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Original article

ASSOCIATION OF INTERLEUKIN-6, INTERLEUKIN-4, AND TUMOR NECROSIS FACTOR-ALPHA GENE POLYMORPHISMS WITH SERUM CYTOKINE PROFILES IN BETA-THALASSEMIA MAJOR PATIENTS

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ABSTRACT

Beta Thalassemia is an inherited condition results from mutation in β globin gene which leads to several complication including, severe anemia and iron overload among others, in which the signs appear in early childhood. This study investigated the possible role of cytokine gene polymorphism on thalassemia patients. Our research involved 72 patients diagnosed with beta thalassemia major and a control group of 40 cases. Both groups' ages ranged from 1 to 30 years. Serum cytokines IL-6, IL-4, and TNF-α level were measured using enzyme linked immunosorbent assay (ELISA) as well as analyzing the genetic profile for the same genes. Blood samples were subjected to DNA isolation and molecular detection for gene polymorphism (interleukin 6 polymorphism at position 174, interleukin 4 polymorphisms at position 589, and tumor necrosis factor alpha polymorphism at position 308) using ARMS-PCR. Results showed significant increase in IL-6 and TNF-α ($p<0.05$), whereas the difference in IL-4 was not significant. High-producing genotypes (GG for IL-6, and GG for TNF-α) showed the highest serum cytokine levels, while low-producing genotypes (CC, and AA respectively) showed the lowest. Serum IL-6 levels were significantly higher in patients across all IL-6 genotypes (GG, GC, CC) compared to their respective genotype-matched healthy controls. IL-4 decreased in CC and slightly increased in CT (not significantly). TNF-α levels significantly increased in GG and GA, but not in AA. For the IL-6 (-174G>C) polymorphism, the odds ratio for the GC genotype was 0.66 (95% CI: 0.27–1.61), whereas the odds ratio for the CC genotype was 1.42 (95% CI: 0.22–15.52). The CC, CT, and TT genotypes displayed varying frequencies among groups in the IL-4 gene polymorphism. In relation to the TNF-α gene polymorphism, the AA genotype displayed an odds ratio of 1.7 (95% CI: 0.13–91.27), while the large confidence interval suggests poor statistical precision, whereas the GG genotype was not substantially linked to thalassemia.

KEY WORDS: Thalassemia, Interleukin-6, Interleukin-4, Tumor necrosis factor-alpha, Single nucleotide polymorphism

1. INTRODUCTION

Beta-thalassemia is a diverse group of hereditary hematologic disorders caused by defective β-globin chain production in hemoglobin. A wide range of symptoms, from severe transfusion-dependent anemia to asymptomatic carrier states, result from this (Galanello & Origa, 2010). β-Thalassemia major is a type of chronic, inherited, and microcytic anemia characterized by impaired biosynthesis of the β-globin chain of hemoglobin, which leads to accumulation of unpaired α-globin chains. Excess presence of the α-globin in chains leads to impaired erythropoiesis and is the primary reason for the cellular oxidative damage (Ali *et al*., 2018). Systematic overload of iron, a major contributing cause to multi system problems like cardiomyopathy, endocrine dysfunction, osteopathy, and increased susceptibility to sickness, is greatly exacerbated by repeated transfusion (Bazi *et al*., 2016). Despite being endemic in areas like the Mediterranean Sea, the Middle East, and Southeast Asia, β-thalassemia has become more widespread worldwide,

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especially in Northern Europe and North America, primarily as a result of growing migration (Kattamis *et al*., 2020). In addition to immunological imbalance that impacts both innate and adaptive immunity, complications of infection were a significant cause of mortality as well as ultimately the greatest reason of death for such patients (Ricerca *et al.*, 2009). The pathophysiology of the disease, along with factors such as blood transfusions, iron excess, splenectomy, and zinc shortage underlie the heightened vulnerability to infection observed in patients with thalassemia (Sari *et al.*, 2016).

Cytokines are vital signals which are crucial for immunity regulation (Naji, 2024). The pro-inflammatory cytokine TNF-α is primarily produced from monocyte as well as macrophage. It contributes towards the pathophysiology of autoimmune illnesses, mediates defense against illness, and stimulates innate and adaptive immunity in chronic inflammatory diseases (Vande Casteele& Hemperly, 2018). In people suffering from thalassemia, elevated TNF-α amounts cause an excess of iron to activate macrophages as well as recurrent transfer therapy to stimulate these erythroid precursors, that in turn leads to the creation of defective red blood cells (Garadah *et al*., 2016). IL-4 is associated with the Th2 pathway that aids in tissues healing and fights infections. People with β-thalassemia have been shown to produce cytokines erratically, which could influence how serious the condition is (Ratha & Altaei, 2013). (IL-6) is a prototype cytokine with repetitive as well as versatile properties. The full understanding of the signaling process initiated by IL6 has given a biological foundation for the distinctive characteristics of cytokines (Tanaka & Kishimoto, 2014). Its excess production is believed to play a role in the pathophysiology of illness. Furthermore, thalassemia patients have been shown to produce excessive IL-6 (Abbas & Zaman, 2019). Genetic variations in cytokine genes could be a significant factor in the pathophysiology of β-thalassemia. The entire creation of cytokines is caused by SNPs, which are single-nucleotide polymorphisms in the promoter portion or related sequences that regulate the cytokine genes (Bagheri *et al.*, 2005). It has been found through several investigations that gene polymorphisms of IL-6at rs1800795 (174 G /C) are linked to the levels of IL-6 in the blood as well as the prevalence, and/or progression of a wide range of diseases (Saeed *et al*., 2024). The IL-6 gene exhibits polymorphisms within both, the 5' and 3' flanking sections. The promoter region-174 single G\C base exchange polymorphism is now believed to have biological significance (Alsadawi *et al*., 2019). The IL-4 gene resides along chromosomal 5q31-33 and encodes the protein that interacts to the IL-4 receptors. Due to the frequent polymorphism, variations in its sequences are linked to an increased risk of developing a number of immune-triggered illnesses (Al-Ahmad *et al*., 2023). Chromosome 6p21.1–21.3 contains the human TNF-α gene. G-308A is one of the many known TNF-α gene polymorphisms that has been the subject of much research (Duan *et al*., 2022). The purpose of this research is to evaluate serum levels of (IL-6, IL-4, TNF- α) as well as analyze the genetic profile for the same cytokines of Beta-thalassemia patients with group of healthy individuals as control.

