ORIGINAL ARTICLE

CONTENTS

The impact of TLR4 gene polymorphisms on the risk of developing diabetic foot syndrome in case of type 2 diabetes mellitus

Olga Bilyayeva[1], Vadim Kryzhevsky[1], Andrii Bitinsh[1], Sergiy Ziablitsev[2]
[1]SHUPYK NATIONAL HEALTHCARE UNIVERSITY OF UKRAINE, KYIV, UKRAINE
[2] BOGOMOLETS NATIONAL MEDICAL UNIVERSITY, KYIV, UKRAINE

ABSTRACT

Aim: To study the effect of *TLR4* gene polymorphisms (rs1927911, rs2149356 and rs4986790) on the risk of developing DFS in T2DM.

Materials and Methods: The study included 58 patients with T2DM who had CVD (case group) and 60 patients with T2DM who did not have CVD (control group). Genetic polymorphisms of the *TLR4* gene were determined by real-time polymerase chain reaction using the Gene Amp® PCR System 7500 amplifier (Applied Biosystems, USA) and TaqMan Mutation Detection Assays Life-Technology (USA). MedStat and MedCalc v.15.1 software packages (MedCalc Software bvba) were used for statistical analysis.

Results: The analysis of the frequency of genotypes and alleles of rs1927911 showed an increase in the ancestral homozygote *G/G* ($p_{Fet} = 0.022$) with a decrease in the frequency of the heterozygote *G/A* ($p_{Fet} = 0.025$) and the minor allele *A* ($p_{Fet} = 0.042$). The rs1927911 allele polymorphism reduced the risk of developing SBS (p=0.040; OR=0.516; 95% CI 0.274-0.974). The rs2149356 polymorphism was not associated with the development of CVD in patients with T2D. In addition, an increase in the frequency of the minor *G/G* rs4986790 homozygote ($p_{Fet} = 0.026$) and the minor *G* allele ($p_{Fet} = 0.047$) was shown in patients with CVD. The rs4986790 allele polymorphism increased the risk of developing SIDS (p=0.047; OR=2.4; 95% CI 0.994-5.812).

Conclusions: The prospects of genotyping *TLR4* gene polymorphisms have been shown and the dependence of the risk of CVD on the polymorphic state of rs1927911 and rs4986790 in patients with T2DM from the Ukrainian population has been confirmed.

KEY WORDS: diabetic foot, risk, rs1927911, rs2149356, rs4986790

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INTRODUCTION

Diabetes mellitus (DM) is a serious metabolic disorder that affects more than 350 million people worldwide, and another billion people are prediabetic and may eventually develop full-blown diabetes [1]. According to other estimates, approximately 462 million people suffer from type 2 diabetes (T2D), which corresponds to 6.28% of the world's population (4.4% aged 15-49, 15% aged 50-69 and 22% aged 70+) with a prevalence of 6059 cases per 100,000 [2]. The global prevalence of T2DM is projected to increase to 7079 per 100,000 by 2030, reflecting a steady increase in all regions of the world.

According to the latest data, approximately 18.6 million people worldwide have diabetic foot syndrome (DFS) every year, which precedes 80% of lower limb amputations in people with diabetes and is significantly associated with an increased risk of death [3]. The mortality rate in people with DFU is 231 deaths per 1000 person-years compared to 182 deaths per 1000 person-years in people with DM but without DFU. At the same time, from 50% to 60% of ulcers become infected, and about 20% of moderate and severe infections lead to lower limb amputation.

Among the many mechanisms of CVD, the most important are peripheral arterial damage (primarily atherosclerosis), peripheral diabetic neuropathy, bacterial infection, immune cell dysfunction, and genetic factors [4, 5].

As key defence molecules, members of the Toll-like receptor (TLR) superfamily play a fundamental role in detecting pathogen invasion or damage and initiating the innate immune system of mammalian cells [6]. In the first line of defence, cells are constantly exposed to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), while TLRs expressed by various immune cells are key molecules for recognising PAMPs and DAMPs and initiating innate immune responses.

Recent systematic reviews and meta-analyses have shown that the effectiveness of the immune

response in SBS is significantly influenced by *TLR4* gene polymorphism, among other factors [7, 8]. The results of bioinformatic analysis identified the *TLR4* gene as one of the central genes in the development of SBS [9, 10]. The association of *TLR4* gene polymorphisms with an increased risk of CVD in patients with T2DM has been shown [11]. The risk genotypes included rs1927911 and rs4986790, which increased the risk of poor wound healing.

