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Immune Response Alterations and Related Biochemical Changes in Iraqi Thalassemia Patients Infected with *Toxoplasma gondii*

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ABSTRACT

Thalassemia is a common inherited blood disorder, and infection with *Toxoplasma gondii* may further alter host immune and biochemical profiles. The study was designed to analyse and compare certain immune and biochemical markers between Iraqi thalassemic patients infected with *Toxoplasma gondii* and healthy control subjects. The anti-toxoplasmic Immunoglobulin M (IgM) and Immunoglobulin G (IgG) were tested using the Chemiluminescent Microparticle Immunoassay (CMIA) assay (Abbott, Germany). As per the protocol provided by the manufacturer, an index ≥1.0 was considered positive, and an index <1.0 was considered negative. Acute toxoplasmosis was diagnosed when IgM was positive, and IgG may be positive or negative. On the other hand, chronic toxoplasmosis was considered when IgG was positive and IgM was negative. Biochemical parameters, including liver and renal function, were measured by the C4000 Ruby automated clinical chemistry analyzer system. Interleukin-10 (IL-10) and forkhead box P3 (FOXP3) were determined through the Elabscience USA donated enzyme-linked immunosorbent assay (ELISA) test kits. The results showed a significant higher serum IgM and IgG levels in IgM and IgG levels in thalassaemic patients infected with toxoplasmosis than in the control group ($p \leq 0.05$). IgM and IgG levels were 0.235 ± 0.12 and 64.95 ± 10.62 IU/mL, respectively, in infected thalassaemic patients, compared with 0.044 ± 0.01 and 1.012 ± 0.50 IU/mL, respectively, in controls. Also, IL-10 and FOXP3 levels were significantly higher in infected thalassaemic patients ($p \leq 0.05$); IL-10 levels were 0.337 ± 0.009 ng/mL and 0.267 ± 0.005 ng/mL, and FOXP3 expression was significantly higher in infected thalassaemic patients than in controls (12.49 ± 0.19% vs 4.57 ± 0.13%; $p \leq 0.01$). Vitamin D3 levels were significantly lower in infected thalassaemic patients than in controls ($p \leq 0.01$), with values of 8.15 ± 0.41 ng/mL and 28.10 ± 0.79 ng/mL, respectively. In some biochemical parameters reflecting kidney and liver function, the study showed a significant increase ($p \leq 0.01$) in urea level (45.13 ± 1.07 mg/dL) and creatinine concentrations (1.316 ± 0.02 mg/dL) in both infected groups compared with control groups (28.10 ± 0.84 mg/dL), (0.749 ± 0.03 mg/dL) respectively, and in liver function tests the results of this study demonstrate that there was a considerable increase ($p \leq 0.01$) in alkaline phosphatase (ALP), alanine aminotransferase (GPT), aspartate aminotransferase (GOT) and total serum bilirubin (TSB) levels in the serum of thalassaemic patients that have been infected with toxoplasmosis compared with the control group, where ALP, GPT, GOT and TSB levels were 424.46 ± 16.20 IU/L, 46.73 ± 0.84 IU/L, 49.76 ± 1.16 IU/L, 7.19 ± 0.48 mg/dL, respectively and their levels in control group were 99.38 ± 6.55 IU/L, 22.98 ± 0.68 IU/L, 19.20 ± 1.12 IU/L, 1.00 ± 0.12 mg/dL, respectively.

1. INTRODUCTION

Hemoglobin molecule hereditary disorders are among the most prevalent and clinically significant genetic illnesses. They fall into two broad categories: those where a mutation affects the quantity of protein generated (thalassemia) and those where the hemoglobin molecule undergoes structural alteration, producing a different protein (hemoglobinopathies) [1]. The β-thalassemia major, also known as transfusion-dependent thalassemia (TDT), is a fatal genetic form of hemoglobinopathy in which production of the β-globin chains is either defective or non-existent, causing chronic anemia and ineffective erythropoiesis necessitating regular blood transfusions for survival [2]. Thalassemia has a significant worldwide illness burden. Depending on the type of thalassemia, the rate of α-thalassemia is larger than that of β-thalassemia; approximately 5–20% of people worldwide have at least one type of α-thalassemia mutation, whereas ≈1.5% have at least one type of β-thalassemia mutation. While prevalence rates vary by area, they are highest in subtropical