2. MATERIAL AND METHOD

Study design & Sampling:

This study was conducted at the Akre thalassemia center in the Kurdistan region of Iraq, from October 2024 to January 2025. The sample of the study included 72 patients (35 male & 37 female) with confirmed beta thalassemia major. Additionally, 40 healthy controls with no history of thalassemia were included in the study. The age intervals for the patients and control group were 1 to 30 years. Every participant had approximately 5 mL of venous blood drawn in a plastic syringe under aseptic conditions.

The blood was then split into two tubes, one containing EDTA for DNA extraction and the other an anticoagulant gel tube. Then, the anticoagulant gel tube was centrifuged at 4000 rpm for 7 minutes to separate the serum for examining the concentration of cytokines.

Cytokine serum level:

In accordance with the manufacturer's instructions, the levels of serum interleukin-6 (IL-6), interleukin-4 (IL-4), and tumor necrosis factor-alpha (TNF-α) were determined by employing enzyme-linked immunosorbent assay (ELISA) kits commercially available from SUNLONG BIOTECH CO., LTD.

DNA extraction & PCR reaction:

Genomic DNA was extracted from 200 µL of whole blood using the AddPrep Genomic DNA Extraction Kit, following the manufacturer's instructions. Using the NanoDropTM 1000 Spectrophotometer, the concentration and purity of DNA were examined for IL-4, IL-6, and TNF-α alleles. ARMS-PCR, or amplification refractory mutational system, was used. The assays were performed using 40 ng of genomic DNA, 1.5 mM dNTPs, 25 mM $MgCl_2$, 1 µL of 10 pmol of each primer, and 0.4 units of Taq polymerase in a 20 µL reaction volume.

Primers and cycling conditions:

Primer description and procedures have been done earlier; the primer sequences were as follows:

Cytokine	Primer name	Primer sequences (5-3)
IL-4	IL-4-589 forward primer	AGCCTAGGCAACATAGTGAGACTCTTATC
	IL-4 -589 reverse primer	CAGGTGGCATCTTGGAAACGTTC
	IL-4 (C)allele primer	AAACACCTAAACTTGGGAGAACATTTTC

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	IL-4 (T)allele primer	TCTCCTACCCCAGCACTGGTGA
TNF-α	TNF-α- 308 forward primer	AGGACTCAGCTTTCCGAAGCCCCTCCCA
	TNF-α- 308 reverse primer	TTCTGTCTCGGTTTCTTCTCCATCGCGG
	TNF-α FI (G) allele	GTAGGACCCTGGAGGCTGAACCCCGTACT
	TNF-α RI (A) allele	GGAGGCAATAGGTTTTGAGGCGCAGGG
IL-6	IL-6 -174 generic primer	GCCTCAGAGACATCACCAGTCC
	IL-6 (G) allele primer	CCCCTAGTTGTGTCTTGCG
	IL-6 (C) allele primer	CCCCTAGTTGTGTCTTGCC

Studied gene polymorphism ARMS-PCR protocol

Cytokine	Position	PCR protocol
IL-4	-589	Initial denaturation at 95°C for 3 minutes, followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds, and finally a final extension at 72°C for 5 minutes
TNF-α	-308	Initial denaturation at 94°C for 5 minutes. This is followed by 10 cycles at 94°C for 15 seconds, 65°C for 50 seconds, and 72°C for 40 seconds, then 25 cycles at 94°C for 20 seconds, 59°C for 50 seconds, and 72°C for 50 seconds. And a final extension at 72°C for 7 minutes
IL-6	-174	Initial denaturation 1 minute at 95°C, followed by 10 cycles of 15 seconds at 95°C, 50 seconds at 58°C, and 40 seconds at 72°C, and 20 cycles of 20 seconds at 95°C, 50 seconds at 54°C, and 50 seconds at 72°C, with the last extension being 5 minutes at 72°C.

Gel electrophoresis:

Analysis of the amplified products was done on a gel composed of 2% agarose. The amplicon sizes were as follows: for TNF-α, M: 100bp size DNA ladder, G allele (162bp), A allele (197bp), common (304bp). For IL-6 amplicon size (230bp): M: 100bp size DNA ladder. And for IL-4, M: 100bp size DNA ladder, C allele (133bp), T allele (382bp) common (466bp).

Statistical analyses:

All statistical analyses were performed using GraphPad Prism 9. When appropriate, mean ± SD was used to express normally distributed values. A p-value of less than 0.05 was deemed statistically significant. The ANOVA test was utilized to evaluate between-group comparisons for categorical variables and serum cytokine concentration. Direct allele counting was used to determine the number of alleles for the TNF-α, IL-4, and IL-6 gene polymorphisms. The χ2-test was used to evaluate the Hardy–Weinberg equilibrium. Means and standard deviations (SDs) are used to display descriptive data. Using 2x2 contingency tables, z statistics, and a χ^2-test of independence, genotype and allele frequencies were compared between groups. The factors were found to be statistically significant at the P<0.05 level.

3. RESULTS

The findings of the current study revealed significant differences in serum levels of IL-6 and TNF-α between patients and the control group (p <). While the difference in IL-4 was not significant, emphasizing that this could reflect its limited role in β-thalassemia pathophysiology p>0.05. Table 1. Figure 1.

Table 1: Shows the patients' and controls' serum levels of TNF-α, IL-6, and IL-4.

