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POLYMORPHISMS OF THE PRO-INFLAMMATORY CYTOKINE GENES IL6 (C174G) AND TNF-A (G308A) IN THE PATHOGENESIS OF DIABETIC FOOT SYNDROME IN PATIENTS WITH TYPE 2 DIABETES MELLITUS

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Abstract

Background. Chronic low-grade inflammation is a central mechanism of vascular complications in diabetes mellitus, and the pro-inflammatory cytokines interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) are key mediators of the impaired wound healing that characterizes the diabetic foot syndrome (DFS). Functional single-nucleotide polymorphisms in their genes may modify cytokine expression and disease susceptibility.

Aim. To determine the distribution of the IL6 C174G (rs1800795) and TNF-α G308A (rs1800629) polymorphisms in patients with DFS and controls, and to assess their association with DFS and with its neuropathic and neuroischemic forms.

Materials and methods. Both polymorphisms were genotyped by polymerase chain reaction in 96 patients with type 2 diabetes and DFS (35 neuropathic, 61 neuroischemic) and in 83 control subjects. Allele and genotype frequencies were compared using the χ^2 test and the odds ratio (OR) with 95 % confidence intervals (CI), and conformity to Hardy–Weinberg equilibrium was verified.

Results. Genotype distributions conformed to Hardy–Weinberg equilibrium. For IL6 C174G, the minor G allele and the heterozygous C/G genotype were more frequent in DFS patients than in controls (G allele 16.7 % vs 12.0 %, OR = 1.5; C/G 25.0 % vs 16.9 %, OR = 1.6), whereas the C allele and C/C genotype were protective. For TNF-α G308A, the minor A allele and the heterozygous G/A genotype increased DFS risk (A allele 9.9 % vs 5.4 %, OR = 1.9; G/A 19.8 % vs 10.8 %, OR = 2.0), and the association of the G/A genotype reached significance in the neuropathic form (OR = 2.8, 95 % CI 1.05–7.73). Carriage of the risk genotypes accompanied higher leukocyte counts and a higher leukocyte intoxication index.

Conclusion. The G allele of IL6 C174G and the A allele of TNF-α G308A, together with their heterozygous genotypes, are associated with susceptibility to DFS, supporting the involvement of genetically determined pro-inflammatory cytokine activity in the pathogenesis of the syndrome.

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Keywords: IL6; rs1800795; TNF-α; rs1800629; cytokine gene polymorphism; chronic inflammation; diabetic foot syndrome; type 2 diabetes mellitus.

1. Introduction

The diabetic foot syndrome (DFS) is one of the most disabling complications of diabetes mellitus and a leading cause of non-traumatic lower-limb amputation [1, 2]. Chronic, low-grade systemic inflammation is now recognized as a fundamental mechanism linking diabetes to its vascular and wound-healing complications [3]. In patients with diabetes the concentrations of pro-inflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-α (TNF-α), are elevated, and their sustained over-expression keeps the wound arrested in the inflammatory phase, impairing the transition to proliferation and repair [4, 5].

Under physiological conditions wound healing progresses through haemostasis, inflammation, proliferation and remodelling. In diabetes this orderly sequence is disrupted: persistent recruitment of inflammatory cells and continued secretion of IL-6, IL-8, IL-1 and TNF-α maintain a chronic inflammatory state that suppresses angiogenesis and granulation-tissue formation and disturbs re-epithelialization [4, 6]. The resulting non-healing ulcers are highly susceptible to infection and are the principal precursors of amputation [1]. Because the magnitude of the cytokine response is, in part, genetically determined, single-nucleotide polymorphisms (SNPs) in cytokine genes are plausible modifiers of DFS risk and severity [7].

The IL6 gene, located on chromosome 7p15.3, encodes a cytokine that acts in inflammation and B-cell maturation and behaves as an endogenous pyrogen; its dysregulation is implicated in the pathogenesis of type 2 diabetes and its complications [8]. The most frequently studied IL6 variant is rs1800795 (C174G) in the promoter region, which influences transcriptional activity and circulating IL-6 levels, although reports of its association with DFS have been inconsistent across populations [9, 10, 11]. The TNF-α gene, located on chromosome 6p21.33, encodes a multifunctional pro-inflammatory cytokine secreted chiefly by macrophages that participates in cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation. SNPs in TNF-α, principally rs1800629 (G308A), have been associated with a range of inflammatory disorders and with susceptibility to type 2 diabetes and its complications [1, 12, 13].

