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The Arg753Gln Polymorphisms in Toll-like Receptor 2 in a Syphilis-Infected and Control Population in The Netherlands: Can Differences in the Number of Self-Reported Sexual Contacts Indicate Protection against Syphilis?

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Abstract: The Arg753Gln polymorphism in Toll-like receptor 2 has been associated with an increased risk of bacterial infections as well as with protection from the late stages of Lyme disease and the acquisition of syphilis. In this study, we determined the presence of this polymorphism in samples collected from men having sex with men/men with women in the Amsterdam Cohort Studies. The presence of the polymorphism was determined by nested PCR, followed by Sanger sequencing. A set of 90 syphilis-seronegative individuals was compared to 95 syphilis-diagnosed participants. A polymorphism allele frequency of 3.9% was found in the control group and 2.63% in the syphilis case group, respectively. None of the individuals showed a homozygous Arg753Gln polymorphism. The number of self-reported sexual contacts was higher in the group of syphilis patients compared to the control group ($p = 0.0063$). Moreover, in the syphilis case group ($n = 49$), participants heterozygous for the TLR2-Arg753Gln reported higher numbers of sexual contacts ($p = 0.037$) compared to wild-type homozygotes. Our findings suggest a possible protective effect of TLR2-Arg753Gln in the acquisition of syphilis. In addition, the determination of the number of self-reported sexual contacts can be used in an evaluation of the protective effect of polymorphism in a population with a low prevalence of it.

Keywords: TLR2; syphilis infection; *Treponema pallidum* ssp. *pallidum*; Arg753Gln polymorphisms; role of TLR2 in syphilis infection

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1. Introduction

Toll-like receptors (TLRs) are proteins of the innate immune system that recognize structurally conserved microbial components (pathogen-associated molecular patterns (PAMPs), e.g., lipopolysaccharides (LPSs), glycolipids, proteins, etc.). Toll-like receptor 2 (TLR2) is the member of the TLR family with the largest number of different 'ligands' (or agonists) identified [1]. TLR2 serves as a sensor and inductor of specific defense processes, including oxidative stress and cellular necrosis, initially triggered by microbial compounds. TLR2 appears to be the major molecular sensor for spirochetes (such as treponemes, borreliae, and leptospirae), activated by their lipoproteins. Protein polymorphisms of these membrane receptors encoded by several non-synonymous single-nucleotide polymorphisms (SNPs) have been shown to alter the susceptibility and course of several chronic infectious diseases, including tuberculosis, leprosy, Lyme disease, and AIDS [2–8].

The Arg753Gln polymorphism in TLR2 (rs5743708, 2258G → A), which is localized in the signaling Toll–interleukin receptor (TIR) domain, is associated with an increased risk of

asymptomatic bacteriuria in women [9], cytomegalovirus infection after liver transplantation [10,11], and life-threatening infections in intensive care unit patients [12]. In addition, the Arg753Gln polymorphism is associated with candida infections [13] and a higher susceptibility to tuberculosis in Turkish, Chinese, and Egyptian populations [14–16]. Several studies have associated the polymorphism with differences in the infection outcomes of several spirochete diseases (e.g., protection from the late stages of Lyme disease [7] and a higher susceptibility to leptospirosis [17]). While the exact mechanism for late-stage protection against Lyme disease is not fully understood, a reduction in the immune response due to decreased TLR2 signaling resulting in an attenuated inflammatory response is likely to be an important mechanism. Schröder et al. [7] showed that mononuclear cells heterozygous for the Arg753Gln polymorphism produced less tumor necrosis factor-α (TNF-α) and interferon-γ after stimulation with borreliae, providing a possible molecular explanation for the reduced immune response. According to the database of polymorphisms (dbSNPs), Arg753Gln is present in the European population, while its prevalence in the rest of the world is close to zero. Despite an overall 3% frequency, its distribution differs greatly among various European populations. The highest frequency was found in the Czech and Slovak populations [18] and among a Turkish population [19], both around 10%. On the other hand, in southern Europe, this SNP has a low frequency [20]. In northern Europe, it varies between 3 and 5% (according to dbSNPs), and a similar low frequency was observed in the Netherlands [21–23].

