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# **Assessing tumor necrosis factor- $\alpha$ (-238 G/A and -308 G/A) genetic polymorphisms in sepsis susceptibility among cystic fibrosis patients**

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**Conflict of interest:** The authors declare that they have no competing interests.

**Ethics approval and consent to participate:** approval was sought and granted by the Institutional Review Board (IRB) at Al-Nahrain University College of Medicine. The ethics approval number is IRB/38/0380.

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**Patient consent for publication:** prior to participation, each patient completed a consent form granting approval for their involvement in the study and the subsequent publication of the findings.

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## Abstract

Patients with cystic fibrosis are prone to recurrent bacterial infections, which eventually can cause critical sepsis and septic shock. Polymorphisms in tumor necrosis factor- $\alpha$  (*TNF- $\alpha$* ), as a pro-inflammatory cytokine, are implicated in its circulating levels and sepsis risk and development, particularly 308 G/A and -238 G/A. This investigation aims to assess the *TNF- $\alpha$*  (-238 G/A and -308 G/A) polymorphisms and sepsis risk in cystic fibrosis. A total of 120 cystic fibrosis patients were categorized into two groups: the septic and non-septic groups. Blood samples were harvested from participants, and DNA was extracted. The frequency of -238 G/A and -308 G/A *TNF* gene polymorphism was determined by polymerase chain reaction–restriction fragment length polymorphism. This investigation reported that heterozygous (GA) genotypes in the -238 G/A were more common among septic patients, 23 (38.33%), than non-septic patients, 13 (21.66%), and the same results were observed in -308 G/A; septic patients were 30 (50%), compared to non-septic patients, 13 (21.66%). Moreover, at the allelic level, the altered allele (A allele) was more commonly found in sepsis cystic fibrosis patients than in non-sepsis patients, with significant differences in both -308 G/A and -238 G/A. Our study concludes that *TNF- $\alpha$* -238 and -308 (GA) polymorphism, as well as mutant (AA) genotypes, may be linked with a higher likelihood of developing sepsis in cystic fibrosis patients relative to those with the GG (wild-type) genotype.

## Introduction

Cystic fibrosis (CF) is a life-threatening, multisystem genetic disorder caused by mutations in the gene responsible for encoding the cystic fibrosis transmembrane conductance regulator (CFTR) proteins. These mutations lead to impaired chloride (Cl) and sodium (Na) ion transport, resulting in abnormally high concentrations of these ions in sweat. The condition is marked by a range of clinical symptoms, including progressive lung disease, increased sweat electrolytes, male infertility, and pancreatic dysfunction.<sup>1</sup>

The most important point that characterizes the course of this illness is repetitive bacterial infection, particularly lung infection, which subsequently affects the development of sepsis and septic shock problems that compromise the patient's quality of life.<sup>2</sup>

Sepsis is a critical organ dysfunction provoked by a host's dysregulation of infection. At present, aside from life support therapy, the eventual of sepsis is so restricted that it is a great cause of death in the intensive care unit.<sup>3</sup> Individuals who are suffering from a chronic debilitating disease, such as chronic obstructive pulmonary disease, distinct types of cancer, and CF, are more prone to developing severe sepsis and septic shock.<sup>4</sup>

Genetic factors related to the host, such as polymorphisms in genes responsible for cytokines and other mediators implicated in innate immunity, coagulation, and fibrinolysis, likely contribute to the occurrence and outcome of sepsis. One of these cytokines that has a pivotal role and has attracted more attention is tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ).<sup>5</sup>

TNF- $\alpha$ , a pro-inflammatory cytokine predominantly released by macrophages, particularly neutrophils, is essential for immune function and maintaining cellular homeostasis.<sup>6</sup> The important role TNF plays in the pathology of the inflammatory response has suggested that genetics may influence its circulating levels as well as the risk and evolution of sepsis.<sup>7</sup>

Several studies discuss the correlation and relationships between polymorphisms in *TNF- $\alpha$*  and susceptibility to sepsis development, in particular 308 G/A and -238 G/A. The results in these studies were conflicting. Some results indicated a relationship between these single-nucleotide polymorphisms (SNPs) in this gene with septicemia, while others were inconsistent.<sup>8-10</sup> This study strives to assess *TNF- $\alpha$*  (-308 G/A and -238G/A) genetic polymorphisms in sepsis susceptibility among CF patients.

