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Predictors of autoimmune hemolytic anemia in beta-thalassemia patients with underlying red blood cells autoantibodies

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ABSTRACT

In beta-thalassemia patients, erythrocyte autoantibodies can remain silent or lead to Autoimmune Hemolytic Anemia (AIHA).The aim of this study was to identify predictors of AIHA in beta-thalassemia patients with positive Direct Antiglobulin Test (DAT), in Tunisia.

This longitudinal prognosis study was carried out on beta-thalassemia patients with a positive confirmed DAT. Predictors of AIHA were identified the Kaplan-Meier method. A Cox model analysis was used to identify independent predictors.

Among 385 beta thalassemia patients, 87 developed positive DAT (22.6%). Autoimmune hemolytic anemia was occurred in 25 patients. Multivariate analysis showed that AIHA was independently associated with beta-thalassemia intermedia and similar family history of AIHA. Splenectomy in patients with positive DAT was independently associated with an increased risk of AIHA (HR = 6.175, CI: 2.049–18.612, $p < 0.001$). The risk of developing AIHA was higher during the first 72 transfusions. Autoimmune hemolytic anemia was significantly associated with polyspecific DAT (anti-complement and anti-IgG), blood group AB and prior alloimmunization. Whereas transfusion by phenotypic and leukoreduced blood was a protective factor.

In summary, splenectomy after autoimmunization, prior alloimmunization, DAT specificity (IgG with complement), thalassemia intermedia, AB blood group and family history of AIHA were strongly associated with AIHA. Leukoreduced blood transfusion had a proven preventive role.

1. Introduction

Homozygous beta thalassemia is one of the most common constitutional diseases in the world, especially in the Mediterranean region, Africa, the Middle East and Southeast Asia [1]. The World Health Organization (WHO) consider thalassemia to be a major health burden and estimates the prevalence of beta thalassemia at 0.46/1000 conceptions worldwide [1].

Transfusion therapy is the primary recommended treatment of homozygous beta-thalassemia but it exposes to a number of complications such as iron overload, transfusion reactions, infections, alloimmunization and autoantibodies formation. Erythrocyte autoantibodies occurred in 6.5% of chronically or intermittently transfused patients [2] against 1.4% in general population [3]. They can remain silent or lead to accelerated clearance of Red Blood Cells (RBC) and hence to Autoimmune Hemolytic Anemia (AIHA), resulting in clinical hemolysis, difficulty in cross-matching blood and shortening of the duration of RBC's survival requiring immunosuppressive therapy or splenectomy. In some patients, there are anti-RBCs autoantibodies present that give a positive Direct Antiglobulin Test (DAT), without notable red cell destruction [3]. It is important for the clinician to know which patients are more likely to develop AIHA with underlying positive DAT in order to develop a therapeutic strategy adapted for patients at risk. Previous studies have been more interested in reporting the incidence of immunologic complications of the transfusion in homozygous beta thalassemia. To our knowledge, this is the first study to identify what factors determine whether or not an anti-RBC autoantibody causes AIHA among patients with homozygous beta-thalassemia.

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The main endpoint of the study was to identify predictive factors of AIHA in homozygous patients with beta thalassemia having underlying anti-erythrocytes autoantibodies in a reference center for hemoglobinopathies in Tunis (Tunisia).

2. Materials and methods

2.1. Research design

This longitudinal prognosis study was carried out in the department of Pediatrics: Immuno-Hematology and Stem Cell Transplantation, Bone Marrow Transplantation Center, Tunis, over a period of 21 years (January 1998–May 2018).

2.2. Subjects

All patients with homozygous beta-thalassemia and having a positive DAT at least two successive times were included.

The diagnosis of homozygous beta-thalassemia was confirmed for all patients by hemoglobin electrophoresis of the patient's and their parents by High Performance Liquid Chromatography (HPLC) method. Beta-plus thalassemia phenotype was defined by the presence of HbA1 in the hemoglobin electrophoresis outside any transfusion. Beta-zero phenotype was defined by the absence of hemoglobin A1 at initial electrophoresis before initiation of transfusion therapy. The distinction between the major and intermediate phenotype has been defined at diagnosis by clinical criteria and red cell transfusion requirements. During beta thalassemia major, the onset of the disease is early (before two years) with a severe anemia (steady state of hemoglobin level < 7 g/dl) that requires life-long regular transfusion therapy for survival. The intermediate form, presents later in childhood (after two years) or even in adulthood with mild/moderate anemia (steady state of hemoglobin level is > 7 g/dl) which only requires occasional or short-course regular transfusions in certain clinical settings [4].

Since 1998, outside urgent situations, all patients needing transfusion received RBCs phenotypically matched for ABO, D, C, c, E, e and Kell, crossmatch compatible and leukoreduced by filtration. If the patient had made an alloantibody, antigen matched crossmatch compatible RBCs was provided. In emergency situations, only ABO and D matched RBCs were offered. The aim of long-term transfusion therapy in the major form was to maintain the hemoglobin level permanently above 9–10 g/dl.

Serum Ferritin (SF) concentration was measured in each patient every three months using standard enzyme immunoassay. An annual lesion report was performed on all patients (liver assessment, blood glucose, phospho-calcium balance, hormonal balance depending on the age and the clinical presentation). Red blood cells alloantibodies screening were performed in all patients before each transfusion.

2.3. Data collection

For each patient, an information sheet has been completed, including patient's identity: date of birth, sex, geographical origin, parental consanguinity, personal and family medical history of AIHA. Beta thalassemia data included age of onset, electrophoretic phenotype and clinical phenotype of disease, genotype, age at diagnosis, and age at first transfusion. Characteristics of RBCs received and number of transfusions had been specified. Laboratory investigations included ABO an D blood grouping, RBC antigen alloantibodies or autoantibodies screening. Data relating to anti-erythrocyte autoimmunization included the age at DAT positivity, clinical and biological signs of hemolysis.