Cytokine	Cytokines serum mean level ± S.D. (Pg./ml)		
	Patients	**Controls**	***P* value**
IL-6	38.92±7.54	16.20± 4.32	0.023
IL-4	24.27±5.67	27.91± 6.73	0.99
TNF-α	160.6±23.43	93.39±9.42	0.03

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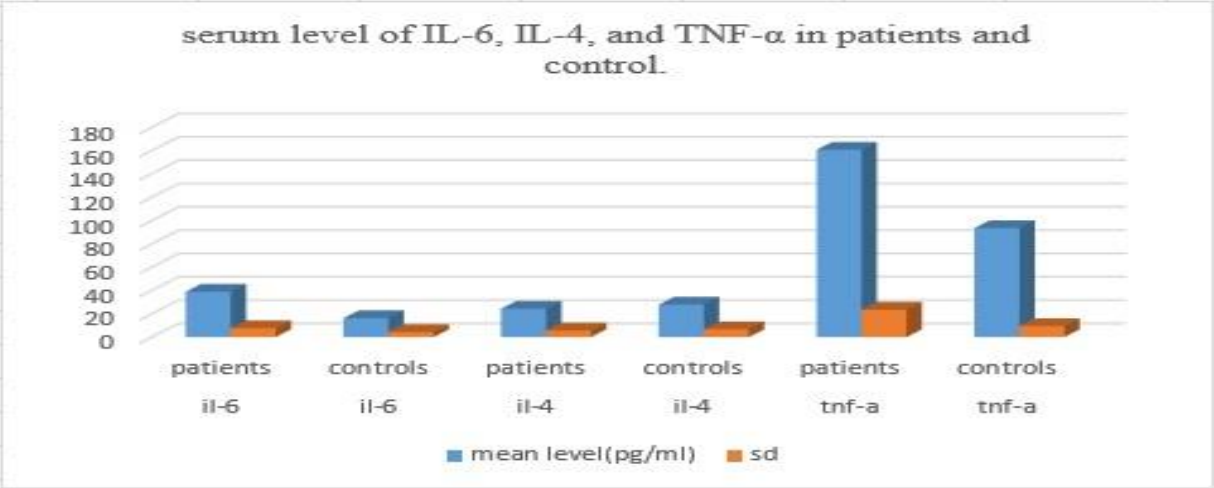

Figure 1: Shows the patients' and controls' serum levels of TNF-α, IL-6, and IL-4.

Serum level of the studied cytokines and ferritin regarding the sex revealed significant increasing in male patients in IL-6, TNF-α and ferritin, $p<0.05$, whereas there was none significant increase in male patients in IL-4 when compared to control group, $p>0.05$. While in females, there was a significant increase in ferritin compared to controls, and a limited difference in IL-6 and TNF-α ($p=0.05$), while in IL-4 there was no significant difference between females of patients and controls groups. Table 2. Figure 2.

Table 2: Serum levels of ferritin, TNF-α, IL-6, and IL-4 in male and female members of the study group.

		Cytokines serum mean level ± S.D. (Pg./ml)		
cytokine	**sex**	**Patients**	**Controls**	**P value**
IL-6	**Males**	46.12 ± 11.71	18.52 ± 5.52	0.03
	Females	31.77 ± 9.63	14.31 ± 4.05	0.05
IL-4	**Males**	26.25 ± 38.21	24.47 ± 7.28	0.65
	Females	22.11 ± 32.57	29.22 ± 6.78	0.76
TNF-α	**Males**	188.90 ± 21.26	87.51 ± 23.16	0.04
	Females	132.21 ± 11.26	94.32 ± 10.23	0.05
FERRETIN	**Males**	3987 ± 2895	44.05 ± 18.89	0.0001
	Females	3325 ± 2319	38.24 ± 16.86	0.0001

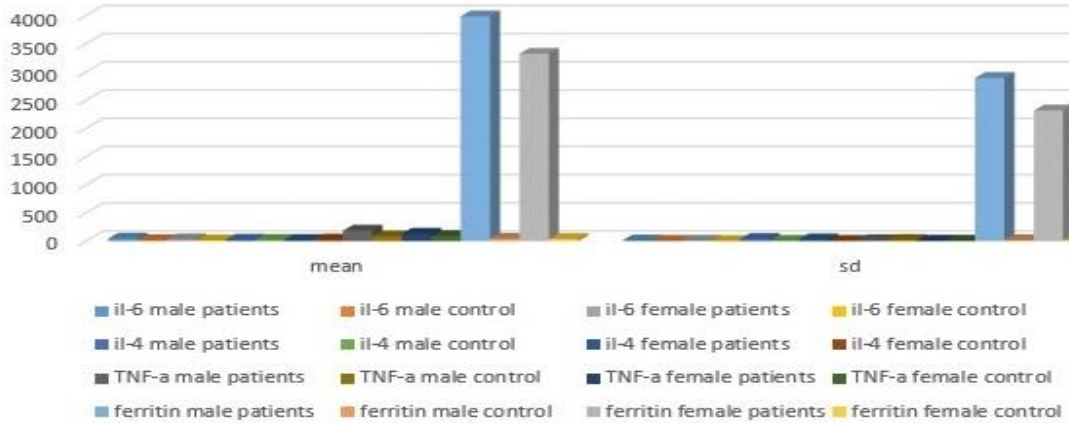

Figure 2: Serum levels of ferritin, TNF-α, IL-6, and IL-4 in male and female members of the study group.

Results of the study showed that the serum level of cytokines varies between the patients and control regarding the age groups. The IL-6 level was increased in all the age groups in patients than in control, $p<0.05$. The lowest level was found in the youngest ages for both groups. The results for IL-4 were different, there was no significant differences between the patients and control in the three age intervals, $p>0.05$. And the lowest level was

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also found in the youngest participants. Regarding the TNF-α, significant differences between patients and controls were observed in the (10-19) years and ≥20 years' age groups ($p<0.05$), while the difference in the ≤ 9 years' age group was not statistically significant ($p=0.06$). Table 3. Figure 3.

Table 3: Serum level of studied cytokines between patients and control regarding the age groups.

