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Haplotypes composed of minor frequency single nucleotide polymorphisms of the *TNF* gene protect from progression into sepsis: A study using the new sepsis classification

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ABSTRACT

Objectives: Several articles have provided conflicting results regarding the role of single nucleotide polymorphisms (SNPs) in the promoter region of the *TNF* gene in susceptibility to sepsis. Former articles have been based on previous definitions of sepsis. This study investigated the influence of *TNF* haplotypes on the development of sepsis using the new Sepsis-3 definitions.
Methods: DNA was isolated from patients suffering from infection and systemic inflammatory response syndrome. Haplotyping was performed for six SNPs of *TNF*. The serum levels of tumour necrosis factor alpha (TNF-α) of these patients were measured using an enzyme immunosorbent assay. Patients were classified into infection and sepsis categories using the Sepsis-3 definitions. Associations between the *TNF* haplotypes and the clinical characteristics and serum TNF-α levels of the patients were examined.
Results: The most common *TNF* haplotype h1 was composed of major alleles of the studied SNPs. Carriage of haplotypes composed of minor frequency alleles was associated with a lower risk of developing sepsis (odds ratio 0.41, 95% confidence interval 0.19–0.88, $p = 0.022$), but this did not affect the 28-day outcome. Serum TNF-α levels were significantly higher among patients homozygous for h1 haplotypes who developed sepsis compared to infection ($p = 0.032$); a similar result was not observed for patients carrying other haplotypes.
Conclusions: Haplotypes containing minor frequency SNP alleles of *TNF* protect against the development of sepsis without affecting the outcome.

Introduction

Sepsis is currently defined as life-threatening organ dysfunction due to a dysregulated host response to infection (Singer et al., 2016). This definition recognizes the heterogeneity of sepsis regarding the pathogenesis (Abraham et al., 2001). This also leads to the question of how the genetic background of the host predisposes that individual to an infection and how it affects the physical course of the infection.

Tumour necrosis factor alpha (TNF-α) is a mediator crucial for an effective immune response, but excessive production may cause harmful effects, as it can lead to cardiovascular collapse (Ulloa and Tracey, 2005). The systematic administration of TNF-α produces sepsis-like symptoms, while high plasma levels of TNF-α have been associated with high mortality rates in septic patients

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(Gordon et al., 2004). Previous studies have shown that the neutralization of TNF prevents endotoxin- and bacteraemia-induced shock despite the presence of endotoxins and bacteria in the circulation (Tracey and Cerami, 1994).

A large number of studies have tried to examine the effect of genetic variation of the *TNF* gene on susceptibility to severe sepsis and mortality due to sepsis using the former sepsis definitions. A study in a Chinese population showed that the −308 G/A single nucleotide polymorphism (SNP) was strongly associated with susceptibility to severe sepsis, but not with a poor outcome (Song et al., 2012). Also, carriage of at least one A allele of the −376 G/A, −308 G/A, and −238 G/A SNPs of the promoter region of *TNF* has been associated with a shorter time to the development of ventilator-associated pneumonia (Kotsaki et al., 2012). On the other hand, other studies have failed to document any association between candidate *TNF* and *TNF* receptor gene polymorphisms and susceptibility to sepsis, illness severity, or the outcome (Gordon et al., 2004).

The new definitions of sepsis lead to the concept that conflicting results from case–control genetic association studies in the past may be reconciled, since these studies were based on the former definitions. The Hellenic Sepsis Study Group (HSSG) has a broad collection of clinical data and genetic material from patients with infections and systemic inflammatory response syndrome (SIRS). Recently these patients were reclassified into infection and sepsis categories using the new Sepsis-3 definitions (Giamarellos-Bourboulis et al., 2017). The study reported here was based on this reclassification and aimed to investigate how *TNF* haplotypes may be associated with infection or sepsis.

Patients and methods

Study design

The study was performed from July 2009 to December 2012 in 20 departments of 10 hospitals in Greece participating in the Hellenic Sepsis Study Group. Of these departments, three are mixed surgical/medical intensive care units (ICUs), five are departments of internal medicine, and two are departments of surgery. Patients with community-acquired sepsis admitted to the general ward or with hospital-acquired sepsis developing in the ICU at least 48 h after ICU admission were enrolled in this study. The study protocol and the written informed consent form were approved by the ethics committees of the participating hospitals.

