

1
2 INTRODUCTION

3 Sepsis is defined as a systemic inflammatory response to infection associated with the
4 activation of a number of host defense mechanisms including the cytokine network,
5 leukocytes and the hemostatic system (1). Proinflammatory cytokines activate coagulation
6 cascade via tissue factor, inhibit fibrinolysis, and cause a decrease in AT-III and PC which are
7 the anticoagulant proteins in plasma. The PC pathway also plays a significant role in
8 inflammatory processes and improves the outcome of patients with sepsis (2).

9 The current use of severity scores, such as the APACHE II or III scoring systems, use
10 age, medical history, and physiological determinants, including body temperature, blood
11 pressure, and serum electrolyte concentrations to quantify the degree of physiologic
12 derangement and to predict outcome (3, 4).

13 The severity of sepsis is related to the intensity of the inflammatory host response.
14 Excessive production of proinflammatory cytokines such as tumor necrosis factor (TNF)- α ,
15 interleukin (IL)1 β , IL-6 and IL-8 by immunocompetent cells can induce systemic
16 inflammatory response syndrome (SIRS) and these cytokines play an important role in the
17 development of acute respiratory syndrome or multiple organ dysfunction syndrome (5).

18 This requires being able to show a correlation between the mediators and severity
19 scores of the disease. Even though many studies associated with prognostic factors in sepsis
20 are available to date, the correlation with severity scores of the disease is not very well
21 known. We investigated whether determination IL-6, IL-8, IL-1beta and TNF-alpha at
22 baseline, total protein C (PC) levels at time of admission and 48 hours after initiation could
23 complement APACHE II scoring system to identify patients with sepsis, severe sepsis or
24 septic shock for clinical outcome.

25 MATERIALS and METHODS

26 The prospective study was conducted at the university hospital. The study was
27 approved by the institution's ethics committee.

28 Patients

29 Patients were included if they had clinical evidence of systemic inflammatory response
30 syndrome due to infection and evidence of sustained hypotension or organ hypoperfusion as a
31 result of SIRS. Sepsis and severe sepsis or septic shock were defined according to recent
32 guidelines (8).

33 The severity of each patient was evaluated on the basis of the APACHE II scoring
34 system (1).

35 60 patients were included in the study and were followed for 28 days or until death.

36 Exclusion criteria were age <18 years, pregnancy, use of cytokines, use of heparine or
37 thrombolytic therapy, end-stage neoplasm or other diseases, history of transplantation,
38 requiring hemodialysis or peritoneal dialysis, treatment for malignant hematological diseases,
39 and patients not expected to survive 24 hours.

40 Demographics and Baseline Characteristics

41 At inclusion, demographic information of the patients (age, gender, underlying
42 diseases such as infection with human immunodeficiency virus (HIV), malignancies or
43 diabetes mellitus, prior treatment with immunocompromising (cytostatic or steroidal) drugs or
44 antibiotics) were recorded. APACHE II scores were calculated. Routine hematologic,
45 chemistry, and coagulation parameters were obtained at study entry and followed throughout.
46 Coagulation assays were performed at study entry and followed daily during the initial five
47 days. Data obtained at this time included platelet count, prothrombine time, partial
48 thromboplastin time and fibrinogen levels. Diagnostic investigations of suspected foci of
49 infection (e.g. cultures of blood and tissue) were done according to the decisions of the
50 attending physician.

51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100

Blood Sampling Procedures

Venous blood samples for the determination of plasma levels of cytokines were obtained at the beginning of the study. In addition, venous blood samples for the determination of plasma levels of total PC were collected in vacutainer tubes containing EDTA at the beginning of the study and 48 hours after initiation. All samples were centrifuged at 3000 rpm for 10 minutes. Serum samples for cytokines levels and plasma samples for PC levels were stored immediately at -70 °C until processing.

Cytokines Assays

Plasma concentrations of IL-6, IL-8, IL-1 β and TNF- α were measured with an enzyme-linked immunosorbent assay (ELISA, Research Diagnostic, BioSource International, Inc., immunoassay kit, California) according to manufacturer's conditions. The sensitivities of the assays were <2 pg/mL for IL-6, <5 pg/mL for IL-8, 1 pg/mL for IL-1 β , 1.7pg/mL for TNF- α respectively. The serum levels of these cytokines are below the detection limit in healthy subjects. Results were related to a dose-response curve obtained with recombinant human IL-6, IL-8, IL-1 β and TNF- α and expressed as picograms per milliliter.

