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Serum Toll-Like Receptor-2 Levels and TLR2 Arg753Gln Polymorphism in Children with Tonsillitis Caused by *Staphylococcus aureus* and *Streptococcus pyogenes*

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Abstract

Tonsils play a vital function in the body's immune system in children. Tonsillitis is one of the most common reasons children seek care in hospital ENT departments worldwide. This study examines the relationship between the presence of *Staphylococcus aureus* and *Streptococcus pyogenes* isolated from tonsillitis patients, the impact of those bacteria on serum TLR2 levels, and the relationship between the TLR2 Arg753Gln (rs5743708) polymorphism risk and susceptibility to tonsillitis. The study was conceived as a case-control study including 118 participants with tonsillitis colonized with *S. aureus* and *S. pyogenes* and 50 uninfected controls. Serum TLR2 levels have been evaluated by utilizing an enzyme-linked immunosorbent assay (ELISA) kit, and TLR2 Arg753Gln polymorphism was detected via ARMS-PCR. Serum TLR2 levels showed a non-significant increase in both the *S. aureus* and *S. pyogenes* groups, whereas they increased significantly in tonsillitis children infected with mixed bacteria ($P3=0.0018$). Moreover, the co-dominant GA genotype and the dominant model GA + AA of TLR2 Arg753Gln polymorphism were significantly elevated in 78% and 84% of tonsillitis patients compared to the control group (46%) (OR = 5.72, 95% CI: 2.30–14.2, $P < 0.0001$) and (OR = 6.38, $P < 0.0001$). Bacterial infections led to significantly increased sTLR2 levels in tonsillitis patients in the mixed bacterial group, suggesting that sTLR2 levels might be useful as a diagnostic tool for tonsillitis in children. Additionally, the GA genotype of TLR2 Arg753Gln showed a significant association with the development of tonsillitis and may serve as a prognostic predictor of tonsillitis risk among children.

Key words: SerumTLR2, Arg753Gln polymorphism, *S. aureus*, *S. pyogenes*, Tonsillitis.

مستويات مستقبل الشبيه بالـ Toll−2 في المصل وتعدد الأشكال الجيني TLR2 Arg753Gln لدى الأطفال المصابين بالتهاب اللوزتين الناتج عن *Staphylococcus aureus* و *Streptococcus pyogenes*.

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الخلاصة

تلعب اللوزتان دورًا مهمًا في الجهاز المناعي لدى الأطفال. التهاب اللوزتين أحد أكثر الأسباب شيوعًا لزيارة الأطفال لأقسام الأنف والأذن والحنجرة في المستشفيات حول العالم.. شملت هذه الدراسة العلاقة بين وجود بكتريا *Staphylococcus aureus* و*Streptococcus pyogenes* المعزولة من المصابين بالتهاب اللوزتين وتأثير هذه البكتريا على مستوى sTLR2 وعلاقة تعدد اشكال جين TLR2 Arg753Gln (rs5743708) بمخاطر و قابيلة الاصابة بالتهاب اللوزتينتم تصميم الدراسة كدراسة حالة–شاهد، حيث شملت 118 مريضًا بالتهاب اللوزتين مصابين بـ *S. aureus* و *S. pyogenes*، إضافةً إلى 50 فردًا من الأصحاء كمجموعة ضابطة. تم قياس مستويات TLR2 في المصل باستخدام عدة المقايسة المناعية المرتبطة بالإنزيم (ELISA)، كما تم الكشف عن تعدد الأشكال Arg753Gln في جين TLR2 باستخدام تقنية ARMS–PCR. أظهرت مستويات TLR2 في المصل ارتفاعًا غير معنوي في كل من مجموعة *S. aureus* ومجموعة *S. pyogenes* بينما لوحظت زيادة معنوية في الاطفال المصابين بالتهاب اللوزتين الناتج من الاصابة المشتركة بال *S. aureus,* و*S. pyogenes* (P3=0.0018). وكذلك أظهر كل من Co–dominant GA genotype و dominant GA + AA للتعدد الشكال TLR2 Arg753Gln أرتفاعا معنويا في 78% و84% في مرضى التهاب اللوزتين مقارنة بالعينات السطرة 46% (OR = 5.72, 95% CI: 2.30–14.2, $P <$ 0.0001) and (OR = 6.38, $P <$ 0.0001). تم الاستنتاج بأن الاصابة البكترية تؤدى الى زيادة في مستوى sTLR2 في مرضى التهاب اللوزتين وبالاخص زيادة معنوية في مجموعة المصابين بالبكتريا المشتركة ولذا من الممكن الاستفادة من هذا العامل لتشخيص الاطفال المصابين بالتهاب اللوزتين. وكذلك GA genotype of TLR2 Arg753Gln له علاقة قوية بتطور مرض التهاب اللوزتين ويمكن أستخدامه كعامل كشف مبكر لتشخيص الاطفال المصابين بالتهاب اللوزتين.

