

# Association of TNF- $\alpha$ -308 G/A promoter polymorphism and postoperative inflammatory cytokine response after inflammatory stimuli—an *in vitro* approach

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## Abstract

**Background:** Postoperative sepsis is one of the major complications after major surgery. In injured patients, tumor necrosis factor- $\alpha$ -308 G/A and TNF- $\beta$  NcoI gene polymorphisms has been found to be associated with the development of sepsis. Genetic factors may also have role in etio-pathogenesis of sepsis following surgery. Both pro- and anti-inflammatory cytokines are major players in the inflammatory response. Much data on blood cytokine-producing capacities in surgical sepsis patients is not available. Thus we investigated the levels of cytokine TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and IL-10 after surgery in with and without LPS stimulated blood sample in vitro.

**Methodology:** 311 patients undergoing major elective surgery were enrolled. Blood sample were drawn 24hour after surgery and then subjected to with and without LPS stimulation. TNF- $\alpha$ -308 G/A polymorphisms was studied in genomic DNA by analyzing restriction fragments of NcoI-digested DNA fragment using Polymerase Chain Reaction. Levels of cytokine TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and IL-10 were measured through Enzyme Linked Immunosorbent Assay.

**Results:** In over all TNF- $\alpha$  genotypes, 214 (68.81%) patients were homozygous dominant GG, 76(24.43%) patients were GA and 21(6.75%) patients were homozygous recessive AA. The overall allele frequency was 0.81 for G and 0.19 for A. In LPS stimulated sample, significantly higher level of TNF- $\alpha$ ( $p < 0.0001$ ) and IL-6( $p < 0.0001$ ) cytokine was observed in AA homozygous genotype subgroup as compared to other genotypes, whereas no such significant difference in level of other studied cytokines (IL-1 $\beta$ , IL-8 and IL-10) was observed between the genotype subgroups.

**Conclusion:** We found significant association of TNF- $\alpha$ -308 G/A polymorphisms with increased expression of cytokine TNF- $\alpha$  and IL-6 in response to inflammatory stimuli to postoperative blood. Our study suggests that an early identification of patients at high risk of developing postoperative severe sepsis can be done with the use of easy-to-collect markers.

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## Introduction

One of the main complications of major surgery is postoperative sepsis. Newest modern therapeutic advancements have enabled clinicians to decrease early postoperative mortality and morbidity.<sup>1,2</sup> Despite these advancements, patients stay at high risk for infection and the related increased morbidity and mortality following surgery.

Cytokine are believed to be of significantly major important factor in the pathogenesis of infectious diseases and tumor necrosis factor (TNF) specifically appears to play a crucial role.<sup>3,4</sup> TNF- $\alpha$ , a pro-inflammatory cytokine is an important component of the host immune response to infection,<sup>5-8</sup> and is responsible for the release of other pro and anti-inflammatory mediators. Furthermore, inherited variability of cytokine production and genetic predisposition for fatal infectious diseases have been suggested.<sup>9</sup> Several polymorphisms have been identified inside the TNF- $\alpha$  promoter,<sup>10</sup> among which the G/A

polymorphism at nucleotide position-308 was found directly affecting TNF- $\alpha$  expression.<sup>11</sup> In sepsis, interest has particularly focused on the promoter TNF-308 G/A SNP. Although association of A allele with susceptibility to septic shock and/or outcome from sepsis have been reported by various studies, the findings have been inconsistent.<sup>12-17</sup> The guanine-to-adenine transition at position-308 in the TNF promoter has been reported to influence TNF promoter activity and also found to be associated with enhanced TNF- $\alpha$  production by some investigators,<sup>18-21</sup> while other could not confirm this association.<sup>14,22,23</sup>

Therefore, in the present study we hypothesize that -308 G/A polymorphism of TNF- $\alpha$  is associated with TNF- $\alpha$  transcriptional activity, affects the production of inflammatory related cytokines and would play an important role in the development of postoperative sepsis. Thus our study aimed to determine whether a relation of the TNF- $\alpha$ -308 promoter polymorphism exists with inflammatory cytokine levels of endotoxin stimulated postoperative blood.

## Material and methods

**Patient characteristics and study protocol:** The ethics committee of the Institution approved the protocol used. Two hundred and thirty-nine patients who underwent major gastrointestinal surgery during October 2010 to January 2015 in the University Hospital were included in the study. Elective major surgery was defined as planned surgery requiring surgical time more than one hour and requiring anesthesia and or respiratory assistance. All patients fulfilled the following criteria: age 18-50 years, absence of preexisting infection, absence of rheumatoid arthritis or a seronegative inflammatory arthropathy, absence of malignancy, absence of diabetes, no steroid medication, and no acquired or inherited immunodeficiencies. Informed consent was obtained to withdraw blood sample for determination of TNF- $\alpha$ -308 polymorphism and for serum cytokine analysis after endotoxin stimulation. All the patients were operated under as similar conditions as possible- either of the two senior authors performing surgery in the same theater and as first case in the theatre. Per-operatively the amount of blood loss and duration of surgery (incision to stitching time) were recorded. All patients were kept on non-steroidal anti-inflammatory drugs for postoperative pain and at the time of induction received broad-spectrum single dose intravenous antibiotic (3rd generation Cephalosporin) for surgical infection prophylaxis.