2.4. Diagnosis procedures of autoimmune hemolytic anemia

The DAT was routinely performed every six months in all patients. Any positive DAT was systematically controlled during the next visit to confirm positivity. For each patient, DAT by gelfiltration, elution and serologic investigations were been systematically achieved. DAT was performed with not washed patients' RBCs, using the gel-filtration test: commercial ID-Card “DC Screening I” DiaMed-ID MicroTyping System, consisting of monospecific antihuman globulin reagents, anti-IgG, anti-IgA, anti-IgM, anti-C3c (rabbit), monoclonal anti-C3d (Cell line C139-9), suspended in gel and a negative control. Elution of antibodies from patients' RBCs was performed by 56 °C heat elution, ether elution technique in AB serum or using commercial acid elution kit (DiacidelDiaMed). Eluates were tested against panel of O test RBCs prepared by the NCBT and a Biotest panel of commercially available O test RBCs, using two different gel test methods: the indirect antiglobulin test with polyvalent anti-human antiglobulin (ID-Card Liss/Coombs DiaMed-ID) and the enzymatic test with broméline treated RBCs (ID-Card NaCl-EnzymDiaMed-ID). Antibodies screening in the serum was realized using the same methods described before for eluates [5,6].

An AIHA was considered if association of the following criteria:

- first, worsening of the baseline clinical signs of haemolysis (jaundice, dark urine) and/or increasing of the baseline biological signs of hemolysis (reticulocytes, LDH and indirect bilirubin level);
- second, drop in baseline pretransfusionnal hemoglobin level (with weekly loss of hemoglobin level exceeding 1.5 g/dl in non-splenectomized and 1 g/dl in splenectomized patients);
- third, a concomitant positive DAT;
- and fourth, a good initial response to steroid.

In this work, a good initial response to steroid was defined by: the improvement of hemoglobin level and the weekly loss of hemoglobin, after seven days of the beginning of corticosteroids therapy with Prednisone at the dose of 2–3 mg/kg/day and followed, if necessary, by high doses of Intravenous methylprednisolone at 1000 mg/m^2/day for three days.

2.5. Statistical methods

Data was analyzed using the Statistical Package for Social Science (SPSS) version 24. The incidence density of AIHA was calculated among beta thalassemia patients with underlying positive DAT. Qualitative variables were quoted as numbers and percentages, and continuous variables as means and standard deviations or as the median and inter quartile range (IQR). The quantitative variables (age at DAT positivity and number of transfusions before DAT positivity), were transformed into qualitative variables with two modalities. To define the threshold at which to cut the quantitative variable, we have established the Receiver Operating Characteristics (ROC) curve. After verifying that the area under the curve is significantly > 0.05, we have chosen as a threshold the value of the variable that corresponds to the best “sensitivity-specificity” pair.

Predictors of AIHA were identified the Kaplan-Meier method. The difference between the curves was assessed with the log-rank method. A Cox model analysis was used to identify independent predictors. The event was defined by the occurrence of AIHA. The risk of AIHA has been calculated from the date of the DAT was first positive (time zero) to the occurrence of the AHAI or last follow-up (delay in months). All variables with a $p < 0.20$ in univariate analysis were entered in the multivariate models. For all tests, a value of $p < 0.05$ was considered significant.

2.6. Compliance to ethical standards

Informed consent was obtained from all individual participants included in the study.

Table 1
Clinical characteristics of beta thalassemia patients having underlying red-blood-cells autoantibodies with and without autoimmune hemolytic anemia.

Data	Total n = 87		Autoimmune hemolytic anemia n = 25		Non clinical significant positive DAT[a] n = 62	
	n	%	n	%	N	%
Sex						
Male	50	57.5	16	64.0	34	54.8
Female	37	42.5	9	36.0	28	45.2
Consanguinity	39	44.8	9	36.0	30	48.0
Hemoglobin electrophoresis						
Beta zero	76	87.4	19	76.0	57	91.6
Beta plus	11	12.6	6	24.0	5	8.1
Clinical phenotype of beta thalassemia						
Major	80	92.0	19	76.0	61	98.4
Intermedia	7	8.0	6	24.0	1	1.6
Mutation (n = 48)						
Codon 39 homozygous	36	75.0	13	76.5	23	37.0
Other	12	25.0	4	23.5	8	12.9
Age at first transfusion > 12 months	49	56.3	11	61.3	38	44.0
Phenotypic and leukofiltrated blood transfusion	63	72.4	16	64.0	47	75.8
Family history of autoimmune hemolytic anemia	8	9.2	6	24.0	2	3.2
Blood group						
A	28	32.2	8	32.3	20	32.0
B	16	18.4	4	16.0	12	19.4
O	39	44.0	10	40.0	29	46.0
AB	4	4.6	3	12.0	1	11.6
Age at DAT[a] positivity < 72 months	41	47.1	18	72.0	23	37.1
Number of transfusions before DAT[a] positivity						
< 72 transfusions	43	49.4	19	76.0	24	38.7
≥72 transfusions	44	50.6	6	24.0	38	61.3
Splenectomy						
Before DAT[a] positivity	38	45.9	2	16.0	36	58.0
After DAT[a] positivity	23	26.4	9	36.0	12	19.3
DAT[a] specificity						
Immunoglobulin G	57	65.5	6	24.0	51	82.0
Immunoglobulin G + Complement	28	32.1	17	68.0	11 (17.7)	17.7
Immunoglobulin M	2	0.2	2	8.0	0	0.0
Alloantibodies prior to DAT positivity	15	17.2	8	32.0	7	11.3

[a] DAT: direct antiglobulin test.