AIM

The aim is to study the effect of polymorphisms (rs1927911, rs2149356 and rs4986790) of the *TLR4* gene on the risk of developing diabetic foot syndrome in type 2 diabetes mellitus.

MATERIALS AND METHODS

The study included 118 patients with T2DM, who were divided into two groups for genetic studies - cases and controls. The case group included 58 patients with T2DM who had DFD with trophic ulcers, foot phlegmon and wounds after amputation of a single toe without primary suture. The control group consisted of 60 patients with T2DM who did not have DFU.

The study design was prospective, cohort, case-control type. All studies were conducted in strict compliance with the bioethical standards and requirements of the Declaration of Helsinki (General Assembly of the World Medical Association) and the Council of Europe Convention on Human Rights and Biomedicine (1977), the International Code of Medical Ethics (1983) and Order of the Ministry of Health of Ukraine No. 690 of 23.09.2009. All patients provided informed consent to participate in the study.

Patients in the case group were aged 25-87 years, with the majority of patients being over 55 years of age (81.0%). The vast majority of patients (86.2%) had atherosclerotic lesions, which was accompanied by the presence of hypertension and coronary heart disease. Obesity was present in 46.6% of patients. In the structure of pathological processes in patients with DFS, gangrene of single toes and gangrene of the distal foot were most often observed (63.8%). Almost all patients (91.4%) had a duration of T2DM exceeding 15 years. Patients with DFS had ischaemic (31.0%) or neuroischemic (69.0%) forms. In terms of age and duration of T2DM, patients in the control group were comparable to the case group.

Genetic polymorphisms of the *TLR4* gene rs1927911, rs2149356 and rs4986790 (*896A/G*; Asp299Gly) were determined by real-time polymerase chain reaction (PCR) using the Gene Amp® PCR System 7500 amplifier (Applied Biosystems, USA) and TaqMan Mutation Detection Assays Life-Technology (USA). Genomic DNA was extracted from venous blood using the PureLink® Genomic DNA Kit For Purification of Genomic DNA (INVITROGEN, USA).

The software packages MedStat and MedCalc v.15.1 (MedCalc Software bvba) were used for statistical analyses. The frequency differences of genotypes and alleles in the groups of cases and controls were compared by Fisher's exact method (p_{Fit}). According to the general table of genotype and allele frequency conjugation, the effect of genotypes and alleles on the development of SBS, odds ratio (OR) and 95% probability interval (95% CI) were calculated [12].

RESULTS

Comparison of the study groups by the frequency of genotypes and alleles of rs1927911 of the *TLR4* gene showed a significant increase in the frequency of the ancestral *G/G* homozygote (1.4-fold; $p_{Fet} = 0.022$) with a decrease in the frequency of the *G/A* heterozygote (2.0-fold; $p_{Fet} = 0.025$) in patients with T2DM with VSD compared with controls (Fig. 1). Accordingly, the frequency of the minor homozygote *A/A* ($p_{Fet} > 0.05$) and the minor allele *A* (1.7-fold; $p_{Fet} = 0.042$) was lower.

The statistically significant difference in the frequencies of genotypes and alleles of rs1927911 in the groups of patients suggested that this polymorphism is associated with the development of CVD. To test this assumption, we determined the effect of the distribution of genotype and allele frequencies of rs1927911 on the development of CVD in patients with T2DM and the degree of their association with CVD (Table 1). The Hardy-Weinberg test for controls and cases was consistent with the random nature of genotype inheritance (respectively, $\chi^2 = 0.002$; p=0.983 and $\chi^2 = 0.035$; p=0.983).

Genotype influence analysis showed that the rs1927911 polymorphism was not significantly associated with the development of SIDS ($\chi^2 = 5.93$; p=0.052), whereas such a relationship was established by allele distribution ($\chi^2 = 4.25$; p=0.040). The presence of the minor *A* allele reduced the risk of SCD (p=0.040; OR=0.516; 95% CI 0.274-0.974).

Thus, the presence of the *A* rs1927911 allele of the *TLR4* gene could be considered as a protective factor for the development of CVD in T2DM, which reduced the risk of CVD in patients with T2DM by 1.9 times compared with carriers of the ancestral *G* allele.

