

# Tumor necrosis factor- $\alpha$ and - $\beta$ genetic polymorphisms as a risk factor in Saudi patients with schizophrenia

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**Background:** Schizophrenia is one of the most common devastating psychiatric disorders that negatively affects the quality of life and psychosocial functions. Its etiology involves the interplay of complex polygenic influences and environmental risk factors. Inflammatory markers are well-known etiological factors for psychiatric disorders, including schizophrenia.

**Objective:** The aim of this study was to investigate the association of proinflammatory cytokine genes, tumor necrosis factor (TNF)- $\alpha$  (-308G/A) and TNF- $\beta$  (+252A/G) polymorphisms with schizophrenia susceptibility.

**Subjects and methods:** *TNF- $\alpha$*  and *TNF- $\beta$*  genes were amplified using amplification refractory mutation system primers in 180 schizophrenia patients and 200 healthy matched controls recruited from the Psychiatry Clinic of Prince Sultan Military Medical City, Riyadh. The frequencies of alleles and genotypes of TNF- $\alpha$  (-308G/A) and TNF- $\beta$  (+252A/G) polymorphisms in patients were compared with those in controls.

**Results:** The frequencies of TNF- $\alpha$  (-308) allele A and genotype GA were significantly higher, while those of allele G and genotype GG were lower in schizophrenia patients as compared to controls, indicating that genotype GA and allele A of TNF- $\alpha$  (-308G/A) may increase susceptibility to schizophrenia, while genotype GG and allele G may reduce it. On the other hand, the distribution of alleles and genotypes of TNF- $\beta$  (+252A/G) polymorphism does not differ significantly in patients from controls; however, the frequency of genotype GG of TNF- $\beta$  (+252A/G) was significantly higher in male patients than in female patients. The distribution of TNF- $\alpha$  (-308G/A) and TNF- $\beta$  (+252A/G) polymorphisms was almost similar in schizophrenia patients with negative or positive symptoms.

**Conclusion:** TNF- $\alpha$  (-308G/A) and TNF- $\beta$  (+252G/A) polymorphisms may increase the susceptibility to schizophrenia in Saudi patients and could be a potential risk factor for its etiopathogenesis. However, further studies are warranted involving a larger sample size to strengthen our findings.

**Keywords:** schizophrenia, tumor necrosis factor, gene polymorphism, genetics, psychiatric disorder

## Introduction

Schizophrenia is one of the most common devastating psychiatric disorders with a lifetime morbidity risk of 0.5%–2.7% and heritability estimated at up to 80%.<sup>1–3</sup> The genetic, neurodevelopmental, neurotransmitter, and neuroimmunological hypotheses have been suggested to explain the etiopathogenesis of schizophrenia.<sup>4,5</sup> It involves the interplay of complex polygenic influences and environmental risk factors operating on brain maturational processes.

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Multiple genes have been associated with the development of schizophrenia. Recent studies have confirmed the association of a large number of single-nucleotide polymorphisms in a variety of genes with schizophrenia susceptibility.<sup>6–11</sup> Genes encoding cytokines seem to be good candidate genes for schizophrenia. Cytokines play an important role in the central nervous system (CNS) as essential mediators of crosstalk between the brain and immune system and regulate neuroinflammatory processes.<sup>12</sup> Some cytokines are normally produced in the healthy brain, where they play critical roles in neurogenesis, migration, differentiation, and synapse formation.<sup>13–15</sup> The concentrations of these cytokines have been reported to be increased or decreased in patients with schizophrenia.<sup>16</sup> Moreover, the concentrations of the cytokines in the blood serum of schizophrenia patients may vary depending on whether the patient is in active or resting phase of the disease.<sup>17</sup> It has also been suggested that schizophrenia may be associated with alterations in the Th1/Th2 cytokine ratios, with a shift toward the Th2 system.<sup>4,18,19</sup>

Cytokines are the key regulators of immune/inflammatory reactions and influence the dopaminergic, noradrenergic, and serotonergic neurotransmission.<sup>20</sup> Tumor necrosis factor (TNF)- $\alpha$ , a proinflammatory cytokine, mediates immune and inflammatory responses and plays a key role in the CNS. It is not only actively transported into the CNS but also released from activated glia cells.

