



## Associations between TNF- $\alpha$ , IL-6 and IL-10 Promoter Polymorphisms and Mortality in Severe Sepsis

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### Authors' contributions

This work was carried out in collaboration between all authors. Author OS participated in the design and the administration of the study and drafted the manuscript. Author LN-Z participated in the design and performed the genetic analyses. Authors AK, LK and ZJ participated in preparation of manuscript. Author JK performed the genetic analyses and took part in the statistical calculations. Author LV participated in the design of the study and the preparation of the manuscript. Author LJB participated in the interpretation of the results and the preparation of the final version of the manuscript. IV participated in the design and the administration of the study and in the preparation of the manuscript. All authors read and approved the final manuscript.

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### ABSTRACT

**Aims:** To determine whether an association exists between TNF- $\alpha$  308 A/G, IL-6 174 G/C, and IL-10-1082 A/G promoter polymorphisms and the corresponding systemic cytokine concentrations and outcome in patients suffering from sepsis.

**Place and Duration of Study:** The study was performed in the Intensive Care Unit (ICU)

of Pauls Stradins Clinical University Hospital, Riga. Between 1 August 2006 and 31 July 2008.

**Methodology:** We enrolled 103 consecutive intensive care unit patients with sepsis into a prospective case control study. Blood samples were obtained for extraction of DNA amplifying regions of interest by means of polymerase chain reaction technique (PCR) using specific primers for TNF- $\alpha$ , IL-6 and IL-10. Simultaneously, plasma cytokines and standard laboratory variables were determined during the first 24 h after the diagnosis. Presence of septic shock, sequential organ failure assessment score (SOFA), demographic data and clinical outcome was noticed  $P < 0.05$  was considered as statistically significant.

**Results:** Non-survivors had significantly higher concentrations of TNF- $\alpha$ , IL-6 and IL-10. The carriage of the IL-6-174C allele and IL-10 -1082G allele were associated with a higher risk of mortality in patients with severe sepsis. Presence of the TNF- $\alpha$ -308 A allele did not influence mortality differently from those lacking this allele.

**Conclusion:** The present study demonstrated an association of the IL-6 -174 and the IL-10 -1082 with increased mortality in patients suffering from severe sepsis. We found no direct association between the examined polymorphisms and the corresponding cytokine levels.

**Keywords:** Sepsis; TNF- $\alpha$ ; IL-6; IL-10; polymorphisms, allele; PCR; SNP.

## ABBREVIATIONS

CI: confidence interval; H-W: Hardy-Weinberg equilibrium; IQR: interquartile range; OR: odds ratio; SD: standard deviation; SOFA: Sequential related organ failure Assessment; TNF- $\alpha$ : Tumor necrosis factor; IL-6: interleukin 6; IL-10: interleukin 10; DNA: deoxyribonucleic acid; PCR: polymerase chain reaction, SNP: single nucleotide polymorphisms; SIRS: systemic inflammatory response syndrome; PaCO<sub>2</sub>: partial tension of carbon dioxide in arterial blood; ELISA: enzyme-linked immunosorbent assay; ICU: intensive care unit.

## 1. INTRODUCTION

Genetic variability plays an important role in the development of sepsis and its response to treatment. A detailed description of the human genome had opened for the possibility to identify associations between single nucleotide polymorphisms (SNPs) and disease. Protein-coding genes involved in the innate immune system are potential candidates for influencing the differences in the activities of pro- and anti-inflammatory cytokines and the outcome of disease in individual patients, including the risks for complications [1]. Although several surveys have demonstrated that genetic factors might influence unfavorably on outcome of disease, a major part of the genetic variations have not yet been fully identified [2,3].

Bacterial products activate the transcription factor nuclear factor kappa B in the cytosol of immune cells, which triggers the production of tumor necrosis factor-alpha (TNF- $\alpha$ ), an initiating cytokine and key mediator of the inflammatory responses in sepsis. Taking into account its role in the pathogenesis [3, 4], genetic variations of TNF- $\alpha$  gene could affect clinical course and the outcome of sepsis. This statement is supported by the demonstration of significant inter-individual differences in stimulated TNF- $\alpha$  production [5, 6].