## Materials and Methods

From February 2022 to February 2024, blood samples were collected from 120 CF patients as part of a cross-sectional study conducted at Al-Imammain Al-Kadhmain Teaching Hospital. This hospital is equipped with a specialized unit dedicated to the care and treatment of CF patients. While the majority of the participants were from Baghdad, the study also included patients from other governorates.

Prior to enrollment, the objectives of the study were thoroughly explained to all patients or their guardians, and informed consent was obtained. The Institutional Review Board of the College of Medicine at Al-Nahrain University granted ethical approval for the research protocol.

The project was carried out in collaboration with the Microbiology Department at the same institution, where all samples were processed and analyzed. All participants had previously been diagnosed with CF through a positive sweat test and confirmed by molecular testing for mutations in the *CFTR* gene.

## Patients' classification

Patients were classified into two groups: i) septic patients' group; ii) non-septic group.

The septic group included 60 CF individuals. The criteria for including participants in this group were based on the Quick SOFA – a simple tool used to quickly identify patients at high risk of sepsis outside the intensive care unit.<sup>11</sup> At the bedside, all septic patients selected for this group were blood culture positive.

The non-septic group included 60 CF individuals with no signs or symptoms of sepsis. Moreover, their blood cultures showed no growth.

### **Exclusion criteria**

1. Pregnant CF patients, as hormonal and physiological changes may affect immune response and sepsis susceptibility.
2. CF patients in the non-sepsis group with a history of acute severe infection (e.g., pneumonia, exacerbations requiring hospitalization) within the last 4 weeks.
3. Recent use of immunosuppressive drugs, chemotherapy, or systemic corticosteroids (within the last 3 months) that might alter immune responses.

### **DNA extraction and genotyping**

Venous blood samples were used to retrieve genomic DNA using the Quick protocol SYNC<sup>TM</sup> DNA extraction kit (Geneaid, New Taipei City, Taiwan).

ensuring high purity. Genotyping was performed to detect SNPs at the -238 G/A and -308 G/A sites in the TNF- $\alpha$  promoter. A specific pair of primers was utilized. The polymerase chain reaction-restriction fragment length polymorphism approach was applied, following the protocol described by Zidi *et al.*,<sup>12</sup> as shown in Table 1.

The PCR conditions for this reaction were as follows: 94°C for 4 minutes, followed by 35 cycles at 94°C for 30 seconds, 61°C for TNF- $\alpha$ -238, 63°C for TNF- $\alpha$ -308 for 30 seconds, and 72°C for 45 seconds, cutoff at 72°C for 5 min. Five  $\mu$ L of PCR product was subjected to 3% agarose gel electrophoresis with Red Safe<sup>TM</sup> Nucleic Acid Staining Solution (0.5  $\mu$ g/ mL) (Intron, Seongnam-si City, South Korea). Amplicons can be seen by using an ultraviolet transilluminator; after that, photographs were taken via a camera. DNA markers (100-1500 bp) were obtained from Promega (Madison, USA).

### **Statistical analyses**

Statistical analyses were conducted using SPSS v25. Significance was set at  $p < 0.05$ . Categorical variables were summarized as frequencies and percentages. Genotype and allele distributions were assessed using Chi-square tests. Hardy-Weinberg equilibrium (HWE) was evaluated, and logistic regression was used to estimate odds ratios (OR) with 95% confidence intervals (CI).

## **Results**

### **Tumor necrosis factor- $\alpha$ -238 polymorphism**

Based on the enzymatic digestion pattern visualized in gel electrophoresis (Figure 1), TNF- $\alpha$ -238 gene polymorphism appeared in three genotypes: GG, AG, and AA. A comparison of genotypes and allele frequencies between both patient groups revealed that the wild-type homozygous genotype (GG) was more common among non-septic CF patients [47(78.33%)] than septic patients [35(58.33%)].

