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IL18 Production and IL18 Promoter Polymorphisms Correlate with Mortality in ICU Patients

TAIHEI YAMADA[1], MICHIKO AOYAMA-ISHIKAWA[1,2], HAYATO YAMASHITA[2], MAYU FUJIWARA[2], MAKOTO USAMI[2], TAKAHIRO UEDA[1], MARIKO TERASHIMA[1], KEISUKE KOHAMA[1], ATSUNORI NAKAO[1] and JOJI KOTANI[1]

[1]*Department of Emergency, Disaster and Critical Care Medicine, Hyogo College of Medicine, Nishinomiya, Japan;* [2]*Department of Biophysics, Kobe University Graduate School of Health Sciences, Kobe, Japan*

Abstract. *Background: Single nucleotide polymorphisms in the promoter of interleukin (IL)-18 (-607C/A and -137G/C) may affect the clinical course of inflammatory diseases. This study examined the relationship between the plasma IL18 levels, IL18 promoter polymorphism, and outcomes in the intensive care unit (ICU) setting. Patients and Methods: Plasma IL18 levels, IL18 promoter genotype, clinical variables, including APACHE II score, and mortality were examined in 70 ICU patients. Results: Plasma IL18 levels were significantly higher in patients who did not survive the ICU stay than in patients who survived, and were correlated with APACHE II score. When examined by IL18 promoter genotype, only patients with the -607CA genotype exhibited differences in IL18 expression between survivors and non-survivors. Conclusion: Plasma IL18 levels may predict outcome in patients with sepsis. IL18 promoter polymorphism, especially at -607, may increase IL18 production in some patients and might be useful in predicting the outcome of patients with sepsis in the ICU.*

Interleukin (IL)-18 is a member of the IL1 cytokine superfamily and is an important regulator of innate and acquired immune responses. IL18 is expressed in many cell types including macrophages, dendritic cells, Kupffer cells, keratinocytes, osteoblasts, intestinal epithelial cells, and microglial cells (1). Single nucleotide polymorphisms (SNPs) in the *IL18* promoter are associated with diabetes (1), cancer (2), human immunodeficiency virus (HIV-1) infection (3), and other diseases. Two SNPs (-607C/A and -137G/C) are of particular interest (4).

Correspondence to: Atsunori Nakao, MD, Department of Emergency and Critical Care Medicine, Hyogo College of Medicine, 1-1, Mukogawa-cho, Nishinomiya, Hyogo, 663-8501, Japan. Tel: +81 798456111, e-mail: atsunorinakao@aol.com

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A hyperacute innate immune response to tissue damage can cause excessive, dysregulated secretion of cytokines (hypercytokinemia) and other inflammatory mediators culminating in systemic inflammatory response syndrome (SIRS) and septic complications. This 'cytokine storm' has been associated with poor outcomes in many different patient populations (5, 6). Several researchers have reported an association of IL18 with sepsis. IL18 induces interferon (IFN)-γ production by Natural Killer (NK) cells and activates helper T (Th1)-cells; this protects against bacterial sepsis (7). Additionally, urinary IL18 levels predicted poor clinical outcomes in patients in the intensive care unit (ICU) (8). However, only a few reports have found an association between *IL18* promoter polymorphism and acute inflammatory diseases (9). The aim of the present study was to elucidate the relationship between plasma *IL18* levels, IL18 promoter polymorphism (at the -607 and -137 loci), and outcomes in patients admitted to the ICU.

Patients and Methods

Study population. The protocol was approved by the Ethics Committee of the Hyogo College of Medicine (approval number 64). Patients admitted to the ICU in the Department of Emergency and Critical Care Medicine, and diagnosed with sepsis, septic shock, trauma, severe pancreatitis, hemorrhagic shock, or burn from June 2007 to December 2012 were enrolled in the study after obtaining informed consent. Patients diagnosed with non-SIRS and giving informed consent were enrolled as controls. Out of the 134 patients enrolled, we excluded 64 patients for the following reasons: *IL18* SNPs were not detected in 10 patients, plasma IL18 was not measured in 44 patients because of sample conditions, eight patients had chronic disease, such as cancer, and two patients were transferred to other hospitals within 24 h of ICU admission. This left us with 70 patients for the study.