The major histocompatibility complex gene cluster IV of chromosome 6 harbors 3 TNF genes, as well as several microsatellites. Variations in the TNF- gene have been intensively studied revealing a relatively constant coding sequence for the gene, but several SNP have been described in the promoter region. Thus, a single nucleotide polymorphism has been identified in the -308 position of the promoter region (G to A, rs1800629) [6]. An association between the TNF - -308A allele and increased production of TNF- has been demonstrated in lipopolysaccharide (LPS) -stimulated human blood cells [7, 8], but searches for genetic associations between the TNF- -308A allele and human sepsis have given inconsistent results. Some researches have noticed an association between the TNF- -308A allele and a higher incidence and mortality of septic shock [9, 10]. However, no such association has been demonstrated in septic patients without shock [11, 12].

Interleukin-6 (IL-6) is instrumental in the regulation of innate and adaptive immune responses [13]. To release of inflammatory cytokines such as IL-6, occurs several hours earlier than the release of other markers of systemic inflammation, suggesting their potential importance as diagnostic parameters in sepsis [13, 14]. The gene coding for IL-6 is localized in chromosome 7 (7p21p14). In vitro studies have revealed one SNP in the promoter region (-174 G/C, rs1800795) in connection with increased production of IL-6 [14], but contradictory results were demonstrated after endotoxin infusion in healthy volunteers with GG and CC genotypes [15, 16].

The anti-inflammatory cytokine IL-10 is produced mainly in monocytes and macrophages and, to a lesser extent, in lymphocytes and epithelial cells. Its role in the pathogenesis of sepsis has been demonstrated in several clinical investigations [17, 18]. Variations in the production of IL-10 in LPS -stimulated blood cultures have been noticed and associations of IL-10 -1082 G/A (rs1800896) polymorphism with the transcription rate have been described [19].

In this study, the aim was to determine whether associations exist between TNF- -308 A/G (rs1800629), IL-6 -174 G/C (rs1800795) and IL-10 -1082 A/G (rs180089) polymorphisms and the systemic cytokine concentrations and outcome in patients with severe sepsis.

## 2. MATERIALS AND METHODS

The study was performed in the Intensive Care Unit (ICU) of Pauls Stradins Clinical University Hospital, Riga.

### 2.1 Patients

Between 1 August 2006 and 31 July 2008, we enrolled 103 consecutive patients into a prospective observational study fulfilling the criteria of severe sepsis according to the International Sepsis Definitions [20]. All patients were of Caucasoids. Patients less than 18 years of age were excluded from the study; as were patients with defined immunodeficiencies and those who refused to participate. Informed consent was obtained from all the patients or from their next of kin in the case they could not respond on their own behalf.

The severe sepsis diagnosis was based on the presence of one of the criteria of systemic inflammatory response syndrome (SIRS) in combination with suspected or proven infection. SIRS criteria are the following: 1) a body temperature more than 38°C or less than 36°C; 2) a heart rate greater than 90 beats per minute; 3) tachypnea, manifested by a respiratory rate

greater than 20 breaths per minute, or hyperventilation, as indicated by a PaCO<sub>2</sub> of less than 32 mm Hg; and 4) white blood cell count greater than 12,000/mm<sup>3</sup> or less than 4,000/mm<sup>3</sup> or the presence of more than 10 percent immature neutrophils ("bands"). To meet the criteria of septic shock, a documented systolic blood pressure of less than 90 mm Hg for at least 30 minutes in the absence of other causes of shock and at least 4 hours of inotropic support after adequate fluid replacement were required. Sepsis with sepsis-induced organ failure - meets the criterion of severe sepsis. Survivors – patients discharged from ICU, non-survivors – who were died in ICU. Basic demographic data (age, sex), primary site of infection and organ failure severity (based on the sequential organ failure assessment (SOFA) score) [see appendix] were noticed for all the patients on the day of inclusion into the study [21].