Comparison of the study groups by the frequency of genotypes and alleles of rs2149356 of the *TLR4* gene

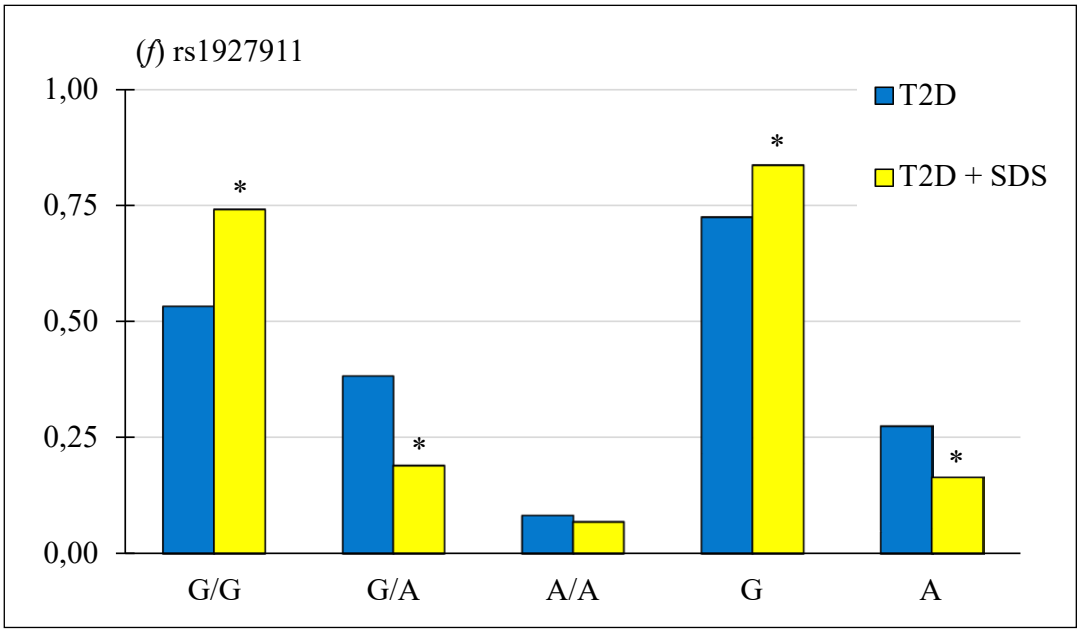

Fig. 1. Distribution of frequencies of genotypes and alleles of rs1927911 of the *TLR4* gene in patient groups; * - $p<0.05$ when compared by Fisher's exact test; vertical axis - frequencies (*f*); horizontal axis - genotypes and alleles

showed no significant shifts (Fig. 2). Also, there was no effect of the distribution of genotype and allele frequencies of rs2149356 on the development of CVD in patients with T2DM (Table 2). The Hardy-Weinberg test for controls and cases corresponded to the random nature of genotype inheritance (respectively, $\chi^2 = 0.00$; $p=1.00$ and $\chi^2 = 0.044$; $p=0.979$).

Thus, the rs2149356 polymorphism of the *TLR4* gene could be considered a neutral factor in the development of SJS in T2DM.

Comparison of the study groups by the frequency of genotypes and alleles of rs4986790 of the *TLR4* gene showed a significant increase in the frequency of the minor *G/G* homozygote in patients with T2DM and SBS (Fig. 3). The *G/G* genotype in the control group was not detected in any patient, while in the case group - in 5 patients (8.6%; $p_{Fet} = 0.026$). Accordingly, the frequency of the minor *G* allele was higher (2.2 times; $p_{Fet} = 0.047$).

The presence of a statistically significant difference in the frequencies of the minor homozygote and rs4986790 alleles in the patient groups suggested that this polymorphism is associated with the development of CVD. Determination of the effect of rs4986790 genotype and allele frequencies on the development of SCD in patients with T2DM and the degree of their association with SCD showed (Table 3) that the rs4986790 genotype polymorphism was not significantly associated with the development of SCD ($\chi^2 = 5.40$; $p=0.068$), whereas the presence of the minor *G* allele increased the risk of SCD (OR=2.4; 95% CI 0.994-5.812). The Hardy-Weinberg test for controls and cases was consistent with the random nature of genotype inheritance (respectively, $\chi^2 =0.004$; $p=0.998$ and $\chi^2 =0.086$; $p=0.958$).

Thus, the presence of the mutant *G* rs4986790 allele of the *TLR4* gene could be considered a risk factor for CVD in T2DM, which increased the risk of CVD in patients with T2DM by 2.4 times compared with carriers of the ancestral *A* allele.