3. Results

3.1. Frequency of anti-erythrocyte autoimmunity and patient's characteristics

According to the homozygous beta thalassemic register including 385 patients, 87 (22.6%) developed autoantibodies against erythrocytes. Anti-RBC autoantibodies caused AIHA in 25 patients. The incidence density rate of the AIHA was 5/100 patients-years.

The general characteristics of patients are summarized in Table 1. Diagnosis of thalassemia in patients with positive DAT was made at a mean of 20 months ± 19 (range, 2–74) with a median of 11 months (IQR: 6–27) and a sex ratio of 1.3 (50 males and 37 females). Molecular analysis was performed in 48 patients. The Cd39 (C➔T) b0 mutation, found in homozygote or compound heterozygote state was the most frequent allele (75%), followed by beta + IVSI-110 (G➔A) mutation which was found in 12.5% and beta +Cd 30 (G > C) in 4%. The beta 0 IVSI-1 (G➔A), beta +IVSI-6 (T➔C), beta 0 IVSII-849 (A➔C), beta 0 Cd47 (+A) were identified exclusively in 2% of cases for each mutation respectively.

The consanguinity was found in 39 (44.8%) families. Mean weekly hemoglobin level drop before DAT positivity was 0.75 g/dl/week ± 0.4 (0–2.4) with a median of 0.71 g/dl/week (IQR: 0.5–0.97). Median age at start transfusion therapy was 11 months (IQR: 3–36).The DAT positivity was observed at a mean age of 88 ± 63 months (range, 7–353) and in all cases after the start of transfusion therapy. The median delay between the first transfusion and the positive DAT was 11 months (IQR: 6–25). Direct antiglobulin test was positive after a mean number of transfusions of 81 ± 74 (1, 375) and a median of 74 (IQR: 23–135) (Fig. 1). Age at initiation of transfusion therapy was not correlate with age at DAT positivity (p = 0.0.27, r = 0.11). Autoimmune hemolytic anemia was triggered by vaccination in two patients and mycoplasma pneumonia infection in one other patient. The frequency of AIHA was inversely proportional to the number of blood transfusion received (p < 10-3) (Fig. 1).

Median age at the occurrence of clinical AIHA was 53 months (Q1 = 26, Q3 = 87). It was concomitant of the age at DAT positivity in 15 cases. Prior alloimmunization was found in 15 patients (17.2%). Splenectomy preceded alloimmunization in eight cases.

All patients were treated with first-line corticosteroid therapy. The median initial dose of prednisone was 2 mg/kg/d. Intravenous methylprednisolone at a 1000 mg/m2/d for three days was indicated for five unresponsive patients to 2 mg/kg/day of prednisone. A good initial response to steroid was obtained in all cases. Immunosuppressive therapy was associated in 11 patients requiring inacceptable high (> 0.25 mg/kg prednisone per day) and protracted corticosteroid therapy. Six patients among them were non-splenectomized (p = 0.57).

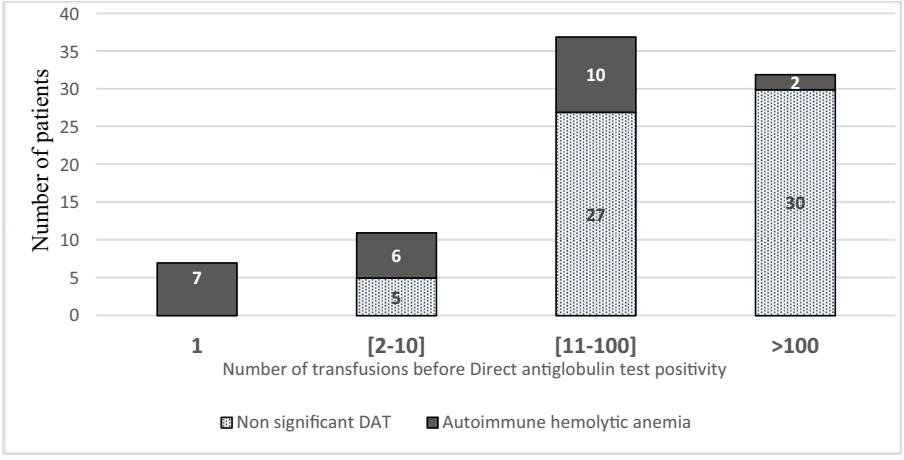

Fig. 1. Transfusion exposure before the development of a positive Direct Antiglobulin Test (DAT) in 87 beta-thalassemia patients according the occurrence or not of autoimmune hemolytic anemia (AIHA). The rate of DAT positivity was higher after 10 blood transfusions received. However, a significant negative correlation between the frequency of AIHA and the number of blood transfusions received was found (p < 10–3).

Table 2
Univariate analysis for predictors of autoimmune hemolytic anemia in beta thalassemia patients with positive Coombs test.

Variables	Risk category	Control category	Hazard ratio	95% confidential interval	p value
Sex	Male	Female	1.3	0.573–2.950	0.48
Parental consanguinity	Yes	No	1.7	0.789–4.060	0.34
Hemoglobin electrophoresis	Beta zero	Beta plus	1.6	0.223–12.272	0.63
Clinical phenotype of thalassemia	Intermedia	Major	8.18	3.080–21.771	**< 10-3**
Mutation (n = 48)	Codon 39 homozygous	Others	0.87	0.615–1.257	0.48
Age at first transfusion	≥12 months	< 12 months	2.03	0.893–4.595	0.14
Phenotypic and leucofiltrated blood transfusion	No	Yes	1.3	0.579–3.008	0.26
Family history of autoimmune hemolytic anemia	Yes	No	5.94	2.28–5.519	**< 10–3**
Blood group	AB	Others	2.7	0.806–2.169	0.1
Rhesus	Positive	Negative	21	0.007–663.93	0.45
Age at DAT[a] positivity	< 72 months	≥72 months	19.48	5.742–66.080	**< 10–3**
Number of transfusions before DAT[a] positivity	< 72	≥72	10.81	3.891–30.078	**< 10–3**
Splenectomy	No	Yes	2.42	0.885–6.625	0.85
Splenectomy preceding DAT[a] positivity	No	Yes	0.104	0.35–0.313	**0.001**
After DAT[a] positivity	Yes	No	6.2	2.681–14.646	**0.017**
DAT[a] specificity	Immunoglobulin G + Complement	Other	3.46	2.173–5.531	**$< 10^{-3}$**
Alloimmunization	Yes	No	5.32	2.864–9.892	**$< 10^{-3}$**
Alloantibodies prior to positive DAT[a]	Yes	No	4.36	1.806–10.552	**0.029**

The bold character means statistically significant associations.
[a] DAT: direct antiglobulin test.