The blood samples for the study of genetic polymorphisms of cytokines (TNF-  $\alpha$  -308A/G, IL-6 -174 G/C, IL-10 -1082 A/G) and for the investigation of the concentration of cytokines in serum, and for other laboratory tests were obtained during the first 24h after the diagnosis. All the patients were observed until discharge from ICU and the clinical outcome was survival or non-survival in ICU.

## 2.2 Cytokine Serum Concentrations

Cytokine serum concentrations (TNF-  $\alpha$ , IL-6, IL-10) were determined. Blood was taken by venopuncture and sampled on vacutainers. The blood was then centrifuged and the serum had been frozen (-70°C) until the analyses.

The serum concentrations of TNF-  $\alpha$ , IL-6, IL-10 were determined by means of enzyme-linked immunosorbent assay ELISA (Biosource, Nivelles, Belgium), according to the manufacturer's description.

## 2.3 Analysis of Gene Polymorphisms

Genomic DNA was extracted from whole (frozen, -20°C) blood samples using standard phenol-chloroform extraction method. DNA of specific region of interest was amplified as follows: specific primers (Fermentas, Lithuania) for IL-6 promoter region harboring -174 C/G were forward 5'-TCGTGCATGACTTCAGCTTT-3' and reverse 5'-GCCTCAGACATCTCCAGTCC-3', for TNF A promoter region harbouring -308 A/G were forward 5'-ACAGGCCTCAGGACTCAACA-3' and reverse 5'-GCACCTTCTGTCTCGGTTTC-3', for IL-10 promoter region harbouring -1082 G/A were forward 5'-TTCCCAGGTAGAGCAACAC-3' and reverse 5'-ATCCTCAAAGTTCCCAAGCA-3'. We used 2xPCR Master Mix (Fermentas, Lithuania). The cycling conditions of PCR were as follows: 5 minutes of initial denaturation at 95°C, following 32 cycles of 15 seconds at 95°C, 30 seconds at 56°C, 30 seconds at 72°C, and final extension – 10 minutes at 72°C.

After amplification the products were purified and analyzed by direct sequencing using 3100 ABI prism DNA sequencer according to the recommendations of the manufacturer. The sizes of the different products were 1, 8 kb (TNF-  $\alpha$ ), 1, 6 kb (IL-10) and 1, 5 kb (IL-6). For sequencing reaction we used the primers for IL-6: – 5'-TCATGGGAAAATCCCACATT-3', for TNFA: 5'-AACACAGCTTTTCCCTCAA-3' and for IL-10: 5'-GATGGGGTGAAGAAGTTGA-3'.

## 2.4 Statistical Analysis

Continuous variables were expressed as mean  $\pm$  standard deviation (SD) if normally distributed or median (interquartile range) if asymmetric distribution was observed. Variables were tested for their association with mortality using Pearson  $\chi^2$  or Fisher's exact test (where appropriate) for categorical data (e.g. gender, polymorphisms) and Mann-Whitney U test for numerical data (e.g. age, SOFA, cytokine concentration). Conformity of genotype distribution to Hardy-Weinberg equilibrium was tested with Pearson  $\chi^2$  test between observed and expected ( $p^2 + 2pq + q^2$ , where p and q are frequencies of the common and the rare alleles, respectively). To examine potential associations between the alternative allele and the ratio of unfavorable outcome, allelic distribution was estimated in various genetic models (e.g. allelic, dominant and recessive models, respectively). The risk of lethal outcome resulting from the presence of individual alleles or genotypes was estimated with the odds ratio with 95% confidence intervals. We used SPSS software (version 16, SPSS, Chicago, IL) for statistical calculations. Two-tailed  $P < 0.05$  was considered as statistically significant.

## 3. RESULTS AND DISCUSSION

Complete clinical information was available from 99 out of 103 patients diagnosed with severe sepsis. Four patients were not genotyped for TNF- $\alpha$ -308A/G polymorphism. Primary sites of infection were the respiratory tract in 71% (n = 73), abdominal organs in 26% (n = 27) of the cases or other locations in 3% (n = 3) of the patients. Microorganisms were identified in blood cultures, tracheal secretions or wound smears in 82 patients (80%). The most common Gram positive microorganism was Staphylococcus aureus and, the most common Gram negative bacterium was Escherichia coli.

