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# Evaluation of the polymorphisms of genes affecting thrombosis throughout sepsis in children

## Sepsisli çocuklarda trombozu etkileyen genlerdeki polimorfizmlerin değerlendirilmesi

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### Summary

**Aim:** Since the clinical course of sepsis exhibits individual variance, its genetic background and the polymorphisms found in the genes which may be responsible for the predisposition to sepsis have been investigated. The interaction between coagulation and sepsis has directed researchers to investigate the genetic polymorphisms of those factors. We aimed to determine the polymorphisms of Factor V Leiden, Factor V 1299, prothrombin, Factor XIII, beta-fibrinogen, plasminogen activator inhibitor 1, glycoprotein III, MTHFR, Apo E in children with sepsis and septic shock.

**Material and Methods:** Ninety-two children with sepsis and ninety-two healthy children were included into the study by using a reverse-hybridization assay. Among the study group 20 cases developed septic shock (10 of nonsurvivors was in the septic shock group, 4 in the sepsis group).

**Results:** Among the sepsis group, a total of 16 cases had (17.4 %) positive blood cultures. The microorganisms were Pseudomonas aeruginosa in 7 cases (43.7 %), Klebsiella pneumoniae in 3 (18.7 %), Eschherichia coli in 2 (12.5 %), Streptococcus pneumonia in 2 (12.5 %), Enterococcus faecalis in 1 (6.3 %) and Candida albicans in 1 (6.3 %). No statistically significant difference associated with all the polymorphisms between the groups of sepsis, septic shock and nonsurvivors compared to healthy group was achieved.

**Conclusion:**The polymorphisms investigated in the proteins having a role in the thrombosis process were not found to have a significant effect on the incidence of developing sepsis or outcome of sepsis in children.

**Key words:** sepsis, children, genes, polymorphism, thrombosis

### Özet

***Amaç:****Sepsisin klinik gidişindeki kişisel farklılıklardan, hastalığın genetik kökeni ve sepsise yatkınlık yarattığı düşünülen genlerde bulunan polimorfizmler sorumlu olabilir. Sepsis ve koagülasyon arasındaki ilişki araştırmacıları koagülasyonda rol oynayan faktörlerin polimorfizmlerini araştırmaya yönledirmiştir. Bu çalışmada sepsisli ve septik şoklu olan çocuk hastalarda Factor V Leiden, Factor V 1299, prothrombin, Factor XIII, beta-fibrinogen, plasminogen activator inhibitor 1, glycoprotein III, MTHFR, Apo E genlerindeki polimorfizmlerin araştırılması hedeflenmiştir.*

***Yöntem ve Gereç:****Sepsisli 92 çocuk hasta ve 92 sağlıklı çocuk çalışmaya dahil edilmiştir. Genotiplendirme reverse hibridizasyon yöntemi ile yapılmıştır. Hasta grubunda 20 çocuk septic şoka girmiştir.*

***Bulgular:****Sepsis grubunda 16 vakada (%17,4) kan kültürü pozitif saptanmıştır. Kültür pozitif olguların 7' sinde (%43,7) Pseudomonas aeruginosa, 3' ünde (%18,7) Klebsiella pneumoniae, 2' sinde (%12,5) Streptococcus pneumonia, birinde (%6,3) Enterococcus faecalis ve birinde (%6,3) Candida albicans saptanmıştır. Tüm genotipler için kontrol grubu ile sepsis, septik şok ve sepsise bağlı ölüm olan gruplar arasında anlamlı bir fark saptanmamıştır.*

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***Sonuç:****Çocuklarda sepsis ya da buna bağlı sonuçların sıklığı üzerinde, tromboz sürecini etkileyen araştırılan genlerdeki polimorfizmlerin rolü olmadığı görülmüştür.*