Diagnostic criteria. SIRS was defined as the presence of two or more of the following conditions: (a) body temperature >38˚C or <36˚C; (b) leukocytosis (>12,000/μl), leukopenia (<4,000/μl), or >10% band neutrophils; (c) heart rate >90 beats/minute; and (d) respiratory rate >20 breaths/min or $PaCO_2$ <32 mmHg. Sepsis,

severe sepsis and septic shock were defined using the 2012 American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference definitions (10). Briefly, sepsis was defined as SIRS with proven or suspected microbial etiology. Microbiological tests were performed on blood samples, sputum (obtained by nasopharyngeal swab or endotracheal suction), urine specimens, removed catheters, or other secretions that were suspected to reflect the infection source. Severe sepsis was defined as acute organ failure complicated by sepsis. Septic shock was defined as acute circulatory failure characterized by persistent arterial hypotension (systolic arterial pressure below 90 mmHg, mean arterial pressure <60 mmHg, or a reduction in systolic pressure of >40 mmHg from baseline despite adequate fluid resuscitation in the absence of other causes of hypotension) (11). During the ICU stay, all patients were treated following the international guidelines for management of severe sepsis and septic shock (10). It was difficult to distinguish severe sepsis from septic shock in our clinical setting. We, therefore, performed combined analysis of patients in severe sepsis and patients in septic shock. The Acute Physiology and Chronic Health Evaluation II (APACHE II) score was used to assess organ dysfunction over time and has been associated with mortality in the ICU (12).

Data collection. Baseline demographic data and clinical variables, including age, sex, presence of severe sepsis/septic shock, APACHE II score during the first 24 h after ICU admission, and hospital mortality, were examined.

Collection of peripheral blood mononuclear cells. Five milliliters of peripheral blood was obtained from 13 of these volunteers (mean age=24.0±3.2 years). Fresh heparinized blood mixed with an equal volume of Hank's balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA) was layered on 4 ml Histopaque 1077 (Sigma-Aldrich). Peripheral blood mononuclear cells (PBMC)-enriched layer was obtained after centrifugation at room temperature for 30 min at 400 ×*g*. The isolated PBMCs were diluted with RPMI-1640 medium (supplemented with 10% fatal bovine serum, 50 U/ml penicillin and streptomycin, 2 μg/ml of bovine insulin, 1 mM oxaloacetate, and 1 mM sodium pyruvate) and cultured in U-bottomed 96-well plates for 24 h at 37˚C with or without lipopolysaccharide (LPS; *Escherichia coli* O55:B5; Sigma-Aldrich). PBMCs were not isolated from 10 volunteers because they had a chronic disease or had taken medication or dietary supplements within 14 days of blood sampling.

Detection of SNPs. DNA was extracted from peripheral blood leukocytes or plasma using the DNA Extractor WB-Rapid Kit (Wako, Osaka, Japan). SNP detection using real-time PCR was performed using a TaqMan® SNP Genotyping Assays, TaqMan® Genotyping Master Mix, and the 7900 HT Fast Real Time PCR System (Applied Biosystems/Life Technologies, Carlsbad, CA, USA).

The following probes were purchased from Applied Biosystems to detect *IL18* promoter SNPs: -137 TGTAATATCACTATTTTCAT GAAAT (C/G) TTTTCTTCCGTAAAAGTTGGGGCTC (rs187238); -607 ACGGATACCATCATTAGAATTTTAT (G/T) TAATAATTTTTA CACTTTCTGCAAC (rs1946518).

In vitro assay of peripheral blood mononuclear cells (PBMCs) from healthy adult volunteers. We assessed the *IL18* -607 genotype in 23 healthy adult volunteers. At the -607 allele, 14 had the CA genotype (60.9%), five had the IL18 -607AA genotype (21.7%), and four had the *IL18* -607CC genotype (17.4%). All the volunteers had the IL18 -137GG genotype.

Table I. *Patient's characteristics*.

Age (years)	64.5±16.8
Gender (male/female)	40 male/30 female
BMI	27.9±5.2
APACHE II score	21.0±8.9
Plasma IL18 (pg/mL)	561.39±685.17
Diagnosis (N)	
Non-SIRS	5
Sepsis	15
Serve sepsis/septic shock	30
Trauma	2
Severe pancreatitis	8
Hemorrhagic shock	7
Burn	3

APACHE, Acute Physiology and Chronic Health Evaluation; BMI, body mass index; IL18, interleukin-18; SIRS, systemic inflammatory response syndrome.