Cytokines serum mean level ± S.D. (Pg./ml)				
cytokine	**Age Group**	**Patients**	**Controls**	**P-Value**
IL-6	**≤ 9**	24.57 ±4.49	12.71 ± 3.00	0.045
	(10-19)	35.68 ± 8.17	14.93 ± 7.42	0.023
	≥ 20	56.51 ± 5.64	20.97 ± 5.32	0.01
IL-4	**≤ 9**	28.04 ± 38.79	15.80 ± 0.00	0.45
	(10-19)	23.95 ± 32.18	30.77 ± 51.16	0.99
	≥ 20	20.82 ± 32.28	37.17 ± 63.61	0.99
TNF-α	**≤ 9**	127.28 ± 18.45	97.87 ± 0.00	0.06
	(10-19)	179.20 ± 26.56	86.52 ± 32.06	0.03
	≥ 20	175.33 ± 22.26	95.79 ± 6.790	0.05

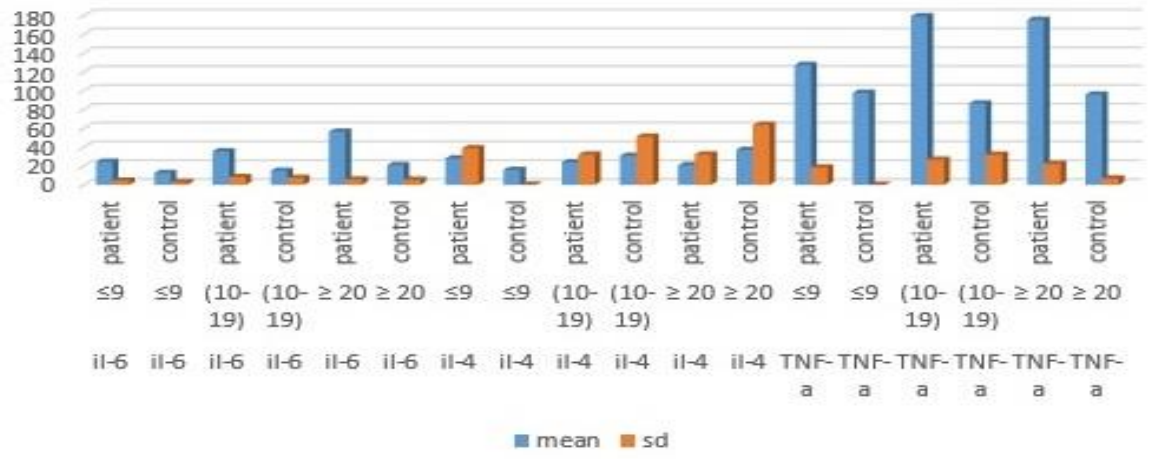

Figure 3: Serum level of studied cytokines between patients and control regarding to age groups.

The results of the IL-4 genotypes were also consistent with H-Weinberg equation during calculation showed none significant difference between the genotypes in patients and control. Highest record was found in CC genotype for studied group, 72.22% and 77.5%. The CT genotype was 22.5% and 26.39%, and the TT genotype was not found in control group and 1.39% of the patients was TT carriers. For the IL-4 polymorphism, no statistically significant association was found between any genotype (CC, CT, TT) or allele (C, T) and beta-thalassemia major risk. For instance, the CC genotype showed an OR of 0.75 (95% CI: 0.27–2.01), indicating a non-significant trend towards a protective effect, Tables (4 and 5)

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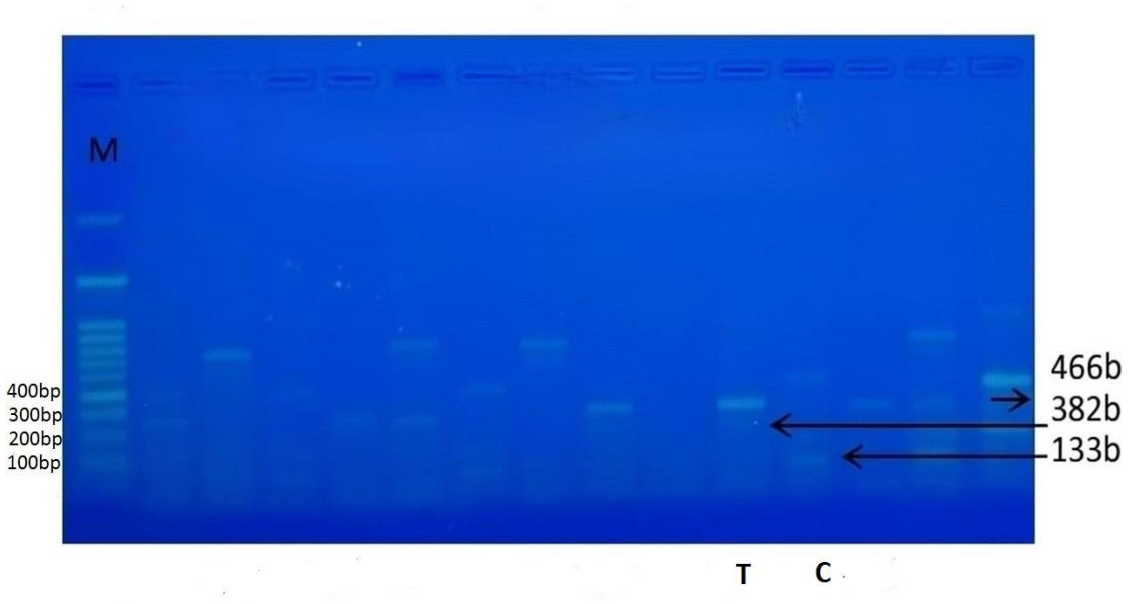

Figure 4: IL-4 product of TARMS-PCR on agarose gel (2%): M: 100bp size DNA ladder, C allele (133bp), T allele (382bp), common (466bp).

Table 4: The H-W equilibrium of IL4-589 genotypes and alleles in Thalassemia patients and controls, as well as their observed numbers and percentage frequencies.