TNF- $\alpha$  (MIM 191160) and TNF- $\beta$  or lymphotoxin-alpha (LT- $\alpha$ ; MIM 153440) are closely related cytokines that share 30% amino acid residues and use the same cell surface.<sup>21</sup> Nedwin et al<sup>22</sup> reported that the *TNF- $\alpha$*  and *TNF- $\beta$*  genes are located in tandem on chromosome 6 between the Class I and Class II cluster of the major histocompatibility complex chromosome (6p21.1–6p21.3) and show close linkage to the genes for human leukocyte antigen (HLA) classes I (HLA-B) and II (HLA-DR). The production of TNF- $\alpha$  is genetically determined.<sup>23</sup> Several polymorphisms in the promoter region of *TNF- $\alpha$*  and the intron 1 of *TNF- $\beta$*  have been associated with changes in the levels of circulating TNF- $\alpha$ .<sup>24</sup> TNF- $\alpha$  (–308G/A) promoter polymorphism (rs1800629) is one of the best described single-nucleotide polymorphisms at the nucleotide position –308, which affects a consensus sequence for a binding site of the transcription factor activator protein 2.<sup>25</sup> TNF- $\alpha$  (–308G/A) polymorphism leads to a less common allele A (2-allele), which has been associated with increased TNF- $\alpha$  production in vitro<sup>26</sup> and a higher rate of TNF- $\alpha$  transcription than that associated with the wild-type GG genotype.<sup>27</sup> Conversely, a polymorphism TNF- $\beta$  (+252A/G)

(rs909253) at the nucleotide position +252 within the first intron of TNF- $\beta$  gene affects a phorbol ester-responsive element. The presence of G at this position defines the mutant allele known as TNF- $\beta$  \* 1 (1-allele), which is associated with higher TNF- $\alpha$  and - $\beta$  production.<sup>28,29</sup> The functional polymorphisms in cytokine genes may result in imbalances in the pro- and anti-inflammatory cytokine production.<sup>11</sup> Keeping in view the potential role of these cytokines as the principal mediators of the immune response and the importance of the chromosomal region containing *TNF- $\alpha$*  and *TNF- $\beta$*  genes, the present study was undertaken to examine the association of TNF- $\alpha$  (–308G/A) and TNF- $\beta$  (+252G/A) polymorphisms with schizophrenia susceptibility in Saudi population. The concomitant analysis of polymorphisms at *TNF- $\alpha$*  and - $\beta$ , given that both are involved in the expression of TNF- $\alpha$ , will help in the development of better strategies for the prevention and treatment of schizophrenia.

## Subjects and methods

### Subjects

The study population consisted of a total of 380 Saudi subjects of either sex. The sample included 180 schizophrenia patients recruited from the Outpatient Psychiatric Clinic of Prince Sultan Military Medical City, Riyadh, Saudi Arabia, and 200 age- and sex-matched healthy subjects as controls. All subjects were biologically unrelated Saudis. To ensure the diagnostic reliability, a systemic search into the case notes of the patients was made. Out of 200 initially selected schizophrenia patients, 20 patients failed to meet the explicit stated criteria and hence were excluded, and only 180 patients were included in this study. Among the confirmed 180 cases of schizophrenia, there were 53 females and 127 males with a mean age of 39 $\pm$ 12.5 years and a mean disease duration of 9 $\pm$ 4.5 years. Age at the onset of disease ranged from 19 to 64 years (mean 29 $\pm$ 2.5 years). Among the confirmed cases of schizophrenia, 94 patients had negative symptoms, while 86 patients had positive symptoms. The female to male ratio of schizophrenia patients in our study was 53:127 (1:2.4). The control group consisted of 60 females and 140 males aged 26–60 years with a mean age of 35 $\pm$ 10.5 years. Power was calculated online (<http://www.stat.ubc.ca/~rollin/stats/ssize/caco.html>).

The diagnosis of schizophrenia was based on the criteria mentioned in the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision* version. The patients were further assessed for positive and negative symptoms using Positive and Negative Syndrome Scale as described by Kay et al.<sup>30</sup>

All subjects in the control group were screened and excluded if they had any history of neurological, psychiatric, or medical disorders or had a past or present involvement in substance abuse. Screening of controls was performed following Johnstone et al.<sup>31</sup> None of the control subjects had a first- or second-degree relative with any mental illness. This study was approved by the research and ethical committee of Prince Sultan Military Medical City (PSMMC). Written informed consent was obtained from all subjects in accordance with ethical guidelines set by the ethical committee.

