provide an early biological marker of sepsis outcome.

MESH Keywords Adult ; Aged ; Antigens, CD ; blood ; Female ; France ; Humans ; Intensive Care Units ; Male ; Middle Aged ; Outcome Assessment (Health Care) ; Receptors, Cell Surface ; blood ; Sepsis ; physiopathology ; Severity of Illness Index ; Vasculitis ; physiopathology

Author Keywords soluble EPCR ; severe sepsis ; gender ; infection ; survival analysis

Severe sepsis is associated with systemic inflammation and exacerbated procoagulant state mediated by the Tissue factor (TF) pathway[1 –2]. The protein C (PC) anticoagulant pathway is a major system that prevents blood coagulation, its impairment influence vital outcome in sepsis. This pathway involves PC and protein S, and two endothelial receptors, thrombomodulin (TM) and the endothelial cell protein C receptor (EPCR). Conversion of PC to activated PC (APC) is initiated by thrombin bound to TM on endothelial cell (EC) surfaces. Binding to EPCR increases the rate of PC activation by thrombin-TM complexes[3]. APC has anticoagulant effects but also exhibits anti-inflammatory[4 –5], and antiapoptotic effects[6 –7]. EPCR also serves as a cellular binding site for Factor VII/VIIa[8 –9].

EPCR (CD201) is a 46-kilodalton type I transmembrane protein, which is expressed mainly on the luminal surface of EC from large vessels and which is homologous to major histocompatibility complex class I/CD1 family proteins[10]. Disruption of the *EPCR* gene in mice causes placental thrombosis and embryonic lethality confirming the key role for EPCR in controlling coagulation[11].

A soluble form of EPCR (sEPCR) has been described in plasma[12]. sEPCR can be generated by ectodomain shedding[13] mediated by TACE/ADAM17[14] or/and, by alternative mRNA splicing in haplotype-A3–carrying cells[15]. sEPCR binds both PC and APC with an affinity similar to that of membrane EPCR(mEPCR)[16]. Binding of APC to sEPCR interferes with binding of APC to phospholipids and inactivation of factor Va. Furthermore, binding of PC to sEPCR does not enhance APC generation, suggesting a procoagulant effect of sEPCR[9 , 17].

EPCR levels vary in a wide variety of pathophysiological conditions, and the consequences depend partly on whether it is the membrane-associated or soluble form that is affected[12 , 18 –19]. While the role of membrane-associated EPCR is clearly antithrombotic and anti-inflammatory in physiological circumstances, the function of the circulating sEPCR remains unclear.

Regulation of circulating sEPCR upon inflammatory disease has not been determined. Thus, whether sEPCR levels may have a predictive value of sepsis outcome is still unknown. In the present study, we investigated the kinetic of soluble EPCR levels in a cohort of 40 patients with severe sepsis upon the 5 first days in our Intensive Care Unit (ICU). The first objective was to compare sEPCR levels in septic patients at admission to healthy volunteers or to patients with active inflammatory disease. The second aim was to investigate the time course of circulating sEPCR at the onset of severe sepsis in correlation with clinical parameters and outcome at D28.

Patients, Materials and Methods

Study population

We recruited consecutive patients with severe sepsis in a 20 beds Medical ICU (Nantes University Hospital, France). Patients were included using the inclusion criteria according to the ACCP consensus definition for severe sepsis [20] and as described in the Electronic Supplementary Material 1 (ESM1) .

The exclusion criteria were age younger than 18 years, patients without informed consent, patients not expected to survive more than 48h (moribund) or not expected to survive more than 28 days given preexisting conditions.

We collected baseline characteristics of the patients, including demographic information, severity score at admission, simplified acute physiology score (SAPS), Sepsis-related Organ Failure Assessment (SOFA) at admission and for 4 days, comorbidities, site and type of infection, and hematologic tests. Patients were followed for 28 days and/or until ICU discharge.

Blood collection

Blood was collected from patients within 24h of meeting the definition of severe sepsis (within the first 24 hours after the first organ dysfunction occurred) and daily in the 4 consecutive following days. For that purpose, blood samples were collected in the entrance in ICU (Day 1) and each morning using daily routine blood puncture (Day 2 to Day 5). After centrifugation, samples were immediately frozen as aliquots (−80°C).

This study also included blood samples from patients with active systemic vasculitis (n=25) including Wegener granulomatosis (WG) or microscopic polyangeitis (MPA) that we previously described[21] and blood samples from healthy adult blood donors (n=40) provided by the Etablissement Français du Sang (Nantes, France).