At the molecular level, IL-6 and TNF-α occupy central positions in the inflammatory network of the diabetic foot. Obesity and insulin resistance, frequent companions of type 2 diabetes, drive the secretion of IL-6, IL-1 and TNF-α from adipose tissue and from infiltrating immune cells, amplifying a self-sustaining inflammatory loop [3, 6]. On binding their receptors, these cytokines activate the JAK–STAT and NF-κB signalling pathways, which govern the transcription of further inflammatory mediators and of factors that regulate tissue remodelling. Persistent activation of these pathways in the diabetic wound favours infiltration by neutrophils, CD8 T-cells and macrophages and impairs the polarization of macrophages towards a reparative phenotype, thereby perpetuating chronic inflammation and delaying healing [4, 5]. The degree to which an individual mounts this response is influenced by regulatory polymorphisms in the cytokine genes, which provides

the rationale for examining IL6 C174G and TNF-α G308A as candidate modifiers of DFS [7].

Previous studies of these polymorphisms in DFS have produced divergent results, reflecting ethnic heterogeneity and differences in study design. A genetic association of IL-6 and TNF-α polymorphisms with serum cytokine levels in DFS was reported in an Indian cohort, in which the TNF-α rs1800629 variant increased DFS risk [9]; the same group later linked IL-6 and TNF-α polymorphisms to severe infection, ulcer grade and amputation [14]. In a Turkish population the IL6 rs1800795 G allele was a risk factor for diabetes but not an independent predictor of DFS [10], and a meta-analysis found no association of rs1800795 with microvascular complications overall, while noting substantial heterogeneity between the pooled studies [11]. These observations justify additional population-specific investigation. The present study therefore examined the IL6 C174G and TNF-α G308A polymorphisms in patients with DFS in Uzbekistan, assessing their association with the disease and with its clinical forms.

2. Materials and Methods

2.1. Participants. The study used a case-control design. The case group comprised 96 patients with type 2 diabetes mellitus and clinically established DFS examined at the clinics of the Andijan State Medical Institute and the Andijan Regional Endocrinology Dispensary between 2020 and 2022; 35 had the neuropathic form and 61 the neuroischemic form, classified according to International Working Group on the Diabetic Foot criteria. The control group comprised 83 subjects without clinical or anamnestic evidence of diabetes and without a family history of the disease. The groups were matched for age, sex and ethnicity, and all participants gave written informed consent.

2.2. DNA extraction and genotyping. Venous blood (5 mL) was collected into EDTA tubes and stored at −70 °C. Genomic DNA was isolated from peripheral-blood leukocytes by a modified phenol–chloroform extraction method; DNA concentration and purity were assessed spectrophotometrically (NanoDrop 2000) at A260/280, with values of 1.7–1.8 confirming suitability for amplification. The IL6 C174G (rs1800795) and TNF-α G308A (rs1800629) polymorphisms were genotyped by polymerase chain reaction (PCR) using commercial detection kits (Sintol and Litekh, Moscow) on Applied Biosystems 2720, Corbett Research CG1-96 and Rotor-Gene Q thermocyclers with optimized amplification programmes. PCR products were separated by electrophoresis in 1–2 % agarose gel containing ethidium bromide and visualized under an ultraviolet transilluminator. Genotyping was performed at the Department of Molecular Medicine and Cell Technologies and the Laboratory of Medical Genetics of the Republican Specialized Scientific-Practical Medical Centre of Haematology, following GRIPS recommendations.

2.3. Statistical analysis. Allele frequencies were calculated by gene counting, and conformity to Hardy–Weinberg equilibrium was tested from observed and expected heterozygosity using the GenePop programme. Differences in allele and genotype distribution were assessed with the Pearson χ^2 test (with Yates' correction for 2×2 tables where any expected frequency was ≤ 5). The strength of association was expressed as the odds ratio (OR) with a 95 % confidence interval (CI); OR > 1 indicated an increased-risk factor and OR < 1 a reduced-risk factor. A two-sided p value below 0.05 was considered significant. Calculations used StatSoft Statistica 10.0 and Open Epi version 2.3.