and tropical nations, especially in Southeast Asia and the Mediterranean [3]. *T. gondii*, an obligatory intracellular parasite that mostly infects mammals and birds, is the cause of toxoplasmosis, a zoonotic disease that is common throughout the world. Eating undercooked or raw meat that contains parasite cysts is the major way that contaminated food and water might spread toxoplasma infections to humans. Yet, there are additional ways to become infected, including blood transfusions, congenital transmission, and transplants. Regular blood transfusions are essential for managing beta thalassemia major, yet there are certain hazards that must be taken into account, like the possibility of contracting bacteria, viruses, or parasites. Indeed, blood transfusions were linked to cases of Chagas disease as well as malaria [4]. Along with their susceptibility to infections, individuals with beta thalassemia major often exhibit endocrine function problems that negatively affect their quality of life and survival [5]. Osteopenia, growth retardation, vitamin D deficiency, osteoporosis, and hypogonadism are among the problems that are highly prevalent and were shown to reduce with age [6]. Theoretical explanations link tissue hypoxia as well as chronic anemia, which raises transforming growth factor-beta (TGF-β) expression and increase oxidative stress due to iron excess [7]. Through changing normal homeostasis, immunological dysregulation brought on through recurrent infections, such as toxoplasmosis, could play a role. Clarifying these relationships may present chances to improve clinical monitoring procedures. In terms of biology, cytokines are active molecules that are essential for regulating immunity and hemopoiesis. The immune response, hemopoiesis, and inflammation are all regulated by such cellular cytokines, which are primarily produced by immunocompetent cells. At least 30 recognized cytokines regulate hemopoiesis [8]. Approximately 5–10% of human CD4+ T cells (1–4% of peripheral blood mononuclear cells) are regulatory T cells (Tregs), which are highly important for the mediation of immune responses, controlling peripheral tolerance to self-antigens, preserving homeostasis, preventing autoimmunity, and limiting inflammatory immune responses towards the commensal pathogens as well as transplants [9]. There are at least 2 subtypes regarding forkhead box P3 (FOXP-3)-expressing Treg cells: induced Treg that differentiate in the periphery upon antigen recognition through naive CD4+ T cells via TGF-β signaling, and thymus-derived, naturally occurring Treg, which are antigen-specific Tregs involved in peripheral tolerance maintenance [10]. In order to suppress effector T cells, Treg cells are known to generate anti-inflammatory cytokines such as Interleukin-10 (IL-10), TGF-β, and IL-35 [11]. The fate of immune cells' lineage development is largely determined by metabolic pathways, which also help various T cell subsets survive [12].

2. METHODS

2.1 Subjects

The 75 samples used in this research were split into two groups: 50 Iraqi patients with toxoplasmosis and β-thalassemia were included in the first group, and 25 samples were taken as a control group from Mar. to Jun. 2024, corresponding to the spring and early summer seasons from the Al-Karama Teaching Hospital in Baghdad, Iraq. The patients' ages ranged between 6 and 45 years. *Toxoplasma gondii* infection status was confirmed using anti-Toxoplasma, and the anti-Toxoplasma Immunoglobulin M (IgM) and Immunoglobulin G (IgG) were tested using the Chemiluminescent Microparticle Immunoassay (CMIA) assay (Abbott, Germany). As per the protocol provided by the manufacturer, an index ≥1.0 was considered positive, and an index <1.0 was considered negative. Acute toxoplasmosis was diagnosed when IgM was positive, and IgG may be positive or negative. On the other hand, chronic toxoplasmosis was considered when IgG was positive and IgM was negative. The control group was also screened for anti-Toxoplasma IgG and IgM antibodies, and only seronegative individuals were included as healthy controls.

Inclusion criteria included patients diagnosed with beta-thalassemia major based on clinical records and hematological assessment. Exclusion criteria included participants with known chronic illnesses, thyroid diseases, or those on drugs that might alter thyroid function, pregnant women, HBV/HCV patients, immunosuppressive therapy, and patients with acute infections, such as bacterial or viral.