1. Introduction

Tonsillitis is an inflammation of the tonsils typically caused by *Staphylococcus aureus* and *Streptococcus pyogenes* [1]. Bacteria are the second most common cause of tonsillitis (about 30%), particularly in recurrent cases, with viruses accounting for around 70% of cases [2, 3]. It is necessary for the immune system's defense in response to microbial invasion to recognize specific molecular patterns on the surface of microorganisms. Toll-like receptors (TLRs) play a key role in initiating both innate and adaptive immune responses [4]. TLRs are present on a diversity of immune and non-immune cells. TLRs are separated into two sets consistent with their position: intracellular TLRs (TLR-3, -7, -8, and -9) on endosome membranes and membrane TLRs (TLR-1, -2, -4, -5, -6, and -10). Every TLR identifies distinct ligands and initiates signaling via pathways that are either TRIF- or MyD88-dependent [5].

Toll-like receptor-2 (TLR2) has a unique capability to identify glycolipids, lipopeptides, lipopolysaccharides, lipoproteins, and lipoteichoic acids from Gram-positive bacteria [6, 7] *S. aureus* lipoproteins are the primary ligands of TLR2 [8]. TLR2 is released into the bloodstream by monocytes, macrophages, dendritic cells, and granulocytes, and their levels increase during inflammatory and infectious diseases [9]. Moreover, TLR2 in mammalian vertebrates triggers immunological responses with TLR1 or TLR6, which can identify microbial patterns as homodimers or heterodimers. Bacterial lipoproteins are strong TLR2 activators that cause the synthesis of cytokines that promote inflammation [10-12].

Single-nucleotide polymorphisms (SNPs) in different genes have been found to be associated with the risk and severity of various diseases, including acute lymphoblastic leukemia patients [13], rheumatoid arthritis [14], and cervical cancer [15]. Additionally, SNPs in TLR families have been connected to increased risk and susceptibility to a range of infections among Chinese AIDS patients [16], gastritis [17], and recurrent or severe infections [18].

Tonsillitis is a leading cause of pediatric ENT visits worldwide. This study was conducted to determine the roles of *S. aureus* and *S. pyogenes* in serum TLR2 levels and the association of the TLR2 Arg753Gln polymorphism with increased risk and susceptibility to bacterial infection in pediatric tonsillitis patients.

2. Materials and Methods

2.1. Blood sample collection

Five mL blood samples were collected from 118 patients with recurrent tonsillitis who had positive cultures for *S. aureus*, *S. pyogenes*, or mixed bacteria. The patients were categorized into three age groups (<5, 5-10, >10 years), including 66 males, 52 females, and 50 healthy controls, who were not suffering from any infections, with a mean age of 6.92±2.346 years, which included 27 males and 23 females. To determine the sTLR2 level, 3 mL of the sample was centrifuged for ten minutes at 3000 rpm, transferred to 1.5 mL, and then kept in an Eppendorf tube at -20°C, and the residual 2 mL was placed in an EDTA tube for TLR2 genotyping. An otolaryngologist assessed the clinical data.

