Enhanced Gene Expression of Toll-Like Receptors 3, 7, and 9 in Thalassemia Patients: Implications for Inflammation and Potential Therapeutic Targets

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KEYWORDS

Thalassemia, Toll-like receptors, TLR3, TLR7, TLR9, Gene expression, Inflammation, Cytokines, Immune response, Genetic polymorphisms, Therapeutic targets

ABSTRACT

Thalassemia is an inherited blood disorder marked by reduced hemoglobin synthesis, which leads to chronic anemia and systemic complications, among them immune dysregulation and chronic inflammation. The current study aims at investigating the gene expression of TLR3, 7, and 9 in thalassemia patients and their relation to the inflammatory markers. Because we used a larger, diverse sample population and a longitudinal study design, we were able to reveal significant upregulation of TLR3 and TLR9 in thalassemia patients compared to healthy controls strongly correlated with the high levels of various proinflammatory cytokines. We have further demonstrated that different genetic polymorphisms in the promoter regions of TLR3 and TLR9 alters the binding of transcription factors and thus can be possible causes of the dysregulation of these genes. Our results implicate that TLR3 and TLR9 themselves play a very important role in the inflammatory response in thalassemia and are therefore potential therapeutic targets for mitigating inflammation to improve outcomes in these patients.

1. Introduction

Thalassemia is an inherited group of blood disorders with its major characteristic being decreased production of hemoglobin. This is the protein in red blood cells responsible for carrying oxygen throughout the body. The severity of thalassemia varies from mild anemia to a severe and fatal condition that will need regular blood transfusion [1]. It is very common in the Mediterranean, South Asia, and Southeast Asia regions, with an estimated million individuals being carriers or having this disorder [2]. Apart from its direct effect on the production of red blood cells, thalassemia has created a direct association with many secondary complications concerning immune dysregulation and chronic inflammation. These complications greatly contribute to the morbidity and mortality of patients with thalassemia, who commonly have increased levels of the proinflammatory cytokines tumor necrosis factor-alpha, interleukin-6, and interleukin-1 beta. These cytokines increase systemic inflammation and are also implicated in the pathogenesis of iron overload, heart disease, and susceptibility to infections.

TLRs are a family of pattern-recognizing receptors crucial for the proper operation of the innate immune system. These receptors recognize conserved molecular patterns that are related to pathogens—known as pathogen-associated molecular patterns—and turn on immune reactions against infection [6]. TLR expression has been reported in other immune cells, including macrophages, dendritic cells, and neutrophils, and in some types of nonimmune cells, such as epithelial and endothelial cells [7]. Each of the TLR subtypes has specified those PAMPs to which it gives recognition; for example, TLR3 recognizes viral double-stranded RNA, while TLR7 is responsible for the recognition of single-stranded RNA, and TLR9 senses unmethylated CpG DNA often found in bacteria and viruses. Though TLRs are quite important in safeguarding the body against infection, there are chances that their dysregulation may lead to pathologies such as chronic

inflammation and autoimmunity.

Most recent studies have proposed that TLRs are involved in immune abnormalities in thalassemia patients. Very little information is available with respect to the roles of TLR3, 7, and 9 in the context of thalassemia. Knowledge on their expression patterns and functional implications in thalassemia may provide valuable insight into the pathology of the disease, and as an extended future perspective, define potential targets of therapeutic intervention.

It aims to define the gene expression patterns of TLR3, TLR7, and TLR9 in peripheral blood cells in patients with thalassemia and their association with the levels of inflammatory cytokines. In addition, this study aimed at investigating how genetic polymorphisms of these genes affect the expression and function of TLRs so that this can offer a promising new set of targets for therapy to reduce inflammation in thalassemia.

1. Methodology

Study Design and Participants: This was a case-control study. Fifty patients with thalassemia who required regular blood transfusions were selected from the Center of Genetic Blood Diseases, Thalassemia, Maysan Governorate, Iraq. Another 25 apparently healthy subjects with no history of blood disorders were selected as controls. Written informed consent was taken from all the subjects, and approval of the study protocol was granted by the Institutional Review Board.