Immunosuppressants included azathioprine (n = 7) and mycophenolate mofetil (n = 4). Splenectomy was effective in two patients.

3.2. Predictive factors for autoimmune hemolytic anemia in beta thalassemia patients with underlying red-blood-cells autoantibodies

Univariate analysis results and patient's characteristics are summarized in Table 2. A significant association was found between the occurrence of clinical hemolysis in DAT positive patients and beta-thalassemia intermediate, family history of autoimmunization, number of transfusions before DAT positivity < 72, early DAT positivity before 72 months of age, splenectomy after DAT positivity, Alloimmunization especially prior to autoimmunization. Splenectomy preceding DAT positivity and phenotypic leukoreduced blood were protective. Age at first transfusion was not associated with AIHA.

Results of multivariate analysis were shown in Table 3. After adjustment, eight independent factors predicting AIHA in homozygous beta thalassemic patients with positive DAT were retained in the regression model.

AIHA in beta thalassemia patients with underlying positive DAT was independently associated with beta-thalassemia intermediate, similar family history of anti-erythrocyte autoimmunity, number of transfusions before DAT positivity < 72, and splenectomy after DAT positivity.

The study showed that influence of splenectomy differs according to its timing: splenectomy preceding DAT positivity was observed more frequently in the group of non-clinical significant DAT than in AIHA group (58% versus 16%, $p < 0.001$). Splenectomy after DAT positivity was associated with an increased risk of AIHA (36% versus 19.3%, $p < 0.001$). Cumulative incidence of AIHA was higher in patients splenectomized after DAT positivity than those splenectomized before DAT positivity (75% versus 20% at five years after DAT positivity respectively, $p < 0.001$) (Fig. 2).

Biological independent factors predicting hemolysis in beta thalassemia patients with underlying positive DAT included presence of both anti-complement (C3d) and anti-IgG polyspecific autoantibodies (Fig. 3a), blood group AB and the presence of prior alloimmunization (Fig. 3b). Whereas, transfusion by phenotypic and leukoreduced blood was a protective factor.

Table 3
Multivariate analysis for predictors of autoimmune hemolytic anemia in beta thalassemia patients with positive Coombs test.

Studied parameters	Risk category	Control category	Hazard ratio	95% confidential interval	p value
Clinical predictors					
History of autoimmune hemolytic anemia	**Yes**	No	**3.062**	1.177–8.628	**0.034**
Splenectomy before hemolysis	**After DAT[a] positivity**	Before DAT[a] positivity	**6.175**	2.049–18.612	**0.001**
Thalassemia	**Intermedia**	Major	**6.842**	2.064–22.683	**0.002**
Age at DAT[a] positivity	< 72 months	≥72 months	4.210	0.901–19.888	0.066
Number of transfusions before DAT[a] positivity	**< 72**	≥72	**7.980**	2.480–25.683	**$< 10^{-3}$**
Age at first transfusion	≥12 months	< 12 months	1.240	0.752–4.712	0.326
Biological predictors and type of RGCs received					
DAT[a] specificity	**Immunoglobulin G with Complement**	Only Immunoglobulin G	**3.830**	2.309–6.355	**$< 10^{-3}$**
Blood group	**AB**	Other	**5.802**	1.537–21.902	**0.009**
Phenotypic and Filtrated blood transfusion	**No**	Yes	**3.110**	1.597–5.504	**0.039**
Alloimmunization	**Preceding anti-red cell autoimmunization**	After or concomitant of anti-red cell autoimmunization	**4.047**	1.483–11.043	**0.006**

The bold character highlights the parameters found as predictive factors of AIHA.
[a] DAT: direct antiglobulin test.

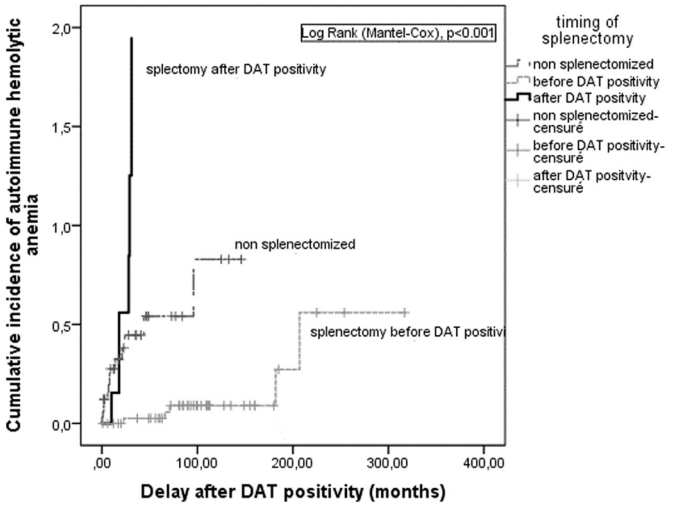

Fig. 2. Cumulative incidence of autoimmune haemolytic anemia (AIHA) after Direct Antiglobulin Test (DAT) positivity in beta thalassemia patients regarding to the time of splenectomy. Splenectomized patients before DAT positivity are less likely to present AIHA. Splenectomy after positivity of DAT increases the risk of occurrence of AIHA I. Thirty months after DAT positivity 85% of patients splenectomized after DAT positivity had already developed AIHA, versus 7% of those splenectomized before DAT positivity and 30% of those not splenectomized ($p < 0.001$).