Thus, the study revealed that *TLR4* gene polymorphisms were associated with the development of SCD in patients with T2DM from the Ukrainian population. The allelic polymorphism of rs1927911 was protective: carriers of the polymorphic allele A had a lower risk of SCD (OR=0.516; 95% CI 0.274-0.974). The rs4986790 allelic polymorphism was risky, as carriers of the *G* polymorphic allele had a higher risk of SCD (OR=2.4; 95% CI 0.994-5.812). In contrast, the rs2149356 polymorphism was not associated with the development of CVD in patients with T2DM.

DISCUSSION

Acute inflammation is essential for the initiation of proper wound healing, and TLRs play a key role in its initiation [13]. Through their signalling pathways, they trigger the production of inflammatory mediators (interleukins, interferons) and activate effector T cells. In the setting of chronic diabetic inflammation in the setting of SJS, there is a cellular immunodeficiency caused by a decrease in leukocyte phagocytic activity due to TLR inhibition and insufficient production of proinflammatory cytokines in the infected diabetic wound [14]. Enhanced TLR4 signalling increases the production of proinflammatory cytokines, restores leukocyte recruitment and stimulates diabetic wound healing.

Our results confirmed the findings of other authors regarding the association of the *TLR4* rs4986790

Table 1. Effect of TLR4 rs1927911 genotype frequency distribution on the development of diabetic foot syndrome

Genotypes Alleles	T2D + SDS, n (f)	T2D, n (f)	χ^2	p	HS	95% OF THE VI
G/G	43 (0,741)	32 (0,533)	5,93	0,052	2,511	1,154-5,452
G/A	11 (0,190)	23 (0,383)			0,377	0,163-0,870
A/A	4 (0,069)	5 (0,083)			0,815	0,208-3,198
G	97 (0,836)	87 (0,725)	4,25	0,040	1,936	1,027-3,652
A	19 (0,164)	33 (0,275)			0,516	0,274-0,974

Notes: n - number; f - frequency; χ2 - Pearson's test with adjustment for continuity; p - statistical significance of differences between groups; OR - odds ratio; 95% CI - 95% confidence interval for OR

Table 2. Effect of the frequency distribution of TLR4 genotypes rs2149356 the development of diabetic foot syndrome

Genotypes Alleles	T2D + SDS, n (f)	T2D, n (f)	χ^2	p	HS	95% OF VI
G/G	27 (0,465)	29 (0,483)	2,86	0,240	0,931	0,452-1,918
G/T	19 (0,327)	25 (0,417)			0,682	0,322-1,445
T/T	12 (0,207)	6 (0,100)			2,348	0,817-6,749
G	73 (0,629)	83 (0,692)	1,02	0,312	3,040	1,789-5,166
T	43 (0,371)	37 (0,308)			0,329	0,194-0,559

Notes: n - number; f - frequency; χ2 - Pearson's correction for continuity; p - statistical significance of differences between groups

Table 3. Effect of TLR4 genotype frequency distribution rs4986790 on the development of diabetic foot syndrome

Genotypes Alleles	T2D + SDS, n (f) 58	T2D, n (f) 60	χ^2	p	HS	95% OF VI
A/A	46 (0,793)	52 (0,867)	5,40	0,068	0,590	0,222-1,569
A/G	7 (0,121)	8 (0,133)			0,892	0,301-2,641
G/G	5 (0,086)	0 (0,000)			-	-
A	99 (0,853)	112 (0,933)	3,97	0,047	0,416	0,172-1,006
G	17 (0,147)	8 (0,067)			2,404	0,994-5,812

Notes: n - number; f - frequency; χ2 - Pearson's test with adjustment for continuity; p - statistical significance of differences between groups; OR - odds ratio; 95% CI - 95% confidence interval for OR

polymorphism with the development of DFS [15]. Patients with T2DM carrying the rs4986790 mutant allele had a triple risk of developing lower limb arteriopathy, neuropathy, and cardiovascular disease.

The Asp299Gly (rs4986790) polymorphism of the *TLR4* gene in patients with T2DM increased the risk of microvascular complications with an OR of 1.42 (95% CI 1.02-1.96), which was more common in the Caucasian population (OR 1.69) [16]. In our studies, this polymorphism increased the risk of SCD with an OR of 2.4 (95% CI 0.994-5.812). It is likely that insufficient activation of the TLR4 signalling pathway, which is inherent in carriers of the rs4986790 polymorphism [17], may contribute to the inhibition of immune cellular responses and cause chronic inflammation. Accordingly, carriers of the rs4986790 polymorphism may be more prone to developing SBS. This assumption is supported by the results of experimental activation of TLR4/NF-κB signal transduction, which promoted macrophage polarisation *in vitro* and improved diabetic wound healing [18].

