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ORIGINAL ARTICLE

Evaluation of Immune System Alterations in Children with β-Thalassemia Major: Single-Center Egyptian Study

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ABSTRACT

Background: Beta-thalassemia is an inherited haemoglobin disorder that is common in the Middle East and Africa. Various studies have attributed the increased susceptibility to bacterial infections in beta-thalassemia patients to changes in their immunological status. This study aimed to evaluate changes in the functions of the immune system in pediatric patients with β-thalassemia major.

Methods: We conducted this case-control study in the Pediatric Haematology Outpatient Department in collaboration with the Department of Clinical Pathology of the Faculty of Medicine, Zagazig University, on 56 patients divided into two groups. The case group included 28 children in whom β-thalassemia major was diagnosed, and the control group consisted of 28 healthy children of the same age and sex. All the children underwent complete blood analysis, immunophenotypic analysis of peripheral blood lymphocyte subsets, assessment of serum immunoglobulin levels, complement 3 (C3) and complement hemolysis activity (CH50) using enzyme-linked immunosorbent assays (ELISAs).

Results: The total white blood cell (WBC), lymphocyte and neutrophil count were significantly higher in the patient group than in the control group. CD19+ expression on lymphocytes,CD3+, CD4+ and CD8+ T-cell subsets were significantly higher in patients than in controls with significant increases in the levels of immunoglobulins, including IgG, IgM, IgA and total IgE. The natural killer cell and C3 levels were significantly lower in the patients than in the controls. CH50 showed a diagnostic accuracy of 64.3%, with a CH50 value of 88.1 pg/ml.

Conclusions: This study demonstrated significant derangements in adaptive and innate immune systems in children with beta-thalassemia major.

Keywords: Immune system alterations, β-thalassemia major, pediatric patients, CH50.

INTRODUCTION

Beta thalassemia is a common inherited disease that affects children worldwide, particularly in Mediterranean countries such as Egypt. β-Thalassemia major (βTM) is a severe form of β-thalassemia in which mutation or deletion of the β-globin gene results in a reduction or absence of the β-globin protein. This inherited defect accelerates the turnover of red blood cells (RBCs) due to ineffective haemoglobin (Hb) synthesis [1]. For most children with βTM, early blood transfusion is essential. Other treatment methods include iron chelation

therapy and splenectomy [2]. Regular blood transfusions help children grow and develop normally and reduce the severity of problems caused by anaemia. One of the many undesirable consequences of regular blood transfusions is iron overload, which can lead to an impaired immune system with an increased likelihood of infection, necessitating the regular use of iron chelation therapy to improve growth and correct immune system disorders in transfusion-dependent βTM patients [3]. Patients with β-thalassemia experience changes in the immune system that can affect various levels of the immune response. These changes include changes in T lymphocyte subsets, such as an increase in the number and activity of suppressor T cells (CD8) and a decrease in the number and activity of helper T cells (CD4), resulting in a reduced CD4/CD8 ratio. Natural killer (NK) cells are functionally impaired and have a reduced ability to proliferate. In addition, the number of B lymphocytes is increased, their activation is enhanced, and their differentiation is impaired. Elevated levels of the immunoglobulins IgG, IgM and IgA indicate impaired immunoglobulin secretion. Abnormal opsonization, phagocytosis, and chemotaxis have also been reported. Decreased C3 and C4 levels have been reported, indicating suppressed function of the complement system [4]. Measuring 50% serum complement haemolytic (CH50) activity could provide insight into the function of classical complement components. CH50 decreases when one or more of the components of the classical signalling pathway are reduced [5]. The purpose of this study was to evaluate immune system changes in children with βTM at Zagazig University Hospital. This included evaluation of T, B and NK lymphocyte subsets, determination of immunoglobulin levels and analysis of the complement system, including CH50.

METHODS

This case-control study was conducted between May 2022 and May 2023 at the Pediatric Haematology Outpatient Clinic in collaboration with the Department of Clinical Pathology of the Faculty of Medicine, Zagazig University Hospital. This study included 56 subjects who were divided into two groups: a case group consisting of 28 children diagnosed with βTM and a control group consisting of 28 healthy children of the same age and sex. Epi software version 6 (Atlanta, Georgia, USA) was used to calculate the sample size with a power of 80% and a confidence interval (CI) of 95%. This study followed the World Medical Association Code of Ethics (Declaration of Helsinki) guidelines for human studies. The parents of all participants provided written informed consent. The Institutional Review Board approved this research (ZU-IRB#9529).