4. Discussion

This original study had identified the predictor factors of the occurrence of an AIHA in homozygous beta-thalassemia, a thematic rarely reported despite its severity once it occurred. There is no consensual definition of AIHA in beta thalassemia in front of the presence of underlying chronic hemolysis in these patients. The imputation of the autoimmune origin of the aggravation of hemolysis was based on the presence of a positive DAT in addition to the clinic and biologic signs of hemolysis with drop in baseline pretransfusionnal hemoglobin level [5]. The good response to steroids and/or immunosuppressive therapy was a supportive evolutionary criteria for AIHA. The DAT positivity has been confirmed on at least two successive reports to eliminate false positives [6].

4.1. Frequency of clinical significant red-blood-cells autoantibodies

In the current study, the rate of RBC autoimmunization among all patients with homozygous beta-thalassemia was 22.6%. The incidence density rate of the AIHA was 5/100 patients-years. According to a Cross-sectional study conducted on this same department in 2010, positive DAT was detected on 40% of all beta thalassemic patients but only the fifth (21%) developed AIHA among patients with underlying erythrocyte autoantibodies [7]. This frequency is higher compared to the general papulation [8]. A positive DAT was found to occur in 7–8% of hospitalized patients. Since AIHA is a rare disease, the predictive value of a positive DAT in the total hospital population was calculated to be only 1.4% [3,5]. The rate of autoimmunization in patients with thalassemia in the other countries has been reported as following: 28.8% in Egypt [9], 23% in Hong Kong [10], 22.8% in Albania [11], 15.8% in India [12] and 6.5% in United States [2].

4.2. Predictors of clinical significant red-blood-cells autoantibodies

4.2.1. Disease phenotype and family history

The RBC autoantibodies can have varied clinical importance, ranging from silent to life-threatening AIHA [12]. The pathophysiology of AIHA complicating beta-thalassemia major is unclear. The hypothesis of a genetic predisposition was suggested in front of the significant higher frequency of AIHA observed among patients with beta thalassemia intermedia in cox model ($p = 0.002$) and the higher rate of similar family history in the group of patients with AIHA ($p = 0.034$). These two factors were identified as independent predictors of AIHA in beta thalassemia homozygous with underlying positive DAT in this study (Table 3). β-thalassemia itself leads directly to a constant immune stimulation. The imbalance in chain synthesis leads to an excess of freed α-globin chains which precipitate in the erythroid cells, resulting structural changes of the cell membrane [13]. The presence of these abnormal erythrocytes leads to a continuous activation of monocytes that responsible for immune clearance. This phenomenon is accentuated in non or poorly transfused patients or in non transfusion dependent thalassemia such as beta thalassemia intermedia. Another immunological mechanism linked to the disease has been suggested. Studies on the frequency of T-lymphocytes in patients with thalassemia intermedia have shown a marked increase in the frequency of the CD4bright CD8dim T-lymphocyte subset in the peripheral blood of β-thalassemia/HbE patients compared to that in healthy controls. These

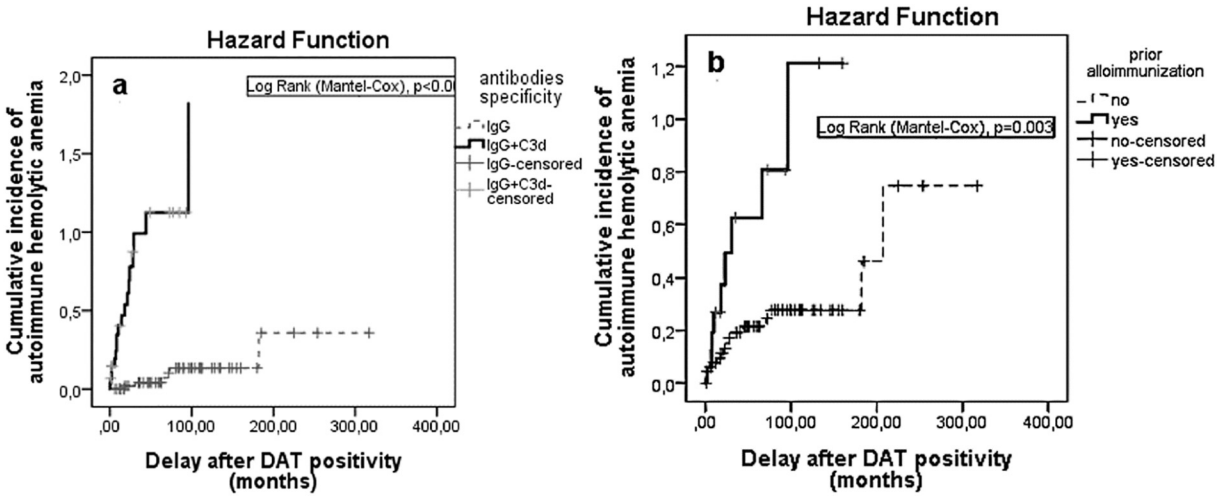

Fig. 3. Cumulative incidence of autoimmune haemolytic anemia(AIHA) after Direct Antiglobulin Test (DAT) positivity in beta-thalassemia patients regarding to DAT specificity (a) and to the presence of prior alloimmunization (b).
a. Patients with a DAT specificity IgG and Complement are more prone to develop AIHA.
b. Patients with prior alloimmunization are more likely to develop AIHA after DAT positivity in beta-thalassemia patients.