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3. Results

3.1. IL6 C174G — Hardy–Weinberg equilibrium and overall association. In both groups the observed genotype distribution of the C174G polymorphism conformed to Hardy–Weinberg equilibrium. In the case group the C and G allele frequencies were 0.83 and 0.17, against 0.88 and 0.12 in controls. The C allele was marginally less frequent in DFS patients than in controls (83.3 % vs 88.0 %), and its presence did not influence DFS risk (χ^2 = 1.5, p = 0.3, OR = 0.7, 95 % CI 0.38–1.25). Conversely, carriage of the unfavourable G allele was associated with a 1.5-fold higher risk of DFS (16.7 % vs 12.0 %; OR = 1.5, 95 % CI 0.8–2.66). At the genotype level the protective C/C genotype was less frequent in patients (70.8 % vs 79.5 %; OR = 0.6, 95 % CI 0.31–1.25), while the heterozygous C/G genotype was more frequent and was associated with a 1.6-fold increase in DFS risk (25.0 % vs 16.9 %; OR = 1.6, 95 % CI 0.79–3.42); the rare G/G genotype was similarly distributed (Table 1, Figure 1).

Table 1. Distribution of the IL6 C174G alleles and genotypes in DFS patients and controls.

Allele / genotype	DFS group (n = 96)	Control (n = 83)	χ^2	p	OR (95 % CI)
C allele	83.3 %	88.0 %	1.5	0.3	0.7 (0.38–1.25)
G allele	16.7 %	12.0 %	1.5	0.3	1.5 (0.8–2.66)
C/C	70.8 %	79.5 %	1.8	0.2	0.6 (0.31–1.25)
C/G	25.0 %	16.9 %	1.8	0.2	1.6 (0.79–3.42)
G/G	4.2 %	3.6 %	0.0	0.9	1.2 (0.25–5.33)

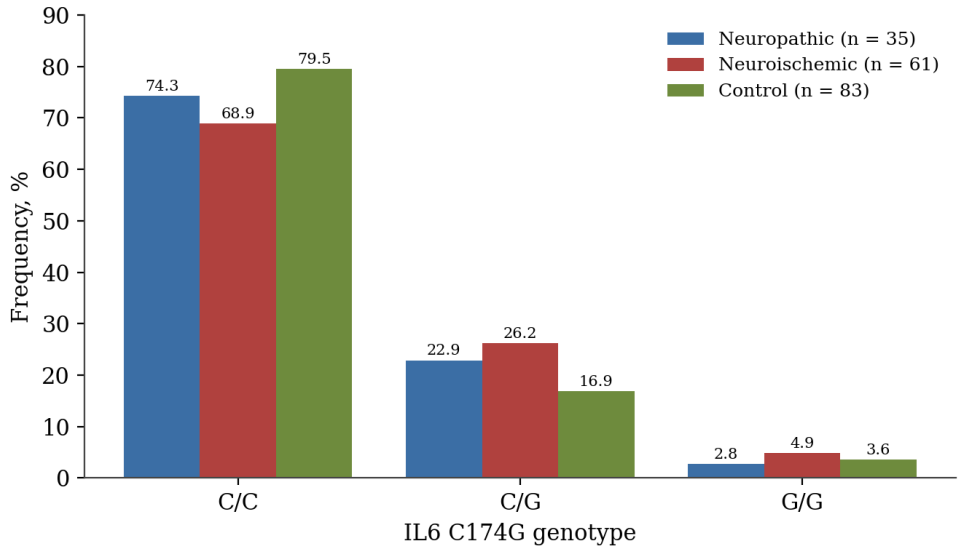

Figure 1. Distribution of the IL6 C174G genotypes across the clinical forms of DFS and controls.

3.2. IL6 C174G — association with the clinical forms. In the neuroischemic form the G allele frequency rose to 18.0 % and carriage of the heterozygous C/G genotype increased the odds of the neuroischemic form approximately 1.8-fold (26.2 % vs 16.9 %; χ^2 = 1.9, p = 0.2, OR = 1.8, 95 % CI 0.78–3.92), while the C/C genotype showed no association.