All enrolled children had a thorough medical history, including information on blood transfusion frequency, chelation therapy, and splenectomy status. A clinical examination, including assessment of signs of iron overload, and laboratory tests were performed. Patients were diagnosed with βTM based on clinical presentation, complete blood count with blood smear examination and reticulocyte count, haemoglobin electrophoresis test and molecular genetic test [6].

Sampling

Under completely aseptic conditions, three millilitres of whole blood were collected from all participants in an EDTA tube for complete blood count (CBC) analysis with Leishman-stained blood smear examination and immunophenotypic analysis. Additionally,

three millilitres of peripheral blood were collected by venepuncture and placed in a plain vacutainer tube for serum separation. The sample was collected at room temperature for 20 minutes until complete coagulation and centrifuged at 3000 rpm for 20 minutes, after which the resulting serum was used to assess the other parameters. Patient samples were collected immediately before transfusion.

CBC was tested on an automatic cell counter, model XN 330 (Sysmex, Japan), paying special attention to the RBC indices. Liver and renal function tests were performed using a Cobas 8000 autoanalyzer and the c702 module via spectrophotometry (Roche Diagnostics, Switzerland). Serum ferritin and total immunoglobulin E (IgE) were measured on a Roche Cobas 8000 autoanalyzer (e602 module) by electrochemiluminescence immunoassay. The immunoglobulin test included analysis of IgG, IgM, and IgA levels with complement assessment for C3 and C4, which was performed on a Cobas 8000 autoanalyzer, c702 module, using an immunoturbidimetric method. Immunophenotypic assessment of T cells (CD3+) and their subset (CD3+/CD4+ and CD3+/CD8+), B-cell (CD19+), and NK cell (CD16/56) markers was performed on a BD FACSCanto II flow cytometer (Becton, Dickinson) and Company, Franklin Lakes, New Gersy, USA) with a negative isotype control for each sample, and the absolute number and percentage of each cell population were calculated. A serum human 50% complement hemolysis (CH50) assay was performed using a double antibody sandwich enzyme immunoassay (ELISA) to analyse the serum CH50 in each sample. The test kit was obtained from Shanghai Sunred Biological Technology Co., Ltd., China (catalogue no. 201-12-0323). To read the ELISA plates, we used a Sunrise™ absorption reader (Tecan Trading AG, Männedorf, Switzerland). Serum CH50 values are reported in pg/ml. All patients underwent serological viral screening tests for human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) on a Roche Cobas 8000 autoanalyzer, e602 module. Seropositivity was confirmed by automated real-time quantitative PCR (Cobas AmpliPrep/Cobas TaqMan; Roche Molecular Diagnostics). All tests were performed using specific reagents provided by the manufacturer and according to the manufacturer's recommendations.

Statistical analysis

We used SPSS version 24 to tabulate and analyse the data we collected (SPSS Inc., Chicago, IL, USA). Categorical data are presented as numbers and percentages. Categorical variables were analysed using a chi-square test ($\chi 2$). The range, median, and standard deviation (SD) of the quantitative data is presented. The normally distributed variables of the two groups were analysed using Student's t test. To assess the degree of association between the variables, we used Spearman's correlation coefficient (rho). To determine the cut-off values with the best sensitivity and specificity, receiver operating characteristic (ROC) curve analysis was used. P values less than 0.05 were considered significant.

RESULTS

The mean age at diagnosis among the studied patients was 1.76± 0.659 years, ranging between 1 and 3.5 years. Among our patients, 35.7% underwent splenectomy. Regarding the history of recurrent infections, 39.3% had a history of recurrent upper respiratory tract infections, 35.7% had a history of urinary tract infections, 14.3% had a history of skin infections or cellulitis, and 10.7% had

previously suffered from gastroenteritis. Two patients were confirmed to be HCV positive by real-time PCR. Otherwise, all other patients were negative for HBV, HCV, and HIV.