There are only two previous studies on the role of the TLR2 Arg753Gln polymorphism in syphilis patients. A study by Grillová et al. [18], which was performed on a Czech and Slovak population, revealed a significantly lower prevalence of the TLR2 Arg753Gln polymorphism among seropositive syphilis patients (4 patients with polymorphism and 133 patients with wild-type sequences) compared to healthy, seronegative individuals (24 individuals with polymorphism and 221 individuals with wild-type sequences) ($p = 0.0065$). These results suggested a protective effect of the TLR2 Arg753Gln polymorphism against the acquisition of syphilis infection. The other study on the TLR2 Arg753Gln polymorphism was performed solely on patients with syphilis, and the results suggested that the TLR2 Arg753Gln polymorphism increased the risk of neurosyphilis [24] (the control group included 2 patients with polymorphism and 254 patients with wild-type sequences; 5 patients with polymorphisms and 86 patients with wild-type sequences in the laboratory-defined neurosyphilis group ($p = 0.015$); 10 patients with polymorphisms and 133 patients with wild-type sequences in the clinically defined neurosyphilis group ($p = 0.001$)). Unlike in Lyme disease, these studies suggest that while the TLR2 Arg753Gln polymorphism could be protective in the early stages of syphilis infection, it could result in disease progression in the later stages of syphilis (found in only about a third of infected and untreated patients).

In our study, we investigated the TLR2 Arg753Gln polymorphism in men who have sex with men (MSM) and men who have sex with men and women (MSMW) from the Amsterdam Cohort Studies (ACS) that visited the Sexual Health Center (SHC) in Amsterdam, the Netherlands. The demographic and clinical characteristics of the 90 individuals without a history of syphilis and the 95 clients with a syphilis diagnosis were compared with respect to the presence of the TLR2 Arg753Gln polymorphism.

2. Materials and Methods

2.1. Study Population: Amsterdam Cohort Study

The Amsterdam Cohort Studies (ACS) is an ongoing prospective cohort study among MSM initiated in 1984 [25,26]. The ACS aims to investigate the prevalence, incidence, associated risk factors, psychosocial factors, the natural course, and pathogenesis of HIV-1 and other STI infections. Men who reported having sex with men (MSM), were ≥18 years old, and lived in Amsterdam or were regularly in the area were eligible to participate. Participation in the ACS was strictly voluntary, and written informed consent was obtained.

The ACS was approved by the Medical Ethics Committee of the Amsterdam University Medical Center of the University of Amsterdam, the Netherlands (MEC-07/182).

During twice-a-year study visits at the Public Health Centre in Amsterdam, participants completed a questionnaire on sexual behavior in the previous six months and were tested (from the start of the study in 1984) for HIV (unless already positive) and, starting in 2008, for syphilis, gonorrhea, and chlamydia. The ACS retrospectively provided samples and requested data for our study. The ACS could only provide previously collected participants' metadata.

2.2. Collection of Samples

Serum samples were retrospectively collected from the ACS participants (n = 185) between 2019 and 2022. Participants included were solely MSM (men having sex with men) and MSMW (men having sex with men and women), and they represent a sex-matched group by inclusion. Those suspected of having syphilis and those without a history of syphilis were also included. A group of 95 participants, which was labeled as the case group, was defined as syphilis-diagnosed, having at least one positive treponemal-specific serological test and/or a PCR test for *Treponema pallidum* ssp. *pallidum* (TPA) DNA. The control group was defined as individuals with no history of syphilis and negative syphilis serology on both treponemal and non-treponemal tests and, if performed, a negative TPA PCR test.