2.2 Collection of blood samples

5 mL of venous blood has been extracted from samples, put right away in a gel tube, and left to coagulate at the temperature of the room (20-25 ℃). The serum has been separated by centrifuging the tube at 3000 rpm for 10 min for analysis. Serum samples were stored at −20 ℃ until analysis. Repeated freeze-thaw cycles were avoided to preserve sample integrity.

2.3 Thalassemic diagnoses

Anti-coagulated samples of blood have been utilized for determining concentrations of Haemoglobin (Hb), Red Blood Corpuscles (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), packed cell volume (PCV), and mean corpuscular haemoglobin (MCH) with the use of the CELL-DYN Ruby Haematology Analyzer system by manufacturer Abbott.

2.4 *T. gondii* diagnosis

Following procedures that have been provided by the kit maker, the operations have been completed. With the use of the Toxoplasma IgM/IgG antibody Immuno-chromatography fast test kit (Qingdao Hightop Bio-tech Company, China), *T. gondii* was first identified. Based on the manufacturer's instructions, the Toxo IgM/IgG kit (Abbott GmbH, Germany) is used in order to measure both IgM/IgG levels utilizing CMIA. Serological interpretation was based on the assay-defined cut-off values. Cases were classified into recent acute infection with the positivity of IgM (with or without IgG) and chronic infection, dependent on the IgG positivity with negative IgM. Liver and renal function parameters were measured using an automated clinical chemistry analyzer (C4000 Ruby, Roche Diagnostics). Serum urea and creatinine levels were expressed in mg/dL according to standard clinical laboratory units.

Serum 25-hydroxyvitamin D3 [25(OH)D3] levels were measured using an automated immunoassay analyzer (Abbott i1000SR, Abbott Laboratories, USA) based on CMIA technology. The results were expressed in ng/mL.

IL-10 and FOXP3 circulating serum levels have been determined through sandwich enzyme-linked immunosorbent

assay (ELISA) utilizing commercial kits based on the manufacturer's instructions: (Elabscience®, product number E-EL-H6154). The test had a sensitivity of 0.94 pg/mL, with a detection range of 1.56–100 pg/mL. Samples and standards were analyzed in duplicate. Intra- and inter-test coefficients of variation ranged from 3.13–5.87% and 3.05–5.73%, respectively. A standard curve was constructed using a four-parameter logistic (4PL) model. Samples were analyzed either undiluted or appropriately diluted when required for IL-10, and Serum FOXP3 levels were measured using a commercially available Human FOXP3 (Forkhead Box Protein P3) Sandwich-ELISA kit (Elabscience, USA; Cat. No. E-EL-H1104) according to the manufacturer's instructions. The kit sensitivity was 0.19 ng/mL, with a detection range of 0.31–20 ng/mL.

2.5 Statistical analyses

To find the impact of different groups (patients and controls) on study parameters, Statistical Analysis System (SAS) (2018) software has been employed. In the presented research, Analysis of variance (ANOVA) was employed to compare the means among the studied groups and assess significant differences in the measured parameters.

3. RESULTS

The results of the current study showed a considerable increase ($p \leq 0.05$) of IgM and IgG levels in the serum of thalassaemic patients that have been infected with toxoplasmosis in contrast to control group, where the level of IgM and IgG were 0.235 ± 0.12 and 64.95 ± 10.62 IU/mL respectively and their levels in control group were (0.044 ± 0.01, 1.012 ± 0.50 IU/mL) respectively. Additionally, Thalassemia patients infected with toxoplasmosis had blood levels of IL-10 that were significantly higher ($p \leq 0.05$) than those of the control group, which had levels of 0.337 ± 0.009 ng/mL and 0.267 ± 0.005 ng/mL, respectively (Table 1) and clarified in Figures 1-3.