In the neuropathic form the differences from controls were smaller and non-significant (C allele 85.7 %, G allele 14.3 %; C/G 22.9 %), indicating a weaker contribution of this polymorphism to the neuropathic phenotype. Direct comparison of the two clinical forms showed that the G/G genotype was relatively more frequent in the neuroischemic than in the neuropathic form (4.9 % vs 2.9 %; OR = 1.8, 95 % CI 0.18–17.11), suggesting that carriage of G/G may favour progression of the milder neuropathic form to the more severe neuroischemic form.

3.3. TNF-α G308A — Hardy–Weinberg equilibrium and overall association. The distribution of the G308A polymorphism conformed to Hardy–Weinberg equilibrium in both groups, and the homozygous mutant A/A genotype was not detected in the study population. In the case group the G and A allele frequencies were 0.90 and 0.10, against 0.95 and 0.05 in controls. Carriage of the mutant A allele was associated with a 1.9-fold increase in DFS risk (9.9 % vs 5.4 %; $\chi^2 = 2.5$, p = 0.2, OR = 1.9, 95 % CI 0.85–4.31), while the G allele was correspondingly protective (OR = 0.5, 95 % CI 0.23–1.17). At the genotype level the G/G genotype was less frequent in patients (80.2 % vs 89.2 %; OR = 0.5, 95 % CI 0.21–1.15) and acted as a protective marker, whereas the heterozygous G/A genotype was significantly associated with DFS, doubling the odds of the disease (19.8 % vs 10.8 %; OR = 2.0, 95 % CI 0.87–4.72) and indicating heightened TNF-α activity (Table 2, Figure 2).

Table 2. Distribution of the TNF-α G308A alleles and genotypes in DFS patients and controls.

Allele / genotype	DFS group (n = 96)	Control (n = 83)	χ^2	p	OR (95 % CI)
G allele	90.1 %	94.6 %	2.5	0.2	0.5 (0.23–1.17)
A allele	9.9 %	5.4 %	2.5	0.2	1.9 (0.85–4.31)
G/G	80.2 %	89.2 %	2.7	0.2	0.5 (0.21–1.15)
G/A	19.8 %	10.8 %	2.7	0.2	2.0 (0.87–4.72)
A/A	0 %	0 %	–	–	–

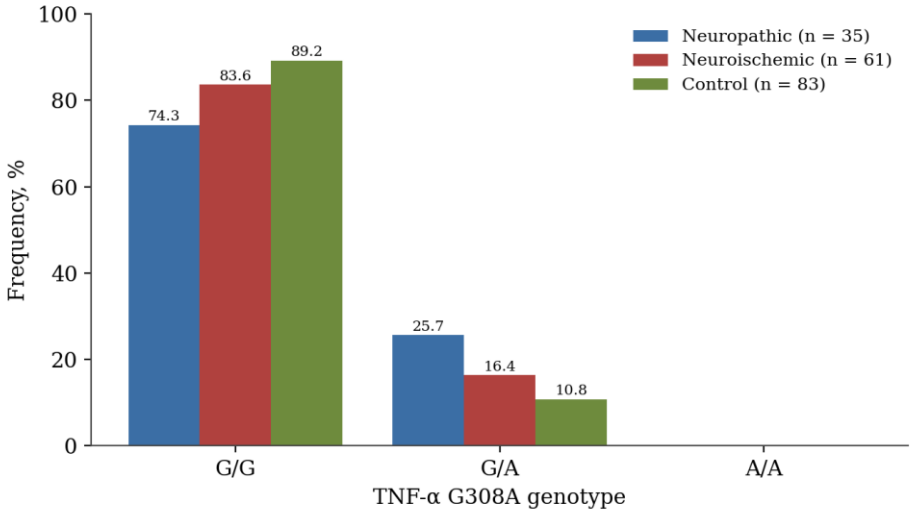

Figure 2. Distribution of the TNF-α G308A genotypes across the clinical forms of DFS and controls.