**Anahtar Sözcükler:** *sepsis, çocuk, gen, polimorfizm, tromboz*

## Introduction

Sepsis is a systemic inflammatory response of the body to infections. It is characterized by 2 or more of the systemic inflammatory response syndrome (SIRS) criteria which are i. changes in the temperature (hypothermia or hyperthermia); ii. tachycardia (>90 bpm), iii. changes in the respiratory system ($\geq$ 20 breaths/min or $PaCO_2$ < 32 mmHg) and iv. changes in the white blood cell count ($\geq$ 12,000 or $\leq$ 4,000 cells/$mm^3$ or > 10% bands). The mortality rate of sepsis varies 28 % to 50 % (1). There are two main factors in the pathophysiology of sepsis; host defense and the pathogen's nature. The genetic diversity in humans has been reported to be responsible for the different clinical presentations of sepsis and for its different responses to treatment (2,3). Innate immune system receptors and related molecules have been widely studied in respect to gene polymorphisms (2). More recently the role of genetic polymorphisms in coagulation factors and pathways have been studied (4-6). The results of the studies relating to the effect of the coagulation system in developing sepsis, septic shock and the mortality of sepsis are controversial, since one of the causes may be the interactions between the different single nucleotide polymorphisms (SNPs) in those gene products involved in the pathways. Some of those SNPs may be modulated by other SNPs therefore unexpected results are observed in those studies. For that reason large-scale association studies evaluating many polymorphisms simultaneously have been suggested (7). In this study we aimed to evaluate the role of polymorphisms in a number of proteins which interfere with the coagulation cascade and thrombosis by using a reverse-hybridization assay (Table 1).

**Table 1.** The list of the genes and polymorphisms

| Genes | Polymorphisms |
|---|---|
| Factor V Leiden | G1691A |
| Factor V 1299 | H1299R |
| Prothrombin | G20210A |
| Factor XIII | V34L |
| beta-fibrinogen | -455 G-A |
| PAI | 4G/4G |
| | 4G/5G |
| | 5G/5G |
| Glycoprotein III | L33P |
| Apo E | E2, E3, E4 |
| MTHFR | C677T |

The evaluation of each factor was not significantly related to the incidence of sepsis, developing sepsis or the final outcome of sepsis in children.

## Materials-Methods

The study was designed retrospectively and 92 children with sepsis, aged 1 month to 168 months, were included in the study. The diagnosis was based on clinical presentation and the laboratory results. Control group included ninety-two age-matched healthy children aged 1 month to 204 months. Patient and control group were unrelated Turkish Caucasian ethnic group. Control group had no history of sepsis, any other disease or exposure to medication.

*Definition of sepsis:*

The diagnosis of sepsis was based on bacterial culture, clinical presentation and the laboratory results. Blood cultures were performed routinely on all cases with clinical presentation suggestive of sepsis. Sepsis was defined as infection plus two or more of the following criteria which were; >38℃ or < 36℃ of temperature; tachycardia w hich is >2 SD above the normal value for age or bradycardia under 1 year old; respiratory rate which is >2 SD above the normal value for age; higher or lower levels of white blood cell count according to the normal range for age (or > 10% band forms). Septic shock was defined as sepsis-induced hypotension plus hypoperfusion abnormalities despite adequate fluid resuscitation (8). All criteria were evaluated specifically according to the normal ranges for age in each case.

*Molecular Analysis:*

Genomic DNA was prepared from whole blood samples by standard techniques following the manufacturer's instructions (Invisorb Spin Blood Kit, Invitek, Berlin, Germany). Multiplex polymerase chain reaction (PCR) was performed. The polymorphisms of Factor V Leiden (G1691A), Factor V 1299 (H1299R), prothrombin (G20210A), Factor XIII (V34L), beta-fibrinogen (-455 G-A), plasminogen activator inhibitor 1 (4G-5G), glycoprotein III (L33P), MTHFR (C677T), Apo E (E2 E3, E4) which are important factors in the thrombosis retrospectively analyzed by reverse-hybridization assay by using a commercially available kit (CVDStripAssay; ViennaLab

Labordiagnostika, Vienna, Austria). PCR products were hybridized by sequence-specific oligonucleotide probes, which were immobilized on nitrocellulose strips (reverse hybridization). The nitrocellulose strips had probes for the wild type and mutated alleles of the each gene locus as well as various control zones. During hybridization, the denatured amplified DNA bound to the gene probes attached to the strips. Streptavidin-coupled alkaline phosphatase bound to the hybrids of gene probes and biotin-labeled amplified DNA. Subsequently those complexes were detected by a color reaction of alkaline phosphatase and the band patterns were evaluated by using the template supplied.

*Consent:*

The university hospital ethics committee approved the study and written informed consents from all families were obtained.

*Statistical Analysis:*

Chi-square analysis was used to compare the allele and genotype frequencies. Odds ratios and the corresponding 95 % CI were estimated by using cross-tabulation. P values less than 0.05 were considered statistically significant. SPSS software (SPSS 10.0, SPSS Inc., Chicago, IL) was used for the calculations.