## Polymerase chain reaction (PCR) amplification

Genomic DNA was extracted from the peripheral blood of Schizophrenia patients and controls using QIAamp<sup>®</sup> DNA Mini Kit (Qiagen, Valencia, CA, USA). TNF- $\alpha$  and TNF- $\beta$  genes were amplified using an amplification refractory mutation system-PCR methodology to detect any polymorphism involved at positions -308 of TNF- $\alpha$  and +252 in intron 1 of TNF- $\beta$  gene. PCR amplification was carried out using PuReTaq Ready-to-Go PCR Beads (GE Healthcare, Buckinghamshire, UK) as described elsewhere.<sup>32</sup> The molecular analysis of the samples was performed in the same laboratory and at the same time. The investigator was blind to the phenotype of the subjects at the time of molecular analysis. Later on, the results were separated for patient and control groups and analyzed for the determination of the frequencies of genotypes and alleles.

## Statistical analysis

The differences in allele/genotype frequencies between patients and controls were analyzed by the Fisher's exact test.  $P$ -values  $\leq 0.05$  were considered significant. Bonferroni correction was applied to minimize error due to multiple comparison test. Therefore, both the  $P$ -values, after Bonferroni correction and Fisher's exact test, are considered in the manuscript and Tables 1–3.

The odd ratio interpreted as relative risk (RR) was calculated following the Woolf's method as outlined by Schallreuter et al.<sup>33</sup> The etiologic fraction (EF) and preventive fraction (PF) were calculated following the formula of Svejgaard et al.<sup>34</sup> as described in our earlier publication.<sup>35</sup>

## Results

Among Schizophrenia patients, the male to female ratio was 127:53 (2.4:1). The number of controls per case was 1.11, which yielded a power of 95%. In this case-control study, the allele and genotype frequencies of both polymorphisms were determined in schizophrenia patients and unrelated matched controls. The results of the genotype and allele distributions of TNF- $\alpha$  (-308) and TNF- $\beta$  (+252A/G) polymorphisms in schizophrenia and control groups are summarized in Tables 1–4. The representative gel pictures for different genotypes are shown in Figures 1 and 2. Allelic frequencies and genotype distributions of both TNF- $\alpha$  and TNF- $\beta$  gene polymorphisms differ between schizophrenia patients and control subjects. The frequency of GA (-308) genotype was significantly higher ( $P \leq 0.0001$ ,  $P = 0.0005$ , Bonferroni corrected), while the frequency of GG (-308) genotype was lower in patients as compared to controls ( $P \leq 0.0001$ ,  $P = 0.0005$ , Bonferroni corrected). AA genotype was absent in the patients but present in 7% of the controls. The frequency of allele A was found to be significantly increased, while that of allele G was decreased in patients as compared to controls ( $P \leq 0.0001$ ). Even after applying Bonferroni correction, the  $P$ -values were statistically significant for both genotypes and alleles (Table 1).

The results of TNF- $\alpha$  (-308G/A) polymorphism for the patient group were then stratified into patients with negative or positive symptoms. The distribution of alleles and genotypes of TNF- $\alpha$  (-308G/A) polymorphism was found to be similar in the two groups of schizophrenia patients (data not shown).

Our study on TNF- $\beta$  gene polymorphism at position +252 of intron 1 showed that although the frequencies of genotypes

**Table 1** Genotype and allele frequencies of TNF- $\alpha$  (-308G/A) variants in schizophrenia patients and matched controls

Genotype/allele	Schizophrenia (n=180)		Control (n=200)		P-value <sup>a,b</sup>	RR	EF <sup>c</sup> /PF
	n	%	n	%			
GG	4	2.22	110	55	<0.0001	0.042	0.444
GA	176	97.78	76	38	<0.0001	71.789	0.688 <sup>c</sup>
AA	0	0.00	14	7	0.0001	–	–
G-allele (TNF- $\alpha$ 1-allele)	184	51.11	296	74	<0.0001	0.367	0.389
A-allele (TNF- $\alpha$ 2-allele)	176	48.89	104	26	<0.0001	2.722	0.397 <sup>c</sup>

**Notes:** <sup>a</sup>Statistically significant using Fisher's exact test. <sup>b</sup> $P = 0.0005$ , Bonferroni corrected. <sup>c</sup>Data for EF.

**Abbreviations:** TNF, tumor necrosis factor; n, number of subjects; RR, relative risk; EF, etiologic fraction; PF, preventive fraction.