3.4. TNF-α G308A — association with the clinical forms. In the neuropathic form the association was strongest: the A allele was significantly more frequent than in controls

(12.9 % vs 5.4 %; χ^2 = 3.9, p = 0.05, OR = 2.6, 95 % CI 1.0–6.61), the protective G/G genotype was reduced (74.3 % vs 89.2 %; OR = 0.4, 95 % CI 0.13–0.95), and the heterozygous G/A genotype was significantly associated with the neuropathic form (25.7 % vs 10.8 %; χ^2 = 4.2, p = 0.05, OR = 2.8, 95 % CI 1.05–7.73). In the neuroischemic form the A allele (8.2 %) and G/A genotype (16.4 %) showed weaker, non-significant trends towards increased risk (OR = 1.6 for both). Comparison of the two forms indicated that the G/G genotype was relatively more frequent in the neuroischemic form (83.6 % vs 74.3 %; OR = 1.8, 95 % CI 0.64–4.85), a finding interpreted as a possible predictor of progression to the neuroischemic phenotype.

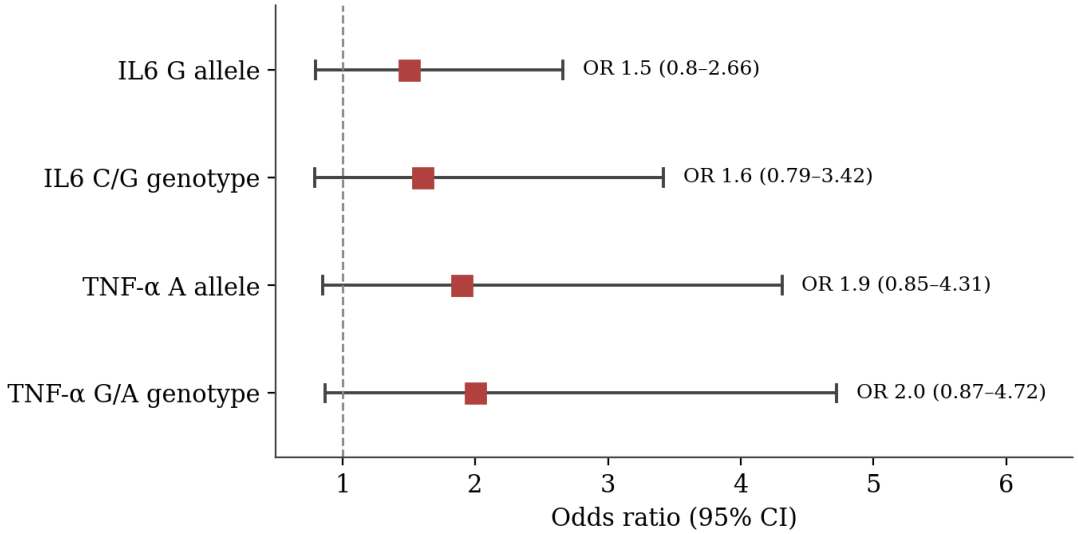

Figure 3. Odds ratios (95 % CI) for DFS risk associated with the unfavourable cytokine alleles and genotypes; the dashed line marks OR = 1.

3.5. Relationship with inflammatory laboratory markers. Carriage of the unfavourable cytokine genotypes paralleled the systemic inflammatory state. In patients with the neuroischemic form, in whom the C/G genotype of IL6 and the G/A genotype of TNF-α were over-represented, the leukocyte count (12.9 ± 0.8 vs 9.6 ± 0.4 ×10^9/L) and the leukocyte intoxication index (6.9 ± 0.3 vs 2.9 ± 0.5) were substantially higher than in the neuropathic form. This concordance links the genetic risk markers to increased activity of the pro-inflammatory cytokines and to progression of the inflammatory process, and is consistent with the more severe trophic and purulent-necrotic lesions observed in the neuroischemic form.

4. Discussion

This study demonstrates that the unfavourable alleles and heterozygous genotypes of two pro-inflammatory cytokine genes are associated with susceptibility to DFS in patients with type 2 diabetes. For IL6 C174G, the G allele and the C/G genotype increased DFS risk, while the C allele and C/C genotype were protective; for TNF-α G308A, the A allele and the G/A genotype increased risk, while the G allele and G/G genotype were protective. These genetic associations are biologically coherent with the central role of IL-6 and TNF-α in

maintaining the chronic inflammatory state that impairs wound healing in the diabetic foot [3, 4, 5].