### Ex vivo endotoxin stimulation of whole blood

A human whole-blood assay was performed. In brief, whole blood was mixed 1:1(vol/vol) with cell culture medium (RPMI 1640, 64 IU of penicillin per ml, 64 $\mu$ g of streptomycin per ml) and was transferred to microtiter plates. The mixtures was incubated at 37°C and 5% CO<sub>2</sub> with endotoxin (lipopolysaccharide [1000ng/ml] from *E. coli* [stock solution, 5mg/ml]) for 4h. After incubation, the supernatants was separated and stored at -20°C.

**Immunologic assays of cytokines:** Commercially available highly sensitive AviBion human TNF- $\alpha$ , IL-6 and IL-8 ELISA kits (Orgenium Laboratories Business Unit, Vantaa Finland) were used for determinations of their concentration according to the manufacturer's instructions.

**Genotyping for TNF  $\alpha$ -308 G/A polymorphism:** The genotype of TNF- $\alpha$ -308 gene polymorphism was determined by polymerase chain reaction (PCR) amplification and enzymatic digestion of the products with NcoI (Fermentas USA). 3ml of venous blood samples

were drawn in ethylenediamine-tetra acid (EDTA) Vacutainer tubes, and each patient's genomic DNA was extracted from EDTA anti-coagulated whole blood using a commercially available DNA isolation kit (QIAmp blood kit, Qiagen, Flexigene, Germany), according to the manufacturer's instructions. A 134-bp PCR product of TNF- $\alpha$  gene was amplified by PCR using the following pair of primers. Forward: 5' AGG CAA TAG GTT TTG AGG GCC AT 3' and Reverse: 5' CAT CAA GGA TAC CCC TCA CAC TC 3'.<sup>24</sup> Reaction was performed with 10–100ng of genomic DNA, 0.2 $\mu$ mol of primers, 200 $\mu$ mol of dNTP, 1.2mmol of MgCl<sub>2</sub> and 1 unit of Taq polymerase in a total volume of 20 $\mu$ l. PCR conditions is of initial denaturation at 95°C for 5minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute and extension at 72°C for 1.30minute. It is further followed by final extension at 72°C for 10 minutes. A total of 10 $\mu$ l of the resulting PCR product was digested with NcoI for 10 minutes at 37°C, and the resulting fragments were analyzed on 3% agarose gel.

### Statistical analysis

Data were expressed as proportion or mean+SD as appropriate. For comparison between groups, Student unpaired t test and One-Way analysis of variance (ANOVA) was performed. P value less than 0.05 is considered as statistically significant.

### Results

Among three hundred and eleven recruited patients, 156(50.16%) were female and 155(49.83%) were male with a mean SD age of 35.60 $\pm$ 11.774 years. Per-operatively the mean amount of blood loss observed was 80.87 $\pm$ 77.15ml. and the mean duration of surgery (incision to closure) was 1.98 $\pm$ 1.40hour.

### Allele frequency and genotype distribution

The overall allele frequency was 0.81 for G and 0.19 for A. In overall TNF- genotype, 68.81%(n=214) patients were homozygous dominant GG, 24.43%(n=76) were heterozygous GA and 6.75%(n=21) were homozygous recessive AA. Patients distributed between the genotype subgroups were compared in terms of parameters like age, gender, length of surgery and blood loss. Insignificant difference for these parameters was observed between the genotype subgroup (p>0.05) (Table 1).

**Table 1** General characteristic and cytokine levels in LPS stimulated postoperative blood in relation to TNF- $\alpha$ -308 G/A polymorphism

	GG (n=214)	GA (n=76)	AA (n=21)	P value
Age (Years)	35.19+10.84	36.72+12.03	33.62+11.53	0.43
Gender (Female/Male)	105/109	39/37	13/8	0.52
blood loss (ml)	81.35+79.52	95.53+79.43	78.57+75.05	0.38
Length of surgery (hour)	1.92+0.66	2.20+2.40	2.14+0.77	0.25
TNF- $\alpha$	208.12 $\pm$ 80.93	232.02 $\pm$ 62.82	308.19 $\pm$ 46.90	<0.0001*
IL-1 $\beta$	57.49 $\pm$ 25.40	60.23 $\pm$ 28.11	70.12 $\pm$ 46.0	0.10
IL-6	205.43 $\pm$ 93.8	245.26 $\pm$ 108.42	326.48 $\pm$ 84.20	<0.0001*
IL-8	212.36 $\pm$ 113.43	232.42 $\pm$ 98.40	252.60 $\pm$ 83.5	0.24
IL-10	113.46 $\pm$ 79.6	122.42 $\pm$ 49.92	156.82 $\pm$ 53.4	0.16

Values are given as frequency and mean $\pm$ SD. Differences were tested by using Chi-square test and ANOVA. (\*, p value<0.05 is considered as statistically significant).