Inclusion criteria were: (1) written informed consent provided by the patient, or by the legal representatives of those patients who were unable to consent, (2) age ≥18 years, (3) either sex, (4) Caucasian ethnicity, (5) at least two signs of SIRS, and (6) sepsis due to acute pyelonephritis, lung infection, intra-abdominal infection, or primary bacteraemia. Exclusion criteria were: (1) infection with HIV, and (2) neutropenia, defined as less than 1×10^9 neutrophils/l, due to causes other than SIRS.

SIRS was defined as the presence of at least two of the following (Levy et al., 2003): (1) core temperature >38 °C or <36 °C, (2) $pCO_2 < 32$ mmHg, (3) pulse rate >90 beats per min, and (4) white blood cell count of $>12 \times 10^9$/l or $<4 \times 10^9$/l, or more than 10% band forms. Acute pyelonephritis, lung infections, intra-abdominal infections, and primary bacteraemia were defined as described elsewhere (Huang et al., 2014; Chastre and Fagon, 2002; Kollef, 2003; Rello et al., 2001; Christ-Crain et al., 2006; Piccoli et al., 2014; Calandra and Cohen, 2005).

The following parameters were recorded for each patient participating in the study: (1) sequential organ failure assessment (SOFA) score, (2) Glasgow coma score (GCS), (3) organ dysfunctions, (4) Charlson comorbidity index (CCI), and (5) acute physiology and chronic health evaluation (APACHE) II score. Patients were originally classified as having sepsis, severe sepsis, or septic shock according to the 2001 international sepsis definitions (Levy et al., 2003) and were reclassified into infection and sepsis categories using the Sepsis-3 definitions (Giamarellos-Bourboulis et al., 2017). Survival status after 28 days was also recorded. Additionally, patients with a CCI ≤2 were categorized as patients with mild comorbidity and those with a CCI >2, which has been associated with higher mortality rates (Huang et al., 2014), were classified as patients with severe comorbidity.

Blood sampling, genotyping, and haplotyping

For the genotyping analysis, 3 ml of whole blood were collected from patients and healthy controls in ethylenediaminetetraacetic acid (EDTA) and stored at −70 °C until processing. Genomic DNA was extracted from all individuals using the Purege Blood Core Kit C (Qiagen) according to the manufacturer's instructions.

Genotyping was done by Sanger sequencing of both strands from PCR products. Primers (Metabion, Martinsried, Germany) were: *TNF* forward 5′-GCC CCT CCC AGT TCT AGT TC-3′; *TNF* reverse 5′-GCA CCT TCT GTC TCG GTT TC-3′. The amplification of individual genomic DNAs was performed in a 25-μl reaction mix containing approximately 25 ng DNA and 10 pmol of each primer, using the BioMix ready-to-use reaction mix (Bioline, Luckenwalde, Germany). PCR cycling conditions were as follows: 95 °C for 1 min, followed by 30 cycles of 95 °C/30 s, 59 °C/30 s, 72 °C/60 s, and a final extension at 72° C for 5 min. PCR products were precipitated by 2 μl of 7.5 mM ammonium acetate and 85 μl of ethanol (96%, v/v), dried and re-dissolved in 10 μl of water. Sanger sequencing was performed using the PCR primers with Dye Terminator 3.1 chemistry and a 3730xl DNA Analyzer (Applied Biosystems). Genotypes of the common SNPs listed in Figure 1 were called by visual inspection of gap4 alignments (Staden package). Haplotype calculations were done using HAPMAX (http://www.uni-kiel.de/medinfo/mitarbeiter/krawczak/download/)

Serum selection and measurement of TNF-α levels

A blood sample was collected from 140 patients in a pyrogen- and anticoagulant-free tube within the first 24 h of signs of SIRS. The tube was centrifuged at 800 *g* at room temperature and the supernatant serum was collected and stored at −80 °C until assayed. TNF-α levels were determined using an enzyme immunosorbent assay. The lower limit of detection was 8 pg/ml.

Study endpoints

The primary endpoint of this study was to identify any possible associations between the carriage of minor frequency *TNF* haplotypes and the development of sepsis. For this purpose, sepsis was defined using the new Sepsis-3 definitions.

The impact of the studied haplotypes on 28-day mortality and TNF-α serum levels were the secondary endpoints of the study.