In contrast, heterozygous (GA) genotypes were more common among septic patients [23 (38.33%)] than non-septic [13 (21.66%)]. Moreover, the mutant homozygous genotype (AA) was detected only in two sepsis CF patients [2(3.33%)], with statistically significant differences (OR=3.108, 95% CI=1.043-9.264,  $p=0.038$ ). The  $p$ -value of HWE was 0.745 in the septic group and 0.642 in the non-septic group, both greater than 0.05, pointing out no significant deviation from expected genotype frequencies.

At the allelic level, the mutant allele (A allele) was more common in sepsis CF patients [27 (22.5%)] than in non-sepsis [13 (10.83%)], with significant differences (OR=2.608, 0.939-7.249,  $p=0.005$ ) (Table 2).

### ***Tumor necrosis factor- $\alpha$ -308 polymorphism***

According to the enzymatic digestion pattern, *TNF- $\alpha$ -308* gene polymorphism appeared in three genotypes: GG, AG, and AA. (Figure 2), A comparison of genotypes and allele frequencies in both groups of patients revealed that the wild-type homozygous genotype (GG) was more common among non-septic CF patients, [47 (78.33%)], than Septic patients [26 (43.33%)].

In addition, the heterozygous (GA) genotype was more common among septic patients [30 (50%)] than non-septic [13 (21.66%)]. Moreover, the mutant homozygous genotype (AA) was detected in four sepsis CF patients [4 (6.66%)] with statistically significant differences (OR=3.108, 95% CI=1.043-9.264,  $p=0.002$ ). The  $p$ -value of HWE in the sepsis group was 0.109 and the non-sepsis group was 0.361, both greater than 0.05, indicating no significant deviation from expected genotype frequencies.

At the allelic level, the mutant allele (A allele) was more common in sepsis CF patients [38 (31.66%)] than in non-sepsis [13 (10.8%)], with statistically significant differences (OR=2.608, 95% CI=0.939-7.249,  $p=0.005$ ) (Table 3).

### **Discussion and Conclusions**

The development of CF is linked to a defect in the gene that encodes the CFTR protein. This condition impacts multiple organ systems, with lung disease and bacterial infections being the primary contributors to morbidity and mortality. Bacterial infections, in particular, pose a significant systemic threat as they can enter the bloodstream, leading to severe complications.<sup>13</sup>

Sepsis has been a major challenge in patients suffering from chronic debilitated disease, particularly those with CF, which has consequently increased morbidity and mortality rates.<sup>13</sup> Many studies support the correlation between gene polymorphisms and the occurrence of sepsis.<sup>14,15</sup> One of the key mediators of the inflammatory response that plays a critical role in sepsis is TNF; excessive release of TNF- $\alpha$  leads to systemic inflammation with poor outcomes in sepsis patients.<sup>16</sup>

For this reason, this study explored the correlation of *TNF- $\alpha$*  gene 238 and 308 polymorphism with sepsis susceptibility in CF patients.

This study was conducted with some limitations. The study notes that the results may not be as broadly applicable due to the small sample size of 120 CF patients (60 septic and 60 non-septic). The specific nature of CF makes it difficult to enroll patients; the study is self-funded, and participant clinical severity varies. Furthermore, the results may not be as applicable to other ethnic groups due to the concentration on a single ethnic population, which is primarily from Baghdad.

Our study reported that the wild-type homozygous genotype (GG) was more common among non-septic CF patients in both *TNF- $\alpha$*  -238 and -308 polymorphisms, compared to septic CF patients. Some studies have denoted an association between TNF- $\alpha$  gene polymorphisms at positions -238 and -308 and susceptibility to sepsis. Hao Wang *et al* investigated that the GG genotype was more prevalent among the control group than among septic patients, which may indicate a protective effect against sepsis.<sup>17</sup> On the other hand, Georgescu *et al.* reported that GG homozygosity was correlated with elevated TNF- $\alpha$  levels in septic patients.<sup>18</sup>

The disagreement results on the sepsis susceptibility regarding the GG genotype of *TNF- $\alpha$*  -308 and -238 polymorphisms can be explained by several factors, such as the complex role of TNF- $\alpha$  in immune response regulation, ethnic variations, study design, and sample size.