## Cytokine levels in LPS stimulated postoperative blood sample of recruited patients in relation to TNF- $\alpha$ -308 G/A polymorphism:

To study the influence of TNF- $\alpha$ -308 G/A polymorphism on cytokine levels of LPS stimulated postoperative blood, we grouped the patients according to their genotypes and compared TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and IL-10 cytokine levels. Significantly higher level of TNF- $\alpha$  ( $p=0.0001$ ) and IL-6 ( $p<0.0001$ ) cytokine was observed in AA homozygous genotype subgroup as compared to other genotypes, whereas no such significant difference in level of other studied cytokines (IL-1 $\beta$ , IL-8 and IL-10) was observed between the genotype subgroups (Table 1).

## Discussion

In patients undergoing elective major surgery, cytokine profiles have previously been proposed as prognostic factors.<sup>25,26</sup> TNF- $\alpha$  secretions show a high degree of inter-individual variability, which is at least partly genetically determined.<sup>27</sup>

This study has focused to explore the association of TNF- $\alpha$  polymorphism with postoperative sepsis development through the postoperative levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and IL-10 cytokines after LPS stimulation. In contrast to certain studies,<sup>14,16</sup> our data showed significantly higher TNF- $\alpha$  cytokine response in LPS stimulated postoperative blood of patients with homozygous AA genotype. Similar results were obtained in Chinese Han population.<sup>28,29</sup> Mira et al. in 1999 reported an association of TNF- $\alpha$ -(308A) SNP and high plasma TNF levels in patients with septic shock.<sup>12</sup> These findings suggest the crucial role of AA genotype in mediating inflammatory response. Dissimilar to one report,<sup>30</sup> our results indicate that AA homozygous genotype is associated with higher IL-6 cytokine production in LPS stimulated blood of patients after surgical trauma. These findings of increased concentration of TNF- $\alpha$  and IL-6 cytokines in response to inflammatory stimuli to postoperative blood are of great significance in potentially forecasting the outcome of septic complications; this has been shown to be associated with TNF- $\alpha$ -308 polymorphism. Additionally, insignificant association and lower production of IL-8 and IL-10 cytokines in response to inflammatory stimuli might be due to their anti-inflammatory property.

A possibility that variation in cytokine levels might be related to surgery or the patient cannot be ruled out. In this study, to minimize surgeon or patient-related variability, surgeons with similar proficiency operated the patients and as the first case of elective operating room. Other patient factors such as age, gender, length of surgery and blood loss is insignificantly different between the genotype subgroup representing no influence on the cytokine levels after postoperative inflammatory stimuli. Though various studies indicate that preexisting patient related factors (e.g., advanced age, male gender) may influence the postoperative complications<sup>31–37</sup> these findings are inconsistent. To the best of our knowledge, this is the first study in a unique cohort that evaluated the association of TNF- $\alpha$ -308 gene polymorphism with cytokine levels following inflammatory stimuli to postoperative blood of patients after major gastro-intestinal surgery.

A limitation of current study is that the influence of type of surgical procedure with TNF- $\alpha$ -308 G/A polymorphism on cytokine level in response to inflammatory stimuli could not be established due to small number of patients in each type of surgery, demanding a study with a larger cohort for exact evidence. But it cannot be unnoticed that in the overall studied patients, homozygous AA genotypes showed

significant increased postoperative level of inflammatory cytokine after LPS stimulation indicating association with postoperative sepsis outcome. Our study suggests that an early identification of patients at high risk of developing postoperative sepsis can be done with the use of easy-to-collect markers. These markers represent an inexpensive way to detect the high-risk patients in whom an early aggressive goal therapy may be evaluated in future studies.

## Conclusion

Our results indicate significant association of TNF- $\alpha$ -308 G/A polymorphisms with increased TNF- $\alpha$  and IL-6 cytokine expression after LPS stimulation to postoperative blood indicating association with septic complication. If this data are supported and confirmed through further pathway studies, it has major implications for the pathogenesis of sepsis and for identifying patients at risk for developing sepsis following elective major surgery.

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## Conflict of interest

The author declares no conflict of interest.

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