## Results

Study group: Forty-three were female and 49 were male. The age of disease onset varied from 1 month to 168 months (mean age: 17.77±27.87 months). Among the study group 20 cases developed septic shock. Fourteen out of 92 cases (15.2 %) died as a result of sepsis. The number of nonsurvivors was 10 in the septic shock group and 4 in the sepsis group.

Control group: Forty were female and 52 were male. The age of disease onset varied from 1 month to 204 months (mean age: 97.45±53.41 months).

Among the sepsis group, 16 cases were proven (17.4 %) by positive blood cultures. Causative microorganisms were Pseudomonas aeruginosa in 7 cases (43.7 %), Klebsiella pneumoniae in 3 (18.7 %), Eschherichia coli in 2 (12.5 %), Streptococcus pneumonia in 2 (12.5 %), Enterococcus faecalis in 1 (6.3 %) and Candida albicans in 1 (6.3 %).

The frequencies of alleles and genotypes are shown in Table 2. There was no statistically significant difference associated with all the polymorphisms between the groups of sepsis, septic shock and nonsurvivors compared to healthy group.

The polymorphisms in the proteins that have a role in the coagulation system were not found to have a significant effect on the incidence of developing sepsis or outcome of sepsis in children.

**Table 2.** The frequencies of alleles and genotypes of the proteins which were studied in children with sepsis 1,00: Wild type; 2,00: Heterozygote mutant; 3,00: Homozygote mutant

| | **Factor V-Leiden** | | | **Total** |
|---|---|---|---|---|
| | **1,00** | **2,00** | **3,00** | |
| **Sepsis** | 69 (95.8 %) | 3 (4.2 %) | 0 | **72** |
| **Septic shock** | 17 (85.0 %) | 3 (15.0 %) | 0 | **20** |
| **Control** | 86 (93.5 %) | 6 (6.5 %) | 0 | **92** |
| **Total** | **172 (93.5 %)** | **12 (6.5 %)** | **0** | **184** |

| | **Factor V-1299** | | | **Total** |
|---|---|---|---|---|
| | **1,00** | **2,00** | **3,00** | |
| **Sepsis** | 66 (91.7 %) | 6 (8.3 %) | 0 | **72** |
| **Septic shock** | 18 (90.0 %) | 2 (10.0 %) | 0 | **20** |
| **Control** | 82 (89.1 %) | 10 (10.9 %) | 0 | **92** |
| **Total** | **166 (90.2 %)** | **18 (9.8 %)** | **0** | **184** |

| | **Protrombine** | | | **Total** |
|---|---|---|---|---|
| | **1,00** | **2,00** | **3,00** | |
| **Sepsis** | 66 (91.7 %) | 5 (6.9 %) | 1 (1.4 %) | **72** |
| **Septic shock** | 20 (100.0 %) | 0 | 0 | **20** |
| **Control** | 85 (92.4 %) | 4 (4.3 %) | 3 (3.3 %) | **92** |
| **Total** | **171 (92.9 %)** | **9 (4.9 %)** | **4 (2.2 %)** | **184** |

| | **Factor XIII** | | | **Total** |
|---|---|---|---|---|
| | **1,00** | **2,00** | **3,00** | |
| **Sepsis** | 53 (73.6 %) | 19 (26.4 %) | 0 | **72** |
| **Septic shock** | 16 (80.0 %) | 4 (20.0 %) | 0 | **20** |
| **Control** | 69 (75.0 %) | 20 (21.7 %) | 3 (3.3 %) | **92** |
| **Total** | **138 (75.0 %)** | **43 (23.4 %)** | **3 (1.6 %)** | **184** |

| | B-Fibrinogen | | | Total |
|---|---|---|---|---|
| | 1,00 | 2,00 | 3,00 | |
| **Sepsis** | 49 (68.1 %) | 20 (27.8 %) | 3 (4.2 %) | **72** |
| **Septic shock** | 12 (60.0 %) | 7 (35.0 %) | 1 (5.0 %) | **20** |
| **Control** | 71 (77.2 %) | 19 (20.7 %) | 2 (2.2 %) | **92** |
| **Total** | **61 (66.3 %)** | **27 (29.3 %)** | **4 (4.3 %)** | **92** |