**Table 2** Genotype and allele frequencies of TNF- $\beta$  (LT- $\alpha$ ) intron 1 +252 variants in Schizophrenia patients with negative or positive symptoms and matched controls

Genotype/ allele	With negative symptoms (n=94)		With positive symptoms (n=86)		Control (n=200)	
	n	%	n	%	n	%
GG	11	11.70	11	12.79	28	14
GA	79	84.04	65	75.58	148	74
AA	4	4.26 <sup>a,b</sup>	10	11.63	24	12
G-allele	101	53.72	87	50.58	204	51
A-allele	87	46.28	85	49.42	196	49

**Notes:** <sup>a</sup> $P=0.034$  as compared to controls (using Fisher's exact test). <sup>b</sup> $P=0.034$ , Bonferroni corrected.

**Abbreviations:** TNF, tumor necrosis factor; LT- $\alpha$ , lymphotoxin-alpha; n, number of subjects.

and alleles differed in patients from controls, the difference was not statistically significant (Table 4). Upon stratification of results of schizophrenia into patients with negative symptoms or with positive symptoms, the distribution of genotypes and alleles of TNF- $\beta$  (+252A/G) polymorphism retained almost the same pattern in the two groups of patients as was in the combined schizophrenia patients except that the frequency of genotype AA was significantly lower in patients with negative symptoms than in those with positive symptoms or controls (Table 2,  $P=0.034$ ). However, the sex difference was clear in the distribution of genotype frequencies of TNF- $\beta$  (+252A/G) polymorphism. The frequency of genotype GG was significantly higher in male patients than in female patients, while the reverse was found for genotype GA, being higher in female patients than in male patients (Table 3).

## Discussion

The higher frequencies of genotype GA and allele A of TNF- $\alpha$  (-308G/A) in schizophrenia patients as compared to controls indicated that the genotype GA and allele A may be susceptible to schizophrenia (RR=71.789, EF=0.688 and RR=2.722, EF=0.397, respectively). The genotype GG and

**Table 3** Genotype and allele frequencies of TNF- $\beta$  (-252A/G) polymorphism in male and female patients

Genotype/ allele	Female (n=53)		Male (n=127)		P-value	RR
	n	%	n	%		
GG	2	3.77	20	15.75	0.025 <sup>a,b</sup>	0.209
GA	49	92.46	95	74.80	0.007 <sup>a,c</sup>	4.126
AA	2	3.77	12	9.45	0.238	0.376
G-allele	53	50.00	135	53.15	0.643	0.881
A-allele	53	50.00	119	46.85	0.643	1.134

**Notes:** <sup>a</sup>Statistically significant using Fisher's exact test. <sup>b</sup> $P=0.05$ , Bonferroni corrected. <sup>c</sup> $P=0.01$ , Bonferroni corrected.

**Abbreviations:** TNF, tumor necrosis factor; n, number of subjects; RR, relative risk.

allele G being higher in controls than in patients show their protective nature for schizophrenia (RR=0.042, PF=0.444 and RR=0.367, PF=0.389, respectively). These results suggested that TNF- $\alpha$  (-308G/A) polymorphism may increase the susceptibility to schizophrenia in the Saudi population and support the possible role of the immune response system in the pathogenesis of schizophrenia as suggested earlier by various researchers.<sup>3,4</sup>