2.2. Bacterial culture and identification

The swab taken from the tonsil tissue was inoculated onto blood agar, DNase agar, and mannitol salt agar, and incubated overnight at 37 °C. After incubation, the clinical isolates were initially identified based on colony characteristics, Gram stain morphology, hemolysis type on blood agar, biochemical tests, bacitracin susceptibility testing, and the Vitek-2 system.

2.3. Assessment of sTLR2 levels

Serum TLR2 levels were measured using a Human TLR2 ELISA kit (ELK 1095, Detection Range 0.32-20 ng/mL, and Sensitivity 0.115 ng/mL). The assays were performed in accordance with the recommended protocols. The optical density at 450 nm was recorded using a spectrophotometer. According to Kit's standard curve, the findings are displayed in ng/ml.

2.4. Genotyping of SNPs in TLR2 Arg753Gln (rs5743708).

Genomic DNA was isolated from whole blood samples using a Blood DNA Preparation Solution Kit (Jena Bioscience/Germany). Concentration and purity of the DNA were assessed by using a NanoDrop spectrophotometer (A260/A280) with a ratio of 1.8-2.0 and a concentration of 70-450 ng/µl, indicating suitable quality for downstream analysis. Members were genotyped for the TLR2 Arg753Gln. The TLR2 Arg753Gln polymorphism was evaluated using a tetraprimer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR). The assays were conducted according to the suggested protocols. Twenty-five µl of PCR master mix reaction volume was utilized, which counted 3 µl of genomic DNA, 12.5 µl of 2X GoTaq Green Master Mix (Promega, USA), and 1 µl of each of the TLR2 gene's forward and reverse primers, the inner primers specific for the G and A alleles were run separately for each sample, with two PCR reactions per sample, due to their identical amplicon size, which could otherwise lead to overlapping bands. Lastly, 6.5 µl of nuclease-free water was added to the mixture. The nucleotide sequences of the primers are displayed in Table 1.

Table 1: Primer sequence, length and product size.

Gene	Primer	Primer Sequence	Length (bp)	PCR product size (bp)
TLR2 gene	F	5′-TATGGTCCAGGAGCTGGAGA-3`	20	430-bp
	R	5′-TGACATAAAGATCCCAACTAGAC-3`	23	430-bp
	G Allele	5′-GGTCTTGGTGTTCATTATCTTCC-3`	23	307-bp
	A Allele	5′-GGTCTTGGTGTTCATTATCTTCT-3`	23	307-bp

The primers TLR2-F, TLR2-R, and TLR2-A were utilized to detect the A allele, while TLR2-F, TLR2-R, and TLR2-G were employed to detect the G allele of the TLR2 Arg753Gln polymorphism [19]. The 430_bp common outcome was increased by utilizing TLR2-F and TLR2-R primers, and the 307_bp product was amplified using either TLR2-F and TLR2-G or TLR2-F and TLR2-A primers. The cycling conditions included an initial denaturation at 95°C for 5 minutes, followed by 35 amplification cycles consisting of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 1 minute, and a final elongation at 72°C for 10 minutes. Additionally, electrophoresis was performed on a 2% agarose gel containing 0.5 µg/ml ethidium bromide to examine PCR products, which were then displayed under UV light.

2.5. Statistical analysis

GraphPad Prism Software 8.0.1 was utilized to perform descriptive analysis and multiple comparisons. Demographic data were expressed as means ± SD or percentage, and the normality of the data was assessed using the D'Agostino-Pearson omnibus test, the Shapiro-Wilk test, and the Kolmogorov-Smirnov test. For serum TLR2 levels, the Kruskal-Wallis test for non-normal distributions was used, and Dunn's post hoc test was used for pairwise comparisons. Additionally, chi-square tests were performed to analyze the genotyping data. P-values < 0.05 were regarded as statistically significant.