Table 1. Comparison between the patient group and the control in IgM, IgG, and IL-10

Group	Mean ± SE		
	IgM (IU/mL)	IgG (IU/mL)	IL-10 (ng/mL)
Patients	0.235 ± 0.12	64.95 ± 10.62	0.337 ± 0.009
Control	0.044 ± 0.01	1.012 ± 0.50	0.267 ± 0.005
p-value	0.0393*	0.0001**	0.0001**
	* ($p \leq 0.05$), ** ($p \leq 0.01$)		

Notes: IgM: Immunoglobulin M, IgG: Immunoglobulin G, IL-10: Interleukin-10

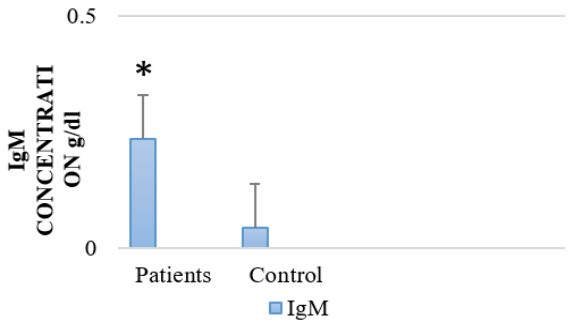

Figure 1. Concentration of Immunoglobulin M (IgM) in thalassemic patients with toxoplasmosis compared with controls

Table 2. Comparison between the patient group and the control group in renal functions

Group	Mean ± SE	
	Urea (mg/dL)	Creatinine (mg/dL)
Patients	45.13 ± 1.07	1.316 ± 0.02
Control	28.10 ± 0.84	0.749 ± 0.03
p-value	0.0001	0.0001
	$p \leq 0.010$	

Concerning the effect of toxoplasmosis thalassemia patients on kidney function, the results demonstrate that there was a significant increase ($p \leq 0.01$) in urea level (45.13 ± 1.07 mg/dL) and creatinine concentrations (1.316 ± 0.02 mg/dL) in both infected groups compared with control groups 28.10 ± 0.84 mg/dL and 0.749 ± 0.03 mg/dL, respectively (Table 2 and Figures 4 and 5).

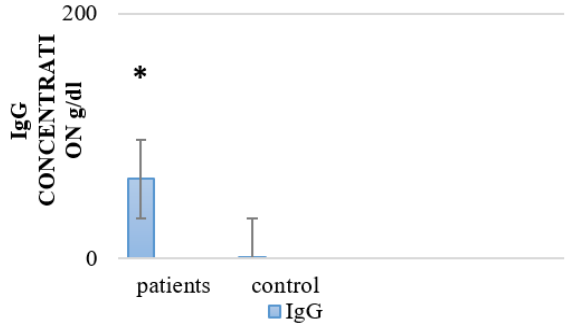

Figure 2. The concentration of Immunoglobulin G (IgG) among patients with thalassemia and toxoplasmosis infection compared to the control group

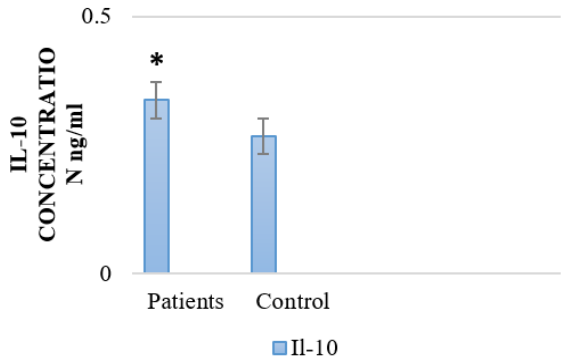

Figure 3. The concentration of Interleukin-10 (IL-10) among patients with thalassemia and toxoplasmosis infection compared to the control group

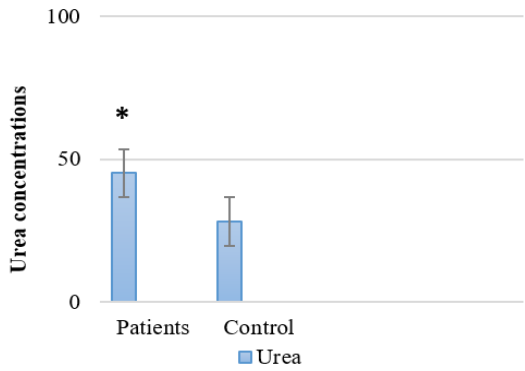

Figure 4. The concentration of urea among patients with thalassemia and toxoplasmosis infection compared to the control group