| | GpIII | | | Total |
|---|---|---|---|---|
| | 1,00 | 2,00 | 3,00 | |
| **Sepsis** | 62 (86.1 %) | 10 (13.9 %) | 0 | **72** |
| **Septic shock** | 16 (80.0 %) | 4 (20.0 %) | 0 | **20** |
| **Control** | 70 (76.1 %) | 19 (20.7 %) | 3 (3.3 %) | **92** |
| **Total** | **148 (80.4%)** | **33 (17.9 %)** | **3 (1.6 %)** | **184** |

| | PAI1 | | | Total |
|---|---|---|---|---|
| | 4G/4G | 4G/5G | 5G/5G | |
| **Sepsis** | 17 (23.6 %) | 32 (44.4 %) | 23 (31.9 %) | **72** |
| **Septic shock** | 7 (35.0 %) | 9 (45.0 %) | 4 (20.0 %) | **20** |
| **Control** | 25 (27.2 %) | 40 (43.5 %) | 27 (29.3 %) | **92** |
| **Total** | **49 (26.6%)** | **81 (44.0%)** | **54 (29.3%)** | **184** |

| | MTHFR129 | | | Total |
|---|---|---|---|---|
| | 1,00 | 2,00 | 3,00 | |
| **Sepsis** | 34 (47.2 %) | 29 (40.3 %) | 9 (12.5 %) | **72** |
| **Septic shock** | 11 (55.0 %) | 8 (40.0 %) | 1 (5.0 %) | **20** |
| **Control** | 43 (46.7 %) | 39 (42.4 %) | 10 (10.9 %) | **92** |
| **Total** | **88 (47.8 %)** | **76 (41.3 %)** | **20 (10.9 %)** | **184** |

| | Apo-E | | | | | | Total |
|---|---|---|---|---|---|---|---|
| | E2E2 | E2E3 | E2E4 | E3E3 | E3E4 | E4E4 | |
| **Sepsis** | 2 (2.8 %) | 5 (6.9 %) | 1 (1.4 %) | 56 (77.8 %) | 7 (9.7 %) | 1 (1.4 %) | **72** |
| **Septic shock** | 0 | 3 (15.0 %) | 0 | 14 (70.0 %) | 3 (15.0 %) | 0 | **20** |
| **Control** | 4 (4.4 %) | 9 (9.9 %) | 0 | 72 (79.1 %) | 4 (4.4 %) | 2 (2.2 %) | **91** |
| **Total** | **6 (3.3 %)** | **17 (9.3 %)** | **1 (0.5 %)** | **142 (77.6 %)** | **14 (7.7 %)** | **3 (1.6 %)** | **183** |

## Discussion

The first contact of the pathogens with the host and the host-pathogen interaction determine the outcome of the subsequent host-microbe relationships. Following the first contact nothing may happen or an infection may start which is defined as an inflammatory response to a pathogen. Sepsis is characterized by an overwhelming systemic response to infection and systemic inflammatory response symptoms. Additional findings of organ dysfunctions contribute to the development of severe sepsis. The differences in host response, activation of inflammatory and coagulation pathways and the role of monocytes and endothelial cells are important elements in investigating the pathophysiology of sepsis. Indeed these last two themes are closely related to the first. There have been a number of studies reporting a significant correlation between the proteins that play a role in the coagulation system and the development of a sepsis (9,10). Therefore the partial or total break up of the coagulation system has

been investigated in the pathogenesis of sepsis and the outcome of sepsis (5,11,12). Previous studies reported the importance of blood clotting in vascular cell activation which contributes to leukocyte activation (13,14). Since genetic epidemiological studies discovered a significant genetic influence in the development and outcome of sepsis, recently host-specific immune response differences have become a target for the studies investigating infectious diseases including sepsis. The differences between the individuals can be explained by the limited DNA changes, namely polymorphisms (15). Among those polymorphisms, the polymorphisms of Factor V (FV), fibrinogen, prothrombin, plasminogen activator inhibitor-1 (PAI), factor XIII, ApoE, Antithrombin III, and MTHFR have been studied to see which have played a role in sepsis. Interestingly, although some studies supported the relationship between the polymorphisms and the incidence and the outcome of sepsis, some were unable to do so (16,17). In our study the importance of the polymorphisms in 9 different proteins were investigated in sepsis and septic shock in children. The importance of those factors in blood clotting will be briefly explained and the literature findings will be compared with the findings of the presented study.