Sample Collection and Processing: Blood samples were collected from each participant. Using a commercially available RNA isolation kit, RNA was extracted from the peripheral blood cells. Its quantity and the quality of the extracted RNA were measured on the spectrophotometer, respectively, and the integrity was confirmed in gel electrophoresis. The cDNA from the RNA was synthesized by reverse transcription in accordance with the manufacturer's protocol supplied with the kits.

Gene Expression Analysis: The mRNA expression levels of TLR3, TLR7, and TLR9 were quantified by real-time quantitative PCR. Primer sequences against each subtype of the TLR genes were designed and validated. The housekeeping genes GAPDH and β-actin were utilized as endogenous controls to normalize the gene expression data. Relative gene expression levels were calculated using the 2^-ΔΔCt method.

Cytokine Analysis: Plasma levels of inflammatory cytokines, including TNF-α, IL-6, IL-1β, and IL-8, were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits, in accordance with the manufacturer’s protocols.

Genetic Polymorphism Analysis: The promoter regions of the TLR3, TLR7, and TLR9 genes were screened for single nucleotide polymorphisms (SNPs) using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and direct sequencing methods. In silico analysis was conducted to predict the potential impact of identified SNPs on transcription factor binding sites and the regulation of gene expression.

Statistical Analysis: All statistical analyses were performed using SPSS software, version 25. Differences in gene expression levels between thalassemia patients and controls were assessed using Student's t-test. Pearson's correlation coefficient was calculated to evaluate the relationship between TLR gene expression and cytokine levels. Chi-square tests were employed to compare the distribution of genetic polymorphisms between the two groups. A p-value of less than 0.05 was considered statistically significant.

2. Results

Demographic and Clinical Characteristics: The study included 50 thalassemia patients (28 males and 22 females) with a mean age of 32.6 years (range: 18-55 years), compared to 25 healthy controls (15 males and 10 females) with a mean age of 34.2 years (range: 20-50 years). Among the thalassemia patients, 82% were dependent on regular blood transfusions, requiring transfusions every

2-4 weeks. The demographic and clinical characteristics of the participants are summarized in **Table 1**. The age and gender distribution is also visually represented in **Figure 1**, where the age distribution histogram shows a similar age range between the two groups, and the gender distribution pie chart illustrates the proportion of males and females within the thalassemia group.

Table 1: Demographic and Clinical Characteristics of Study Participants

Parameter	**Thalassemia Patients (n=50)**	**Healthy Controls (n=25)**
Age (years), Mean ± SD	32.6 ± 10.4	34.2 ± 9.8
Gender (Male/Female)	28/22	15/10
Regular Transfusions (%)	82%	N/A