Plasma and supernatant IL18 measurement. Plasma and supernatant IL18 levels were assessed using an enzyme-linked immunosorbent assay (ELISA) kit that was specific for the 18 kDa bioactive form of IL18 (MBL, Nagoya, Japan), according to the manufacturer's instructions. The ELISA plate was read at 405 nm using a plate reader (Model 680 Microplate Reader; Bio-Rad, Hercules, CA, USA).

Statistical analysis. Data are shown as the mean±standard deviation. Multivariate analysis was performed by logistic regression to predict ICU mortality using age, sex, body mass index (BMI), APACHE II score, plasma IL18 level, *IL18* -607 and *IL18* -137 genotype as factors. The correlation between plasma IL18 and APACHE II score was assessed using Pearson's correlation coefficient. The difference between plasma IL18 levels in survivors and non-survivors were analyzed by *t*-test. The other comparisons were performed by ANOVA (analysis of variance) with Tukey-Kramer's post hoc test. The statistical analyses were performed using JMP software, version 9 (SAS Institute Incorporated, Cary, NC, USA), and a value of $p<0.05$ was considered statistically significant.

Results

Patients' characteristics. Out of 70 patients admitted to ICU, 15 were diagnosed with sepsis; 30 were diagnosed with septic shock; 20 were diagnosed with severe pancreatitis, hemorrhagic shock, trauma, or burn; and five did not have SIRS (Table I). There were no significant differences in age, BMI, APACHE II score, or plasma IL18 levels between the diagnoses. Fifty-one patients survived the ICU stay. Patients who did not survive were diagnosed with septic shock (n=14, 73.7%), burn (n=2, 10.5%) or severe pancreatitis (n=3, 15.8%).

Table II. *Multivariate analysis of predictors of mortality.*

	p-Value	Odds ratio (95% CI)
Age	0.8938	0.996 (0.946-1.052)
Gender (male/female)	0.0019	16.471 (2.501-234.298)
BMI	0.9133	0.989 (0.806-1.225)
APACHE II score	0.0013	1.155 (1.053-1.300)
Plasma IL18 level (pg/ml)	0.0080	1.002 (1.000-1.003)
IL18 -607		
CA *vs*. AA	0.1813	0.301 (0.041-1.721)
CA *vs*. CC	0.8409	0.786 (0.075-10.002)
AA *vs*. CC	0.3947	3.325 (0.581-24.195)
IL18 -137		
GC *vs*. GG	0.4004	2.126 (0.362-13.809)

APACHE, Acute Physiology and Chronic Health Evaluation; BMI, body mass index; CI, confidence interval; IL18, interleukin-18.

Table III. *Survival by interleukin (IL)18 promoter genotype.*

	Number of patients (ICU survivors/non-survivors)
IL18 -607/-137	
AA/CC	2 (2/0)
AA/GC	7 (4/3)
AA/GG	14 (9/5)
CA/GC	12 (7/5)
CA/GG	22 (18/4)
CC/GG	13 (11/2)
Total	70 (51/19)

IL18 , Interleukin-18; SIRS, systemic inflammatory response syndrome.