Elisa assays for soluble EPCR

Quantification of sEPCR in plasma samples was carried out using ELISA kits (Asserachrom, Stago-Diagnostica, France).

Research ethics

The study was approved by medical research committee (Nantes University Hospital, France). Informed consent was obtained from the patient/substitute decision-maker before inclusion in this study. All data were anonymous.

Statistical Analysis

The means and SD or the median [IQR], in case of non-normality of the distributions, are reported for continuous variables. The number of patients in each category and the corresponding percentages are given for categorical variables. Non parametric ANOVA was used for the comparison of sEPCR levels at admission between groups.

Multivariate analyses were performed to study the evolution of sEPCR levels over time. To account for the correlation between measurements from the same individual, repeated measures ANOVA using linear mixed models allowing for random effects with restricted maximum-likelihood estimation were used to examine changes in sEPCR levels over time and potential interactions when appropriate (“time effect”), as well as the effect of covariates of interest (“group effect”). Several covariance structures among the repeated measurements (autoregressive, unstructured etc.) were compared using Akaike’s Information Criterion and Schwarz Bayesian Criterion. Random intercept models with autoregressive covariance structures often provided the best fit to the data. Residual analysis was used to evaluate the validity of the models assumptions including normality and homoscedasticity. An iterative stepwise selection procedure was used to select the variables that were significantly associated with the time course of sEPCR levels (variable candidates for the model were those associated with sEPCR evolution in univariate analyses with $p<0.20$ criterion and subsequently selected in the model using $p<0.05$ criterion). All measurements were transformed by calculating the natural logarithm and all analyses were adjusted on sEPCR levels at day1 in repeated measures models.

Multivariate analyses were subsequently performed using exact logistic regression and a similar strategy was used with an iterative stepwise selection procedure to select the variables that were significantly associated with day-28 survival, as assessed by the likelihood ratio test. The area under the receiver operating characteristic (ROC) curve was estimated for sEPCR levels at day 2.

Statistical analyses were performed with SAS 9.1 statistical software (SAS Institute, Cary, NC, USA). P-values <0.05 were considered to be statistically significant.

Results

Study population

Between December 2004 and September 2005, 82 patients were admitted for severe sepsis in our Medical ICU, 27 patients were not eligible because they had developed first organ dysfunction more than 24h before and 15 patients refused inclusion or delayed their response after inclusion “dead line". Thus, the present study includes 40 consecutive adult patients with severe sepsis. Most of them (n=37, 93.5%) were in septic shock at admission but all of them at day 2. The patients ranged in age from 24 to 81 years (58.3±14.4), and 24 (60%) were female. The mean admission SAPS II score was 51 (SD, 21), and the mean SOFA score at Day1 was 10.5 (SD, 3.4). The overall 28-day mortality rate was 22.5 %. Demographic and clinical characteristics of these patients are summarized in Table 1 . The most common site of infection was pneumonia (27.5%). Infecting organism isolation was available for 35 patients (87.5%) with. a Gram-negative bacillus for 18 patients (45%). Hemoculture was positive for 35% of patients.

Soluble EPCR levels in patients with severe sepsis at admission (Day1): comparison with patients with inflammatory disease and healthy volunteers

Soluble EPCR was quantified concomitantly in all samples (n=182 for sepsis and n=65 for healthy controls and vasculitis) using a dedicated Elisa assay. When sEPCR levels were compared at the time of admission (D1), no significant difference (Figure 1) was found between patients with severe sepsis (Med [IQR]:115 [145]; Mean±SD: 174±117 ng/mL) compared with healthy control subjects (156 [65]; 170±63 ng/mL; p =0.37) and with patients with active systemic vasculitis (WG and MPA)(159[94]; 157±60 ng/mL). As compare to the healthy controls, sEPCR values in septic patients exhibit larger variability, especially above the median value (115 ng/mL). For further analysis, septic patients will be divided in 2 groups (High sEPCR> 115 ng/mL and Low sEPCR≤ 115 ng/mL) according to their basal sEPCR levels at Day1.