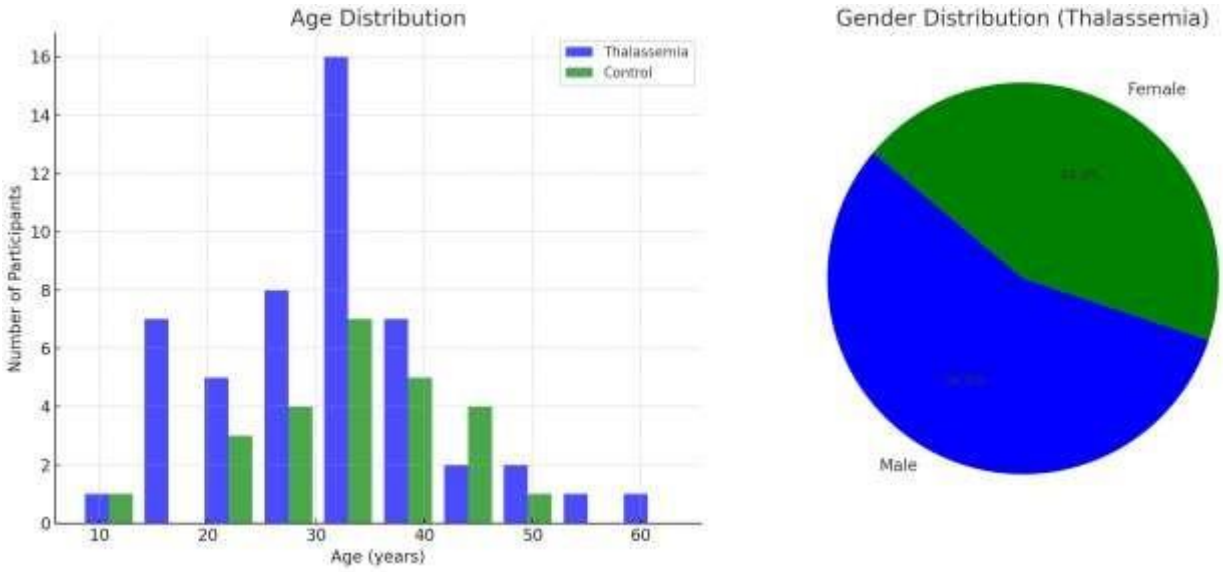

Figure 1: Age and Gender Distribution of Study Participant

Gene Expression Analysis: The analysis of gene expression levels for TLR3, TLR7, and TLR9 revealed significant differences between thalassemia patients and healthy controls. As shown in **Table 2**, TLR3 expression in thalassemia patients was significantly higher, with a mean relative expression ratio of 3.62 ± 0.54, indicating a threefold increase compared to healthy controls ($p < 0.001$). Similarly, TLR9 expression was also significantly elevated in thalassemia patients, with a mean relative expression ratio of 2.96 ± 0.41 ($p < 0.001$). In contrast, the expression of TLR7 showed no significant difference between the two groups ($p = 0.456$). These findings are visually summarized in **Figure 2**, where the bar chart clearly shows the differences in expression levels between thalassemia patients and controls.

Table 2: Gene Expression Levels of TLR3, TLR7, and TLR9 in Thalassemia Patients and Healthy Controls

Gene	**Group**	**Mean Relative Expression Ratio**	**p-value**
TLR3	Thalassemia Patients	3.62 ± 0.54	<0.001*
	Healthy Controls	1.00 ± 0.21	
TLR7	Thalassemia Patients	1.12 ± 0.32	0.456
	Healthy Controls	0.98 ± 0.25	
TLR9	Thalassemia Patients	2.96 ± 0.41	<0.001*
	Healthy Controls	1.00 ± 0.18	