Compared with the control group, the patient group had lower haematocrit (HCT) values, mean corpuscular volume (MCV), and mean corpuscular haemoglobin (MCH) levels, and platelet counts, and higher total white blood cell (WBC) counts and lymphocyte and neutrophil counts and ferritin levels.

Regarding cellular and humoral immunity, all patients had increased CD3+, CD4+, and CD8+ T lymphocyte counts and expression percentages. All the control groups had normal counts and expression percentages.Although all patients had normal CD19 levels, the percentage of CD19 expression was significantly higher in patients than in controls. Nearly 82.1% of patients had a decrease in NK cells, and there was a statistically significant difference between patients and controls ($p < 0.001$) (Table 1).

A total of 67.9%, 85.7%, 39.3% and 85.7% of the patients had significantly increased IgG, IgM, IgA and total IgE levels, respectively, compared to those in the control group. According to the quantitative data, the IgG and total IgE levels in the patient group were significantly higher than those in the control group ($p<0.001$) (Table 2).

Fifty percent of patients had low C3 levels, and C3 levels were significantly lower in patients than in controls ($p<0.001$). All patients had normal C4 levels. The mean CH50 value was significantly greater in patients than in controls ($p = 0.009$) (Table 3). CH50 showed a sensitivity of 60.7%, a specificity of 67.9%, a positive predictive value of 65.4%, a negative predictive value of 63.3% and a diagnostic accuracy of 64.3%, with a CH50 value of 88.1 pg/ml. At this cut-off value, 17 patients had high CH50 values, 9 of the controls had high CH50 values, and the difference between the two groups was statistically significant ($p=0.032$). Receiver operating characteristic (ROC) curve analysis was used to assess the diagnostic performance of the CH50 in the patient group (Figure 1).

Correlation analysis revealed that total lymphocyte count, CD4+ T lymphocyte count, serum ferritin, IgG, and total IgE levels were significantly positively correlated with CH50, while Hb, HCT, MCV, MCH, and C3 levels were negatively correlated with CH50 (Table 4).

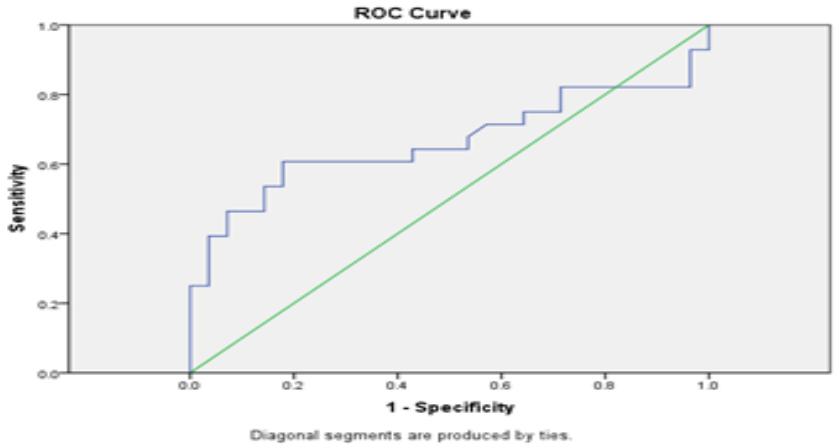

Figure 1: Receiver operating characteristic curve for CH50 for the diagnosis of patients

Table 1: Comparison between patients and controls regarding CD3, CD4, and CD8 expression concerning CD reference range, CD-expressing lymphocyte count, and CD expression percentage