Therefore, a practical aspect arising from these results may be the justification for the use of TLR4 signalling pathway activators in chronic sluggish SJS in carriers of the rs4986790 polymorphism of the *TLR4* gene.

In our opinion, the results obtained in the study of the rs1927911 promoter polymorphism, which, according to our data, had a protective effect, should be interpreted in a completely different way. It has been shown that blocking the PAMP-induced TLR4/MyD88 pathway suppressed macrophage polarisation and inflammatory cytokine production in diabetic wounds in an experiment [19]. Inhibition of TLR4 signalling reduced the hyperimmune response and tissue damage when repair processes were activated, for example, as shown in this study, through the enhancement of autophagy mechanisms.

The results of bioinformatic analysis identified the *TLR4* gene as one of the risk genes for the development

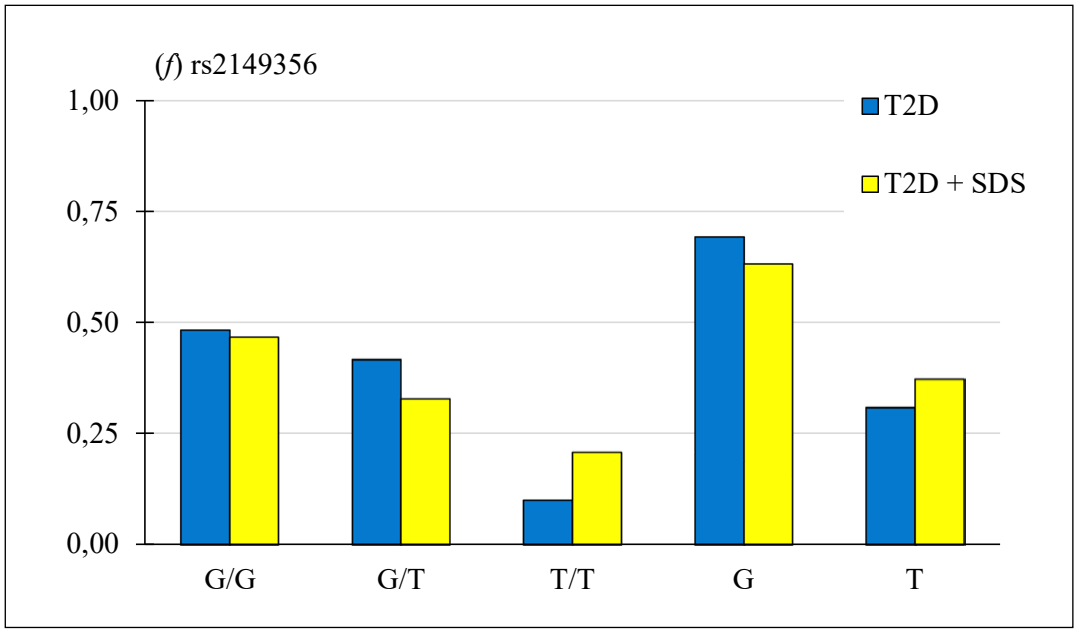

Fig. 2. Distribution of frequencies of genotypes and alleles of rs2149356 of the *TLR4* gene in patient groups; vertical axis - frequencies (*f*); horizontal axis - genotypes and alleles