3. Results

3.1. Demographic data and clinical status of tonsillitis patients and healthy controls

A total of 118 patients with tonsillitis (48 infected with *S. aureus*, 30 with *S. pyogenes*, and 40 with mixed bacteria) and 50 healthy controls took part in the study. Among the *S. aureus* group, there were 24 males and 24 females. While 16 males and 14 females were included in the *S. pyogenes* group. In the mixed bacteria group, males prevailed with 26 patients, while females accounted for 14. The healthy control group consisted of 27 males and 23 females.

Most patients in the *S. aureus, S. pyogenes,* and mixed bacteria groups were between 5 and 10 years old, with proportions of 77%, 70%, and 60%, respectively. Patients younger than 5 years accounted for 14.5%, 16.5%, and 30% in these groups, while those older than 10 years accounted for smaller proportions of 8.5%, 13.5%, and 10%, respectively. The healthy control group predominantly fell in the 5–10 years category (80%), with fewer patients below 5 years and above 10 years. The mean ages and standard deviations were as follows: *S. aureus* (7±2.423 years), *S. pyogenes* (7.33±2.682 years), mixed bacteria (6.7±2.848 years), and healthy controls (6.92±2.346 years).

Tonsillitis history was recorded in 64.58% of the *S. aureus* group, 80% of the *S. pyogenes* group, and 72.5% of the mixed group. Difficulty swallowing was severe in 54.2%, 40%, and 42.5%, respectively. Severe breathing difficulty occurred in 25%, 36.6%, and 27.5%, while snoring was severe in 58.3%, 43.4%, and 45% (Table 2).

Table 2: Demographic and clinical characteristics of healthy controls and patients with tonsillitis infected with *S. aureus*, *S. pyogenes*, or mixed bacterial infections.

Demographic data	Patient (n=118)			Control (n=50)
	***S. aureus* (n.%)**	***S. pyogenes* (n.%)**	***Mixed* (n.%)**	
Gender				
M	24(50%)	16(53.33%)	26(65%)	27(54%)
F	24(50%)	14(46.67%)	14(35%)	23(46%)
Total number of participate	48(100%) 40.6%	30(100%) 25.4%	40(100%) 33.8%	50(100%)
Age <5 5-10 >10	7(14.5%) 37(77%) 4(8.5%)	5(16.5%) 21(70%) 4(13.5%)	12(30%) 24(60%) 4(10%)	8(16%) 40(80%) 2(4%)
Age (Mean +SD)	(7±2.423)	(7.33±2.682)	(6.700±2.848)	(6.920±2.346)
History of Tonsillitis				
Yes	31(64.58%)	24(80%)	29(72.5%)	0(0%)
No	17(35.42%)	6(20%)	11(27.5%)	0(0%)
Difficulty of Swelling				
Poor	10(20.8%)	12(40%)	15(37.5%)	0(0%)
Moderate	12(25%)	6(20%)	8(20%)	0(0%)
Severe	26(54.2%)	12(40%)	17(42.5%)	0(0%)
Hard Breathing				
Poor	24(50%)	13(43.3%)	18(45%)	0(0%)
Moderate	12(25%)	6(20%)	11(27.5%)	0(0%)
Sever	12(25%)	11(36.6%)	11(27.5%)	0(0%)
Snoring				
Poor	13(27.2%)	10(33.3%)	16(40%)	0(0%)
Moderate	7(14.5%)	7(23.3%)	6(15%)	0(0%)
Sever	28(58.3%)	13(43.4%)	18(45%)	0(0%)

3.2. Analysis of sTLR2 levels in tonsillitis patients and healthy controls

A significant increase in sTLR2 levels (ng/mL) was noted in the mixed bacteria group compared to the healthy control group ($p3 = 0.0018$). The sTLR2 levels were higher in the *S. aureus* group than in the control group (p1 = 0.6996); the difference was not significant. While the *S. pyogenes* group shows a lower level of sTLR2 compared to the control group (p2 > 0.9999) (Table 3, and Figure1).