PC Antigen Assays

Total PC antigen levels in the heparinized plasma were measured with a commercially available antigen assay Asserachrom Protein C (Stago Diagnostica, Asniers, France) following the manufacturer's instructions. This sandwich-based ELISA uses rabbit antihuman PC F(ab) 2 fragments as the capture antibody. Dilutions in the ratio of 1/50 of plasma samples were made. Reference range was 70-140 % in a normal adult. Reported range was 14.6-max %. Values of PC levels above the detection limit of assay (max %) were calculated logarithmically with respect to their optic densities.

Clinical Outcome

Patients were observed for clinical outcomes, with 28-day all cause mortality as the primary outcome measure. The relationship among PC levels at baseline and after 48 hours, cytokines and APACHE II score to 28 -day all cause mortality, were investigated.

Statistical Analysis

Values of cytokine levels below the detection limit of assay were replaced by 0. Values of cytokine levels above the detection limit of assay were calculated logarithmically according to optic density. The distributions of all continuous variables for normal distribution were tested using Kolmogorov-Smirnov test. The variables with normal distributions were compared between groups by Student's t-test. Mann-Whitney U tests were applied for non-normally distributed variables. For normally distributed variables, mean and standard deviation were used, for non-normally distributed variables median and 25th-75th percentiles were used. Categorical variables were compared between groups with chi-square analysis. Correlations between variables were determined by using Pearson correlation analysis for normally distributed variables, and by Spearman correlation analysis for non-normally distributed variables. Forward multiple logistic regression analysis was used for PC 48 hours after initiation, baseline IL-levels and APACHE II score to predict mortality. The power of the study was calculated as 0.595. This power value was obtained using SigmaStat 3.5 programme according to APACHE II score with 60 sample size.

RESULTS

We prospectively enrolled 60 patients meeting the criteria for sepsis (n=41), severe sepsis or septic shock (n=19). 24 patients were female and 36 male, with mean age 58 \pm 18.2 years. Infections were microbiologically proven in 39 of 60 infected patients (65%). 12% of septic patients and 58% of septic shock or severe sepsis had positive blood cultures. In total, 21 patients were deceased. The mean APACHE II score was 17.3. APACHE II score, the mean baseline IL-6 level and PC level at 48 hours after initiation were significantly higher in

101 the severe sepsis or septic shock group than in the sepsis group ($p=0.000$, $p=0.016$ and
102 $p=0.044$, respectively). The mean baseline IL-1 β , IL-8, TNF- α and PC did not differ between
103 sepsis and severe sepsis or septic shock patients. The main patient characteristics, cytokine
104 levels, PC levels baseline and 48 hours after initiation, APACHE II scores and mortality rates
105 are shown in Table 1.

106 Cytokine levels did not differ according to source of infection. Cytokine levels also
107 did not differ with positive blood culture results. Table 2 shows focus of infection and
108 causative organism and blood culture results.

109 APACHE II score, baseline IL-6 levels and PC level at 48 hours after initiation were
110 significantly higher in deceased than in survived ($p=0.017$, $p=0.016$ and $p=0.049$
111 respectively). Demographic data, cytokine levels, PC levels, and APACHE II scores of both
112 survived and deceased patients are shown in Table 3.

113 In the present study, forward multiple logistic regression analysis were used for IL-6
114 levels, PC levels at baseline, 48 hours after initiation and APACHE II score to predict
115 mortality. Only the APACHE II score (OR:1.098, $p:0.022$) was found to be an independent
116 predictor for mortality of septic patients. In addition baseline IL-6 and PC levels 48 hours
117 after initiation measurements did not reveal any predictive value of mortality in combination
118 with APACHE score tested (Figure 1).

119 The correlation between the APACHE score and blood IL-6, IL-8, IL-1 β and TNF- α
120 were analyzed. A significant positive correlation was observed between them except IL-
121 1 β ($p=0.002$, $p=0.007$, $p=0.851$, $p=0.018$, and $p=0.326$ respectively) (Table 4). The
122 correlation between the APACHE score and PC baseline and 48 hours after initiation also
123 were analyzed. No correlation between the APACHE score and PC levels (baseline and 48
124 hours after initiation) was detected. PC levels baseline and 48 hours after initiation were not
125 associated with cytokines.

126 Despite the significant positive correlation between IL-6, IL-8, TNF- α and APACHE
127 II score, our multivariate analysis of these did not reveal any predictive value in combination
128 with APACHE II score.