The results of this study demonstrate that there was a considerable increase ($p \leq 0.01$) in alkaline phosphatase (ALP), alanine aminotransferase (GPT), aspartate aminotransferase (GOT) and total serum bilirubin (TSB) levels in the serum of thalassaemic patients that have been infected with toxoplasmosis compared with the control group, where ALP, GPT, GOT and TSB levels were 424.46 ± 16.20, 46.73 ± 0.84, 49.76 ± 1.16, and 7.19 ± 0.48 IU/L respectively and their levels in control group were 99.38 ± 6.55, 22.98 ± 0.68, 19.20 ± 1.12, and 1.00 ± 0.12 IU/L respectively (Table 3 and Figure 6).

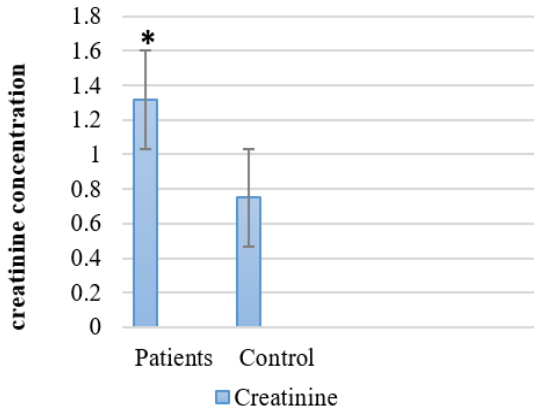

Figure 5. The concentration of creatinine among patients with thalassemia and toxoplasmosis infection compared to the control group

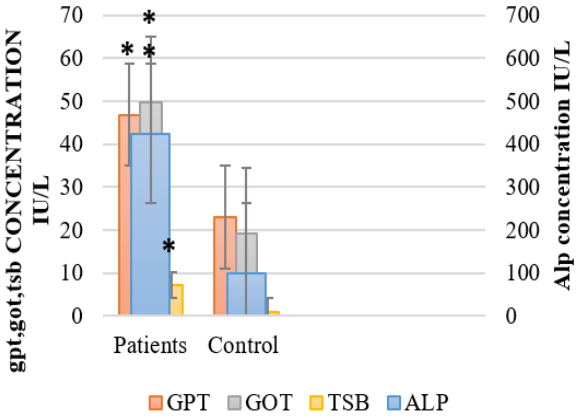

Figure 6. The concentration of liver function enzymes (ALP, GPT, GOT, TSB) among patients with Thalassemia and Toxoplasmosis infection compared to the control group.
Notes: The results show a significant difference in comparison with the control group (*p < 0.01). ALP: alkaline phosphatase, GPT: alanine aminotransferase, GOT: aspartate aminotransferase, TSB: total serum bilirubin.

Table 3. Comparison between the patient group and controls in liver enzymes

Group	Mean ± SE			
	ALP (IU/L)	GPT (IU/L)	GOT (IU/L)	TSB (mg/dL)
Patients	424.46 ± 16.20	46.73 ± 0.84	49.76 ± 1.16	7.19 ± 0.48
Control	99.38 ± 6.55	22.98 ± 0.68	19.20 ± 1.12	1.00 ± 0.12
P-value	0.0001	0.0001	0.0001	0.0001
		p ≤ 0.010		

Notes: ALP: alkaline phosphatase, GPT: alanine aminotransferase, GOT: aspartate aminotransferase, TSB: total serum bilirubin.

The present work's findings indicated that the amount of D3 in the serum of thalassaemic patients infected with toxoplasmosis has been significantly lower ($p \leq 0.01$) than that of the controls, which had a level of D3 (8.15 ± 0.41) ng/mL, and in the control group (28.10 ± 0.79) ng/mL. Additionally, in comparison to the control group, thalassaemic patients infected with toxoplasmosis had a significantly higher level of FOXP3 in their serum (12.49 ± 0.19%), $p \leq 0.01$, while its level in the control group was 4.57 ± 0.13% (Table 4 and Figures 7 and 8).