Our findings showed that heterozygous (GA) genotypes on -238 polymorphisms were more common among Septic patients (38.33%) than non-septic (21.66%) patients.

In line with this result, heterozygous (GA) genotypes were also more common among septic patients (50%) than non-septic (21.66%) on the *TNF- $\alpha$ -308* gene polymorphism, which indicates that the heterozygous genotype (GA) might have an increased risk of developing sepsis in CF patients compared to those with the GG (wild-type) genotype.

Many studies declare that a heterozygous genotype (GA) in the *TNF-α* gene is associated with an increased risk of sepsis. A possible explanation for such results is that genotype (GA) may be connected to the immune regulation of many cytokines, particularly *TNF-α*, interleukin (IL)-6, IL-10, or may affect pattern recognition molecules such as (NOD2, TLR2, and TLR4), which at the last events alter the host response to infection.<sup>19,20</sup>

Furthermore, the HWE related to heterozygous (GA) genotypes on -238 polymorphisms in the sepsis group was 0.066, which is greater than 0.05. However, in the non-sepsis group (HWE was 0.618) highlighted that GA genotypes at the -238 polymorphisms distribution appear normal.

This result may indicate that genotype frequencies might be slightly deviating from HWE, possibly due to environmental factors such as age, hospital setting, treatment protocol, and CF severity related to sepsis susceptibility. Apart from that, the p-value of HWE on heterozygous (GA) genotypes of -308 showed no significant deviation from expected genotype frequencies, suggesting that these polymorphisms are not under selective pressure and that genetic drift does not appear to affect our study groups.

Our result aligns with a study by Song Z *et al.*, who reported that the -308G/A polymorphism enhanced the risk for severe sepsis, but its genotype distribution remained consistent with HWE speculation.<sup>21</sup>

Moreover, the mutant homozygous genotype (AA) of -238 was detected in two sepsis CF patients (3.33%). The result was statistically significant with an OR of 3.108 and a 95% CI of 1.043-9.264, suggesting an increased likelihood of the AA genotype being associated with sepsis in CF patients.

While analysis of genotype using HWE revealed that the sepsis group does not significantly deviate from HWE (0.066), it is close to the p-value threshold (0.05). However, in the non-sepsis group, the result was the opposite (0.618), which indicates that the frequency of the (AA) genotype has no evidence of population stratification, selection, or genotyping errors because the result is in line with HWE.

On top of that, the mutant homozygous genotype (AA) of -308 was recognized in 4 (6.66%) sepsis CF patients with an OR higher than three times that of the non-sepsis group. The p-value for HWE in these patients did not show significant deviation, which supports the observed association of the AA genotype with an increased likelihood of developing sepsis in CF patients.

Our results are in line with some studies that reported that individuals with the AA genotype presented with higher *TNF-α* levels, leading to a hyperinflammatory state.<sup>22,23</sup>

Our results suggested that the A allele is strongly associated with an increased risk of developing sepsis in CF patients because it was reported that the mutant allele (A) was more common in sepsis CF patients than non-sepsis with statistically significant differences (OR=2.608, 95% CI=0.939-7.249, p=0.005), which is in agreement with other studies.<sup>24,25</sup>

This study concludes that *TNF-α*-238 and -308 (GA) polymorphism, as well as the mutant (AA) genotypes, may be linked to risen risk of developing sepsis in CF patients compared to those with the GG (wild-type) genotype. Notably, the presence of the A allele appears to be strongly associated with an increased susceptibility to sepsis in CF patients.

However, these results implications may be overstated due to statistical power is limited by the small sample size, particularly for rare genotypes (*e.g.*, -238 AA); potential confounders like the severity of the CFTR mutation, previous infections, and treatments were not taken into account; and there is no mechanistic evidence to support a causal relationship in this study.

Future research should: i) include larger, multi-center cohorts with diverse populations; ii) use functional assays, like *TNF-α* expression analyses, to establish genotype–phenotype correlations; and iii) do power calculations to ensure sufficient sample sizes for detecting significant effect sizes in order to validate and strengthen these findings.