			Cases (No.=28)	Controls (No.=28)	χ2	P. value
CD3 normality level	**increased**	**No.**	28	0	56.000	<0.001**
		%	100.0%	0.0%		
	normal	**No.**	0	28		
		%	0.0%	100.0%		
CD3 expressing lymphocyte count(/mm^3)	**Mean ± SD**		3674.39± 1355.19	1337.75± 356.78	**t. test** 8.823	<0.001**
CD3 expression percentage	**Mean ± SD**		58.07± 11.41	52.00± 8.60	2.247	0.029*
CD4 normality level	**increased**	**No.**	28	0	56.000	<0.001**
		%	100.0%	0.0%		
	normal	**No.**	0	28		
		%	0.0%	100.0%		
CD4 expressing lymphocyte count(/mm^3)	**Mean**		2017.82±779.05	635.10±245.83	**t. test** 8.956	<0.001**
CD4 expression percentage	**Mean ± SD**		38.17± 7.91	33.87±8.41	2.211	0.031 *
CD8 normality level	**increased**	**No.**	28	0	56.000	<0.001**
		%	100.0%	0.0%		
	normal	**No.**	0	28		
		%	.0%	100.0%		
CD8 expressing lymphocyte count(/mm^3)	**Mean ± SD**		1731.57± 487.27	664.89± 141.55	**t. test** 11.124	<0.001**
CD8 expression percentage	**Mean ± SD**		20.75± 3.99	18.46± 4.06	2.123	0.038*
CD19 normality level	**normal**	**No.**	28	28	0	1
		%	100.0%	100.0%		
CD19 expression percentage	**Mean ± SD**		20.14± 4.89	16.32± 4.20	**t. test** 3.132	0.003*
CD16/56 normality level	**Decreased**	**No.**	23	0	35.292	<0.001**
		%	82.1%	0.0%		
	normal	**No.**	5	28		
		%	17.9%	100.0%		
CD16/56 levels	**Mean ± SD**		12.21± 5.92	13.32± 3.51	**t. test** -0.850	0.399

CD: cluster differentiation; No: number; χ2: chi-square test; t-test: Student's t-test; *: Statistically significant; **: Statistically highly significant. Normality regards reference range (normal, increased, decreased)

Table 2: Comparisons between patients and controls regarding the normality of IgG, IgM, IgA, and total IgE levels

			Cases (No.=28)	Controls (No.=28)	χ2	P. value
IgG: mg/dl normality level	**increased**	**No.**	19	0	28.757	<0.001**
		%	67.9%	0.0%		
	normal	**No.**	9	28		
		%	32.1%	100.0%		
IgG: mg/dl	**Mean ± SD**		1297.75± 482.00	811.64± 179.02	**t. test** 5.003	<0.001**
IgM: mg/dl normality level	**increased**	**No.**	24	0	42.000	<0.001**
		%	85.7%	0.0%		
	normal	**No.**	4	28		
		%	14.3%	100.0%		
IgM: mg/dl	**Mean ± SD**		164.35± 31.75	161.53± 20.95	**t. test** 0.392	0.696
IgA: mg/dl normality level	**increased**	**No.**	11	0	10.606	0.001*
		%	39.3%	0.0%		
	normal	**No.**	17	28		
		%	60.7%	100.0%		
IgA:mg/dl	**Mean ± SD**		153.67± 18.69	151.53± 11.47	**t. test** 0.517	0.607
Total IgE:IU/ml normality level	**increased**	**No.**	24	0	42.000	<0.001**
		%	85.7%	0.0%		
	normal	**No.**	4	28		
		%	14.3%	100.0%		
Total IgE:IU/ml	**Mean ± SD**		153.28± 41.44	55.57± 13.56	**t. test** 11.858	<0.001**

Ig: immunoglobulin; No: number; χ2: chi-square test; t-test: Student's t-test; *: Statistically significant; **: Statistically highly significant.Normality as regards reference range (normal, increased, decreased)

Table 3: Comparison between cases and controls regarding C3 and C4 normality as regards the reference range and CH50 concerning the resultant ROC curve cut-off point

			Cases (No.=28)	Controls (No.=28)	χ2	P. value
C3 normality level	**Decreased**	**No.**	14	0	15.389	<0.001**
		%	50.0%	0.0%		
	normal	**No.**	14	28		
		%	50.0%	100.0%		
C3 mg/dl	**Mean ± SD**		86.50± 17.03	111.42± 18.65	**t. test** -5.222	<0.001**
C4 normality level	**normal**	**No.**	28	28	0	1
		%	100.0%	100.0%		
C4 mg/dl	**Mean ± SD**		22.60± 8.97	23.67± 6.60	**t. test** -0.509	0.613
(CH50) pg/ml	**High (≥88.1)**	**No.**	17	9	4.595	0.032*
		%	60.7%	32.1%		
	Low (<88.1)	**No.**	11	19		
		%	39.3%	67.9%		
(CH50) pg/ml	**Mean ± SD**		164.19± 136.38	93.01± 26.98	**t. test** 2.709	0.009*

C3: complement 3; C4: complement 4; CH50: 50% component hemolysis; No: number; χ2: chi-square test; t test: Student's t test; *: Statistically significant; **: Statistically highly significant.Normality as regards reference range (normal, increased, decreased).