Time course of soluble EPCR levels at the onset of severe sepsis

A retrospective and concomitant determination of circulating sEPCR was performed on blood samples collected from 40 patients with severe sepsis during the 5 first days in ICU. Quantification of circulating sEPCR in the overall population of patients (Figure 2a) mainly showed only slight variations in the sEPCR levels at onset of sepsis. No significant change was observed from D1 to D4 while a significant decrease was found at D5 (p< 0.05 versus D1, D2 and D3). Overall changes in sEPCR levels from D1 to D4 were low and remained below 10% of basal (D1) sEPCR rate. Similarly, although significant, decrease at D5 was moderate and around 15% of basal rate.

This pattern of release was consistently observed whatever the level of sEPCR in patients at D1 (ESM2a); higher levels (H group) were maintained during the 5-days follow-up (group effect, p<0.01).

Next, uni- and multivariate statistical analysis were performed to identify factors influencing sEPCR kinetics. These parameters were selected according to their relevance to demographics and severe sepsis (early disease severity scores, disease evolution and infection). Variables analyzed include gender, age, outcome at D28, SAPS II, SOFA D1, SOFA D2, Gram staining, site of infection (pneumoniae), septicemia. First that time course of sEPCR release was clearly affected in septic patients according to patient survival at D28 (Figure 2b). Univariate analysis showed a significant difference in sEPCR levels (Group effect “GE", p=0.029), and in kinetics (Time effect “TE" p= 0.016) between the 2 groups. These results were confirmed in multivariate analysis (GE, p=0.003 and TE, p=0.005). At Day 2 : patients that do not survive over D28 had significantly elevated sEPCR (Med 268[169], mean 268±126) as compared to septic patients that do survive (Med 120[92], mean 143±92 ng/mL; p<0.01). Increase at D2 was only transient since sEPCR return to control level at D3.

A difference between males and females was observed in septic patients at D1 (respectively Med 196[149], mean 225±143 *versus* 107[98], 140±84 ng/mL, p<0.05) as well as in healthy individuals (respectively Med 177[63], mean 201±67 *versus* 129[41], 145±47 ng/mL, p<0.05). Nevertheless, no significant impact of gender on sEPCR profiles was found in our cohort using our statistical analysis model (ESM2b) and similar time courses were observed for males and females. Similarly, age had also no influence on sEPCR profile.

Considering variables related to severity of sepsis, SAPS II and Mc Cabe scores had no impact. Interestingly, a weak effect of SOFA score at D1 and D2 was found on sEPCR levels (GE respectively p=0.045 and 0.034) but with no significant impact on the kinetic from D1 to D5. To further investigate these results, correlations were examined considering individual time points. Interestingly, a slight correlation was only found between sEPCR D2 and SOFA D2 (ρ= 0.293, $p<0.05$).

Finally considering parameters related to infection, we found that the presence of a positive blood culture (not shown) or pneumonia as septic localisation (ESM2c) had no effect on sEPCR profiles. In contrast, a significant impact of gram negative bacterial infection was observed (ESM2d). Univariate analysis showed higher D1-D5 plasma levels (GE p=0.034) without clear effect on kinetic (TE p=0.376) while multivariate analysis confirmed the influence of gram negative on both, group level and kinetic (GE p=0.045 and TE p=0.005).

Overall, our findings show only slight variations in the course of sEPCR at the onset of sepsis. It appears that most of parameters affecting basal sEPCR rate have no incidence on the kinetic profile of sEPCR upon sepsis. However, we demonstrated that both level and kinetic of sEPCR are clearly influenced by Gram negative infection and, importantly, by D28 mortality: an early significant increase in sEPCR rate at D2 being associated with a poor outcome.

Consequently, to sustain this conclusion, uni- and multi-variate analyses were performed for eleven parameters (including sEPCR at D2) relevant to D28 mortality in septic patients. These parameters include sEPCR D2, sEPCR D1 H/L, SAPS II, Gender, SOFA score at D1 and D2, Age, McCabe Score, Sepsis localisation (Pneumoniae), septicemia and Gram negative infection. Major findings are reported in Table 2 . Plasma concentration of sEPCR at D2 was the only variable found significantly associated with D28 mortality in multivariate analysis (p=0.0095). Our data indicate that an increase in sEPCR at D2 of 100 ng/mL corresponds to an Odd Ratio of 2.44 [1.22–5.95].

We undertook a specific analysis to further investigate the accuracy of sEPCR value at D2 to predict D28 mortality in our cohort. Sensitivity and specificity are presented in the ROC curve (ESM3). The accuracy estimated by area under the ROC curve seemed satisfactory (AUC = 0.819; IC [0.658–0.971]). The moderate size of the samples (dead=9, alive=31) is, however, a limit for further extrapolation.