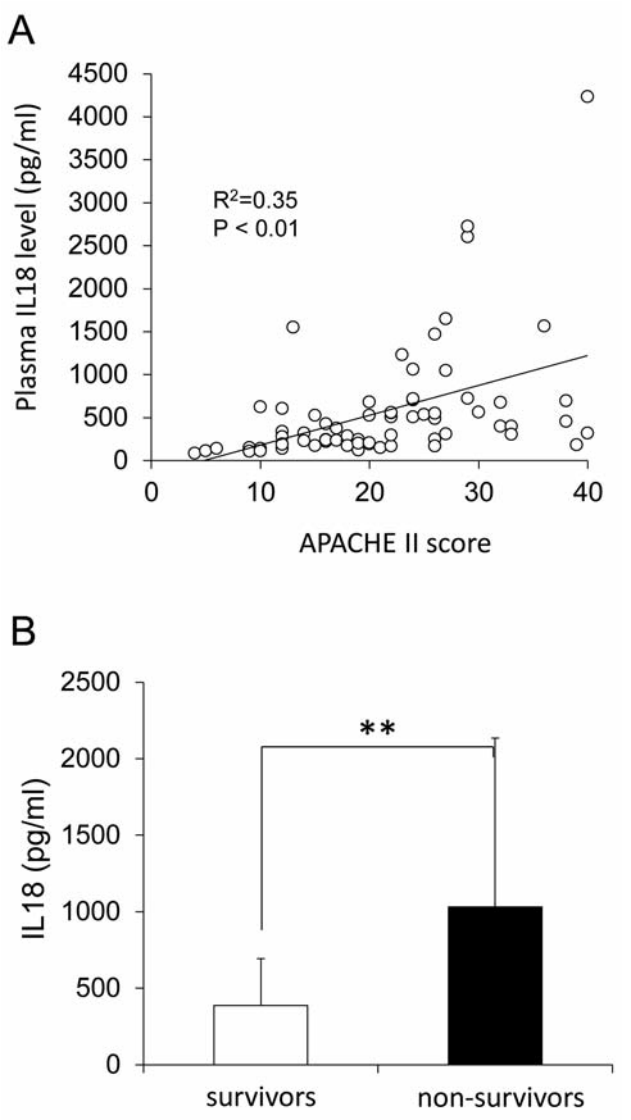

Figure 1. *A: The correlation between plasma interleukin (IL)18 level and the Acute Physiology and Chronic Health Evaluation II (APACHE II) score on admission (n=70). B: Plasma IL18 level in patients who survived the intensive care unit stay (survivors) and patients who did not (non-survivors) (**p<0.01).*

Plasma IL18 levels on admission predicted mortality in patients in ICU. We performed multivariate analysis using age, sex, APACHE II score, BMI, plasma IL18 level on admission, *IL18* -607 and -137 genotypes to identify independent predictors of outcome in the patients in ICU. Only sex, APACHE II score, and plasma IL18 levels were identified as independent predictors of mortality (Table II. The plasma IL18 levels were correlated with APACHE II score ($p<0.01$, $R^2=0.35$, Figure 1A), and the patients who did not survive the ICU stay showed significantly higher levels of plasma IL18 on admission compared to survivors ($p<0.01$, Figure 1B).

Correlation of IL18 -607CA genotype with higher plasma IL18 levels in patients who did not survive the ICU stay. Because SNPs in the *IL18* promoter can alter IL18 expression, we examined the frequency of each *IL18* promoter genotype and the distribution of the genotypes between survivors and non-survivors (Table III). At position -137 in the *IL18* promoter, most patients carried the -137GG genotype (49 patients, 70%); 27.1% (19 patients) had the -137GC genotype and 2.9% (two patients) had the -137CC genotype. At the -607 position, 48.5% (34 patients) had the -607CA genotype; 32.9% (23 patients) -607AA, and 18.6% (13 patients) -607CC. The most common genotype combination was *IL18* -607CA with -137GG (CA/GG, 22 patients, 31.4%), and the rarest was *IL18* -607AA with -137CC (AA/CC, two patients, 2.8%).