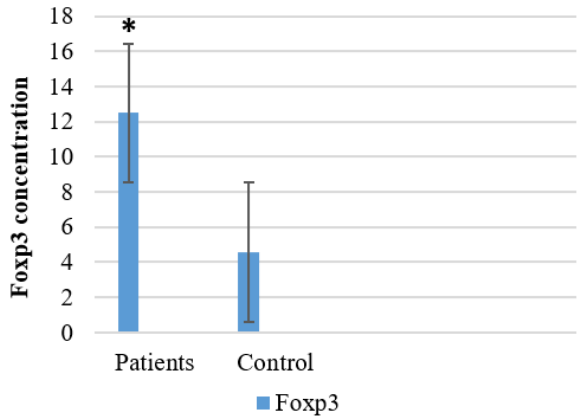

Figure 7. The concentration of FOXP3 among patients with thalassemia and toxoplasmosis infection compared to the control group

Table 4. Comparison between the controls and patient groups in D3 and forkhead box P3 (FOXP3)

Group	Mean ± SE	
	D3 (ng/mL)	FOXP3 (%)
Patients	8.15 ± 0.41	12.49 ± 0.19
Control	28.10 ± 0.79	4.57 ± 0.13
P-value	0.0001	0.0001
	p ≤ 0.010	

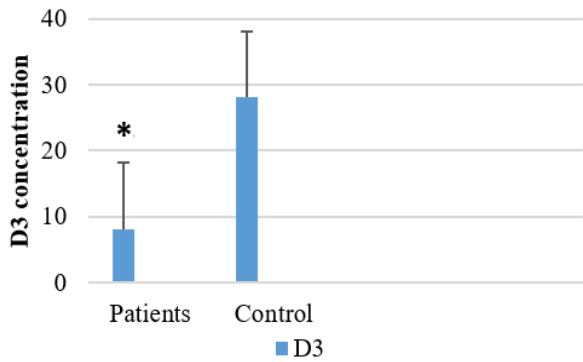

Figure 8. The concentration of vitamin D3 among patients with thalassemia and toxoplasmosis infection compared to the control group

4. DISCUSSION

Patients who have β-thalassemia have a higher susceptibility to infections, which are regarded as a serious and deadly consequence. Furthermore, the illness is linked to other consequences, like splenomegaly, growth retardation, liver disease, immune system problems, iron overload, heart failure,

and bone deformities. An aberrant immune system, as well as a higher risk of infection, could be linked to elevated iron levels in β-thalassemia patients [13]. The abnormalities of the immune system related to conditions that involve increased iron load (such as thalassemia and hemochromatosis) include decreased phagocytosis through the monocyte–macrophage system, altered T-lymphocyte subsets (as it is evidenced by increased CD-8 and decreased CD-4), decreased immunoglobulin secretion, and impaired complement system function. In addition to iron overload, which is a key factor contributing to the immune deficiency in β-thalassemia, protein compounds containing iron have immunoregulatory characteristics, so their elevated levels could negatively impact immune balance [14]. Whereas 50% of patients had raised IgM and IgG levels, a significant portion of patients had normal IgA and IgE levels, according to research by Amin et al. [15]. Additionally, there has been a substantial correlation between immunoglobulin levels and age [15], which was consistent with our research's findings. In research involving 106 thalassemia patients, it was discovered that iron chelator agents significantly strengthen the immune systems of those with thalassemia major because high IgM and IgG immunoglobulin levels in these patients are caused by frequent procedures of blood transfusion, which stimulate immune response [12]. In Iran, anti-toxoplasma IgG antibody levels have been positive in 51.90% of thalassemia patients as well as 34.80% of the healthy individuals, while anti-Toxoplasma IgM antibody levels were positive in 3.40% of thalassemia patients and 2.10% of healthy persons [16]. The high seroprevalence of anti-Toxoplasma IgG (32.46%) as well as IgM (8.68%) has been indicated in another research that was carried out in Zakoh, Iraq. The seroprevalence of both anti-Toxoplasma antibody types that have been reported in this research is high compared to the rates of seroprevalence of earlier studies that have been carried out in the same city [17].