Discussion

During sepsis, blood coagulation is activated by TF expressed on the endothelium and monocytes/macrophages in response to cytokines[22]. Inflammatory processes can shift the hemostatic balance toward thrombus formation not only by stimulating TF-dependent coagulation but also by inhibiting anticoagulant and fibrinolytic pathways[23]. EPCR is a key factor controlling coagulation through the binding of both PC and APC. Inhibition of EPCR binding of PC or APC was also found to exacerbate the septic response[24 –25]. However, clinical and functional impact of its soluble counterpart remains to be established. Here, we examined for the first time the kinetic of plasma sEPCR in a cohort of patients at the onset of severe sepsis.

First, when comparing baseline levels of sEPCR in septic patients at admission we found no significant difference with healthy controls and patients with active vasculitis, although variation among the septic patients was important. In previous studies, sEPCR levels in septic patients were found significantly higher[12 , 26], similar[27] or even lower[28], than in healthy volunteers. These discrepancies may reflect the distribution of the A3 haplotype (15–20%) among the cohorts since this haplotype is associated with higher baseline levels of sEPCR[18].

Moreover, we observed here that gender ratio may be another key factor affecting D1 sEPCR in septic patients. A similar difference was found in healthy donors. These results confirm our previous data indicating that gender was a critical parameter determining basal level of sEPCR [29]. Although clinical impact of gender on sepsis outcome is still controversial[30 –32], whether males and females differently respond to infection is important for both clinical care and the design of clinical trials. However, probably due to the small size of this cohort and to relative variability of basal sEPCR levels, this study was unable to demonstrate an impact of gender on the sEPCR time course. Since no DNA samples were available for genetic analysis, the respective impact of EPCR haplotype and gender was not investigated.

Next, profiling sEPCR during the 5 first days of the disease showed no change in the level of sEPCR from D1 to D4 before a significant decrease at D5. Overall variations in sEPCR concentrations remain below 15% of baseline. Interestingly, this kinetic was found consistently in septic patients regardless of basal level and gender. Another interesting observation is that, overall, sepsis only induces a moderate variation of sEPCR plasma level as suggested by initial reports[12 , 27]. Together these data suggest that level of sEPCR is tightly controlled even upon inflammation *in vivo* . This hypothesis is comforted by our results obtained using cultured vascular EC. Indeed, we found that *in vitro* inflammation downregulates the cell surface expression of EPCR without affecting the production rate and kinetic of its soluble form (Data not shown and [29]).

All available data concerning simple coagulation disorders were noted (platelet count, Prothrombin time, activated partial thromboplastin time (aPTT) ratio patient/witness, and fibrinogen). The impact of these parameters on sEPCR was examined for each time point using a Spearman test (data not shown). No major correlation was found except for platelet count and aPTT at D4. Considering the type of infection, multivariate analysis highlighted a significant impact of gram-negative infection on both sEPCR level and kinetic. Despite data of sepsis studies large cohorts, the link between severity of coagulation disorders and type of causative microorganism had still remained controversial. [33 –34].

We identified a statistically significant difference between D28 survivors and nonsurvivors in terms of sEPCR levels at D2 compared to baseline. This new finding suggests that sEPCR level at D2 may be an early prognostic marker of adverse outcome in septic patients. It could also suggest an implication of elevated sEPCR in the pathogenesis of severe sepsis. While EPCR is considered as a protective molecule[24], the function of its soluble form remains to be determined. Functionally, sEPCR bind PC and APC with high affinity (Kd= 75nM)[35] and thus may counteract (or balance) the anticoagulant action of mEPCR. On another hand, sEPCR also bind the neutrophil proteinase PR3 with a similar high affinity, this interaction results in the proteolytic degradation of the receptor[36]. Thus, we can speculate that a significant increase in plasma sEPCR may indeed reflect uncontrolled EC activation and/or irreversible EC injury. This pathogenic link remains to be examined in vitro and in vivo. Whatever the mechanism, a functional impairment of the endothelial protein C activation pathway reflected by an increase in sEPCR level will most probably exacerbate the preexisting state of hypercoagulability in septic patients.

It is to notice that death was associated with higher sEPCR level in septic patients at Day 2 but not at the time of death. Five patients died within the 5 first days of the study. Although it seems difficult to drawn a firm conclusion from only 5 patients, the available values for the last sEPCR dosage before death showed no significant change.