Table 3: Comparison of TLR2 serum levels in tonsillitis patients infected with *S. aureus*, *S. pyogenes*, mixed bacteria, and controls.

Serum TLR2 (ng/mL)	***S.aureus* (n = 24)**	***S.pyogenes* (n = 20)**	**Mixed (n = 20)**	**Control (n = 24)**	**H**	**P-value**	**SBG**
Mean ±SD	5.71 ± 2.60	5.10 ± 4.39	8.42 ± 3.63	4.47 ± 2.26	13.52*	0.0036*	$p1 = 0.6996$ ns
Median	6.40	3.30	9.340	4.390			$p2 > 0.9999$ ns
Min-Max	1.17 - 9.16	1.16 – 13.5	1.16 - 13.7	1.30 – 8.47			$p3 = 0.0018$**

H stands for the Kruskal-Wallis test, and Dunn's post hoc test was used for pairwise group comparisons. p1: p-value for comparing between Control and S. aureus. p2: p-value for

comparing between Control and S. pyogenes. p3: p-value for comparing between Control and Mixed. SBG: Significant differences between groups.

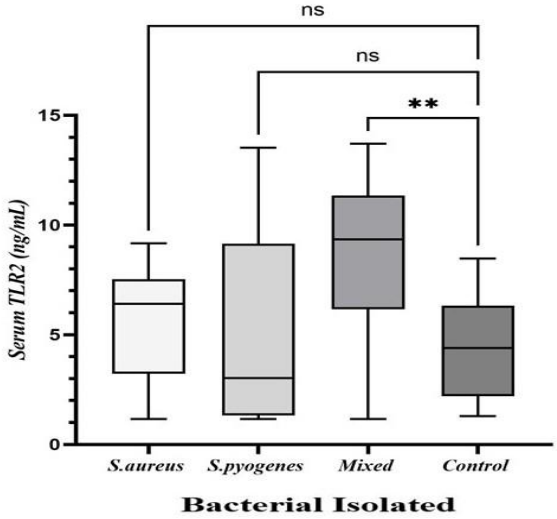


Figure. 1: Serum TLR2 levels (ng/mL) were significantly higher in the mixed bacterial infection group (p<0.01) with no significant differences between the *S. aureus* and *S. pyogenes* groups and the control group.

3.3. Role of TLR2 Arg753Gln polymorphism on tonsillitis susceptibility

Genotype frequencies of the co-dominant GA genotype were significantly enhanced in 78% of tonsillitis patients compared to controls (46%) (OR = 5.72, 95% CI: 2.30–14.2, P < 0.0001). The dominant model (GA + AA vs. GG) showed an OR of 6.38 (P < 0.0001), indicating an increased risk and susceptibility to tonsillitis. The AA genotype was only found in 6% of the patients, while the G allele was more common in controls than in tonsillitis patients (77%, 55%, OR = 2.81, 95% CI: 1.48–5.32, P = 0.001) (Table 4, and Figure 2).

Table 4: Genotypic and allelic distribution of the TLR2 Arg753Gln (rs5743708) SNP (G/A) polymorphism in tonsillitis patients and healthy controls.

TLR2 Arg753Gln (G/A)	Patients N=50 (NO.%)	Control N=50 (NO.%)	OR	95% CI	*P-value*
	Genotype Frequencies				
Co-dominant					
GG	8 (16)	27 (54)	1	-	-
GA	39 (78)	23 (46)	**5.72**	2.30 - 14.2	**<0.0001**
AA	3 (6)	0 (0)	-	-	-
	Genetic Models Analysis				
Dominant					
GG	8 (16)	27 (54)			
GA + AA	42 (84)	23 (46)	**6.38**	-	**<0.0001**
Recessive					
GG + GA	47 (94)	50 (100)			
AA	3 (6)	0 (0)	-	-	-
	Alleles				
G	55 (55)	77 (77)	**2.81**	1.48 - 5.32	**0.001**
A	45 (45)	23 (23)			

The statistical analysis was performed via chi-square (χ^2) test. TLR2 Arg753Gln (rs5743708) SNP: single nucleotide polymorphism, CI: confidence interval, N: sample size, and OR: odds ratio.