Groups			*IL4-589* Genotype or Allele					H-W
			CC	**CT**	**TT**	***C***	***T***	*P*-value
Thalassemia (N=72)	**Observed**	**No.**	52	19	1	127	17	Not significant
		%	72.22	26.39	1.39	88.2	11.8	
	Expected	**No.**	52.53	17.94	1.53	Not Estimated		
		%	72.96	24.92	2.12			
Controls (N=40)	**Observed**	**No.**	31	9	0	71	9	Not significant
		%	77.5	22.5	0	88.75	11.25	
	Expected	**No.**	31.51	7.99	0.51	Not Estimated		
		%	78.77	19.97	1.26			

Table 5: Shows statistical analyses of the relationships between patients with Thalassemia and IL4-589 genotypes or alleles.

***IL4*-589 Genotype or Allele**	**Statistical Evaluations**			
	Relative Risk	**Etiological or Preventive Fraction**	**Fisher's Exact Probability**	**95% Confidence Intervals**
CC	0.75	0.19	0.35	0.27 to 2.01
CT	1.23	0.05	0.41	0.46 to 3.49
TT	1.7	0.08	0.64	0.07 to 41.21
C	0.95	0.04	0.54	0.35 to 2.39
T	1.06	0.06	0.54	0.42 to 2.84

The results of this study for IL-6 applying the H-Weinberg equation were also consistent with the law. None significant differences showed for patients and control. GG genotype was found in more than 50% of both patients and control. GC genotype was found decreased in patients with 10%. Lowest numbers of the participants in both groups was CC genotype. Although GG genotype had an OR of 1.36 (95% CI: 0.58-3.21), and GC genotype had an OR of 0.66(95% CI: 0.27-1.61), these associations were not significant. Similar non-significance was observed for CC genotype and G and C alleles. Figure 5. Tables (6 and 7).

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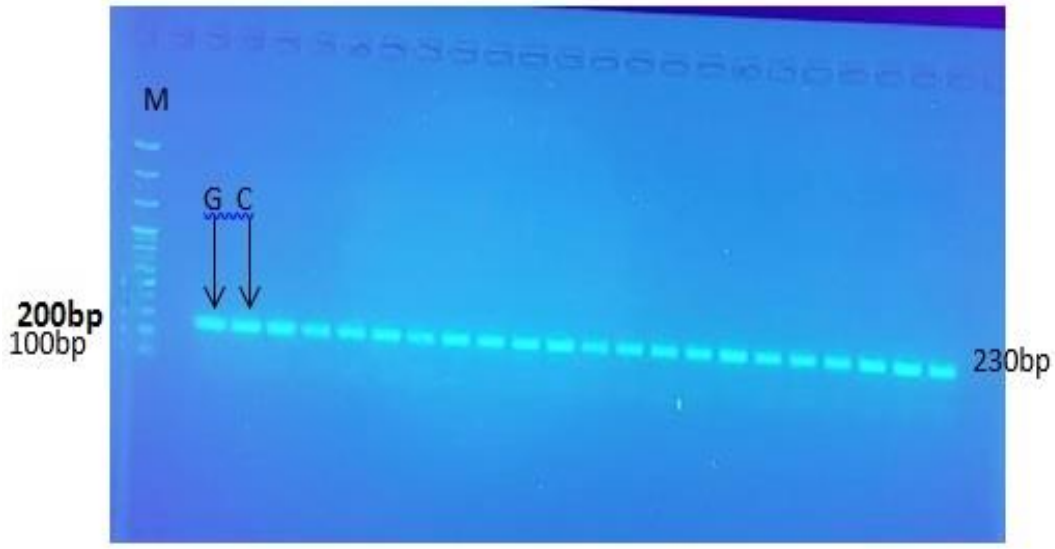

Figure 5: IL-6 (174G/C) product of ARMS-PCR on agarose gel (2%) amplicon size (230bp): M: 100bp size DNA ladder, double reaction PCR was done to each sample due to same genotype product bp.

Table 6: Shows the observed frequencies, percentages, and H-W equilibrium of IL6-174 genotypes and alleles in controls and patients with thalassemia.

Groups			*IL6*-174 Genotype or Allele					H-W P ≤
			GG	**GC**	**CC**	***G***	***C***	
Thalassemia (N=72)	**Observed**	**No.**	45	22	5	112	32	Not significant
		%	62.6	30.55	6.9	77.78	22.22	
	Expected	**No.**	43.56	24.89	3.56	Not Estimated		
		%	60.5	34.56	4.94			
Controls (N=40)	**Observed**	**No.**	22	16	2	60	20	Not significant
		%	55	40	5	75	25	
	Expected	**No.**	22.5	15	2.5	Not Estimated		
		%	56.25	37.5	6.25			

Table 7: Statistical analyses of the relationships between patients with Thalassemia and IL6-174 genotypes or alleles.

IL6-174 Genotype or Allele	Statistical Evaluations			
	Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
GG	1.36	0.16	0.28	0.58 to 3.21
GC	0.66	0.13	0.21	0.27 to 1.61
CC	1.42	0.02	0.51	0.22 to 15.52
G	1.17	0.11	0.37	0.58 to 2.31
C	0.86	0.03	0.37	0.43 to 1.73

Regarding to TNF-α, the H-Weinberg equation of the TNF-α revealed none significant difference and it was consistent with the calculation. High producer homozygous genotype GG recorded the highest occurrence with 70%, mutant heterozygous GA 20% and mutant homozygous AA recorded only 2%. Allele frequency were 83.33% and 16.67% for G and A allele. The same results were almost found in control group. Regarding the effect of the genotype on the disease progression and protection, no statistically significant associations were observed between any genotype (GG, GA, AA) or allele (G, A) and disease risk. The GG genotype showed non-significant odds ratio (OR: 1.17, 95% CI: 0.46-2.90), with the wide CI indicating considerable uncertainty. Similarly, the GA genotype suggested a non-significant trend (OR: 0.78, 95% CI:

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0.30–2.04), while the AA genotype, despite an OR of 1.7, had an extremely wide and non-significant confidence interval (95% CI: 0.13–91.27), precluding definitive conclusions about its role as a risk factor. Allele frequencies (G: 83.33%; A: 16.67%) also lacked significant associations, with the G allele showing no clear protective effect (OR: 1.25, 95% CI: 0.58–2.65) and the A allele demonstrating no robust risk association (OR: 0.8, 95% CI: 0.38–1.74). These results suggest that neither genotypes nor alleles of TNF-α have a statistically meaningful impact on disease progression in this study. Figure 6. Tables (8 and 9)