To conclude, severe sepsis, at the onset, only triggers moderate quantitative changes in plasma sEPCR concentration. Our findings also suggest that in pathological conditions such as severe sepsis, an early (at day2 post admission), transient but significant increase in circulating sEPCR may be detrimental. These new findings suggest that measuring sEPCR at Day2 could provide an early biological marker of sepsis outcome.

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Footnotes:

Authorship: Contribution: C.G., N.G., O.Z. and C.B. performed research assays and analyzed data; V.S. performed the statistical analysis. D.V. provided critical review of the manuscript; and; B.C. and C.G. conceived and designed the study and wrote the manuscript.

Conflict of interest disclosure: The authors declare no competing financial interests.

Abbreviations

APC : activated protein C
EC : endothelial cell
EPCR : endothelial protein C receptor
ICU : intensive care unit
MPA : microscopic polyangeitis
PC : protein C
SAPS : simplified acute physiology score
mEPCR : membrane EPCR
sEPCR : soluble EPCR
SIRS : systemic inflammatory response syndrome
SOFA : Sepsis-related Organ Failure Assessment
TF : tissue factor
TM : thrombomodulin
TNFα : Tumor necrosis factor-alpha
WG : Wegener granulomatosis

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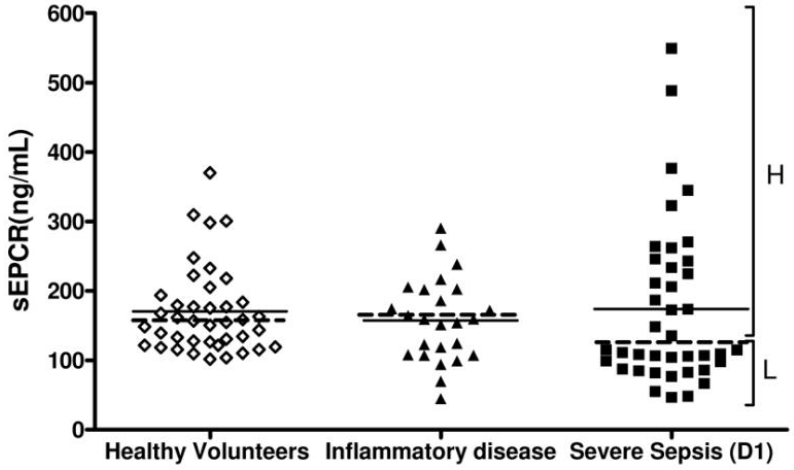
Figure 1

Baseline sEPCR in septic patients and controls

sEPCR was quantified in blood samples from healthy volunteers (n=40), patients with vasculitis (n=25) and in samples from 40 patients with severe sepsis harvested at admission (D1). No significant difference (p=0.37). *Individual results are shown, bars represent mean values, dashed lines represent the median value. H; high and L; low referred to sEPCR levels compare to the median value.* .



n:	40	25	40
Med (IQR):	156 (65)	159 (94)	115 (145)
Mean (SD):	170 (63)	157 (60)	174 (117)

Figure 2

Time-course (from D1 to D5) analysis of sEPCR in septic patients (n=40)

sEPCR was measured daily from admission in ICU (D1) to D5. Statistical difference ($p<0.05$) is indicated as **#** for "group effect" (GE) and $ for "time effect" (TE). (**a**) Overall population [TE p=0.037, § $p<0.05$ in comparison to D5]. Number of available samples is indicated under box plot for each time point (**b**) According to outcome at D28, alive (n=31), dead (n=9) [GE p=0.029; TE p=0.016; * difference at day2, $p<0.01$].