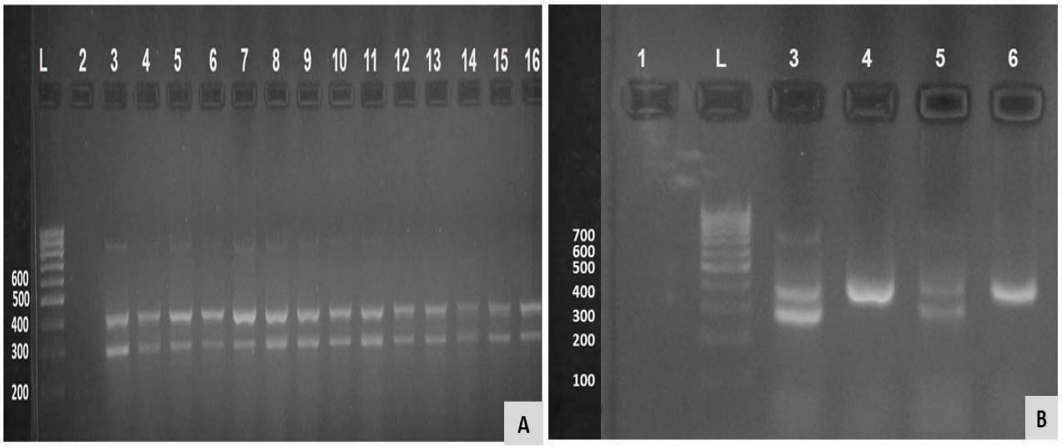


Figure 2 : Agarose gel electrophoresis showed the TLR2 polymorphism gene: (A) Lane 1: Ladder of 100 pb. Lane 2: Negative control. The 430 bp is an internal control. Lanes 3-9: Amplified the Arg753Gln for allele G (307 bp). Lane 10-16: Allele A target amplification (307 bp). (B) Lane 1: Negative control; Lane 2: Ladder of 100 pb. The 430 bp is an internal control. Lanes 3 and 5 are heterozygous carriers of alleles G and A (307 bp). Lane 4: Target amplification for Allele A and Lane 6: Allele G (307 bp).

4. Discussion

Tonsils are small masses of lymphoid tissue at the back of the throat that participate in immune system functions by monitoring and trapping bacteria and viruses that enter the body through oral intake and cause tonsillitis, which may have a negative effect on the quality of life in children. Peak susceptibility to tonsillitis occurs between 5 and 10 years. Vintilescu and his colleague concluded that children aged 5 to 8 with RT are the most common reasons for visiting a pediatrician [20].

A history of difficulty swallowing was most frequent in the *S. aureus* group. A distinct investigation in Brazil and Somaliland of children and adolescents with chronic tonsillitis who underwent tonsillectomy identified *S. aureus* in tonsillar tissue, demonstrating its ability to form biofilms, which are recognized contributors to chronic symptoms such as pain and dysphagia [21, 22]. Kostić and his team confirmed that these symptoms are common among patients with tonsillitis caused by *S. aureus* [1]. While severe breathing issues were predominantly observed in the *S. pyogenes* group, abscesses, which are often caused by *S. pyogenes*, may progress to laryngeal edema and airway obstruction [23].

Snoring was present in both the *S. aureus* and *S. pyogenes* groups (Table 2). A comprehensive analysis highlights that *S. aureus* and *S. pyogenes* in tonsillar biofilms contribute to tissue hypertrophy and subsequent upper airway obstruction, including snoring and sleep apnea [21, 24]. In pediatric obstructive sleep apnea syndrome (OSAS), *S. pyogenes* was frequently found colonizing OSAS tonsils (via immunohistochemistry and molecular profiling), suggesting a direct link between bacterial presence and chronic tonsillar hypertrophy that underlies snoring and airway obstruction [25].