CD4brightCD8dim T-lymphocytes has been observed in patients with unrelated diseases, including various autoimmune diseases [14]. In a recent review, the association between autoimmune diseases and beta thalassemia trait has been reported. This associations can be explained by the close proximity between the hemoglobin β-chain locus 11p15.5 and eight genes with profound roles in immune regulation: STIM1, CD151, TC21/RRAS2, SIGIRR/TOLL/IL1R8, pp52/LSP1 (lymphocyte specific protein), TRIM21, toll interacting protein (TOLLIP) and SLEN3 [15].

4.2.2. Timing of splenectomy compared with the DAT positivity

It is known that splenectomized patients are more prone to alloimmunization and autoimmunization [16]. In the current study, splenectomy preceded alloimmunization and autoimmunization in 53.3% (8 of 15 patients) and 52.8% (46 of 87 patients) respectively. Splenectomy prior to positive Coombs test was more observed in the group of patients who did not present AIHA (p < 10-3). In agreement with our study in Egyptian and Indian studies, autoantibodies formation after splenectomy were not interfering with compatibility testing in most cases neither with AIHA [9,16]. However, performing splenectomy in patients with underlying erythrocytes autoantibodies was an independent predictive factor for AIHA in patients with beta-thalassemia in the present report (*HR = 6.1; CI 95% 2.046–18.612;* $p = 10^{-3}$). This could be explained by the increase of the synthesis of anti-erythrocyte auto antibodies in other lymphoid organs, and the liver replacing of the spleen's function of RBC destruction [17]. The absence of a spleen may further enhance the immune response to the infused foreign antigens which are not effectively filtered [8,9]. In patients with a previously positive DAT, splenectomy could increase the amount of autoantibodies leading to AIHA. In previous studies, it has been shown that the most obvious factor that could determine the clinical significance of an autoantibody is the quantity in which it is present [3]. On the other hand, splenectomy markedly increases antigen and microparticles exposure in AIHA. This splenectomy-induced antigen overload likely increases alloimmunization rate which is a proven predictor of autoimmunization during beta thalassemia [2]. In addition, the pathogenesis of AHAI in splenectomized patients may be associated with additional factors that influence the clinical characteristics of AIHA such as the autoantibody pathogenicity and the activity of the reticuloendothelial system [17]. From an immunological point of view, it has been reported in splenectomized beta thalassemic patients, a significant increase of double negative T lymphocytes which play an important role in the down regulation of the immune response [14].

4.2.3. Red blood cells alloimmunization

In this study, the DAT positivity was observed after the start of transfusion therapy in all patients included. Red blood cell autoimmunization after transfusion was described in patients with multiple transfusions, suggesting the probable association between autoimmunization and alloimmunization [8,16,18]. Effectively, this work found that prior alloimmunization was an independent predictor of AIHA (*HR = 4.047; CI 95%: 1.483–11.043; p = 0.006*). The pathogenesis of RBC autoantibody formation after RBC alloimmunization is not well understood. One possible hypothesis is that alloantibody binding to the transfused RBCs could lead to conformational changes in antigenic epitopes that then stimulate production of an autoantibody [18]. A previous study have reported the coexistence of autoantibodies and alloantibodies in 38.9% of Chinese thalassemia major patients [10]. Prior alloimmunization affected 84% of thalassemic patients with autoantibodies in a large longitudinal cohort study in United States [2]. The relatively low rate of alloimmunization in the current study can be explained by the homogenization of ethnicity between recipients and donors of RBCs.

4.2.4. Transfusion therapy

A previous Tunisian work outlined the effect of leukofiltration in thalassemic patients on the risk of RBCs autoimmunization [7]. In continuation with the previous work, the current study had demonstrated the protective role of the administration of phenotyped and leucodepleeted blood to prevent AIHA in patients with preceding erythrocyte autoimmunity. The role of leucoreduction in preventing allo and autoimmunization has been reported in several studies showing that patients receiving leukodepleted blood appeared to have a lower red cell alloimmunization rate, suggesting that it is the removal of leucocytes that reduces immune activation due to allogeneic transfusion [2,8,19]. Other studies have raised the interest of in-line filtration or pre-storage leukodepletion to decrease the rate of cytokines and soluble biologic mediators that can be released in case of post-storage leukofiltration [20].

The immune response may also be affected by the number of blood units received. The relation between the number of blood units transfused and antibody formation is unknown in thalassemia [8]. In this study, the rate of erythrocyte autoimmunization was higher after exposure to a number of blood transfusion between 10 and 100 (Fig. 1). In accordance with this result, some previous studies reported a higher erythrocyte immunization rate in patients who receive more units of blood [21]. However, our data found that the risk of AIHA is inversely proportional to the number of blood transfusion received before DAT positivity with a risk multiplied by 7.98 during the first 72 transfusions (CI = 2.480–25.683, p = $< 10^{-3}$). Multiple blood transfusions lead to a continuous allo-antigen stimulation, which leads to disruption of the immune balance in patients with beta thalassemia [22]. Red blood cells transfusions had specific effects on innate and adaptive recipient's immune system that play a critical role in loss of self-tolerance in the development of anti-erythrocyte autoantibodies and AIHA [17,23]. Paradoxically, another study by Bozdogan et al. found that β-thalassemia major patients who are exposed to repeated blood transfusions had increased T regulatory cells, which might suppress immune activation status [24]. This could explain the secondary decrease in the AHAI rate after chronic blood exposure in our patients.