FV is an important coagulation factor which is responsible for the conversion of factor II to the active form together with factor X. Mutations in the FV leads to the increased risk of thrombosis by gaining resistance to inactivation by activated protein C. The data associated with the harmful or beneficial effects of FV mutations in sepsis is contradictory because of conflicting results (7,18). In our study we analyzed FVL (G1691A) and FV 1299 (H1299R) polymorphisms and observed neither a significant effect on the development or on the outcome of sepsis. In the literature FVL in association with PC or protein S deficiency has been linked to the severity of sepsis (19), therefore further studies evaluating the PC and PS levels with the FV polymorphisms may be required.

G20210A polymorphism in the prothrombin gene is related to hyperprothrombinemia which also causes thrombophilia. This polymorphism has also been suggested as an excellent SNP candidate to be studied in severe sepsis and septic shock patients (7); However the results in our study regarding G20210A polymorphism showed no such causal relationship.

The formation of fibrin and its stability is crucial in the last stage of coagulation and should be in correspondence with the fibrinolytic system. High levels of Factor XIII and fibrinogen are associated with Val34Leu and -455G-A polymorphisms respectively. In the literature there are a number of studies evaluating those polymorphisms in septic conditions (17). Val34Leu polymorphism in factor XIII causes a difference in the structure of the fiber in the clot. In the Leu34 genotype, the fiber is thinner which lets fewer platelets to incorporate itself to the clot; therefore Leu34 genotype may prevent sepsis hindering the occurrence of thrombosis. 455G/A polymorphism in the fibrinogen gene is associated with high levels of fibrinogen which may also play a role in the susceptibility to clotting. In theory sepsis and severe sepsis are very closely related to blood coagulation disorders, and any effect which disturbs the balance between the coagulation and fibrinolytic system may facilitate a pathogen invasion. Therefore it is also highly probable that PAI polymorphisms may have an effect on those cases with sepsis together with Fc XIII and fibrinogen polymorphisms. High plasma PAI-1 concentration is associated with a deletion of a G residue giving rise to 4G/4G genotype, and this high level has been reported to be associated with poor outcome in sepsis and septic shock (17,20,21). Since the contribution of clotting to the leukocyte activation via vascular cell activation was shown (13), we expected to see a significant correlation between those polymorphisms and the development of sepsis, particularly in children with septic shock. However regarding the effects of those polymorphisms on the development of sepsis, no significant differences were observed between the control group and the study group in the study presented.

The *677T* allele of MTHFR is associated with elevated plasma homocysteine levels, which have been shown to cause an increased risk of cardiovascular disease and birth defects (22). Homocysteine may also have an effect on promoting blood clots (23). Apolipoprotein E (apoE) is a type of lipoprotein which is responsible for carrying cholesterol and other fats through the bloodstream. There are three major human isoforms of apoE, designated apoE2, apoE3, apoE4. The presence of the ApoE2 allele is associated with high levels of factor VIII in plasma and E3 is associated with a decreased incidence of severe sepsis (24). L33P polymorphism of the glycoprotein IIIa is associated with an increased risk of arterial thrombosis. It was also reported that platelets and fibrinogen play an important role in mediating neutrophil-endothelial cell adherence in septic shock (25). In the literature there is no study reporting a relationship between the polymorphisms

in homocysteine, glycoprotein IIIa and sepsis. We also evaluated the association between homocysteine, glycoprotein IIIa and ApoE polymorphisms with the sepsis. No significant correlation was noted.

Despite significant findings regarding the role of all those proteins in the pathogenesis of sepsis and severe sepsis, the effect of polymorphisms on sepsis studied were found to be neither harmful nor beneficial. Factors which are related to the clotting mechanism are expected to have an important role in the sepsis or septic shock development. Analysis of different proteins in this study did not reveal a significant association either. On the other hand there are a number of limitations in our study; the first one is the retrospective nature of the study and the small sample size. The second one is the failure in identifying the causative microorganisms in the patient group due to the some factors like administration of antibiotics before blood collection or technical reasons. There are a number of studies showing that hematological molecular markers may be useful in predicting the severity and the outcome of sepsis, therefore prospectively designed large-scale association studies based on different age groups investigating the polymorphisms of the innate immune system and clotting system simultaneously in well-defined patients' groups may provide reliable results.

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