2.3. Isolation of Human DNA and PCR Amplification

Serum samples were used for the isolation of human DNA using a QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Briefly, 200 µL of serum was mixed with 20 µL of protease. After that, 200 µL of lysis buffer AL was added, and the mixture was incubated at 56 °C for 10 min. After incubation, 200 µL of ethanol was added. Samples were then transferred to isolation columns (Qiagen, Hilden, Germany) and spun for 1 min at 8000 rpm (rotations per minute). In the next step, 500 µL of washing buffer AW1 was added to the samples, and they were centrifuged at 8000 rpm for 1 min. In the following step, 500 µL of washing buffer AW2 was added, and samples were spun at 14,000 rpm for 3 min. In the next round, without adding any buffer, samples were centrifuged for 1 min at 14,000 rpm to discard extra liquid. Elution was carried out by adding 100 µL of AE buffer and spinning for 1 min at 8000 rpm. Polymorphisms were detected using nested PCR amplification of the TLR2 region spanning the Arg753Gln region and subsequent Sanger sequencing. Briefly, DNA was amplified in two steps,: the first used outer primers (F 5′–3′: CTACTGGGTGGAGAACCTTATG; R 5′–3′: CTAGGACTTTATCGCAGCTCTC; resulting in PCR products of 400 bp in length), and the second used inner primers (F 5′–3′: TCTTCATAAGCGGGACTTCATT; R 5′–3′: ATCGCAGCTCTCAGATTTACC, resulting in 327 bp long PCR products) (primer sequences were used according to Grillová et al., 2020 [18]). Sanger DNA sequence electropherograms were individually evaluated for the presence of the Arg753Gln polymorphism (2258G → A; rs5743708), the Tyr715Asn polymorphism (1892C → A; rs5743706), and the Glu738Gln polymorphism (2029C → T; rs142286429), representing non-synonymous SNPs of TLR2 that have been shown to alter susceptibility to different infectious diseases. The PCR mix in one reaction (25 µL) contained 14.9 µL of sterile water, 5 µL of Flexi GoTaq buffer, 2 µL of $MgCl_2$ (25 mM), 0.5 µL of 10 mM dNTPs, 0.25 µL of each primer (100 pmol/µL), 0.1 µL of *Taq* polymerase (GoTaq Flexi DNA polymerase, Promega), and 2 µL of DNA template. In every PCR run, water was used as a negative control. The PCR protocol was carried out using a C1000 BioRad thermocycler with the following conditions: 1 cycle: 50 °C for 2 min; 1 cycle: 95 °C for 2 min; 45 cycles: 95 °C for 15 s, 60 °C for 45 s. The same PCR mixture and PCR conditions were used in both steps, i.e., outer and inner primers. The positivity of PCR products was analyzed using a QIAxcel system. Positive products were purified using a DyeEx 2.0 104 Spin Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Spin columns containing gel were vortexed, unsealed, and centrifuged in

collection tubes for 3 min at 750 rpm. After this, the gel solidified, and spin columns were transferred to Eppendorf tubes. On the gel inside of the spin column, 20 µL of PCR product was applied, and the column was spun for 3 min at 750 rpm. Purified PCR products were Sanger-sequenced by Eurofins Genomics (Constance, Germany). PCR products were separately mixed with 20-times-diluted inner primers (with original concentration of 100 pmol/µL), separately for F and R primers. Sanger sequencing was performed from both sides to confirm the final sequence. Moreover, all examined samples were tested and sequenced in biological (blinded) duplicates. None of the duplicates showed different sequencing results. Analyses of the sequences were performed using Lasergene software (DNASTAR v.7.1.0; DNASTAR, Madison, WI, USA).

2.4. Statistical Methods

All analyses were performed using R Statistical Software (v4.2.0, [27]). Several tests, including Fisher's exact two-sided test, the Mann–Whitney U test, and the exact permutation test for a two-sample comparison (as a nonparametric alternative to the Student's *t*-test), based on the characteristics and distribution of the data, were used. Fisher's exact two-sided test was used for categorical data, while the Mann–Whitney U test, Student *t*-test, and exact permutation test were used for continuous data. We used a natural logarithmic transformation because of data dispersion (outliers) for the number of partners in the individual categories. After transformation, the data were tested for their normal distribution using the Shapiro–Wilk normality test and for homogeneity of variance by the F-test. For data with a non-normal distribution (comparisons of the number of contacts in control and case groups), the distributions of two independent groups were compared using the Mann–Whitney U test. For data with normal distributions (comparisons of homozygotes and heterozygotes) and homogeneity of variance, differences between groups were tested with Student's *t*-test and the exact permutation test. p values less than 0.05 were considered significant. To test for Hardy–Weinberg equilibrium, we used a standard formula published online (https://gene-calc.pl/hardy-weinberg-page, accessed on 14 February 2023); the significance level was set to 0.05.

3. Results

In this study, we tested a total of 185 serum samples collected from 2019 to 2022 from ACS participants by the Public Health Service of Amsterdam, the Netherlands. Since all the samples came from MSM and MSMW, both the case group and control group participants were sex-matched (Table 1). The case group participants were slightly older compared to the control group (age median: 46 (IQR: 12.5) and 44 (IQR: 20.75), respectively, Table 1); however, this difference was not significant ($p = 0.074$), and the tested groups were therefore considered age-matched. Most participants were of Dutch ethnicity. Both groups contained the same number of non-Dutch or non-specified-nationality participants ($n = 22$), representing 24.4% of the control group and 23.2% of the case group (Table 1). There were 10 people living with HIV (PLWH) in the case group and one PLWH in the control group ($p = 0.004$).