Numerous works have assessed the levels of cytokines in patients who have β-thalassemia. As a result, persons with thalassemia were found to have a wide range of immunological diseases. Iron overload brought on by frequent blood transfusions increases cytokine production. In line with our research's findings, 16 investigations involving 805 β-thalassemia patients as well as 624 healthy individuals found that thalassemia patients had significantly higher IL-10 levels than the control group [18]. In this Iraqi cohort, 28% of individuals with persistently transfused thalassemia had seroprevalence of *T. gondii* infection. This is similar to rates that have been reported in various geographic locations, ranging from 5 to 39% [19]. The statistically substantial increase in prevalence compared to only 5% among healthy controls is consistent with previously documented trends [20]. They are particularly vulnerable to transfusional infections due to frequent blood transfusions and immunocompromised conditions. Iron overload brought on by frequent blood transfusions will result in oxidative stress to the kidneys and direct cytotoxicity. Additionally, a transient, non-progressive excess in serum creatinine levels is linked to the administration of specific iron-chelating medications. Renal damage is one of the less well-known side effects that is more noticeable when blood transfusions are given. The most common appearance in thalassaemic individuals with type β-major renal injury is tubule and glomerular dysfunctions [21]. There has been a statistically significant difference between patients and controls in the present research in the case when the vital and commonly used indices of kidney function, such as creatinine and urea, have been examined. However, the results remained within normal bounds, indicating no signs of renal impairment. In contrast to those that have been reported by Aldudak et al. [22] and Belsare et al. [23], who had not found any obvious differences in creatinine and urea in the patient population. Additionally, one possible reason for the elevated levels of urea and creatinine in the infected group is that the parasite Toxoplasma creates anomalies in the urine and glomerular lesions, both of which can result in renal failure, and this outcome was consistent with research [24].

Individuals with β-thalassaemia may not necessarily have cell death, but rather high blood levels of ALT and AST activity as a result of damage. Their cell membranes become altered chemically as a result of this damage, which increases cellular permeability and releases these enzymes into the circulation, which intensifies their activity [25].

Frequent blood transfusions are particularly linked to liver damage [26], and liver fibrosis is one of the consequences of thalassemia, causing such enzymes to become more active owing to iron excess. High ALT, AST, ALP, and TSB levels were observed by a number of writers in thalassemia patients. The results are in line with another research [27] that discovered thalassemia patients had higher ALT levels, AST, ALP, and TSB compared to the control group. A well-researched consequence of thalassemia is liver injury, which could be indicated by increased levels of such markers. Additionally, patients with thalassemia have low amounts of ALP, according to earlier studies. This conflicts with the study's findings, which indicated that patients with thalassemia had higher ALP levels compared to those in the controls. Low ALP levels have been found in Snider's patients, which is concerning since it could render them even more vulnerable to dehydration and provide them with fewer defines against infection [28]. The findings provide additional evidence of the impact of thalassemia on the body and highlight the importance of monitoring biochemical markers in people with the condition. Further studies are necessary in order to determine the underlying causes of such changes as well as to develop management and prevention methods for thalassemia patients. Additionally, the present research's findings show that patients who have thalassemia toxoplasmosis have noticeably lower levels of vitamin D. Vitamin D is necessary for the homeostasis of calcium (Ca) as well as skeletal mineralization, in particular, throughout times of rapid growth. Research from several nations has revealed that various thalassaemic patients experience vitamin D deficiency and insufficiency even in the presence of plenty of sunshine and a daily prescription of 400 IU–1,000 IU of vitamin D, which supported the findings of our investigation. Prior research [29] has noted that individuals with β-thalassemia have deficits in micronutrients, particularly immune-related vitamins as well as minerals such as selenium, zinc, E, C, and D. Increased endogenous requirements for essential micronutrients, significant losses, or inadequate food intake could be the source of such vitamin and mineral deficits [30]. Although people live in a country like Iraq, which is rich in sunlight, there is a high prevalence of vitamin D deficiency among people who have β-thalassemia major. There may be several reasons for this phenomenon. Low physical activity and lack of exposure to sunlight because of illnesses and tiredness might be some of these reasons. Furthermore, repeated blood transfusions in thalassemia patients lead to iron overload, particularly in the liver, which may impair hepatic hydroxylation of vitamin D and subsequently reduce

circulating levels of 25-hydroxyvitamin D [31]. In addition, iron deposition in endocrine glands involved in calcium and vitamin D regulation may further disrupt normal metabolic processes.