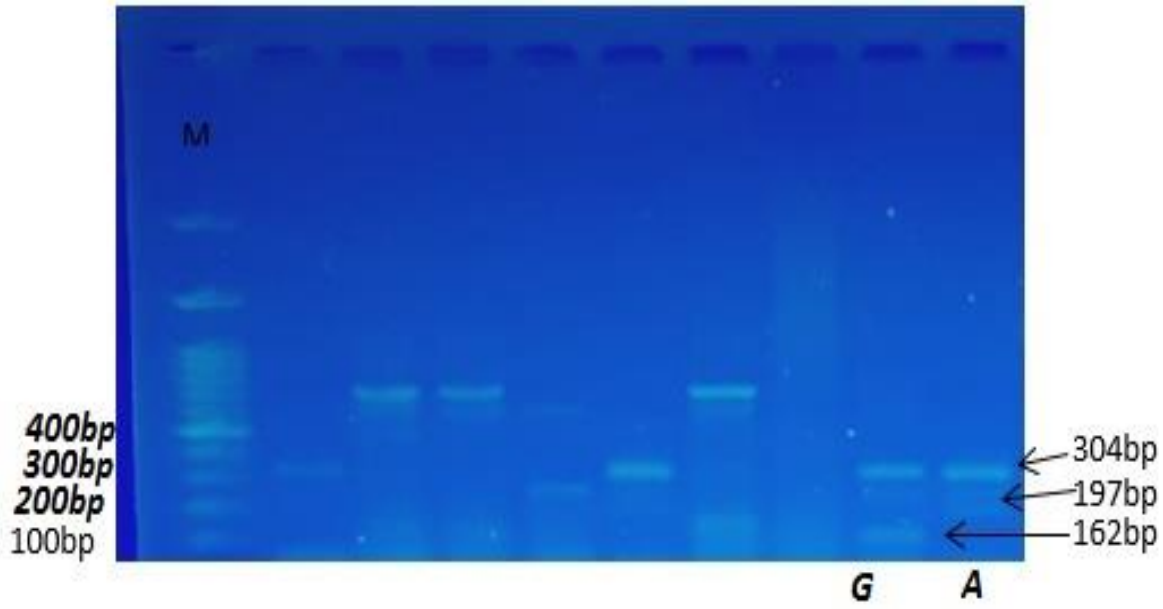

Figure 6: TNF-α product of TARMS-PCR on agarose gel (2%): M: 100bp size DNA ladder, G allele (162bp), A allele (197bp), common (304bp).

Table 8: Shows the H-W equilibrium and observed numbers and percentage frequencies of TNF-α-308 genotypes and alleles in controls and patients with thalassemia.

Groups			*TNF-α* -308 Genotype or Allele					H-W
			GG	**GA**	**AA**	***G***	***A***	P ≤
Thalassemia (N=72)	**Observed**	**No.**	51	18	3	120	24	Not significance
		%	70.8	25	4.2	83.33	16.67	
	Expected	**No.**	50	20	2	Not Estimated		
		%	69.44	27.78	2.78			
Controls (N=40)	**Observed**	**No.**	27	12	1	64	16	Not significance
		%	67.5	30	2.5	80	20	
	Expected	**No.**	27.22	11.55	1.23	Not Estimated		
		%	68	28.9	3.1			

Table 9: Statistical evaluations of associations between TNF-α-308 genotypes or alleles and Thalassemia patients.

	Statistical Evaluations			
TNF-α-308 Genotype or Allele	**Relative Risk**	**Etiological or Preventive Fraction**	**Fisher's Exact Probability**	**95% Confidence Intervals**
GG	1.17	0.1	0.43	0.46 to 2.90
GA	0.78	0.06	0.36	0.30 to 2.04
AA	1.7	0.01	0.55	0.13 to 91.27
G	1.25	0.16	0.32	0.58 to 2.65
A	0.8	0.4	0.32	0.38 to 1.74

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Studies on SNPs and their protein expression revealed that the higher producers GG, and GG for IL-6, and TNF-α produced the highest serum concentrations respectively. The lowest level was found in low producer's CC for IL-6, and AA of TNF-a. There was significant increase in IL-6 genotypes when compared to control within the same genotype. The IL-4 level showed no significant decrease in the CC genotype, while a non-significant increase was observed in the CT genotype. Notably, the TT genotype for IL-4 was absent in controls. Regarding the TNF-α serum level for the three genotypes, the GA genotype showed a borderline significant increase (p=0.05), while GG and AA genotypes did not show statistically significant differences. (Table 10). Figure 7.

Table10: Effects of genotype on cytokine serum levels in patients and controls.

Cytokines serum mean level ± S.D. (Pg./ml)				
Cytokine	**Genotype**	**Patients**	**Controls**	**P-Value**
IL-6	**GG**	43.23 ±4.49	20.71 ± 3.00	0.05
	GC	36.12 ± 8.17	14.93 ± 7.42	0.03
	CC	31.51 ± 5.64	11.64 ± 5.32	0.04
IL-4	**CC**	30.11 ± 38.79	34.18 ± 0.00	0.87
	CT	25.23 ± 32.18	21.63 ± 51.16	0.9
	TT	18.22 ± 32.28	-----	NA
TNF-α	**GG**	187.31± 18.45	100.55 ± 0.00	0.056
	GA	164.32 ± 26.56	91.16 ± 32.06	0.05
	AA	130.21 ± 22.26	88.43 ± 6.790	0.23