Regarding the TLR2 Arg753Gln polymorphism, only heterozygotes were found in this study (GA), and no homozygotes (AA) were detected. Most serum samples (92.2% of the control group and 94.7% of the case group) contained TLR2 wild types (GG) (Table 1). Given the low number of alternative alleles (Table 1), this finding did not deviate from the Hardy–Weinberg equilibrium ($p = 0.901$). The heterozygous TLR2 Arg753Gln polymorphism was found in seven people in the control group (with a polymorphism allele frequency of 3.9%) and five people in the case group (with a polymorphism allele frequency of 2.63%) (Table 1); this difference was not statistically significant ($p = 0.566$).

Table 1. Characteristics of study participants and TLR2 polymorphism gene frequencies.

Characteristics	Control Group (n = 90)	Case Group (n = 95)	p-Values
Median age in years (IQR)	44 (20.75)	46 (12.5)	0.074
Age range (years)	22–80	24–73	
Different ethnicity *	22 (24.4%)	22 (24.4%)	0.864
HIV status (pos./neg.)	1/73	10/61	0.004
Mean no. of sexual contacts (no. of participants) **	9.97 (n = 39) 95%CI [5.539–14.401]	17.69 (n = 49) 95% CI [10.301–25.079]	0.006
Arg753Gln polymorphism frequency	7/180 (3.9%)	5/190 (2.63%)	0.566
GG genotype frequency	83 (92.2%)	90 (94.7%)	
GA genotype frequency	7 (7.8%)	5 (5.3%)	0.559
AA genotype frequency	0 (0.0%)	0 (0.0%)	
Hardy–Weinberg equilibrium	0.929	0.966	0.901
Tyr715Asn polymorphism frequency ***	0 (0.0%)	0 (0.0%)	not tested
Glu738Gln polymorphism frequency ***	0 (0.0%)	0 (0.0%)	not tested

* participants reporting different nationality than Dutch or non-specified nationality for themselves or their parents; ** reported known and unknown casual sexual contacts, not including stable partner contact(s) as casual contacts (usually random), represent higher risk for acquiring syphilis. *** while these additional polymorphisms were tested, neither was found.

The numbers of self-reported sexual contacts in the last six months were collected for almost half (47. 57%) of the participants (n = 88 in total, out of 185). The numbers of casual sexual contacts were those reported in addition to steady partner contact(s). Nine participants reported more than one steady partner in the last six months, eight reported two steady partners, and one reported three steady partners. The number of sexual partners (i.e., reported casual contacts, excluding steady partners) was higher in the case group (group mean: 17.69; 95% confidence interval: 10.3–25.1) compared to the control group (group mean: 9.97; 95% confidence interval: 5.4–14.4) (Figure 1, Mann–Whitney U test, p = 0.006).

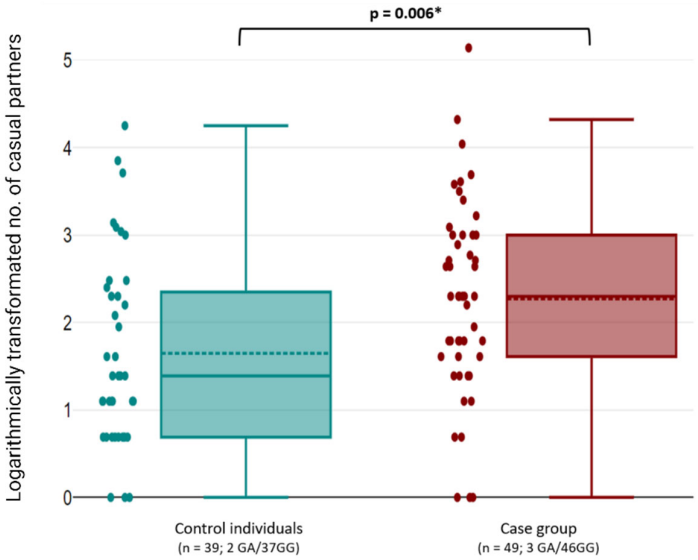

Figure 1. The $\log_e$ numbers of casual sexual contacts in the control group and case group. Altogether, the number of casual sexual contacts was known for 88 participants. The case group reported significantly more contacts than the control group (Mann–Whitney test, p = 0.006). * Statistical significance lesser than p = 0.05.