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**Table 1. Genotyping conditions and primer sequences for the found of TNF- $\alpha$  gene SNP.**

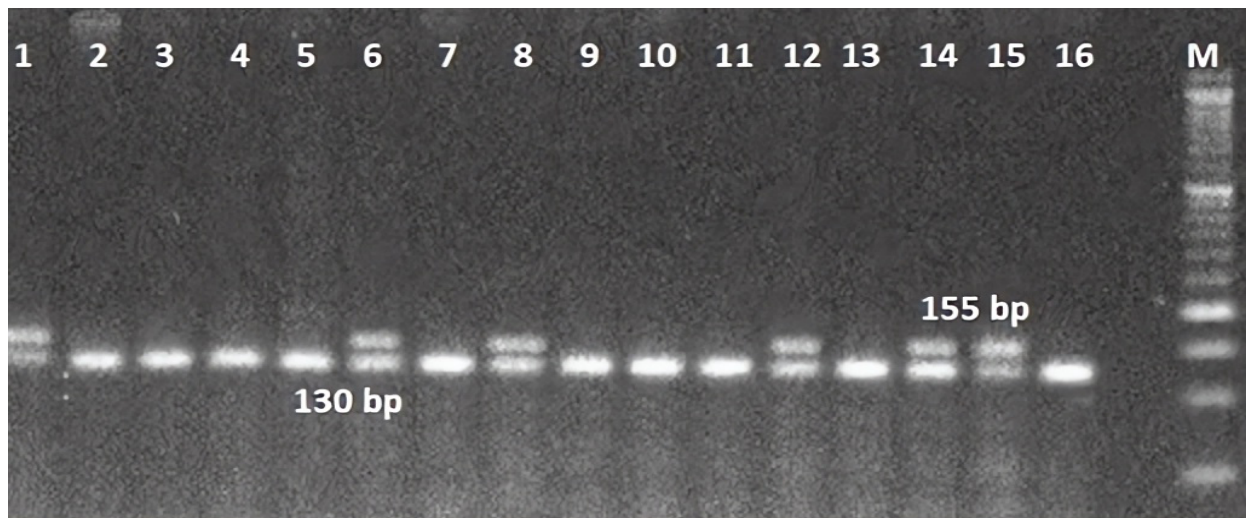
| SNP Locus | Primers (5'→3')                           | Ta (°C) | Restriction enzyme | Product size (bp)  |
|-----------|-------------------------------------------|---------|--------------------|--------------------|
| -308      | <b>F:</b> GAGGCAATAGGTTTTGAGGGCCAT        | 63      | <i>NcoI</i>        | <b>A:</b> 147      |
| G/A       | <b>R:</b> GGGACACACAAGCATCAAG             |         |                    | <b>G:</b> 126 + 21 |
| -238      | <b>F:</b> 5'-AAACAGACCACAGACCTGGTC-3'     | 61      | <i>BamHI</i>       | <b>A:</b> 155      |
| G/A       | <b>R:</b> 5'-CTCACACTCCCCATCCTCCCGGATC-3' |         |                    | <b>G:</b> 130 + 25 |

**Table 2. Allocation of genotypes and alleles for the TNF- $\alpha$ -238 polymorphism in cystic fibrosis patients, categorized into sepsis and non-sepsis groups.**