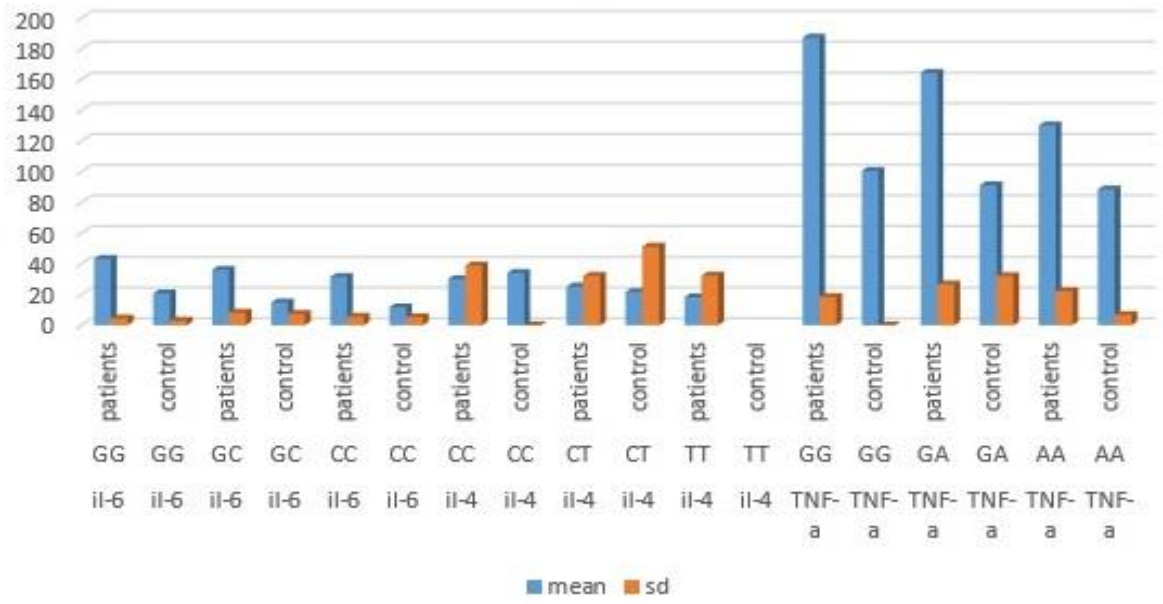

Figure 7: Effects of genotype on cytokine serum levels in patients and controls.

DISCUSSION

Despite observing differences in cytokine serum levels, our study did not find statistically significant associations between the investigated IL-4, IL-6, and TNF-α gene polymorphisms and beta-thalassemia major risk in our cohort (Tables 5, 7, 9). This could be attributed to several factors, including the relatively small sample size, which may limit the power to detect subtle genetic associations. Furthermore, the genetic background of the studied population, environmental factors, or other modifying genes may play a more dominant role in disease susceptibility than these specific SNPs. The immune system, a complicated and smart combination of humoral and cellular elements, might have a role in organ malfunction in thalassemia major patients. Just in the past few years attention has been drawn to the potential impact of elements of the immune system on the medical manifestations of thalassemia major (Elsayh *et al*., 2016). The pathophysiology of β-thalassemia may be significantly influenced by cytokines and cytokine gene polymorphisms, according to findings. The entire production of cytokines is caused by polymorphisms of a single nucleotide (in the region of the promoter or other

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regulatory regions of cytokine genes (Bagheri *et al*., 2005). According to the present research, thalassemia patients' serum levels of TNF-α and IL-6 were higher than those of normal people. Since thalassemia is a chronic inflammatory disease, excess iron could be an important player in the release of IL-6. An elevated serum level of IL-6 in thalassemia patients is related to the pathophysiology of beta thalassemia patients, and it is likely that the increased production of IL-6 is caused by macrophages excessive stimulation, which leads to irregularities in metabolism of iron (Kohgo *et al*., 2008). According to a study by Abd *et al*. (2022) there was a significant difference ($P<0.01$) in serum IL-6 levels comparing patients with β-thalassemia and normal controls. Serum IL-6 levels were capable of distinguishing between thalassemia individuals and healthy volunteers as two different groups. Subsequent investigation of the IL-6 levels of the major and moderate thalassemia subtype under study also revealed significant differences ($P<0.01$). Patients with thalassemia major had a greater mean IL-6 level than those with thalassemia moderate. These findings are in line with ours from our latest recent investigation. Once TM patients were compared with normal subjects, the findings showed a significant increase in IL-6 levels; this outcome could be explained by the inflammation brought on by multiple blood transfusions. Overdose of iron linked to macrophage activation during transfusion and ongoing antigenic stimulation may cause elevated IL-6 output. Additionally, our results are in line with those of El-Rasheidy and coworkers (El-Rasheidy *et al*., 2016). In contrast to our results a research done by Surchi & Ali (2018) found thalassemia patients had lower levels of IL-6 compared normal. While according to Aggeli *et al*. (2005) β-thalassemia patients' IL-6 levels were much higher than those of healthy volunteers. Elevated serum level of IL-6 in thalassemia patients may result from many factors among which the destruction of RBC causes chronic inflammation, oxidative stress caused by iron overload which activate macrophage in addition to long term anemia which leads to bone marrow expansion and further inflammation. As with IL-6, a research study by Hassan & Rahman (2022) found that those with thalassemia had significantly higher average levels of TNF-alpha ($p < 0.01$). TNF-α belongs to the big cytokine family and is a pro-inflammatory cytokine. Macrophages and other innate immune system cells are the main producers of it (Shnawa & Taha, 2023). According to a different study by Malallah *et al*. (2023) thalassemia patients had significantly ($P < 0.05$) higher serum levels of TNF-α compared to the controls, and this is in line with our findings. Increased TNF-α levels were a cause of iron overload-induced triggering of macrophages and repetitive transfusion-induced macrophages stimuli, which are erythrocyte progenitors and help generate dormant red blood cells (Garadah *et al*., 2016). Irons chelation therapy and frequent transfusions of blood helped thalassemia patients, but excessive iron and its toxic effects caused other health issues since too much iron causes inflammation and oxidative stress in thalassemia patients (Kohgo *et al*., 2008). This research revealed that the difference in serum level of IL-4 between patients and healthy group was not significant ($p>0.05$) as the IL-4 dose not contribute to the core pathological triad of thalassemia which primarily involves iron overload, chronic inflammation, and ineffective erythropoiesis. These processes activate macrophage and monocyte which are major sources of IL-6 and TNF-α not IL-4. According to sex, the current study showed both male and female patients exhibited significantly elevated levels of IL-6, TNF-α, and ferritin compared to healthy controls, suggesting a systemic inflammatory and iron overload state independent of sex. In healthy males and females, plasma IL-6 levels are thought to rise over ages; however, the issues surrounding gender variations in these levels are still open (Legrand *et al*., 2013).