Table 4: Correlations between CH50 and other studied variables

Correlation with (CH50) pg/ml	Pearson's correlation	
	R	p
Age(year) at the time of study	-0.112	0.413
RBCs: ($x10^6/mm^3$)	-0.262	0.051
Hb: (gm/dl)	-0.305	0.022*
HCT:(%)	-0.327	0.014*
MCV:(fl)	-0.407	0.002*
MCH:(pg)	-0.367	0.005*

Correlation with (CH50) pg/ml	Pearson's correlation	
	R	p
RDW :(%)	0.190	0.161
WBCs: ($x10^3/mm^3$)	0.034	0.801
Lymphocyte count ($x10^3/mm^3$)	0.322	0.016*
Neutrophil count ($x10^3/mm^3$)	0.168	0.216
Platelets: ($x10^3/mm^3$)	0.053	0.700
AST: U/L	-0.087	0.525
Serum albumin: g/dl	0.120	0.385
Total protein: g/dl	-0.090	0.510
Total bilirubin: mg /dl	0.125	0.357
ALT: U/L	-0.174	0.200
Urea: mg/dl	-0.258	0.055
Serum creatinine: mg/dl	-0.036	0.790
Serum ferritin:ng/ml	0.304	0.023*
CD3 expressing lymphocyte count($/mm^3$)	0.424	0.001*
CD3 expression percentage	0.249	0.064
CD4 expressing lymphocyte count($/mm^3$)	0.278	0.038*
CD4 expression percentage	-0.182	0.180
CD8 expressing lymphocyte count($/mm^3$)	0.247	0.066
CD 8 expression percentage	0.015	0.912
CD19 expression percentage	0.167	0.220
CD16/56 levels	-0.145	0.287
IgG: mg /dl	0.427	0.001*
IgM: mg /dl	0.062	0.648
IgA: mg /dl	-0.126	0.355
Total IgE:IU/ml	0.387	0.003*
C3:mg/dl	-0.309	0.020*
C4:mg/dl	0.207	0.127

*: Statistically significant.

DISCUSSION

Patients with βTM often experience several complications, including heart failure and recurrent infections. Infectious complications are the second leading cause of death and a major cause of morbidity in these patients [7]. The susceptibility to infections in βTM patients is influenced by the disease itself, frequent blood transfusions, splenectomy, iron overload, chelation therapy, exposure to allogeneic antigens in the blood, and impaired immune system function. [7-9]

Both humoral and cell-mediated immune system abnormalities have been observed in patients with βTM [8]. We conducted the current study to highlight immunological changes in pediatric patients with βTM. The most common types of infections revealed in our patients were upper respiratory tract and urinary tract infections, which were reflected in increased total WBC, neutrophil and lymphocyte counts. Kadhim et al. [10] reported in their study on βTM patients who sepsis was one of the most serious bacterial infections, and draining otitis media was also identified as a significant bacterial infection. Soft tissue infections, osteomyelitis, lung, kidney or liver abscesses and fever of unknown origin were also previously reported in those patients [11].

All patients in our study had increased CD3+, CD4+, and CD8+ T lymphocyte counts, and expression percentages compared to those in the control group. These results are consistent with previous studies by Mahmoud et al. [12], Gharagozloo et al. [13] and Zhou et al. [14]. In contrast, Noulsri et al. [15] and Del Vecchio et al. [16] concluded that CD3+ cell counts and CD4+ and CD8+ subsets were not significantly different between patients and controls. Our findings can be explained by the fact that lymphocytes are the cornerstone of cell-mediated immunity, and it is better to check the percentages of these lymphocyte subpopulations rather than absolute numbers, which can fluctuate daily and with age in

children. In addition, the proper function of B cells depends on T cells, so B lymphocytes are affected by T-cell deregulation [12]. Frequent transfusions have been found to lead to chronic infections, which are followed by immune system stimulation with T-cell induction [14].