As shown in Table 1, no significant differences in demographical characteristics (age, gender), organ dysfunction and length of ICU stay were observed between survivors and non-survivors of sepsis. A higher incidence of septic shock was observed among the non-survivors. Cytokines TNF- $\alpha$ , IL-6 and IL-10 displayed significantly higher values in non-survivors compared with survivors.

**Table 1. Demographical, clinical and laboratory characteristics of survivors and non-survivors of severe sepsis**

Variable	Survivors (n=59)	Non-survivors (n=44)	P
Male patients, n (% of total)	45 (76.3%)	32 (72.7%)	NS
Age, years ( $\pm$ SD)	58 ( $\pm$ 14)	59 ( $\pm$ 13)	NS
SOFA, median (IQR)	6 (4-8)	9 (6-12)	NS
ICU stay, days ( $\pm$ SD)	11 ( $\pm$ 9)	13 ( $\pm$ 10)	NS
Septic shock, n (%)	10 (16.9%)	15 (34.1%)	0.045
TNF- $\alpha$ , median (IQR), pg/ml	26 (18/40)	40 (28/63)	0.002
IL-6, median (IQR), pg/ml	190 (95/430)	450 (235/790)	0.003
IL-10, median (IQR), pg/ml	1 (1/39)	2 (1/7)	0.027

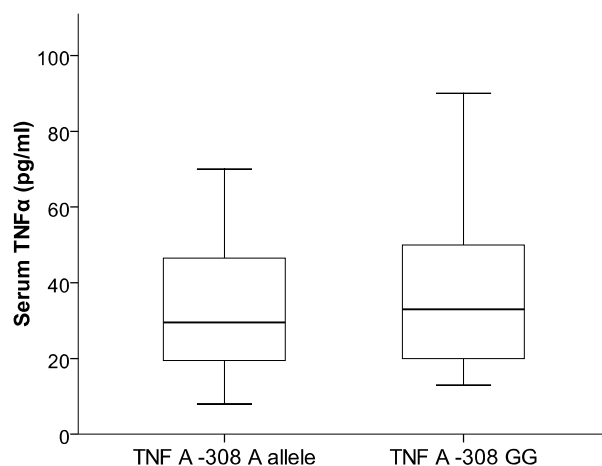
SOFA = Sequential organ failure assessment score, ICU = Intensive care unit; TNF- $\alpha$  = tumor necrosis factor  $\alpha$ ; IL-6 = interleukin-6; IL-10 = interleukin 10; data given as mean  $\pm$  standard deviation (SD) or as median and interquartile range (IQR), NS – not significant;  $P < 0.05$  is significant.

### 3.1 Allele and Genotype Frequencies

Table 2 summarizes the allele and the genotype distributions of SNPs in survivors and non-survivors.

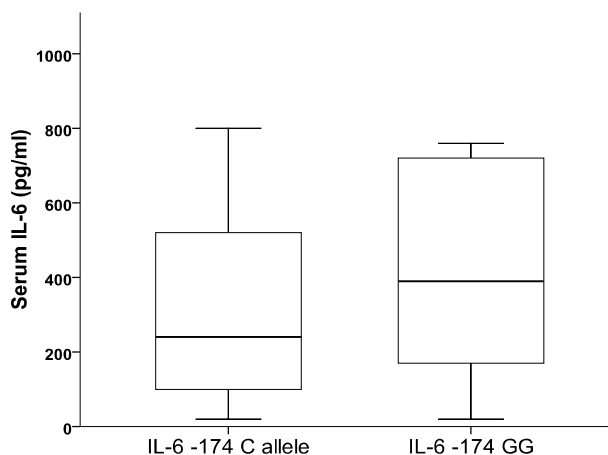
The occurrence of the IL-6 -174C allele and IL-10 -1082G allele were associated with a higher risk of mortality in septicemic patients. However the distribution of the TNF- -308 A allele did not differ significantly between survivors and non-survivors (Table 2).