4.2.5. Age at first transfusion and age at direct antiglobulin test positivity

Immune tolerance as a factor in the rate of red cell alloimmunization has previously been suggested [2,25]. The risk of alloimmunization will increase with increasing exposure to transfusions. In addition to the differences that exist between different RBC antigens in terms of their immunogenicity, there are differences between individuals in terms of their sensitivity to alloimmunization. Younger age at the time of initial RBC exposure correlates with a lower likelihood of alloimmunization [25]. The concept of transfusion immune tolerance before 12 months of age does not confirmed as an independent predictor in the cox model in this work. Similarly, in Egyptian and Malaysian patients, authors found that there was no association between autoantibodies and age of start of transfusion (p = 0.5, p = 0.8 respectively) [9,21]. Genetic factors influencing immune tolerance and RBC antibodies formation have been identified [25,26]. In a study in sickle cell patients, it was outlined that mean age at first transfusion of patients that developed at least one anti-RBC antibody was significantly lower in patients with rs660C/T than in patients with T/T genotype in patients less than five years old. The age of five years represented the turning point, beyond which the risk becomes comparable [25].

This study had also shown that the risk of AHAI was correlated with the age of positivity of DAT in univariate analysis. In the multivariate study the p value was substantially close to 0.05, this could be due to the small number of patients. This information is relevant in order to alert the clinician that the early positivation of the DAT may be clinically significant and result in an AIHA, so these patients should be closely monitored for worsening clinical and biological signs of hemolysis.

4.2.6. Erythrocytes autoantibodies specificity

Direct antiglobulin test specificity IgG + Complement was retained

as independent predictors of clinical significant hemolysis in beta thalassemia patients with underlying RBC autoimmunity in this report. Another study in Kuwait reported AIHA in 11% (21/109), among them 52.38% of patients had only IgG. Similar results were reported by Singer et al who found autoimmunization in 25% (16/64) of patients with thalassemia major. Three patients developed a clinically significant AIHA: IgG-induced in one, and IgG with complement in the other two [8]. The strength of the polyspecific DAT with anti-IgG and anti-C3 together was shown to give the best predictive value for hemolysis in several reports [3,27,28].

This study identified predictors of clinical hemolysis in homozygote beta thalassemia patients with positive DAT. It is limited particularly by the sample size. However, this is an overlooked complication with limited bibliography, which allows considering this cohort relatively broad. These results suggest conducting a multicenter study to validate these predictive factors and to develop a new scoring of the risk of AIHA among beta thalassemia patient. In fact, identifying predictors of AIHA will allow adapting therapeutic management of these patients such as:

-Better evaluating the risk-benefit balance of splenectomy especially in patients with underlying RBCs autoantibodies, as well as the risk-benefit balance of transfusion in patients with beta thalassemia intermedia especially in the presence of familial cases of AIHA.

- Watch for the occurrence of AIHA in patients with polyspecific positive DAT.

- Ensure transfusion with leukocyte-depleted phenotypic Red blood cell concentrates as recommended for all polytransfused patients especially in the presence of RBCs immunization [29].

- Extended matching and typing of donor's blood against the patient's for all the possible antigens [30], especially for D, C, E, c, e, K, FYa, FYb, JKa and JKb antigens in order to prevent alloimmunization in patients at risk.

5. Conclusion

In summary, erythrocyte autoantibodies is a frequent complication in homozygote beta thalassemia comparing to general population. They may cause clinically significant hemolysis leading to life threating AIHA and worsen the course of the disease. This study identified predictive factors of AIHA in homozygote beta thalassemia patients with positive DAT. Autoimmune hemolytic anemia was independently associated to thalassemia intermedia, similar family history of autoimmunization, splenectomy after autoimmunization, prior alloimmunization, DAT positivity during the first 72 transfusions, DAT specificity (IgG with complement) and AB blood group. Whereas, leukoreduction has a proven preventive role. Further multicenter studies on a larger population or a metaanalysis would improve the quality of the findings and validate a predictive score of AIHA in homozygote beta thalassemia patients with underlying positive DAT.

Authorship contributions

All authors have contributed to the manuscript in significant ways. M.BK designed the research, interpreted data, and wrote the first manuscript draft. N.S collected data and wrote the first manuscript draft. M.O, N.D, S.R, R.K, H.S, F.M and H.K collaborated in the research design, data interpretation and clinical management of the patient. A.BA performed statistical analysis and provided supervision and advice on design, analysis and interpretation of the results. M.B offered supervision in design, analysis, interpretation of study results and reviewed the manuscript. All authors reviewed and agreed on the final manuscript.

Declaration of Competing Interest

The authors have declared that no competing interests exist.

References

[1] B. Modell, M. Darlison, Global epidemiology of haemoglobin disorders and derived service indicators, Bull. World Health Organ. 86 (2008) 480–487.

[2] E. Vichinsky, L. Neumayr, S. Trimble, P.J. Giardina, A.R. Cohen, T. Coates, J. Boudreaux, E.J. Neufeld, K. Kenney, A. Grant, A.A. Thompson, CDC Thalassemia Investigators, Transfusion complications in thalassemia patients: a report from the Centers for Disease Control and Prevention (CDC), Transfusion 54 (2014) 971–972.

[3] E.M. Meulenbroek, D. Wouters, S.S. Zeerleder, Lyse or not to lyse: clinical significance of red blood cell autoantibodies, Blood Rev. 29 (2015) 369–376.

[4] A.T. Taher, M.D. Cappellini, How I manage medical complications of beta-thalassemia in adults, Blood (2018), https://doi.org/10.1182/blood-2018-06-818187.

[5] N.D. Zantek, S.A. Koepsell, D.R. Tharp, C.S. Cohn, The direct antiglobulin test: a critical step in the evaluation of hemolysis, Am. J. Hematol. 87 (2012) 707–709.

[6] Z. Bıçakçı, S. Öztürkmen, A. Akyay, L. Olcay, False positive result of the direct antiglobulin test (DAT): the role of the elevated level of immunoglobulin G, Pediatr. Hematol. Oncol. 29 (2012) 611–619.