In recurrent tonsillitis, the TLR2 expression on CD8+ T-cells is upregulated compared to uninfected tonsil tissue, suggesting that TLR2 may link innate recognition of bacterial

components directly to T-cell activation [26]. Numerous lipoproteins and lipoteichoic acid expressed by the *S. aureus* and *S. pyogenes* cell walls activate numerous immune cells through a variety of receptors, especially TLR2 [27]. According to our data analysis, sTLR2 levels were elevated in tonsillitis patients and may be useful for diagnosing tonsillitis, especially in children infected with mixed bacteria (Table 3). An immunohistochemistry analysis conducted by Gazi and co-authors showed higher TLR2 expression in the tonsillar surface epithelium and the lymphoid interior [28].
Numerous studies have found the sTLR2 level to be significantly elevated in many diseases, such as metastatic and non-metastatic breast cancer [29], Hashimoto's disease [30], myeloproliferative neoplasm [31], and recurrent myocardial infarction [32]. Yuan *et al*., reported that downstream organ failure in severe bacterial disease is supported by the finding that increased sTLR2 in early-stage sepsis [33]. Paarnio and his team show that systemic inflammation in colorectal cancer patients was inversely correlated with sTLR2 levels [34]. Furthermore, it has been shown that serum TLR2 levels are lower in individuals with acute dengue virus infection than in healthy individuals [9].

Our results show that the TLR2 Arg753Gln (rs5743708) variant and polymorphism make children more susceptible to tonsillitis. The GA genotype showed the strongest association with disease susceptibility (OR = 5.72, 95% CI = 2.30–14.2, $p < 0.0001$). The AA genotype was detected in 6% of patients but was absent in the healthy control group. Moreover, the dominant GA + AA genotype was significantly associated with the development of tonsillitis in children (OR = 6.38, $p < 0.0001$). In the allele frequency analysis, the G allele was significantly more frequent in the control group and thus associated with a lower risk of infection (OR = 2.81, 95% CI = 1.48–5.32, $p = 0.001$) than to the A allele, which was more common in patients (Table 4). Kutukculer and his colleague concluded that Arg753Gln polymorphisms are favorably connected with several recurrent febrile infectious diseases in children [35]

A study by Ruifeng and his colleagues on the TLR2 rs5743708 polymorphism found a substantial association with an increased risk of tuberculosis [36]. On the other hand, some studies showed a correlation between lower TLR2 Arg753Gln polymorphisms and protective effects against inflammation and infectious diseases [37] and some sexually transmitted diseases (syphilis) [38]. A study by Vuononvirta J and his colleagues [39] confirmed that the A allele and the TLR2 (rs5743708) mutant (G/A) genotype were associated with a higher risk of pneumonia infection in children. Teräsjärvi and his colleagues concluded that the TLR2 Arg753Gln polymorphism may influence baseline susceptibility to bacterial infections by impairing innate immune activation. Suggesting its effect may vary with age, local immunity, or environmental factors [18].

In conclusion, bacterial infections led to significantly increased sTLR2 levels in tonsillitis patients in the mixed bacterial group, and thus, they might be useful as a diagnostic tool for tonsillitis in children. Additionally, the GA genotype of TLR2 Arg753Gln **(**rs5743708) was significantly associated with the progression of tonsillitis. It may serve as a prognostic predictor of tonsillitis risk in children and as a possible diagnostic biomarker to identify children at risk of recurrent bacterial tonsillitis.

Institutional Review Board Statement: The study was conducted at a Teaching Hospital in Erbil city from July 2024 to December 2024 and was approved by the Human Ethics Committee of the College of Science, Salahaddin University-Erbil, which authorized and approved the current study (Approval No.: 45/270 Date: 30/6/2024).

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Conflict of Interest: The authors report no conflicts of interest.

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