Comparing the serum cytokine concentrations for carriers of polymorphism and common allele homozygote carriers showed no significant differences (Fig. 1-3).



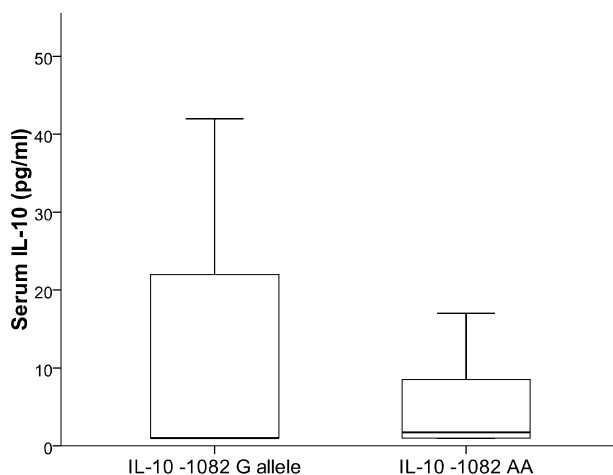
**Fig. 1. Serum concentrations of TNF- in septicemic carriers of TNF A -308 polymorphism (A allele) and G allele homozygote carriers**

*P = 0.6, Mann-Whitney U test, line = median, box = interquartile range*



**Fig. 2. Serum concentrations of IL-6 in septicemic carriers of IL-6 -174 polymorphism (C allele) and G allele homozygote carriers**

*P = 0.2, Mann-Whitney U test, line = median, box = interquartile range*



**Fig. 3. Serum concentrations of IL-10 in septicemic carriers of IL-10 -1082 polymorphism (G allele) and A allele homozygote carriers**

*P = 0.7, Mann-Whitney U test, line = median, box = interquartile range*

The present study demonstrates a slightly increased risk of mortality in the homozygote carriers of the IL-6 -174 C allele in comparison with IL-6 -174 G allele carriers. Correspondingly, carriage of the IL-10 -1082 G allele was associated with a slightly, but significantly increased mortality rate. In contrast, the presence of the TNF-  $\gamma$  -308 rare allele (A) did not influence mortality significantly (Table 2).

Our observation of an association between IL-6 -174 polymorphism and mortality in a recessive genetic model, is different from the demonstration of previous investigators who showed such an association in a dominant model [22]. Based on this finding we suggest that, at least, one copy of the IL-6 -174 G allele may improve clinical outcome [22, 23].

The TNF-  $\gamma$  -308 polymorphism has been studied in several diseases, including sepsis, but the results are contradictory. The TNF-  $\gamma$  -308 A allele is associated with a higher mortality rate, mostly in patients with septic shock [9, 10, 23, 24]. No such association has been observed in septicemic patients without shock [24, 25]. The observed lack of association is not surprising since our study population in the major part consisted of patients without septic shock (75%). Possibly, this could indicate a functional significance of TNF-  $\gamma$  -308 polymorphism in cases of more severe inflammatory responses [25].

The functional significance of IL-10 -1082 A/G polymorphism in patients with sepsis has been sparsely focused on. Our findings agree with those of previous investigators, who observed an association of the IL-10 -1082 G allele with a worse clinical outcome [26-28]. However, this observation has not been unequivocally accepted. According to earlier studies, genetic predisposition to high IL-10 production may be protective of admission to the ICU, but once admitted, any protection provided by this genotype seems to be lost, and the authors found no relationship between IL-10 genotype and mortality [29]. Thus, the results of these studies are still contradictory and the functional significance of IL-10 -1082 polymorphism and its association with a higher mortality is still unclear.