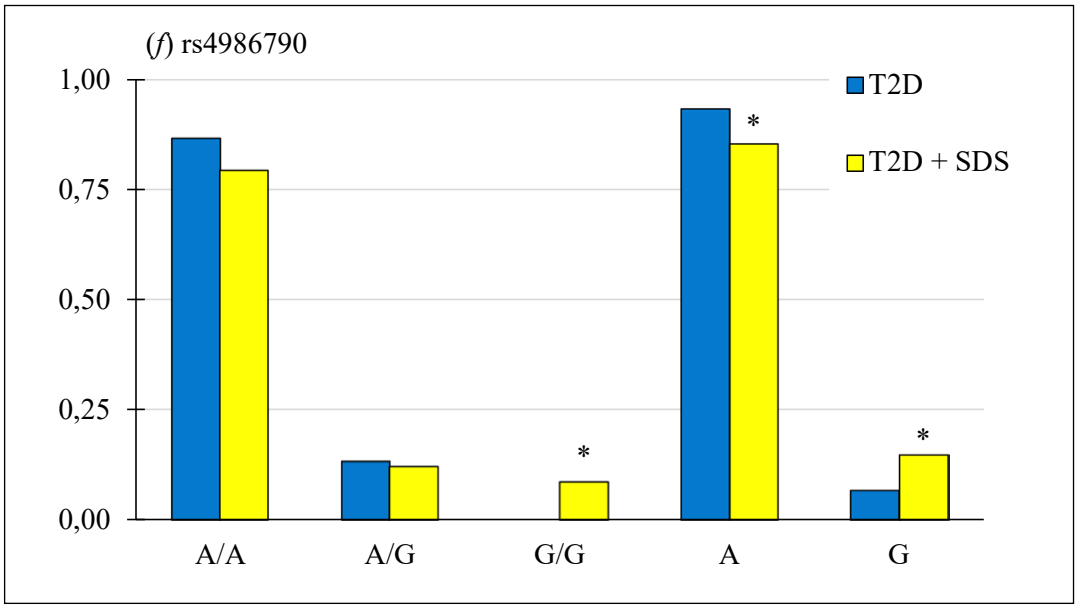

Fig. 3. Distribution of frequencies of genotypes and alleles of rs4986790 of the *TLR4* gene in patient groups; * - $p<0.05$ when compared by Fisher's exact test; vertical axis - frequencies (*f*); horizontal axis - genotypes and alleles

of DFS [9]. Accordingly, reducing its activity may be a promising way to inhibit hyperimmune reactions that cause widespread tissue damage at the first stage of inflammation, for example, in wound infection in patients with T2D. An experiment showed that hypoxia stimulates the regulation of TLR4 protein expression, and this effect is enhanced by hyperglycaemia [20]. Knockout of the *TLR4* gene or the use of TLR4-neutralising antibodies attenuated the effects of hyperglycaemia and hypoxia in the experiment and improved the healing of ischaemic wounds of the tender extremity.

This assumption was confirmed in our results: carriers of the rs1927911 allele polymorphism had a reduced risk of SBS compared with carriers of the ancestral allele (OR=0.516; 95% CI 0.274-0.974). Thus, hyporeactivity of the innate immune system, which is inherent in carriers of the rs1927911 polymorphism, may be a protective factor for the development of SBS and may be important in the initial stages of its development. Of course, in the case of a prolonged course of DFS, this factor, in our opinion, can also cause chronicity of the process and poor wound healing results.

Thus, impaired formation of a full-fledged immune response in carriers of *TLR* gene polymorphisms is an important risk factor for CVD in T2DM and its complications, including DR [17]. The rs4986790 polymorphism, which leads to a change in the structure of the TLR protein, probably contributes to the inhibition of the innate immune response and chronic inflammation. The protective effect of the

rs1927911 promoter polymorphism may be explained by limiting hyperimmune inflammation and tissue damage in the initial stages of the development of SBS, but it may also be negative in the setting of chronicity. Of course, such assumptions require further research on a larger population of patients with different stages of the pathological process and, possibly, taking into account the results of their treatment. At this stage, we can state that genotyping is promising and that the risk of CVD depends on the polymorphic state of the *TLR4* gene in patients with T2DM from the Ukrainian population.

CONCLUSIONS

1. In patients with T2DM, the allelic polymorphism rs1927911 of the *TLR4* gene was associated with the development of SCD, and its carriers had a lower risk of SCD (p=0.040; OR=0.516; 95% CI 0.274-0.974).
2. The rs2149356 polymorphism of the *TLR4* gene was not associated with the development of SJS in patients with T2DM.
3. In patients with T2DM, the allelic polymorphism rs4986790 of the *TLR4* gene was associated with the development of SCD, and its carriers had a higher risk of SCD (p=0.047; OR=2.4; 95% CI 0.994-5.812).

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CONFLICT OF INTEREST
The Authors declare no conflict of interest

CORRESPONDING AUTHOR
Andrii Bitinsh
Shupyk National Healthcare University of Ukraine
9 Dorohozhytska St, 04112 Kyiv, Ukraine
e-mail: bitinshandrew@gmail.com

ORCID AND CONTRIBUTIONSHIP
Olga Bilyayeva: 0000-0003-2862-0423 E F
Vadim Kryzhevsky: 0000-0002-8538-2583 E F
Andrii Bitinsh: 0000-0003-3867-5524 A B D
Sergiy Ziablitsev: 0000-0002-5309-3728 A C D

A – Work concept and design, B – Data collection and analysis, C – Responsibility for statistical analysis, D – Writing the article, E – Critical review, F – Final approval of the article

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