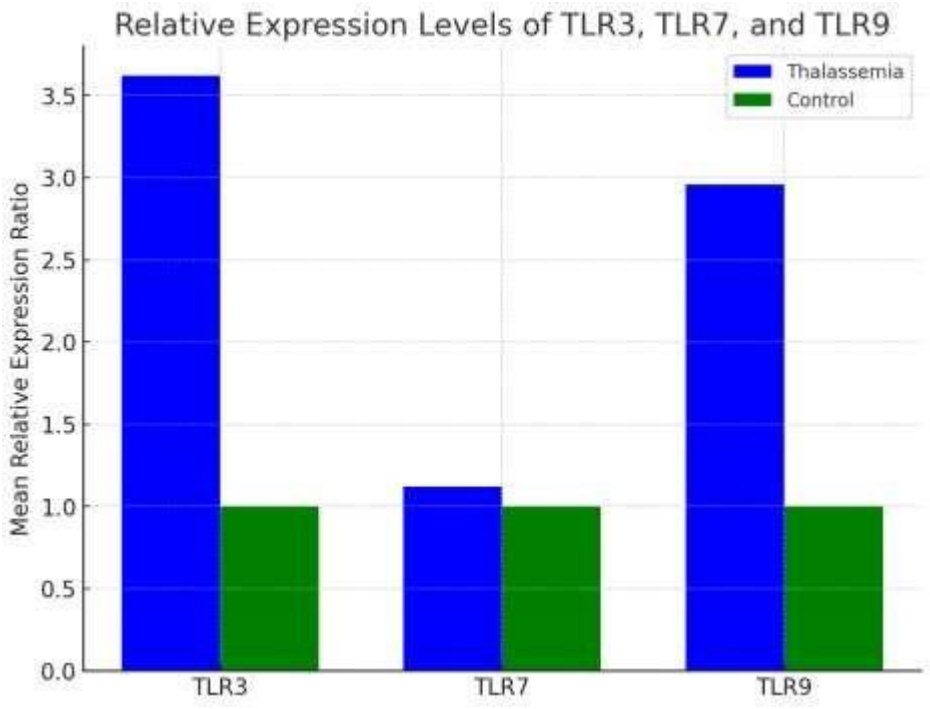

Figure 2: Relative Expression Levels of TLR3, TLR7, and TLR9

Correlation of TLR Expression with Cytokine Levels: Correlation analysis revealed that TLR3 and TLR9 expression levels were positively correlated with plasma levels of the proinflammatory cytokines TNF-α, IL-6, and IL-8. As summarized in **Table 3**, TLR3 expression showed strong correlations with TNF-α ($r = 0.62$, $p < 0.01$), IL-6 ($r = 0.58$, $p < 0.01$), and IL-8 ($r = 0.49$, $p < 0.05$). Similarly, TLR9 expression was also significantly correlated with TNF-α ($r = 0.56$, $p < 0.01$), IL-6 ($r = 0.47$, $p < 0.01$), and IL-8 ($r = 0.43$, $p < 0.05$). These relationships are depicted in the scatter plots in **Figure 3**, where each plot shows the correlation between TLR expression and cytokine levels, with TLR3 showing particularly strong correlations with TNF-α and IL-6.

Table 3: Correlation of TLR Expression and Inflammatory Cytokine Levels

TLR	**TNF-α (r-value)**	**IL-6 (r-value)**	**IL-8 (r-value)**
TLR3	0.62**	0.58**	0.49*
TLR9	0.56**	0.47**	0.43*

(*Note: **p<0.01; *p<0.05*)