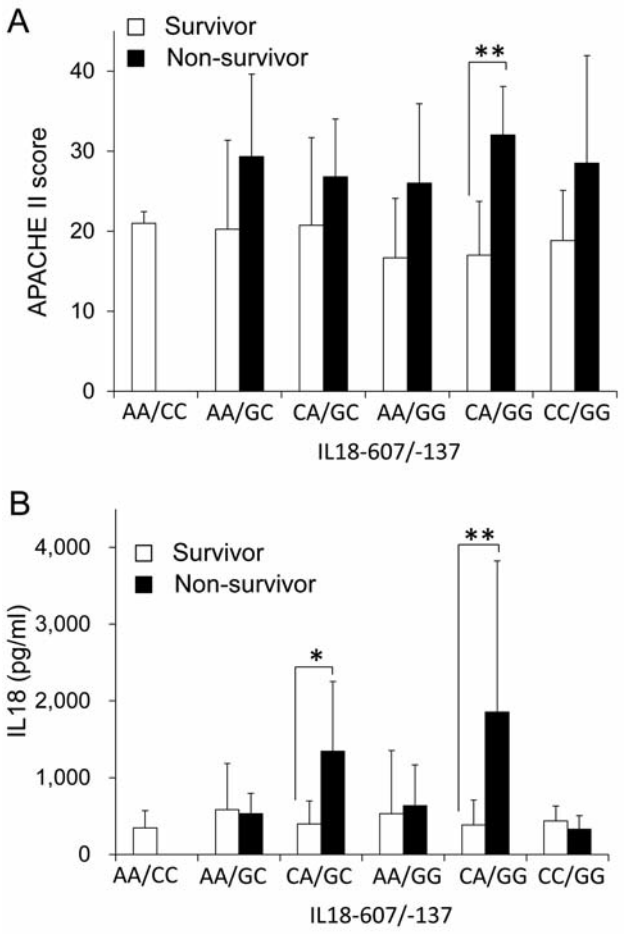

Figure 2. *A: The Acute Physiology and Chronic Health Evaluation II (APACHE II) score by interleukin (IL)18 promoter genotype. Non-survivors tended to have higher APACHE II score than survivors. B: Plasma IL18 levels by IL18 promoter genotype (*p<0.05, **p<0.01).*

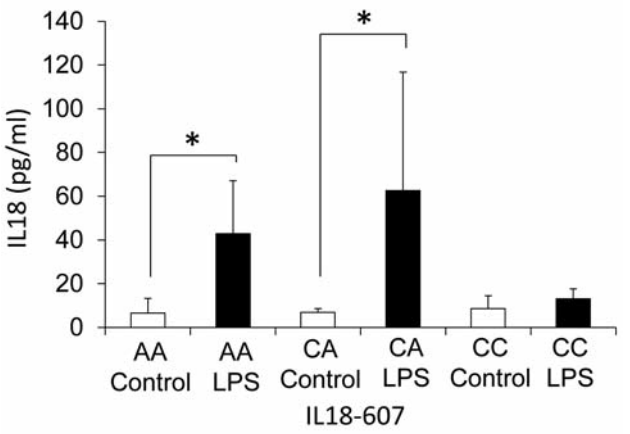

Figure 3. *Interleukin (IL) 18 level in supernatant of lipopolysaccharide (LPS)-stimulated Peripheral blood mononuclear cells (PBMCs) with different IL18 promoter genotypes. Control: PBMCs were incubated for 24 hours in medium only. LPS: PBMCs were incubated with 1 μg/ml LPS (Escherichia coli O111:B4) for 24 h (*p<0.05).*

The APACHE II score at the time of ICU admission was not significantly different among patients with different *IL-18* promoter genotypes either in survivors or non-survivors (Figure 2A). Although APACHE score was an independent predictor of mortality in these patients, when examined by *IL18* promoter genotype, only patients with the CA/GG genotype showed significantly higher APACHE II scores in non-survivors than in survivors ($p<0.01$, Figure 2A).

The plasma IL18 level was also not significantly different among the genotype combinations (Figure 2B). However, patients who did not survive the ICU stay and had the -607 CA genotype (CA/GC or CA/GG) had significantly higher plasma IL18 levels than survivors with the same genotype (CA/GC; $p<0.05$, CA/GG; $p<0.01$, Figure 2B).

The IL18 production from PBMCs from healthy adult volunteers. To further examine the impact of *IL18* promoter SNPs on IL18 expression in response to critical illness, we exposed isolated PBMCs from 13 volunteers (-607 CA, n=6; AA, n=3; CC, n=4; IL18 -137 GG, n=13) to LPS and measured IL18 expression. In cultured PBMCs with the -607 AA and -607 CA genotypes, LPS significantly increased IL18 production as compared with controls incubated with culture medium (Figure 3). In contrast, IL18 production was not increased by LPS in PBMCs with the -607CC genotype (Figure 3). IL18 production in PBMCs with the -607CA genotype tended to be higher than in PBMCs with the -607AA genotype, but the difference was not significant (Figure 3).

Discussion

This study demonstrated that patients who did not survive their ICU stay had higher plasma IL18 levels than patients who survived, and that the *IL18* -607CA genotype may increase IL18 expression in these non-survivors compared ICU survivors. In PBMCs challenged with LPS, the -607CA and AA genotypes allowed IL18 production, while the -607CC genotype did not, further supporting the role of *IL18* promoter SNPs in modulating expression during the inflammatory response. Plasma IL18 levels correlated with APACHE II score, suggesting that plasma IL18 involved in pathophysiology od critical illness.