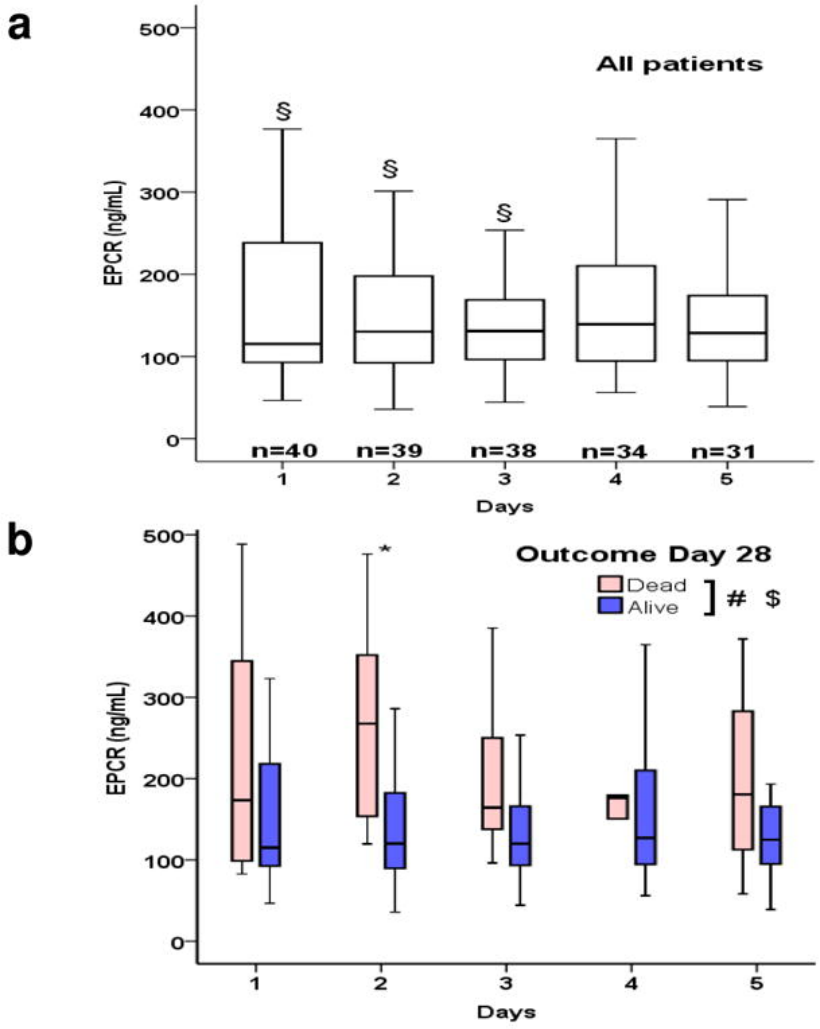


Table 1

Characteristics and outcome of patients with severe sepsis(n= 40), description of infections.

Age, y	58.3 ± 14.4
Gender Ratio : Female, n (%) :	24 (60%)
Intensive Care : Medical/Surgical, n (%):	22 (55%)/18 (45%)
Mc Cabe : 1/2/3, n (%)	11 (27%)/24 (60%)/5 (13%)
Immuno depression, n (%)Hematology, Cancer, Corticotherapy, HIV, Cirrhosis	23 (57%)
SAPS II	51 ± 21
SOFA score Day 1	10.5 ± 3.4
Septic Shock at inclusion, n (%)	37 (93.5%)
Mechanical ventilation * **, n(%)**	34 (85%)
Hemodiafiltration * **,** n (%)	12 (30%)
Hydrocortisone * **,** n (%)	30 (75%)
Drotrecogin * **,** n (%)	2 (5%)
Site of Infection , n	
Pneumoniae	11
Abdominal	8
Skin or Soft tissues	6
Bones or Joints	4
Urologic	4
Endocarditis	3
Septicemia	2
Meningitis	1
Pleural empyema	1
Positive blood culture , n (%)	14 (35%)
Results of Gram's staining culture	
Pure Gram-negative	14
Pure Gram-positive	17
Mixed	4
Culture negative	5
ICU length of stay, d	17±13
Mortality, n (%) ICU	12 (30%)
Day 28	9 (22.5%)

ICU: Intensive care unit; SAPS: Simplified Acute Physiology Score: SOFA: Sepsis-related Organ Failure Assessment

* during 5 first days of ICU hospitalisation

Table 2

Univariate and multivariate analysis of factors associated with mortality at Day 28.

	Odd Ratio	Confidence Interval	p
Univariate analysis			
EPCR D2	1.009	1.002–1.018	0.0095
SAPS II	1054	1.012–1.107	0.0087
SOFA D1	1.249	0.981–1.642	0.0740
SOFA D2	1.243	1.007–1.598	0.0417
Age	1.054	0.994–1.131	0.0845
Pneumoniae	4.948	0.800–34.047	0.0936
Multivariate analysis			
EPCR D2	1.009	1.002–1.018	0.0095

The odds ratio are expressed *for an increase of 1ng/ml for s EPCR at D2, 1 point for SAPS II and SOFA D1, 1 year for age. IC Interval confidence. Factors with p<0.20 are noted.*