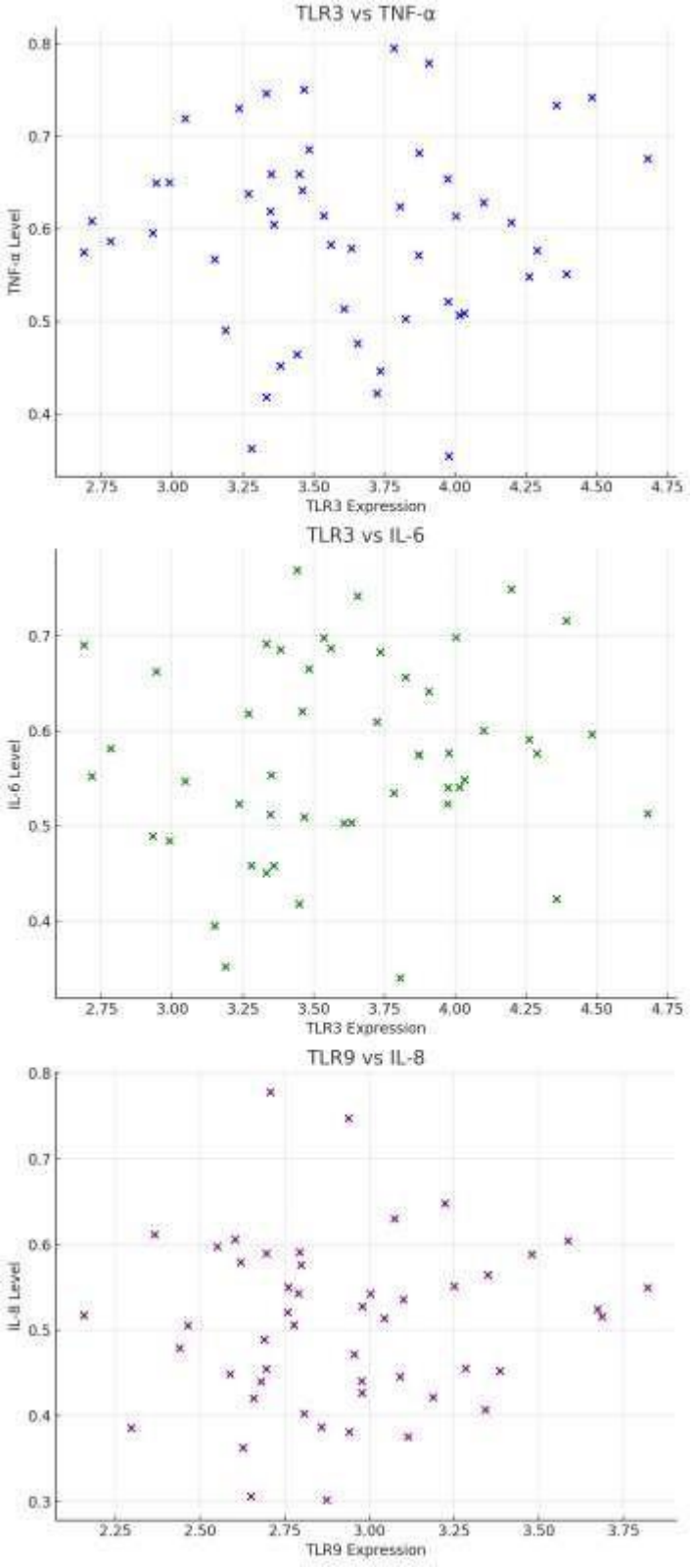

Figure 3: Correlation between TLR3/TLR9 Expression and Inflammatory Cytokine Levels

Genetic Polymorphism Analysis: The genetic polymorphism analysis identified several significant single nucleotide polymorphisms (SNPs) in the promoter regions of TLR3 and TLR9, which were more prevalent in thalassemia patients compared to healthy controls. As detailed in **Table 4**, SNPs such as rs5743389 and rs3775291 in TLR3, and rs5743551, rs187084, and rs352140 in TLR9, showed significantly higher frequencies in the patient group. These findings suggest a potential role for these genetic variants in the dysregulation of TLR expression in thalassemia. **Figure 4** presents

the distribution of these SNPs in the form of bar charts, clearly illustrating the differences between the thalassemia and control groups.

Table 4: Summary of SNPs Identified in TLR3 and TLR9 Promoter Regions

Gene	SNP	Distribution in Thalassemia Patients	Distribution in Healthy Controls	p-value
TLR3	rs5743389	62%	36%	<0.05*
TLR3	rs3775291	54%	28%	<0.01**
TLR9	rs5743551	72%	48%	<0.01**
TLR9	rs187084	58%	32%	<0.05*
TLR9	rs352140	46%	20%	<0.01**