129 DISCUSSION

130 The interrelationships between endogenous mediators and their roles in the
131 pathophysiology of systemic inflammation are extremely complex. Multiple mediators have
132 been identified, both other proinflammatory mediators and anti-inflammatory mediators.
133 (9,10). Proinflammatory cytokines activate coagulation cascade via tissue factor, inhibit
134 fibrinolysis, and cause a decrease in AT-III and PC which are the anticoagulant proteins in
135 plasma. (11)

136 Previous studies have shown that there was a similar positive correlation between the
137 APACHE II score and blood IL-6 and TNF- α level on admission to ICU in patients with
138 SIRS and sepsis (2,12). A significant correlation was found among initial serum levels of IL-
139 6, IL-8, TNF- α and the APACHE II score in the present study as in previous studies
140 (12,13,14). In the study of Presterl et al, TNF- α was not found to be correlated with any
141 scoring system, while IL-6 was found to be correlated with MPM II and APACHE III score
142 on day 1 and 2 respectively. The authors suggested that IL-6 should be included in the
143 assessment models of outcome prediction patients with sepsis, either associated with or
144 included in a clinical scoring system (14,15). In addition, Dhainaut et al reported that the
145 predictive power of APACHE II plus IL-6 was higher with respect to the predictive value of
146 APACHE II alone (13). Oberholzer and colleagues investigated the relationship of several
147 cytokine and physiologic scoring systems for predicting clinical outcome in patients with
148 severe sepsis. They have demonstrated that of the cytokines examined, only baseline IL-6 and
149 TNF receptor concentrations (TNF-R1) discriminated among patients who survived or died,
150 and only IL-6 concentrations remained significant when combined with age and the baseline

151 APACHE II and MOD scores (14). A significant correlation of initial serum levels of TNF- α ,
152 IL-6 and other cytokines and the APACHE II scores in 13 patients with meningococcal and
153 pneumococcal septic shock was reported (16). Recently Uras and colleagues have presented
154 that serum CRP and IL-6 levels no significant difference between neonatal bacterial sepsis
155 and transient tachypnea of newborn(17). This study suggests that IL-6, IL-8 and TNF- α
156 baseline levels correlate with the severity of physiologic insult, as determined by APACHE II
157 score. However, our multivariate analysis of these did not reveal any predictive value in
158 combination with APACHE II score.

159 The normal range of circulating levels of PC has been established by several studies
160 and varies over approximately a two-fold range between the upper and lower limits (70%-
161 140%) (18-20). Numerous studies have shown that acquired PC deficiency is prevalent in the
162 majority of septic patients and is associated with increased morbidity and mortality (21-23).
163 In the present study, only PC levels 48 hours after initiation in patients who died were below
164 the lower limit. PC levels 48 hours after initiation were found to be a prognostic marker for
165 fatal clinical outcome. But our results showed no correlation between total PC concentrations
166 48 hours after initiation and APACHE II score. In this study, total PC concentration did not
167 offer any predictive advantage over the APACHE II score alone in septic shock patients, as in
168 the study of Oberholzer and colleagues (14).

169 In recent years, activation of cytokine cascade and subsequent coagulation
170 abnormalities has begun to attract close attention as a possible factor responsible for multiple
171 organ dysfunction (24). The effectiveness of recombinant human activated PC against severe
172 sepsis has been demonstrated in a multi-center study (25). These findings suggest that
173 sequential monitoring of blood IL-6 levels allows for precise prediction of activation of the
174 coagulation system induced by cytokine activation and subsequent onset of organ
175 dysfunction, thus enabling anti-coagulant therapy and anti-cytokine therapy to be performed
176 at an optimum timing (5). Our data suggests that there are prognostic values of baseline blood
177 IL-6 levels and PC levels 48 hours after initiation in patients with sepsis, and severe sepsis or
178 septic shock at an early stage. Baseline IL 6 levels and PC levels 48 hours after initiation
179 showed no correlation.

180 In conclusion, in this prospective evaluation of several different sepsis indicators in a
181 consecutive series of patients, APACHE II score proved to be the best indicator of sepsis in
182 newly admitted critically ill patients. Although determination of baseline IL-6 and total PC
183 concentrations at 48 hours after initiation have a prognostic value for the outcome of septic
184 patients, this did not significantly increase the predictive power of the APACHE scoring
185 system to identify patients with sepsis for fatal clinical outcome. Future studies need to
186 evaluate these approaches in patients with sepsis.