In prior studies, the frequencies of the *IL18* -137 SNP genotypes in the Japanese population were reported as GG, 75%; GC, 23%; and CC, 2% (13, 14). We saw a similar distribution at the -137 locus in our patients in ICU with frequencies of *IL18* -137 GG, 70%; GC, 27%; and CC, 3%. Takada *et al.* reported the frequencies of the *IL18* -607 genotypes in a Japanese cohort as CC, 17%; CA, 39%; and

AA, 44% (n=130) (13). However, Ide *et al*. reported the frequencies of the *IL18* -607 genotypes in a Japanese cohort as CC, 16%; CA, 58%; and AA, 26% (n=114) (14). In our patients in ICU, the distribution was intermediate between those of the previous reports (*IL18* -607 CC, 19%; CA, 48%; and AA, 33%); thus, we believe the frequencies of *IL18* -607 and -137 SNPs in our patients were representative of the frequencies in the Japanese population.

The *IL18* -137 SNP is part of a transcription factor binding site in liver cells (15), and Liang *et al*. reported that the *IL18* -137C, +113G, +127T genotype increased *IL18* transcriptional activity as compared with the *IL18* -137G, +113G, +127T genotype in the HepG2 liver hepatoma cell line (15). In our study, the *IL18* -137 SNP did not significantly affect plasma IL18 levels in patients. Because IL18 is produced in multiple cell types (1), plasma IL18 levels most likely reflect transcriptional activity in more than one tissue. Moreover, the effects of different *IL18* promoter SNP on IL18 expression may vary by tissue. Dolinary *et al*. reported that plasma IL18 levels were higher in patients with sepsis who suffered from acute lung injury than in patients with sepsis without lung injury (16). IL18 expression in lung tissue and IL18 levels in bronchoalveolar lavage fluids were significantly elevated in a mouse model of ventilator-induced lung injury (16). We hypothesize that in patients in ICU, the lungs may be more important for IL18 production than the liver. The *IL18* -607CA genotype is significantly associated with susceptibility to chronic obstructive pulmonary disease in male smokers (17), and *IL18* -607CA and AA genotypes convey a 2.6-fold and 3.2-fold increased risk of lung cancer, respectively (18). The association of lung disease with the *IL18* -607 SNP may explain why plasma levels of IL18 were higher in patients with the -607CA genotype who did not survive their ICU stay.

The current study also supports the notion that the effects of *IL18* promoter SNPSs on IL18 expression may vary depending on the ethnicity of the studied patients. In this study, we performed *in vitro* analysis using PBMCs from health Japanese volunteers. LPS induced IL18 production from PBMCs with the -607AA or -607CA genotype but not in those with the -607CC genotype. This finding was similar to the findings of Khripko and colleagues, who reported that in PBMCs obtained from healthy Russian volunteers, the *IL18* -607CA genotype induced higher production of IL18 than did the CC genotype (19). In Chinese patients with systemic lupus erythematosus, patients with the *IL18* -607 CA or CC genotype had higher plasma IL18 levels than patients with the AA genotype (2). In contrast, in a study of Americanpatients in ICU with severe trauma (allelic distribution: CC, 22%; CA, 55%; AA, 3%; n=69), Stassen *et al*. reported that the *IL18* -607CA genotype lowered susceptibility to post-injury sepsis (9). Additionally, the *IL18* -607 polymorphism is associated with increased overall cancer risk in Asians but not Europeans and Africans (20-22). Thus, the *IL18* -607 SNP may be more strongly associated with inflammation in Asian individuals. In our study, the *IL18* -607CA genotype was associated with significantly higher IL18 production only in patients who did not survive the ICU stay. The non-survivors also had higher APACHE II scores than did the survivors, suggesting that non-survivors had more severe inflammation than survivors. Thus, severe inflammation and *IL18* -607CA genotype may lead to poor outcomes, particularly in Asian patients in ICU.

In conclusion, plasma IL18 levels may be predictive of outcome in patients with sepsis. *IL18* promoter polymorphism, especially at *IL18* -607, may increase IL18 production under certain circumstances and might be useful in predicting patient outcomes in ICU.

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