The results of the present study are in accordance with earlier reports indicating the association of allele A of TNF- $\alpha$  (-308G/A) in the etiology of schizophrenia in Italian,<sup>36,37</sup> Brazilian,<sup>38</sup> Singaporean,<sup>39</sup> Pakistani,<sup>40</sup> and Polish population.<sup>3</sup> On the other hand, some reports indicated the association of allele G of TNF- $\alpha$  (-308G/A) with schizophrenia in German,<sup>41</sup> Finnish male,<sup>42</sup> Australian,<sup>43</sup> and Polish populations.<sup>44</sup> Contrarily, several reports indicated no association of TNF- $\alpha$  (-308G/A) polymorphism with the susceptibility or pathogenesis of schizophrenia in the German,<sup>45</sup> Korean,<sup>46</sup> Australian, Indian Fijian, Indigenous Fijian, and Brahmin populations of India<sup>47</sup> and in Taiwanese,<sup>48</sup> Japanese,<sup>49,50</sup> Chinese Han,<sup>51</sup> Finnish,<sup>42,52</sup> American Caucasian,<sup>53</sup> and Canadian populations.<sup>54</sup> These variations in the association of TNF- $\alpha$  (-308G/A) polymorphism with schizophrenia in various ethnic populations may be due to ethnic differences in the distribution of this polymorphism worldwide. The distribution of TNF- $\alpha$  (-308G/A) polymorphism is not uniform in healthy populations, and the frequencies vary from 50 to 98, 1.96 to 40.5, and 0 to 11.9% for GG, GA, and AA genotypes, respectively, in different ethnic populations, showing ethnic variations.<sup>55</sup> However, the association of the TNF- $\alpha$  (-308G/A) promoter polymorphism with schizophrenia in the Saudi population and other ethnicities has complemented the clinical findings of increased levels of TNF- $\alpha$  in schizophrenic patients, with some support for a functional consequence of the variant as allele A has been associated with increased TNF- $\alpha$  production in vitro<sup>26</sup> and a higher rate of TNF- $\alpha$  transcription than the wild-type GG genotype.<sup>27</sup> Alterations in the serum TNF- $\alpha$  level of chronic schizophrenia patients have been reported previously,<sup>56-58</sup> implicating the role of TNF- $\alpha$  and TNF- $\alpha$ -related signaling pathways in the pathophysiology of schizophrenia. The roles of TNF- $\alpha$  in controlling neuronal excitability and metabolisms of glutamate, dopamine, and serotonin neurotransmitters have made it an outstanding candidate for etiology and pathophysiology of schizophrenia.<sup>59</sup> It has been suggested that TNF- $\alpha$  may be a trait marker of schizophrenia and may have an important role in the psychopathology of schizophrenia.<sup>60,61</sup> The increased level of TNF- $\alpha$  may increase

**Table 4** Genotype and allele frequencies of TNF- $\beta$  (LT- $\alpha$ ) intron I +252 variants in schizophrenia patients and matched controls

Genotype/allele	Schizophrenia (n=180)		Control (n=200)		P-value	RR	EF <sup>a</sup> /PF
	n	%	n	%			
GG	22	12.22	28	14	0.650	0.855	0.744
GA	144	80.00	148	74	0.181	1.405	0.142 <sup>a</sup>
AA	14	7.78	24	12	0.230	0.618	0.185
G-allele (TNF- $\beta$ 1-allele)	188	52.22	204	51	0.771	1.050	0.023 <sup>a</sup>
A-allele (TNF- $\beta$ 2-allele)	172	47.78	196	49	0.771	0.952	0.229

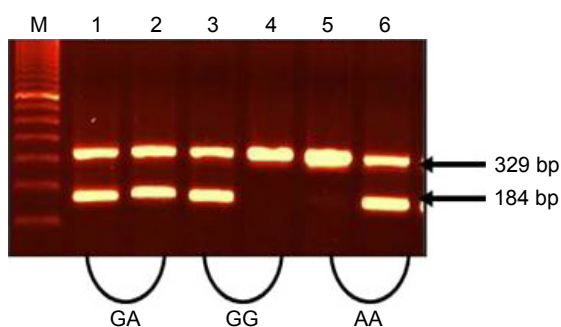
**Note:** <sup>a</sup>Data for EF.

**Abbreviations:** TNF, tumor necrosis factor; LT- $\alpha$ , lymphotoxin-alpha; n, number of subjects; RR, relative risk; EF, etiologic fraction; PF, preventive fraction.

the production of 3-hydroxykynurenine. The increase in ratio between neurotoxic 3-hydroxykynurenine and neuroinhibitory/neuroprotective kynurenic acid may account for altered neurogenesis and structural abnormalities characteristic of schizophrenia.<sup>62</sup> However, Morar et al<sup>43</sup> besides supporting the association with the TNF- $\alpha$  (-308G/A) promoter polymorphism emphasized a linkage to the major histocompatibility complex region where the TNF- $\alpha$  gene is located and suggested linkage disequilibrium rather than direct involvement in the disorder. Paul-Samojedny et al<sup>3</sup> investigated the combined impact of gene polymorphisms in three proinflammatory cytokines, namely, IL-2, IL-6, and TNF- $\alpha$ , on susceptibility to schizophrenia and hypothesized that IL-6 and TNF- $\alpha$  gene polymorphisms that contribute to changes in the cytokine levels may impair the immune response to infections and also affect the development of the normal brain. The precise neurobiological mechanism explaining these increased risks in relation to infections is not clear, although the role of cytokines and an impaired immune response to these infections during the critical period of brain development are documented.<sup>63</sup> Because of the fact that particular schizophrenia subtypes are characterized by different clinical pictures, it is reasonable to perform genetic

association studies on homogenous groups of patients. Therefore, we stratified schizophrenia patients into schizophrenia with positive symptoms and schizophrenia with negative symptoms. However, the distribution of alleles and genotypes was similar in the two groups.