Statistical analysis

Comparisons between patients with infection and sepsis were done using the Chi-square test for qualitative variables and Mann–Whitney *U*-test for quantitative variables. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated according to Mantel and Haenszel. Logistic regression analysis was conducted to confirm observed differences in haplotypes between patients with infection and sepsis: sepsis was entered into the equation as the

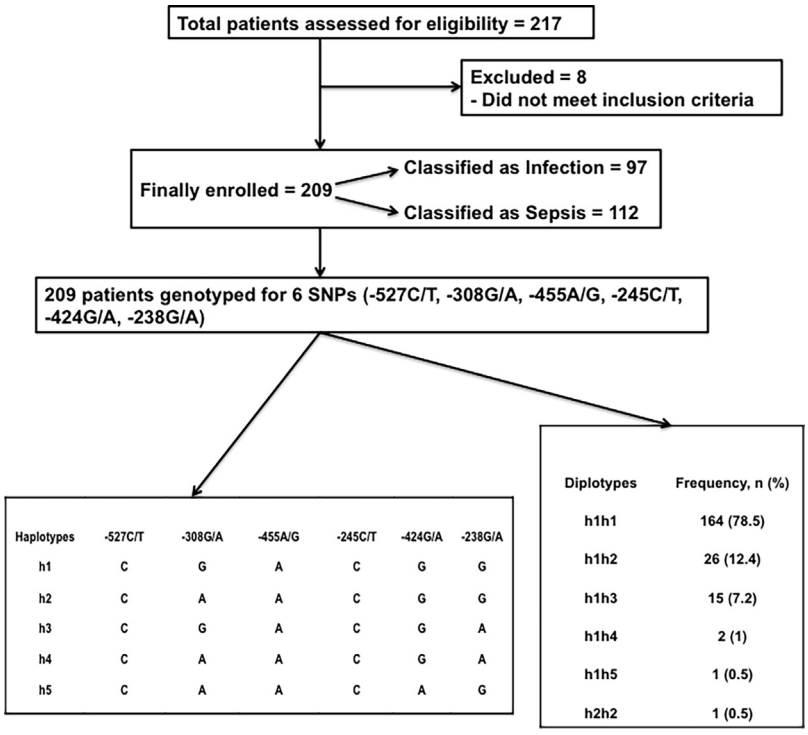

Figure 1. Study flow chart.

dependent variable and all parameters with a p-value <0.1 for the difference between patients with infection and patients with sepsis were included as independent variables. TNF-α levels were expressed as the median and 95% confidence intervals (CI) and compared between groups using the Mann–Whitney U-test. A nominal p-value of <0.05 was considered significant.

Results

Two hundred and eighteen patients were screened for eligibility, of whom 209 were enrolled in the study: 97 with infection and 112 with sepsis (Figure 1). The clinical and demographic characteristics of the patients are shown in Table 1.

Table 1
Demographic and clinical characteristics of the patients.

	Infection ($n=97$)	Sepsis ($n=112$)	p-Value
Male sex, n (%)	36 (37.1)	59 (52.7)	0.036
Age (years, mean ± SD)	61.12 ± 21.79	72.06 ± 15.19	<0.0001
WBC ($\times 10^9$/l, mean ± SD)	11.915 ± 4.666	14.937 ± 8.886	0.012
SOFA score (mean ± SD)	0.3 ± 0.46	4.98 ± 2.93	<0.0001
APACHE II score (mean ± SD)	8.05 ± 4.99	18.04 ± 7.56	<0.0001
ARDS, n (%)	0 (0)	19 (17.0)	<0.0001
Acute kidney injury, n (%)	0 (0)	22 (19.6)	<0.0001
Acute coagulopathy, n (%)	5 (5.2)	34 (30.4)	<0.0001
Cardiovascular failure, n (%)	1 (1.0)	27 (24.1)	<0.0001
Underlying infection, n (%)			
Acute pyelonephritis	56 (57.7)	46 (41.1)	0.019
Community-acquired pneumonia	2 (2.1)	12 (10.7)	0.013
Intra-abdominal infection	25 (25.8)	27 (24.1)	0.873
Primary bacteraemia	13 (13.4)	19 (17.0)	0.565
Other	1 (1.0)	8 (7.1)	
Pathogen in blood cultures, n (%)			
Escherichia coli	19 (19.6)	16 (14.3)	0.355
Klebsiella pneumoniae	3 (3.1)	9 (8.0)	0.147
Pseudomonas aeruginosa	1 (1.0)	6 (5.4)	0.125
Acinetobacter baumannii	2 (2.1)	5 (4.5)	0.454
Underlying disease, n (%)			
Diabetes mellitus type 2	17 (17.5)	34 (30.4)	0.036
Chronic heart failure	13 (13.4)	23 (20.5)	0.201
COPD	6 (6.2)	13 (11.6)	0.229
Chronic renal disease	1 (1.0)	14 (12.5)	0.001

SD, standard deviation; WBC, white blood cells; SOFA, sequential organ failure assessment; APACHE, acute physiology and chronic health evaluation; ARDS, acute respiratory distress syndrome; COPD, chronic obstructive pulmonary disease.