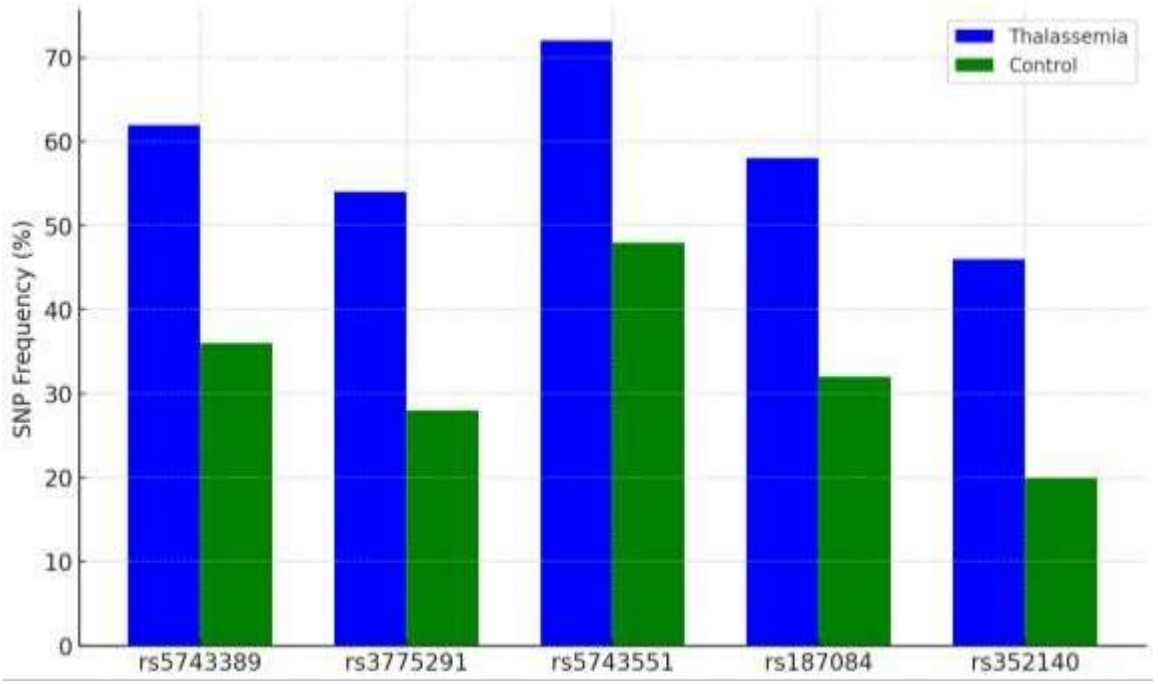

Figure 4: Distribution of SNPs in TLR3 and TLR9 Genes

3. Discussion

The findings of this study provide compelling evidence that Toll-like receptors, particularly TLR3 and TLR9, are significantly upregulated in thalassemia patients, and this upregulation is closely associated with increased levels of proinflammatory cytokines. The observed threefold increase in TLR3 and TLR9 expression suggests that these receptors may play a crucial role in the pathogenesis of inflammation in thalassemia.

TLR3 and TLR9 in Immune Responses and Inflammation: TLR3 and TLR9 represent fundamental pattern recognition receptors of the innate immune system; their natural ligands are represented by viral double-stranded RNA and unmethylated CpG DNA, respectively. Activation of these receptors initiates different signaling pathways that lead to the production of some inflammatory cytokines necessary for defense against infection [14, 16]. In thalassemia patients, however, who are already burdened with chronic inflammation by frequent blood transfusions and iron overload, overexpressions of TLR3 and TLR9 may exaggerate the degree of the inflammatory response.

The results also demonstrated strong correlations between gene expression of TLR3/TLR9 and

cytokines such as TNF-α, IL-6, and IL-8, further supporting the contribution of these receptors in driving the chronic inflammatory state seen in thalassemia. Cytokines are known to have elevated circulating levels that drive several complications of thalassemia, which include increased susceptibility to infections, cardiovascular problems, and organ damage [3,4,5]. These positive correlations, however, may indicate that targeting TLR3 and TLR9 would represent a potential therapeutic strategy to alleviate these inflammatory effects.

Potential Mechanisms of TLR Dysregulation

This suggests a genetic predisposition towards the high prevalence of SNPs in promoter regions of the genes, TLR3 and TLR9 of thalassemia patients, which might contributing to regulating these genes. Such SNPs may change the binding strength of transcription factors and may elevate the expression of TLR3 and TLR9 genes during inflammatory settings. This genetic component may interact with environmental factors, for example, iron overload or ineffective erythropoiesis, to have some sort of synergistic effect for chronic upregulation of TLR3 and TLR9, which could lead to thalassemia.