CD19 expression was significantly greater in our patients than in the controls. These results were consistent with those of AI-Consolini et al. [17], Pattanapanyassat et al. [18] and Mahmoud et al. [12]. However, Ahmadiafshar et al. [19] and Gharagozloo et al. [13] reported no statistically significant differences in CD19+ cells. Consistent with our results regarding NK cell numbers, Mahmoud et al. [12].Ahmadiafshar et al. [19] reported that the percentage of CD16/56+ cells was significantly lower in patients than in controls. The NK cell count has been found to be inversely proportional to the number of transfusions, leading to susceptibility to severe systemic infections [3,12].

Regarding immunoglobulin levels, we found that the levels of IgG, IgM, IgA and total IgE were greater in our patient group than in the control group, which was consistent with the results of Amin et al. [20] and Ehsanipour et al. [21], who reported that the serum levels of immunoglobulins are significantly greater in patients with thalassemia than in controls, especially with increased age. This could be attributed to chronic blood transfusion resulting in increased susceptibility to various blood-borne pathogens that stimulate the production of IgG, IgM and IgE [21]. A possible explanation for the increased serum IgA levels observed in thalassemia patients is an excess of iron in the skin, which leads to an increase in mucocutaneous IgA production. Excess iron triggers increased migration of T helper cells into the gastrointestinal tract and lymph nodes, resulting in increased serum immunoglobulin levels, particularly those of IgA, the major immunoglobulin isotype on most mucosal surfaces [22].

Iron overload in βTM patients results in the suppression of both classical and alternative complement pathways with reduced C3 and C4 levels [21]. Fifty percent of patients had decreased C3 levels, which was significantly different from those of the control group, and these results were consistent with those of other studies [12,20,23]. However, Ehsanipour et al. [21] and Aleem et al. [24] reported that the majority of patients with severe β-thalassemia had normal C3 and C4 levels, with no significant difference in C3 or C4 between patients and controls.

CH50 is a screening test for activation of the classical complement pathway and may indicate reduced or absent function of an element of this pathway [25]. In the present study, 60.7% of patients had elevated CH50 values, 32.1% of healthy controls had elevated CH50 values, and the mean CH50 was significantly greater in patients than in controls. Ghaffari et al. [26] reported that CH50, C3 and C4 did not differ significantly between patients and controls. In a previous study of patients with βTM and sickle cell anaemia, high CH50 levels were reported, and the authors attributed this elevation to the certainty of CH50 activity as an acute phase reactant. The authors stated that a high CH50 value is an indicator of an over-functioning complement system due to an increased susceptibility to infection after splenectomy [27].

The inconsistency of results between different studies could be attributed to differences in the characteristics of the study population in terms of regional and demographic data, methodological disparities, and differences in treatment strategies. Our results showed that CH50 had a significant positive correlation with serum ferritin, IgG, IgE, total lymphocyte count and CD4+ T lymphocyte count. CH50 was significantly negatively correlated with Hb concentration, haematocrit, MCV, MCH and C3. Ghaffari et al. reported that the most notable finding about the complement system in patients with thalassemia was the significantly reduced amounts of C4 and CH50 in patients whose ferritin levels were above 3000 ng/ml. Possible explanations for this finding include excessive complement activity due to iron overload, which is a key component in the regulatory mechanisms of complement activity [26].

Limitations

There are certain limitations in our study.

First, the sample size might be relatively small. For this reason, the results may not be applicable to a broader population. Second, there was a possibility of selection bias because the study was conducted in a single outpatient clinic of a specific hospital. The patient population may not fully represent the diversity and characteristics of all individuals with βTM.

Conclusions

This study demonstrated significant defects in the adaptive and innate immune system in children with βTM. In particular, patients show a significant increase in T lymphocyte subsets (CD4+ and CD8+), a decrease in NK cells (CD16/56), increased serum immunoglobulin levels (IgG, IgM, IgA and IgE) and activation of the complement system (low C3) and high CH50. These immune effects correlate with anaemia severity and are modulated by transfusion frequency and iron overload. Therefore, further longitudinal studies with larger sample sizes are needed to clarify immune system dysregulation in βTM patients.

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