**Table 2. Association of unfavorable clinical outcome with SNPs**

SNP	Genetic model	Number		P value (chi-square)	OR [95% CI]
		Non-survivors	Survivors		
rs1800896 IL10 (-1082)	Allelic (G/A)	40/48	47/71	0.418	1.26 [0.72-2.20]
	Dominant (GG+GA/AA)	35/9	36/23	<b>0.044</b>	2.49 [1.01-6.11]
	Recessive (GG/GA+AA)	5/39	11/48	0.313	0.56 [0.18-1.75]
rs1800629 TNF (-308)	Allelic (A/G)	15/67	23/93	0.787	0.91 [0.439-1.86]
	Dominant (AA+AG/GG)	14/27	22/36	0.699	0.84 [0.37-1.95]
	Recessive (AA/AG+GG)	1/40	1/57	0.803	1.43 [0.09-23.46]
rs1800795 IL6 (-174)	Allelic (C/G)	47/41	46/72	<b>0.039</b>	1.79 [1.026-3.13]
	Dominant (CC+CG/GG)	34/10	39/20	0.217	1.74 [0.72-4.23]
	Recessive (CC/CG+GG)	13/31	7/52	<b>0.024</b>	3.11 [1.12-8.64]

SNP = single nucleotide polymorphism; TNF- = tumor necrosis factor ; IL-6 = interleukin-6; IL-10 = interleukin 10, OR (odds ratio); CI (confidence interval); p-value (probability)  $P < 0.05$  is significant.



As the IL-6 -174C and the IL-10 -1082G alleles are concerned, we did not find any significant differences in the serum concentrations of the examined cytokines between the carriers of the rare alleles and the homozygotes of the common alleles. Therefore the exact pathophysiological mechanisms of the studied polymorphisms remain unclear, but the observed differences in cytokine concentrations between survivors and non-survivors indicate that they were pathogenetically relevant.

We admit that the present study has limitations. One of the possible causes, for lack of association between the investigated polymorphisms and the cytokine serum levels, is the timing of the blood sampling. According to the protocol, we determined concentrations of cytokines during the first 24h after diagnosing sepsis. Assuming that the gene transcription rate could be different due to time variations in the intensity of SIRS, we may have lost the periods of most active gene transcription with corresponding peak cytokine concentrations. The functional significance of SNP may have appeared more clearly in these periods. Many factors contribute to mortality in severe sepsis, such as age, co-morbidities and number of failing organs. A possible reason for rather weak effect of the polymorphism on outcome in this study may be due to limited number of patients included.

Possibly, other gene regions that are in linkage disequilibrium with the studied polymorphism may be responsive for the observed association with mortality.

#### **4. CONCLUSION**

Although the present study give no clear answers concerning the functional significance of the examined polymorphisms, we have demonstrated an association of the IL-6 -174 and the IL-10 -1082 polymorphisms with an increased mortality in patients suffering from severe sepsis.

*Key messages: In severe sepsis, IL-6 -174C allele and IL-10 -1082G allele polymorphisms are associated with increased mortalities.*

#### **ETHICAL APPROVAL**

The study was approved by the Central Medical Ethics Committee of Latvia. And the written informed consent for participation in the study from participants was obtained.

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#### **COMPETING INTERESTS**

The authors declare they have no financial or non-financial competing interests.

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## APPENDIX

## Sequential organ failure assessment (SOFA) score

SOFA score	1	2	3	4
Respiration PaO <sub>2</sub> /FiO <sub>2</sub> , mm Hg	<400	<300	<200 (respiratory support)	<100 (respiratory support)
Coagulation Platelets x 10 <sup>3</sup> /mm <sup>3</sup>	<150	<100	<50	<20
Liver Bilirubin, µmol/l	20-32	33-101	102-204	>204
Cardiovascular Hypotension	MAP<70	Dopamine≤5 or dobutamine (any dose)	Dopamine>5 or epinephrine≤0.1 or norepinephrine ≤0.1	Dopamine>15 or epinephrine>0.1 or norepinephrine >0.1
CNS Glasgow Coma Score	13-14	10-12	6-9	<6
Renal Creatinine, mmol/l or Urine output	0.11-0.17	0.171-0.299	0.3-0.44 or <500 ml/day	>0.44 or <200 ml/day

*Record the worst values. Record adrenergic agents administered for at least 1 h ( g/kg/min)*

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*Peer-review history:*

*The peer review history for this paper can be accessed here:  
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