The analysis of the number of TLR2 Arg753Gln polymorphism heterozygotes in the case group (n = 49) showed that the heterozygous participants reported more sexual contacts compared to wild-type homozygotes (exact permutation test = 0.037) (Figure 2).

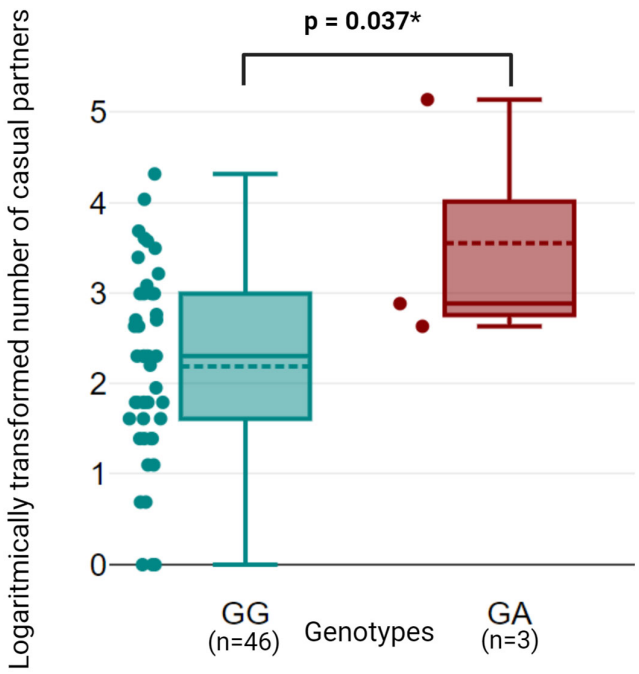

Figure 2. The comparison of the $\log_e$ numbers of casual sexual partners in the case group. The number of self-reported sexual contacts was higher in heterozygous (GA) participants than homozygous participants without a polymorphism (GG); the difference was statistically significant (exact permutation test, $p = 0.037$). The normal distribution of data within groups GG and GA was tested with Shapiro–Wilk normality test, and both groups did not differ from a normal distribution ($p = 0.55$ and $p = 0.17$, respectively). The homogeneity of variance between the GG and GA groups was tested by the F-test, and there was no difference in variance between the groups ($p = 0.38$). * Statistical significance lesser than $p = 0.05$.

Neither of these two additionally detected polymorphisms (Tyr715Asn (rs5743706, 1892C > A) nor Glu738Gln (rs142286429, 2029C > T)) were found in our sample set.

4. Discussion

While the previous work (Grillová et al. (2020) [18]) on the Czech and Slovak populations identified the TLR2 Arg753Gln polymorphism as a possible protective factor against syphilis, our current study did not find this association. Nevertheless, a similar, but not statistically significant, trend was noticed in this study, where the control group had a higher prevalence of Arg753Gln heterozygotes (GA, Table 1) compared to the syphilis-positive participants. Grillová et al. (2020) [18] also described one heterozygote for the polymorphisms Tyr715Asn (rs5743706, 1892C > A) and Glu738Gln (rs142286429, 2029C > T) [18], but we failed to find these in our samples, most likely due to a low general frequency of these two polymorphisms, which, according to the dbSNP database, is less than 1% in the uploaded studies for human genome sources (e.g., 1000 Genomes).

The allele frequency of the Arg753Gln polymorphism in the ACS population tested in this study was 3.9%, which was similar to another study performed in a Dutch population, where the allele frequency of the TLR2 Arg753Gln SNP was 4% based on 262 tested individuals [23]. A study by Krediet et al. (2007) [22], which showed that the Arg753Gln polymorphism was associated with preterm births in a Dutch population, reported an overall allele frequency of 2.8% based on an analysis of 524 individuals. Interestingly, these frequencies are lower than the one reported in the dbSNP database [28], where it is 5.1% (based on 998 individuals) [21]. Ours and previous studies suggest an inherently lower frequency of the TLR2 Arg753Gln polymorphism in the Dutch population compared to the Czech and Slovak populations, with a control group allele frequency of 10.9% [18]. In addition to the effect of our sample size, the relatively low allele frequency of the TLR2

Arg753Gln polymorphism may explain why no mutant Arg753Gln homozygotes (AA, Table 1) were found in our study and also why the difference between the case group and control group did not reach statistical significance.