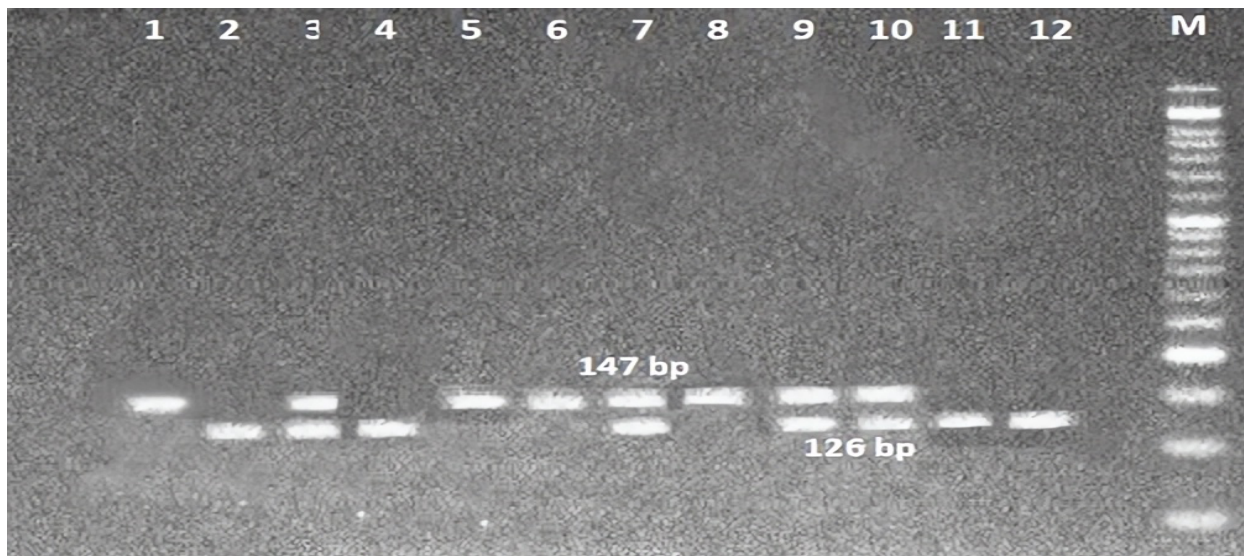
| TNF- $\alpha$ -238 | Sepsis (n=60) | Non-sepsis (n=60) | p-value | OR (95% CI)         |
|--------------------|---------------|-------------------|---------|---------------------|
| Genotypes          |               |                   |         |                     |
| GG                 | 35 (58.33%)   | 47 (78.33%)       | 0.038   | 1.0 (Reference)     |
| GA                 | 23 (38.33%)   | 13 (21.66%)       | —       | 3.108 (1.043-9.264) |
| AA                 | 2 (3.33%)     | 0 (0%)            | —       | —                   |
| HWE                | 0.745         | 0.642             | —       | —                   |
| Alleles            |               |                   |         |                     |
| G                  | 93 (77.5%)    | 107 (89.16%)      | 0.005   | 1.0 (Reference)     |
| A                  | 27 (22.5%)    | 13 (10.83%)       | —       | 2.608 (0.939–7.249) |

**Table 3. Genotypes and allele frequencies of TNF- $\alpha$ -308 polymorphism in sepsis and non-sepsis cystic fibrosis patients.**

| TNF- $\alpha$ -308 | Sepsis (n=60) | Non-sepsis (n=60) | p-value | OR (95% CI)         |
|--------------------|---------------|-------------------|---------|---------------------|
| Genotypes          |               |                   |         |                     |
| GG                 | 26 (43.33%)   | 47 (78.33%)       | 0.002   | 1.0 (Reference)     |
| GA                 | 30 (50.00%)   | 13 (21.66%)       | —       | 3.108 (1.043–9.264) |
| AA                 | 4 (6.66%)     | 0 (0%)            | —       | —                   |
| HWE                | 0.109         | 0.361             | —       | —                   |
| Alleles            |               |                   |         |                     |
| G                  | 82 (68.33%)   | 107 (89.16%)      | 0.005   | 1.0 (Reference)     |
| A                  | 38 (31.66%)   | 13 (10.83%)       | —       | 2.608 (0.939–7.249) |



**Figure 1.** Gel electrophoresis for TNF- $\alpha$ -238 PCR yields, viewed below UV light after staining with Red Safe Stain. The marker lane (M) corresponds to a 50-1000 bp DNA ladder. 3, 5, 7, 9, 10, 11, 13, and 16 represent the homozygous genotype (GG), while 1, 6, 8, 12, 14, and 15 indicate the heterozygous genotype (GA). Lanes 2 and 4 show the mutant genotype.



**Figure 2.** TNF- $\alpha$ -308 PCR fragment according to UV light, subsequently stained with Red safe Staining. M: 50- 1000bp ladder; lanes 2,4,11, and 12: homozygous genotype (GG). 3,7,9, and 10 heterozygous genotypes (GA). Lane 1,5,6, and 8 mutant types.