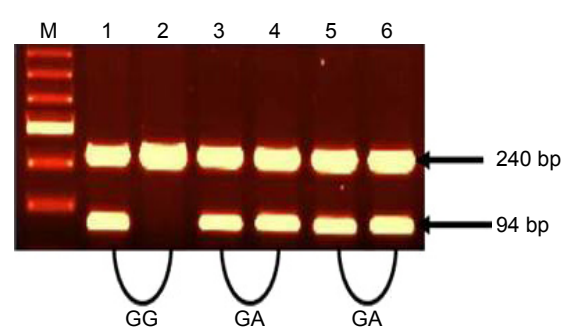
On the other hand, our results for another gene TNF- $\beta$  indicated that TNF- $\beta$  (+252A/G) polymorphism is not directly associated with schizophrenia in Saudi patients; however, the fact that this polymorphism might be in linkage disequilibrium with TNF- $\alpha$  or other gene located in the same region cannot be ruled out. The TNF- $\beta$  gene is known to play a central role in neurodevelopment, synaptic plasticity, and the response to neural injury.<sup>64</sup> Repeatedly associated with various brain activities and having immunologic, neurochemical, neuroendocrine, and behavioral effects, the TNF- $\beta$  gene has also been associated with symptoms of schizophrenia.<sup>65</sup> In contrast to studies of TNF- $\alpha$ , few reports are available on the association between TNF- $\beta$  gene polymorphisms and susceptibility to schizophrenia.<sup>41,66-68</sup> The immunomodulatory functions of TNF- $\beta$  is well known and could impact the pathophysiology of schizophrenia. It affects and modulates production of TNF- $\alpha$ , which is supposed to be involved in the development of schizophrenia. In addition, TNF- $\beta$  is



**Figure 1** Amplification of TNF- $\alpha$  (-308G/A) alleles (G and A).

**Notes:** Lane M: 100 bp DNA marker; lanes 1 and 3: amplification of allele G; lanes 2 and 6: amplification of allele A; 184 bp band for target DNA and 329 bp band for internal control.

**Abbreviation:** TNF, tumor necrosis factor.



**Figure 2** Amplification of TNF- $\beta$  (+252A/G) alleles (A and G).

**Notes:** Lane M: 100 bp DNA marker; lanes 1, 3, and 5: amplification of allele G; lanes 2, 4, and 6: amplification of allele A; 94 bp band for target DNA and 240 bp band for internal control.

**Abbreviation:** TNF, tumor necrosis factor.

effective in the protection of neuronal cells against glutamate and N-methyl-D-aspartate toxicity, which is considered a neurodevelopmental hypothesis of schizophrenia. Moreover, it is also found to be associated with the regulation of glial cells and stimulation of the synthesis and secretion of nerve growth factors in the CNS.<sup>69</sup>

Our results also showed significantly higher frequency of genotype GG of TNF- $\beta$  (+252A/G) in male patients as compared to female patients (Table 3). The higher frequency of genotype GG in male patients, which is known to be associated with increased production of TNF- $\alpha$ , might be responsible for the higher prevalence of schizophrenia in male subjects than in female subjects. As far as we know, this is the first study that examines the impact of TNF- $\alpha$  and TNF- $\beta$  gene polymorphisms among Saudi patients with schizophrenia. The main limitation of this study is the small sample size, while the strengths of this study lie in the fact that it is the first report on Saudi patients with Schizophrenia, showing association of polymorphisms in TNF- $\alpha$  and - $\beta$  genes. The errors in genotyping both cases and controls have been avoided carefully by using a standard protocol with positive and negative controls. Statistical analysis was performed to obtain *P*-values, RR, EF and PF, and power.

## Conclusion

We suggest that TNF- $\alpha$  (-308G/A) and TNF- $\beta$  (+252G/A) polymorphisms may be associated with schizophrenia susceptibility in Saudi patients and could be potential risk factors for its etiopathogenesis. However, further studies are required to strengthen these findings.

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## Disclosure

The authors report no conflicts of interest in this work.

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