A study done by Abd *et al*. (2022) found that the levels of IL-6 were greater in both males and females with thalassemia, and that the thalassemia subtype appeared to be having an impact on their amounts; men as well as women within the major subgroup had greater IL-6 levels than intermediary and control subjects. According to this research, thalassemia patients had elevated plasma IL-6 levels despite their gender. Ferritin, which is the main type of iron that the human body retains, is released into the plasma in tiny quantities by the organism. In the absence of inflammation, there is a positive correlation between the amount of iron all body reserves and the quantity of this plasma (or serum) ferritin. Age and sex have an impact on natural ferritin levels. At birth, levels are elevated; they then increase over the first two months of life before declining for the remainder of infant (Mishra & Tiwari, 2013). Results of our study show a significant difference in serum ferritin between patient and controls in both male and female, frequent blood transfusion, ineffective erythropoiesis and inflammation are the main causes of this elevation of ferritin concentration. These results are similar to results of Gombar *et al*. (2018); Mohammed & Yenzeel. (2024); and Abdulla. (2018). Regarding the age groups, results of this study revealed significant difference in IL-6 and TNF-α between patients and controls, the lowest level was in younger group as the youngest group may have lower iron overload, milder disease complication and higher anti-inflammatory cytokine like IL-10 which suppers IL-6 and TNF-α. It is asserted that both the circulation amount and transcriptional activity of the IL-6 gene are correlated with the promoter 174 polymorphism. This has rendered it possible to utilize this SNP as a genetic tool to investigate the relationship between elevated IL-6 levels and a variety of disorders (Woo & Humphries, 2013). The current study showed that

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none significant differences showed for patients and control. And GG genotype was found in more than 50% of both patients and control. As well as the GG genotype produced the highest serum concentration of IL-6 while CC genotype was the lowest producer. A few studies have examined the IL-6 polymorphism in thalassemia. One of which is Bagheri *et al*. (2005) that found that Iranian thalassemia patients had a higher frequency of GG and described how the GG, GC genotype results in increased IL-6 output, which exacerbates the condition. And this is similar to our findings. This finding is also consistent with the conclusion put forth by Vicari *et al*. (2015) who describe a significant relationship among 174 G/C between IL-6 SNP and sickle cell anemia, and the GG genotype was more frequent among patients compared to normal individuals. According to Du *et al. (*2015*)* the allele G is an unsafe allele, and there is a substantial correlation between IL-6 rs1800796 and the risk of cancer. The most frequent nucleotide change across most people is the one at position -308, which changes guanine (G) to adenine (A). It has been demonstrated that this change impacts TNF-α expression (Luaibi & Mohammed, 2023). Regretfully, no prior research has addressed the TNF-α 308 (rs1800629) polymorphism in thalassemia patients, with the exception of studies by Mohammed (2022) and Luaibi & Mohammed (2023) which concurred with the current study when it investigated the relationship between TNF-α gene polymorphism and β-thalassemia patients and discovered that risks associated with β-thalassemia susceptibility were associated with genotype AA and allele A. and the O.R. value for the A allele was 1.3, while the O.R. value for the mutant genotype AA was 1.83. But numerous investigations regarding the significance of this gene variant were conducted. According to a study by Zhuang *et al*. (2013) the TNF-α 308 G/A polymorphism was strongly linked with a lower rate of psoriasis under three genetic comparison models. Furthermore the research conducted by Kim *et al*. (2004) mentioned that the TNF-α 308 polymorphism did not correlate with the incidence of Nephrotic Syndrome. In the Chinese population with acute pancreatitis, a research by Zhang *et al*. (2003) found no association among acute pancreatitis and TNF-α 308. However, they proposed that the TNF-α 308 A allele is linked to an increased risk of acute pancreatitis being complicated by deadly sepsis. Chromosome 5q31–33 contains the IL-4 gene, which produces a protein that attaches itself to the IL-4 receptor. is extremely variable, and variations within the nucleotide are related to an increased risk of developing a number of immune-driven illnesses, such as autoimmunity, allergies, and asthma (Al-Ahmad *et al*., 2023). Research conducted by Bagheri *et al*. (2005) examining the impact of IL-4 in thalassemia found that patients had a significant rise in the C allele at position -589 in the IL-4 gene, which suggests that those with thalassemia disease are going to produce more IL-4 as well as an elevated IgE activity. The results of our study revealed that the highest record were found in CC genotype for studied groups. And none significant differences showed for patients and control. However, the results of IL-4 gene polymorphism were varying; the wild homozygous genotype CC showed an OR of 0.75 (CI: 0.27-2.01), the association was not statistically significant. According to another study by Naji (2024) those individuals suffering from thalassemia were more probable to carry the C allele at IL-4 gene position -590. This further suggests that individuals with thalassemia could have higher IL-4 amounts and an improved IgE activity.

CONCLUSION

Our findings indicate that patients with beta-thalassemia major exhibit significantly elevated serum levels of IL-6 and TNF-α compared to healthy controls, while IL-4 levels show no significant difference. Regarding cytokine gene polymorphisms, we observed varying effects on serum levels for different genotypes. For example, the IL-6 GG genotype was associated with the highest serum IL-6 levels. However, no statistically significant associations were found between the investigated IL-4, IL-6, and TNF-α gene polymorphisms and beta-thalassemia major risk in this cohort.

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Ethical statement:

This study received full approval from the HRECs at Salahaddin University-Erbil. Code no. 106 (1/9/2024)

Author contribution:

Each author has agreed to be held accountable for every part of the article after reviewing the completed version that will be published.

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