While PLWH were enrolled in both studies, our study had a somewhat lower number of PLWH than the study by Grillová et al. (2020) [18], in which more than 50% of the patients with a known HIV status represented PLWH. One possible explanation for the lower prevalence of HIV-positive patients in our study was the self-reported pre-exposure prophylactic (PrEP) use of antiretrovirals in both the case and control groups (28 and 19 individuals, respectively); PrEP could substantially limit HIV transmission, even though PrEP only became available in 2015 [29]. However, it is unknown whether and to what extent this could contribute to the differences observed between this study and the Grillová et al. [18] syphilis TLR2 polymorphism study. HIV positivity could affect participants' distribution in the control group or case group, as HIV coinfection can cause serofast or non-responding serology [30]. Despite that, a low prevalence of HIV positivity and no discrimination between active and past infection in participants limit the effect of HIV coinfection in our study. Another important difference with the previous study by Grillová et al. (2020) [18] was related to the analyzed study samples. The study population used by Grillová et al. (2020) [18] was younger (27.5 years for the control group and 33.9 years for the case group) compared to our study (44 years and 46 years, respectively); thus, there was a greater potential for exposure to *Treponema pallidum* infection in our sample. Additionally, our study tested only men, specifically men having sex with men (MSM) and men having sex with men and women (MSMW), while the Grillová et al. study tested mainly heterosexual males and females. This difference could impact differences in sexual networks and the number of sexual contacts.

Despite the small number of participants and the lack of information as the biggest limitations of our study, we found—as expected—that the reported number of sexual partners was higher in the syphilis case group compared to the control group. Moreover, we found that TLR2 Arg753Gln heterozygotes were reported more frequently among syphilis patients who reported more casual sexual contacts (exact permutation test; $p = 0.037$), suggesting that, for TLR2-Arg753Gln heterozygote syphilis patients, more casual contacts are necessitated to acquire syphilis compared to wild-type homozygous syphilis patients. Our definition of syphilis cases (i.e., having at least one positive treponemal-specific serological test and/or a PCR test for *Treponema pallidum* ssp. *pallidum* DNA) did not distinguish between past and active syphilis infections, and we considered any detected syphilis infection as a case. Focusing on only active syphilis, i.e., patients positive for the first time for treponemal-specific tests, RPR-positive, and/or PCR positive, the number of syphilis cases decreased to 26 participants. Despite that, the comparison of the number of casual sexual contacts between participants with heterozygotes in Arg753Gln polymorphisms and those with homozygotes not carrying polymorphism remained statistically significant (Mann–Whitney test, $p = 0.046$), with the mean number of casual sexual contacts being 92 (standard deviation = 78, $n = 2$) for those with polymorphic heterozygotes and 15.6 (standard deviation = 13.4, $n = 24$) for those with non-polymorphic homozygotes, respectively. These findings, even though limited by the small number of participants in the individual groups, in addition to the observed higher prevalence of TLR2 Arg753Gln heterozygotes in the control group, support the idea that the TLR2 Arg753Gln polymorphism could partially protect against syphilis acquisition. Participants with heterozygotes for the TLR2 Arg753Gln polymorphism appear to have more sexual contacts before contracting syphilis compared to those with non-polymorphic homozygotes. We thus suggest that the use of the number of self-reported sexual contacts may be seen as a proxy to detect the protective effect of the Arg573Gln polymorphism, which can be particularly useful in a population with a low prevalence of the TLR2 Arg753Gln polymorphism. In such populations, the number of contacts can significantly differ, despite the fact that no difference between the numbers of polymorphisms between the case and control groups was found. However, our conclusions have to be verified by future research on a larger set of samples.

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Institutional Review Board Statement: The ACS study (source of samples) was conducted in accordance with the Declaration of Helsinki and approved by the Medical Ethics Committee of the Amsterdam University Medical Center of the University of Amsterdam, the Netherlands (MEC-07/182).

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Data Availability Statement: The data presented in this study are available upon reasonable request from the corresponding author.

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