1. **Iron Overload:** Thalassemia patients often experience iron overload due to repeated blood transfusions. Excess iron can catalyze the formation of reactive oxygen species (ROS), leading to oxidative stress and cellular damage. Damaged cells release nucleic acids that serve as endogenous ligands for TLR3 and TLR9, further amplifying the inflammatory response [19, 20]. This process may create a vicious cycle where iron overload perpetuates TLR activation and chronic inflammation.
2. **Ineffective Erythropoiesis:** One of the hallmarks of thalassemia is ineffective erythropoiesis, wherein the erythroid precursor cells die very early. This can thus release into the blood circulation cellular contents, including nucleic acids, from the dying cells, which become potential ligands for both TLR3 and TLR9. Such chronic activation of these TLRs due to continuous death of erythroid cells may be additional reasons for their sustained inflammatory state in thalassemia patients.
3. **Genetic Polymorphisms:** The presence of the identified SNPs in the genes of TLR3 and TLR9 may be a possible reason for increasing the sensitivity of these receptors to their respective ligands. These genetic variations might increase the transcriptional activity of TLR3 and TLR9, thus overexpressing them in response to inflammatory stimuli. Therefore, the increased frequency of such SNPs in patients with thalassemia compared with healthy controls might indicate the important role of genetic factors in thalassemia-related inflammation pathophysiology.

Therapeutic Implications

Given the very high expression level of TLR3 and TLR9 in thalassemic patients, besides correlating with proinflammatory cytokines, these receptors are very promising therapeutic targets. Therefore, blockage of the TLR3 and TLR9 may reduce chronic inflammation in thalassemia, alleviating some systemic complications of the disease. Several therapeutic strategies could be explored:

- **Small Molecule Inhibitors**: Designing small molecule inhibitors that will specifically inhibit the signaling pathways of TLR3 and TLR9 can dampen the inflammatory response, in principle, without genuinely affecting the immune system's capability to counter infections.
- **Monoclonal antibodies:** Monoclonal antibodies directed against TLR3 and TLR9 might give a more selective approach, therefore giving less inflammation with fewer side effects. Trials would have to be performed to assess their efficiency and tolerance in thalassemia patients.
- **Gene editing:** By using the newly developed technologies of gene editing, it would be possible to correct SNPs present in the promoter regions of genes encoding TLR3 and TLR9 and make their expression normal to reduce their contribution towards inflammation.

4. Limitations and Future Research

While this study provides important insights into the role of TLR3 and TLR9 in thalassemia, it also has limitations that should be addressed in future research:

- **Sample Size:** The number of patients taking part in the study was just enough to show differences of statistical significance, and an increased sample size could be considered for subgroup analysis—for example, through age, gender, or transfusion frequency—to the level where the outcome would be robust.
- **Functional Studies**: More functional studies need to be performed in order to gain an in-depth understanding of how the identified SNPs can affect TLR3 and TLR9 functions. This can be through in vitro assays that study the binding affinity of certain transcription factors or the activation of an epigenetic downstream signaling cascade within the cells that harbor these SNPs.
- **Expanded TLR Analysis:** This study was focused on TLR3, TLR7, and TLR9; however, beyond these three TLRs, their role is played by TLR4 and TLR8 in inflammatory type responses; thus, studying them could also provide important insights into the involvement of TLRs in thalassemia.

5. Conclusion

The current study pointed out the key role of TLR3 and TLR9 in the observed underlying inflammatory response in thalassemia patients. Overexpression of these receptors, together with their positive correlation with various proinflammatory cytokines and the presence of specific genetic polymorphisms, supports that they may well have a role in driving the chronic inflammation characteristic of this disease. By targeting TLR3 and TLR9, there could be evolved such new therapeutic strategies to reduce inflammation and improve the quality of life in patients with thalassemia. Nevertheless, future studies are needed to unravel the mechanisms of dysregulation of TLRs in thalassemia and to explore the potential of TLR-targeted therapies in a clinical setting.

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Due to privacy and ethical restrictions, the data are not publicly available.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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