The genotype frequencies and the allele frequencies of the patients are shown in the **Supplementary Material** (Table S1). All six studied alleles were in Hardy–Weinberg equilibrium.

Five haplotypes of the *TNF* locus were recognized (Figure 1). The most common haplotype, named h1, was composed of the major alleles of the studied SNPs, and it was found to be homozygous in 164 of the 209 patients (h1h1 diplotype, 78.5%). Carriers of the other haplotypes carried at least one minor frequency allele of the six studied SNPs.

This finding was confirmed by logistic regression analysis (Table 2). The results revealed that the carriage of haplotypes other than h1 was independently associated with the development of sepsis (OR 0.41, 95% CI 0.19–0.88, $p = 0.022$). The presence of a severe comorbidity and the presence of community-acquired pneumonia were also independently associated with the development of sepsis.

There was no association between carriage of the other *TNF* haplotypes and the development of sepsis or the disease outcome.

When using the 2001 definitions, no association was found between the carriage of minor frequency haplotypes of *TNF* and the development of severe sepsis or septic shock (33/136 patients with sepsis (24.3%) vs. 12/73 (16.3%) patients with severe sepsis/septic shock, $p = 0.189$).

Secondary endpoints of the study

Mortality after 28 days was 18.8% (31/164 patients) among homozygous carriers of the major frequency haplotype h1 and 15.9% (7/45 patients) among carriers of the minor frequency haplotypes h2 to h5 ($p = 0.453$).

As shown in Figure 2, among patients homozygous for the h1 haplotype, TNF-α serum levels were significantly higher in patients who developed sepsis as compared to patients with infection ($p = 0.032$). No similar difference was detected in patients carrying the other haplotypes (comparison between infection and sepsis, $p = 0.186$).

Discussion

This study showed, for the first time, the protective role of minor frequency haplotypes of *TNF* in the development of sepsis in a patient population with SIRS developing against the background of an infection. There are unique differences in design between the present study and previous case–control studies that have investigated the role of SNPs within the promoter region of *TNF* in susceptibility to sepsis. These differences are the following: (1) previous studies have focused on the study of candidate SNPs across the entire gene and not of haplotypes of its promoter; (2) former studies have compared the distribution of genotypes of patients with severe sepsis, i.e., sepsis aggravated by organ dysfunction, with healthy matched controls.

The current study analyzed a study population of patients all of whom had systemic inflammatory response due to an infection. However, only some of them progressed to sepsis as defined in the new 2016 definitions. With these new definitions, the classification of sepsis takes into account the SOFA score, which is an estimate of the global organ dysfunction of the patient. This allows limited mismatching of patients to a lower grade of severity (Giamarellos-Bourboulis et al., 2017). The importance of the minor frequency haplotypes was confirmed after logistic regression analysis taking into consideration patient comorbidities and the type of infection. Based on these findings it can be postulated that the common h1 haplotype dominated the transition of infection into sepsis in patients with SIRS. However, a similar association was not found when classifying the patients with the former definitions, highlighting the significance of the new Sepsis-3 classification to the results of this study.

It was also found that the presence of the h1 haplotype has a major impact on circulating levels of TNF-α. The study demonstrated an association between elevated TNF-α serum levels and susceptibility to the development of sepsis in patients carrying the h1 haplotype. This is in line with the results of former studies that have found increased serum levels of TNF-α in patients with septic shock (Ulloa and Tracey, 2005). The present study finding that there was no significant difference in TNF-α level between patients with sepsis and infection bearing the minor frequency h1 haplotypes might imply that minor frequency SNP alleles could eliminate the increase in TNF-α production that is observed in patients carrying wild-type alleles.

Several studies have identified genetic variants of *TNF* that have been associated with either susceptibility to severe sepsis or the outcome of the disease. A number of studies have linked the −308 G/A and −252 G/A SNPs of *TNF* with the sepsis risk and outcome, while other trials have failed to confirm these associations (Song et al., 2012; Gordon et al., 2004). A recently published meta-analysis of 26 studies that included Caucasian and Asian populations focused on the functional −308 G/A and −238 G/A *TNF* SNPs, revealing an association between the presence of each of the two SNPs and an increased risk of sepsis without affecting sepsis mortality (Zhang et al., 2017). The results of this study are in agreement with the previous findings of another meta-analysis, which showed that the −308G/A polymorphism was associated with sepsis but not with sepsis-related mortality (Teuffel et al., 2010). The contradictory results of these studies may be attributed to limited statistical power, heterogeneous patient populations, and differences in genotyping techniques (Peters et al., 2003).