187 REFERENCES

- 188 1. Members of the American College of Chest Physicians/Society of Critical Care Medicine
189 consensus conference: definitions for sepsis and organ failure and guidelines for the use of
190 innovative therapies in sepsis. *Crit Care Med* 1992; 20:864-74.
- 191 2. Satran R, Almog Y. The coagulopathy of sepsis: pathophysiology and management. *IMAJ*
192 2003; 5:516-20.
- 193 3. Knaus WA, Draper EA, Wagner DP, Zimmerman JE. APACHE II: A severity of disease
194 classification system. *Crit Care Med* 1985; 13:818-829.
- 195 4. Knaus WA, Wagner DP, Draper EA, Zimmerman JE, Bergner M, Bastos PG, et al. The
196 APACHE III prognostic system. Risk prediction of hospital mortality for critically ill
197 hospitalized adults. *Chest* 1991; 100:1619-1636.
- 198 5. Oda S, Hiroyuki H, Shiga H, Nakanishi K, Matsuda K, Nakamura M. Sequential
199 measurement of IL-6 blood levels in patients with systemic inflammatory response syndrome
200 (SIRS)/sepsis. *Cytokine* 2005; 29:169-175.

- 201 6. Meduri GU, Headley S, Kohler G, Stentz F, Tolley E, Umberger R, et al. Persistent
202 elevation of inflammatory cytokines predicts a poor outcome in ARDS: plasma IL-1 β and IL-6
203 levels are consistent and efficient predictors of outcome over time. *Chest* 1995; 107:1062-73.
- 204 7. Damas P, Canivet JL, Grootte DD, Vrindts Y, Albert A, Franchimont P, et al. Sepsis and
205 serum cytokine concentrations. *Crit Care Med* 1997; 25:405-12.
- 206 8. Levy MM, Fink MP, Marshall JC, et al. 2001 SCCM/ESICM/ACCP/ATS/SIS International
207 sepsis definitions conference. (*Intensive Care Med* 2003;29:530-538.) S1
- 208 9. Terregino CA, Lopez BL, Karras DJ, Killian AJ, Arnold GK. Endogenous Mediators in
209 Emergency Department patients with presumed sepsis: are levels associated with progression
210 to severe sepsis and death? *Ann Emerg Med* 2000; 35:26-34.
- 211 10. Bellomo R. The cytokine network in the critically ill. *Anesth Intensive Care* 1992; 20:288-
212 301.
- 213 11. Pober JS, Cotran RS. Cytokines and endothelial cell biology. *Physiol Rev* 1990; 70:427-
214 51.
- 215 S2, 24 Oda S, Hiroyuki H, Shiga H, Nakanishi K, Matsuda K, Nakamura M. Sequential
216 measurement of IL-6 blood levels in patients with systemic inflammatory response syndrome
217 (SIRS)/sepsis. *Cytokine* 2005;29:169-175.
- 218 12. Gardlund, B., Sjölin J, Nilsson A, Roll M, Wickerts C. J., Wretling B. Plasma levels of
219 cytokines in primary septic shock in humans: correlation with disease severity. *J. Infect Dis.*
220 1995; 172:296-301.
- 221 13. Dhainaut JF, Shorr AF, Macias WL, Kollef MJ, Levi M, Reinhart K, et al. Dynamic
222 evolution of coagulopathy in the first day of severe sepsis: Relationship with mortality and
223 organ failure. *Crit Care Med* 2005; 33:341-348.
- 224 14. Oberholzer A, Souza SM, Tschoeke SK, Oberholzer C, Abouhamze A, Pribble JP, et al.
225 Plasma cytokine measurements augment prognostic scores as indicators of outcome in patients
226 with severe sepsis. *Shock* 2005; 23:488-93.
- 227 15. Presterl E, Staudinger T, Pettermann M, Lassnigg A, Burgmann H, Winkler S, et al.
228 Cytokine profile and correlation to the APACHE III and MPM II scores in patients with
229 sepsis. *Am J Respir Crit Care Med* 1997; 156:825-832.
- 230 16. Gardlund, B., Sjölin J, Nilsson A, Roll M, Wickerts C. J., Wretling B. Plasma levels of
231 cytokines in primary septic shock in humans: correlation with disease severity. *J. Infect Dis.*
232 1995; 172:296-301.
- 233 17. Uras N, Karadag A, Tonbul A, Mete E, Kara S, Karabel M, et al. Serum Interleukin-6
234 Levels in differential Diagnosis of Sepsis and Transient Tachypnea of Newborn. *Trakya Univ*
235 *Tip Fak Derg* 2010;27:257-260.
- 236 18. Philippe J, Offner F, Declerck PJ, Leraux-Roels G, Vogelaers D, Baele G, et al.
237 Fibrinolysis and coagulation in patients with infectious disease and sepsis. *Thromb Haemost*
238 1991; 65:291-295.
- 239 19. Lorente JA, Garcia-Frade LJ, Landin L, de Pablo R, Torrado C, Renes E, et al. Time
240 course of hemostatic abnormalities in sepsis and its relation to outcome. *Chest* 1993;
241 103:1536-1542.
- 242 20. Carvalho AC, Freeman NJ. How coagulation defects alter outcome in sepsis: survival may
243 depend on reversing procoagulant conditions. *J Crit Illness* 1994; 9:51-75.
- 244 21. Fourrier F, Chopin C, Goudemand J, Hendrycx S, Caron C, Rime A, et al. Septic shock,
245 multiple organ failure and disseminated intravascular coagulation: compare patterns of
246 antithrombin III, protein C and protein S deficiencies. *Chest* 1992; 101:816-823.
- 247 22. Fisher CJ, Yan SB. Protein C levels as a prognostic indicator of outcome in sepsis and
248 related diseases. *Crit Care Med* 2000; 28(Suppl):49-56.
- 249 23. Macias WL, Nelson DR: Severe protein deficiency predicts early death in severe sepsis.
250 *Crit Care Med* 2004; 32(suppl):223-228.