Our findings are broadly concordant with reports linking cytokine gene polymorphisms to DFS, while also reflecting the population-dependent nature of these associations. The TNF-α rs1800629 variant was shown to increase DFS risk and to associate with serum cytokine levels and with severe infection, higher ulcer grade and amputation in an Indian cohort [9, 14]. For IL6 rs1800795, the evidence is more variable: the G allele was a risk factor for diabetes but not an independent predictor of DFS in a Turkish population [10], and a meta-analysis reported no overall association with microvascular complications, though with marked between-study heterogeneity arising from the pooling of different complications and ethnic groups [11]. Our observation of a modest, consistent risk effect of the IL6 G allele and a significant association of the TNF-α G/A genotype with the neuropathic form adds region-specific data to this debate and supports a genuine, if moderate, contribution of these variants to DFS in the Uzbek population.

The mechanistic basis of these associations lies in the dysregulated inflammatory response of the diabetic wound. In type 2 diabetes, hyperglycaemia, continued recruitment of inflammatory immune cells and elevated expression of IL-6, IL-1, IL-8 and TNF-α create a chronic inflammatory environment that prevents the transition from the inflammatory to the reparative phase of healing [4, 5]. Persistent inflammation not only holds the wound in the inflammatory phase but also suppresses angiogenesis and granulation-tissue formation, while impairing keratinocyte and fibroblast function and re-epithelialization [4, 6]. Genotypes that enhance the transcription or activity of IL-6 and TNF-α would be expected to intensify this maladaptive response, and the parallel elevation of the leukocyte count and leukocyte intoxication index in carriers of the risk genotypes supports this interpretation.

From a translational perspective, the involvement of IL-6 and TNF-α in DFS aligns with growing interest in resolving, rather than merely suppressing, the inflammatory response of chronic wounds. Strategies based on specialized pro-resolving lipid mediators and on the delivery of resolvins and cytokine modulators are being explored as means of restoring the orderly progression of wound healing in diabetes [2, 4]. In this context, genotyping of the cytokine genes could contribute to the identification of patients whose wounds are most likely to remain trapped in a persistent inflammatory state, and thus to the selection of candidates for early, mechanism-directed therapy. Because the effect of any single polymorphism is moderate and DFS is a polygenic, multifactorial condition, the practical value of IL6 and TNF-α genotyping will depend on its integration with other genetic markers, with metabolic control and with the clinical and instrumental assessment of the foot [1, 7].

The concentration of the strongest TNF-α association in the neuropathic form, and the relative enrichment of the protective G/G genotype together with a higher overall inflammatory burden in the neuroischemic form, illustrate the complexity of genotype–phenotype relationships in DFS. They are compatible with the view that, while the neuropathic phenotype may be driven more directly by inflammatory cytokine activity, the neuroischemic phenotype reflects the additional and dominant contribution of impaired perfusion. Identification of patients carrying the unfavourable cytokine genotypes could help to detect, before the appearance of clinical symptoms, individuals at high risk of

chronic inflammatory complications who might benefit from closer surveillance and earlier anti-inflammatory or wound-directed intervention [7, 12]. The principal limitations of the study are its single-centre design, the modest sample size — which limited the power to detect associations of the rarer genotypes and to attain significance for several point estimates — and the absence of direct measurement of circulating cytokine concentrations. Larger, multi-centre studies with functional correlates are needed to confirm these associations.

5. Conclusion

In patients with type 2 diabetes, the G allele and the heterozygous C/G genotype of the IL6 C174G (rs1800795) polymorphism, and the A allele and the heterozygous G/A genotype of the TNF-α G308A (rs1800629) polymorphism, are associated with increased susceptibility to DFS, whereas the corresponding C and G alleles and the homozygous C/C and G/G genotypes are protective. The TNF-α G/A genotype was significantly associated with the neuropathic form, and carriage of the risk genotypes was accompanied by a higher inflammatory burden. These results support the involvement of genetically determined pro-inflammatory cytokine activity in the pathogenesis of DFS and justify the inclusion of the IL6 and TNF-α polymorphisms in a multi-marker panel for risk assessment.

Declarations

Funding. This work received no specific external funding.
Conflict of interest. The authors declare no conflict of interest.
Ethical approval. The study was conducted in accordance with the Declaration of Helsinki; all participants provided written informed consent.

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