Although the functional importance of the carriage of minor frequency alleles of *TNF* has not been elucidated, their protective role in sepsis may have a plausible explanation. As is known, TNF-α produces most of the deleterious results of sepsis (Gordon et al., 2004). A recent meta-analysis on the beneficial effects of anti-TNF monoclonal antibodies in sepsis that included 8971 patients (Lv et al., 2014), raised evidence that high TNF-α levels worsen the course of sepsis. The current results are also in line with the recent finding that genome-wide rare deleterious genetic variations are protective for the disease course after sepsis (Taudien et al., 2016).

In conclusion, this study showed that patients bearing haplotypes composed of minor frequency SNPs of *TNF* are

Table 2
Step-wise logistic regression analysis of variables associated with the development of sepsis.

	Infection, *n* (%) (*n* = 97)	Sepsis, *n* (%) (*n* = 112)	OR	95% CI	*p*-Value
Carriage of at least one haplotype other than h1	27 (27.8)	18 (16.1)	0.41	0.19–0.88	0.022
Male sex, *n* (%)	36 (37.1)	59 (52.7)	0.52	0.31–1.08	0.081
Charlsons's comorbidity index >2	51 (52.6)	92 (82.1)	4.45	2.28–8.69	<0.0001
Acute pyelonephritis	56 (57.7)	46 (41.1)	0.57	0.31–1.07	0.081
Community-acquired pneumonia	2 (2.1)	12 (10.7)	6.31	1.16–34.42	0.033

OR, odds ratio; CI, confidence interval.

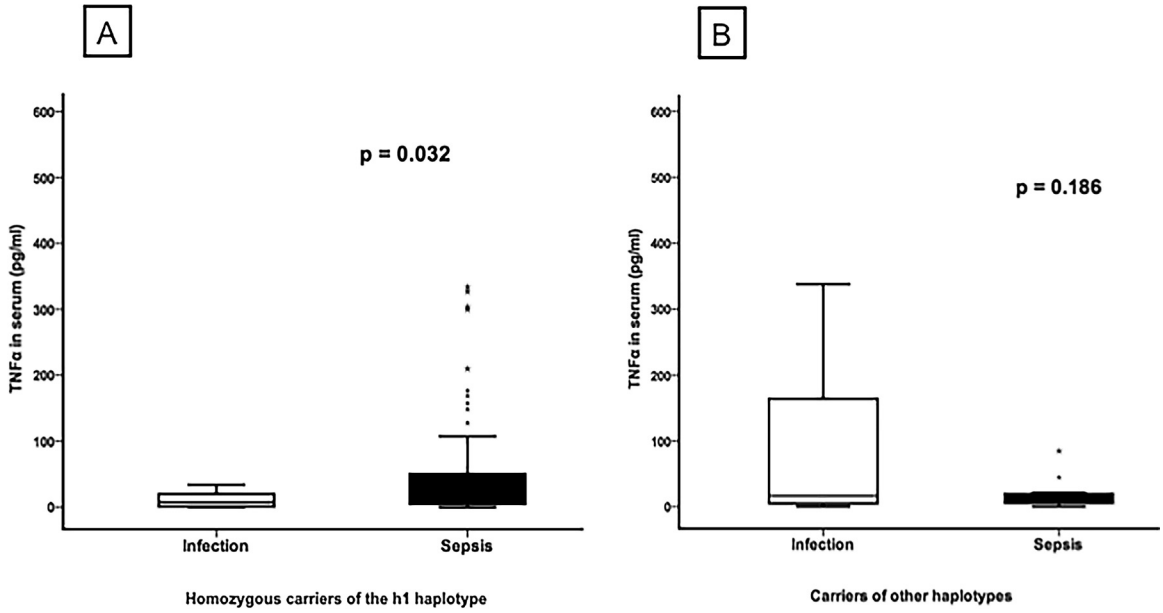

Figure 2. (A) Serum levels of tumour necrosis factor alpha (TNF-α) were significantly higher in patients who developed sepsis than in patients with infections among homozygous carriers of the h1 haplotype. (B) No similar difference was found for patients carrying other haplotypes.

protected from the development of sepsis. Despite this protective effect, carriage of these haplotypes does not influence disease severity or patient survival.

Conflict of interest

None of the authors have any conflict of interest related to this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2017.12.008.

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