The immune system in β-thalassemia individuals is also affected owing to the micronutrient deficiency along with the oxidative stress due to excessive iron [32]. This predisposes the individual to infections like those caused by *Toxoplasma gondii*. The results obtained have been supported in the literature by other researchers. For instance, a research study carried out in Egypt found the mean serum 25(OH)D concentrations in patients to be lower than 30 ng/mL, with 22.5% patients suffering from vitamin D insufficiency [33]. Some other factors that can cause vitamin D deficiency in thalassemic patients include darkened skin, improper dietary intake, lack of sufficient sunlight exposure, and lack of physical activity due to the disease [34]. Another reason can be that vitamin D is not converted to 25(OH)D because of excess hepatic iron. Moreover, the next step of converting 25(OH)D into its active form (1,25(OH)2D3) may also be impaired [35]. Overall, vitamin D deficiency in β-thalassemia major appears to be multifactorial, involving disease-related, metabolic, and environmental factors.

The average (± SD) of levels of vitamin D in 57 independent *T. gondii* seropositive cases has been found to be 16.20 ± 7.50 ng/mL, while mean (± SD) of the levels of vitamin D in 50 seronegative cases has been 29.90 ± 8.1 ng/mL, according to one of the latest researches that measured vitamin D levels in samples of serum that have been obtained from patients. These new findings demonstrated that vitamin D levels have been considerably lower in *T. gondii* seropositive patients than in non-infected seronegative women ($p < 0.050$) [36]. In β-thalassemia, the FOXP3 ratio as well as Treg cells might change as a result of increased antigenic stimuli brought on by repeated blood transfusions. The FOXP3 is exclusively expressed in regulatory T cells, which are essential for the maintenance of immunological self-tolerance. In a study of patients with β-thalassaemia, the percentage of regulatory T cells (Tregs; CD4+ CD25+ FOXP3+) and peripheral blood lymphocyte immune function were assessed in 20 healthy children, 30 children with β-thalassaemia major, and 30 children with β-thalassaemia trait. The findings of the research showed that CD4+ $CD25^{bright}$ and CD4+ CD25+ FOXP3+ Treg cells' percentages have been higher in β-thalassaemia major patients than in the controls, and that has been in agreement with our findings [37].

5. LIMITATIONS OF THE STUDY

The study is limited in some aspects, which must be kept in mind while analyzing its results. While its single-center design may restrict its generalizability to the entire population of β-thalassemia major, at the same time, it ensures that all variables are measured in a standardized manner in one center, eliminating any center-to-center variability. Moreover, a lack of full data regarding some of the clinical factors, like the rate of transfusions, serum ferritin, iron chelation treatment, and splenectomy, may have restricted the conduct of more detailed multivariate analysis.

Finally, due to its cross-sectional design, the study was not able to establish any temporal or causal relationship between the toxoplasmosis infection and the immune and biochemical changes observed in the study participants. However, despite all these limitations, the present study does provide valuable insights into the relation between toxoplasmosis and β-thalassemia major and serves as a base for further research in the field.

6. CONCLUSION

Based on results of this study and other recent studies, concluded that thalassemic patients may be at increased risk for infections and it's found a significant increase in the immunological markers, immunoglobulins and in the biochemical molecules such as IgM, IgG, IL-10, FOXP3, liver enzymes, urea, and creatinine, alongside significantly reduced vitamin D3 levels in thalassemia patients co-infected with *Toxoplasma gondii* compared with healthy controls These findings may reflect the complex pathophysiological changes associated with thalassemia. However, factors such as iron overload, chelation therapy, and transfusion-related effects were not directly assessed in this study; therefore, no causal relationship can be established.

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