251 24. Levi M, Ten Cate H, van der Poll T. Endothelium: interface between coagulation and
252 inflammation. Crit Care Med 2002; 30(Suppl):S230-4.
253 25. Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-odriquez A, et al.
254 Efficacy and safety of recombinant human activated protein C for severe sepsis. N Eng J Med
255 2001; 344:699-709.

256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300

301
 302 Table 1: Demographic data, Cytokine levels, PC levels, APACHE II scores and mortality
 303 rates of patients

	Sepsis	Severe sepsis	
		Septic shock	
	(n=41)	(n=19)	P value
307 Age	56.5±18.2	61.5±18.4	0.329
308 Gender (M/F)	24/17	12/7	0.955
309 APACHE II score	15±6.9	22±6.1	0.0*
310 Protein C baseline %	106±43.2	84±51.6	0.098
311 PC 48 hours %	67.8±17.1	56.7±19.6	0.044*
312 IL-1 β pg/mL	23.7±14.4	25.5±19	0.696
313 Il-6 pg/mL	106.4	244.2	0.016*
314 (27th-75th percentiles)	(49.2-302.3)	(113.5-544.2)	
315 IL-8 pg/mL	11.9	9.6	0.435
316 (27th-75th percentiles)	(1.8-52.6)	(6.6-67)	
317 TNF-α pg/mL	4	5.5	0.393
318 (27th-75th percentiles)	(2-8.6)	(2.9-10)	
319 Mortality (n)	12	9	0.282

320
 321
 322
 323
 324
 325
 326
 327
 328
 329
 330
 331
 332
 333
 334

369 Table 3: Demographic data, Cytokine levels, PC levels, APACHE II scores survived and
370 deceased patients.

371

	Survived (n=39)	Deceased (n=21)	P value
374 Age	56.2±20.9	61.6±11.6	0.204
375 Gender (M/F)	24/17	12/7	0.955
376 APACHE II score	15.6±7.4	20.4±6.6	0.017*
377 PC Baseline %	106±48.3	85.5±41	0.095
378 PC 48 hours %	71.9	64	0.049*
379 (27th-75th percentiles)	(54.6-80.3)	(54-73)	
380 IL-1 β pg/mL	23.7±14.6	25.4±18.3	0.707
381 IL-6 pg/mL	106.7	164.4	0.016*
382 (27th-75th percentiles)	(57.3-343)	(81.8-492.3)	
383 IL-8 pg/mL	9	18.7	0.435
384 (27th-75th percentiles)	(2.3-51.8)	(7-66.2)	
385 TNF-α pg/mL	4.8	4	0.393
386 (27th-75th percentiles)	(2-9.5)	(3-8.9)	

387

388

389

390