[7] N. Guirat-Dhouib, M. Mezri, H. Hmida, F. Mellouli, H. Kaabi, M. Ouderni, B. Zouari, S. Hmida, M. Bejaoui, High frequency of autoimmunization among transfusion-dependent Tunisian thalassaemia patients, Transfus. Apher. Sci. 45 (2011) 199–202.

[8] S.T. Singer, V. Wu, R. Mignacca, F.A. Kuypers, P. Morel, E.P. Vichinsky, Alloimmunization and erythrocyte autoimmunization in transfusion-dependent thalassemia patients of predominantly Asian descent, Blood 96 (2000) 6.

[9] A.M. Ahmed, N.S. Hasan, S.H. Ragab, F.A. Kuypers, P. Morel, E.P. Vichinsky, Red cell alloimmunization and autoantibodies in Egyptian transfusion-dependent thalassaemia patients, Arch. Med. Sci. 6 (2010) 592–598.

[10] C.K. Cheng, C.K. Lee, C.K. Lin, Clinically significant red blood cell antibodies in chronically transfused patients: a survey of Chinese thalassemia major patients and literature review: RBC antibodies in thalassemia major, Transfusion 52 (2012) 2220–2224.

[11] I. Seferi, M. Xhetani, M. Face, G. Burazeri, E. Nastas, G. Vyshka, Frequency and specificity of red cell antibodies in thalassemia patients in Albania, Int. J. Lab. Hematol. 37 (2015) 569–574.

[12] A. Jain, A. Agnihotri, N. Marwaha, R.R. Sharma, Direct antiglobulin test positivity in multi-transfused thalassemics, Asian J Transfus Sci 10 (2016) 161–163.

[13] Arthur W. Nienhuis, D.G. Nathan, Pathophysiology and clinical manifestations of the β-thalassemias, Cold Spring Harb Perspect Med 2 (12) (2012) a011726.

[14] E. Noulsri, S. Lerdwana, S. Fucharoen, K. Pattanapanyasat, Phenotypic characterization of circulating CD4/CD8 Tlymphocytes in β-thalassemia patients, Asian Pac. J. Allergy Immunol. 32 (2014) 261–269.

[15] M.A. Altinoz, G. Gedikoglu, G. Deniz, β-Thalassemia trait association with autoimmune diseases: β-globin locus proximity to the immunity genes or role of hemorphins? Immunopharmacol. Immunotoxicol. 34 (2012) 181–190.

[16] H.K. Dhawan, V. Kumawat, N. Marwaha, R.R. Sharma, S. Sachdev, D. Bansal, R.K. Marwaha, S. Arora, Alloimmunization and autoimmunization in transfusion dependent thalassemia major patients: study on 319 patients, Asian J Transfus Sci 8 (2014) 84–88.

[17] W. Barcellini, New insights in the pathogenesis of autoimmune hemolytic anemia, Transfus. Med. Hemother. 42 (5) (2015) 287–293.

[18] P.P. Young, A. Uzieblo, E. Trulock, D.M. Lublin, L.T. Goodnough, Autoantibody formation after alloimmunization: are blood tranfusions a risk factor for autoimmune hemolytic anemia? Transfusion 44 (2004) 67–72.

[19] J.E. Hendrickson, C.A. Tormey, Red blood cell antibodies in hematology/oncology patients, Hematol. Oncol. Clin. North Am. 30 (2016) 635–651.

[20] L.Y. Wang, D.C. Liang, H.C. Liu, F.C. Chang, C.L. Wang, Y.S. Chan, M. Lin, Alloimmunization among patients with transfusion-dependent thalassemia in Taiwan, Transfus. Med. 16 (2006) 200–203.

[21] M.N. Noor Haslina, N. Ariffin, I. Illuni Hayati, H. Rosline, Red cell autoantibodies among thalassaemia patients in Hospital Universiti Sains Malaysia, Singap. Med. J. 48 (2007) 922–925.

[22] C.D. Asadov, Immunologic abnormalities in β-thalassemia, J Blood Disorders Transf 5 (2014) 7.

[23] J.C. Zimring, Regulation of immune responses by RBC transfusion, ISBT Science Series 8 (2013) 181–184.

[24] G. Bozdogan, E. Erdem, G.Y. Demirel, Y. Yildirmak, The role of Treg cells and Foxp3 expression in immunity of B-thalassemia major and B-thalassemia trait patients, Pediatr. Hematol. Oncol. 27 (2010) 534–545.

[25] Z. Tatari-Calderone, N.L.C. Luban, S. Vukmanovic, Genetics of transfusion recipient alloimmunization: can clues from susceptibility to autoimmunity pave the way? Transfus. Med. Hemother. 41 (2014) 436–445.

[26] Z. Tatari-Calderone, C.P. Minniti, T. Kratovil, M. Stojakovic, A. Vollmer, I. Barjaktarevic, E. Zhang, A. Hoang, N.L. Luban, S. Vukmanovic, rs660 polymorphism in Ro52 (SSA1; TRIM 21) is a marker for age-dependent tolerance induction and efficiency of alloimmunization in sickle cell disease, Mol. Immunol. 47 (2009) 64–70.

[27] W. Barcellini, Immune hemolysis: diagnosis and treatment recommendations, Semin. Hematol. 52 (2015) 304–312.

[28] R. Chaudhary, S. Das, Autoimmune hemolytic anemia: from lab to bedside, Asian J. Transfus. Sci. 8 (2014) 5.

[29] C. Wickremaarachchi, E. McGill, A. Bosco, G. Kidson-Gerber, Improving transfusion practice in transfusion dependent thalassaemia patients, Thalassemia Reports 7 (2017) 30–31.

[30] S. Jansuwan, O. Tangvarasittichai, S. Tangvarasittichai, Alloimmunization to red cells and the association of alloantibodies formation with splenectomy among transfusion-dependent β-thalassemia major/HbE patients, Indian J. Clin. Biochem. 30 (2015) 198–203.