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Association of Activated Circulating Endothelial Cells with Vascular Complications in Egyptian Beta-Thalassemic Patients

Afaf Abd Elaziz Abd Elghaffar[1], Soha Raouf Youssef[1], Deena Mohamed Mohamed Habashy[1*], Mona Ali Mohamed Hassan[1], Nevine Nabil Mostafa[2], Gihan Mohamed Kamal[2] and Amira Nashaat Abd El-Gawad[3]

[1]Department of Clinical Pathology, Hematology Unit, Faculty of Medicine, Ain Shams University, Cairo, Egypt

[2]Department of Internal Medicine, Clinical Hematology and Bone Marrow Transplantation Unit, Faculty of Medicine, Ain Shams University, Cairo, Egypt

[3]Department of Pediatrics, Faculty of Medicine, Ain Shams Univeristy, Cairo, Egypt

[*]**Corresponding author:** Deena Mohamed Mohamed Habashy, MD, Department of Clinical Pathology, Hematology Unit, Faculty of Medicine, Ain Shams University, Cairo, Egypt, Tel: + 02 2473 4377; E-mail: deena_habashy@yahoo.com

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Abstract

Background: There is evidence of an association between circulating endothelial cells (CECs) and vascular complications in thalassemic patients.

Objectives: To quantify CECs and its activated fraction (AECs) in Egyptian ß-thalassemic patients and to investigate their association with incidence of vascular complications.

Subjects and methods: Eighty pediatric patients and 30 healthy pediatric volunteers were studied for the proportion of CECs and AECs using cellular expression of endothelial adhesion receptors: CD146 and CD106 by flow cytometry.

Results: CECs, AECs were higher in patients' group than control group (p=0.001). AECs, serum ferritin, total leucocyte count (TLC), platelet (PLT) count were higher in patients with vascular complications and splenectomized patients (p=0.001for all). Percent of AECs was positively correlated to each of serum iron (p=0.001) and serum ferritin (p=0.001). No correlation was found between AECs (percent/absolute count) or MFI of CD106 and serum ferritin neither in group with vascular complications nor in splenectomized group (p>0.05). Also, no correlation was found between red blood cell (RBC) and PLT counts neither in group with vascular complication and who underwent splenectomy, nor in those without vascular complications and did not undergo splenectomy or those who underwent splenectomy but has no vascular complications (p=0.05). Size of the AEC compartment, intensity of CD106 expression were positively correlated to each of TLC, PLT count (p=0.001 for all). Risk of vascular complications was identified if percent of ACEs was ≥58.3% of CECs, absolute count of AECs was ≥0.059 x 10^3 /µl, MFI of CD106 was ≥7.9, with an effectiveness of 95%, 91.3%, 95% respectively.

Conclusion: Measurement of AECs presents an effective quantitative method for assessment of risk of vascular complications in thalassemia.

Keywords: Circulating endothelial cells; Thalassemia; Vascular damage

Introduction

Thalassemia is a hereditary hemolytic anemia caused by mutation in globin gene complex. Circulatory disturbances, including arterial and venous thrombosis, have been reported in these patients [1]. Activation of vascular endothelium is considered as an important factor of thrombosis and vasculitis [2]. As with many other cell types, endothelial cells (ECs) may be resting or activated. Functionally, Activated endothelial cells (AECs) are characterized by the production of nitric oxide, prostacyclin and toxic oxygen radicals and by procoagulant activity and increased leukocyte adhesion and phagocytosis [3]. AECs express a number of immunologically relevant surface markers which are not detected in dormant condition. These surface markers on ECs may be involved in adhesion reaction and migration of blood cell components [2].

Evidence of activation of vascular endothelium in the thalassemic patients in terms of over expression of adhesion molecules has been reported earlier [4]. The reasons for this phenomenon is not well investigated, but iron overload, transfusions, infectious agents and factors derived from increased red blood cell (RBC) destruction may play a significant role [5].

Quantification of circulating endothelial cells (CECs) in peripheral blood (PB) is developing as a reproducible method of assessing endothelial damage/dysfunction. The CECs are thought to be mature cells that have detached from the intimal monolayer in response to endothelial injury [6]. Ultimate clinical utility of CEC detection rely on accurate detection of CEC subsets (activated versus resting or live versus dead) rather than a gross quantification of all CECs [7].

Increased number of CECs was demonstrated in α and β-thalassemic patients. Appearance of CECs with markers for endothelial cell activation may indicate a propensity towards vascular perturbation in thalassemic subjects [8].

We aimed to quantify the compartments of endothelial cells, CECs and AEC, in relation to incidence of vascular complications in Egyptian β-thalassemic patients in order to evaluate its subsequent utility in assessment of thrombotic risk.

Subjects and Methods

Subjects

Eighty β-thalassemia patients were enrolled during their routine follow up with the Hematology clinic at the Pediatric and Internal Medicine Hospitals of Ain-Shams University. All patients have undergone the routine diagnostic work–up for hemolytic anemia diagnosis. Sixty of them were diagnosed as having β-thalassemia major and 20 as β-thalassemia intermedia. They were 40 (50%) males and 40 (50%) females, with a male to female ratio of 1:1 and age ranging from 2-18 years, with a mean of 12.44 ± 4.98 years. Patients were followed up for 36 months period.

Thirty healthy volunteers were enrolled as a control group. They were 17 (56.7%) male and 13 (43.3%) female with a male to female ratio of 1.2:1. Their ages ranged from 6-18 years, with a mean of 12.83 ± 3.61 years. The procedures applied in this study were approved by the Ethical Committee of Human Experimentation of Ain Shams University and are in accordance with the Helsinki Declaration of 1975. All participants and their caregivers were informed about the objectives and procedures of the study and written consents were obtained prior to enrollment.

Therapeutic regimen

Patients with ß-thalassemia major received blood transfusions at a rate of two units of packed RBCs/15 days, and they were on regular chelation therapy with subcutaneous infusions of Desferrioxamine (Desferral), its initial dose is 20 mg/kg body weight 5 nights per week with 100 mg of oral vitamin C on the day of transfusion. In patients who were heavily iron loaded, continuous intravenous infusion of Desferral was given at a dose of 50 mg/kg body weight [9].

Exclusion criteria

-Thalassemic patients other than β-thalassemia.

- Thalassemic patients also having abnormal hemoglobins (Hb).

-Concurrent infections or inflammations or chronic illness or thrombotic episode at time of sampling.

-Pregnancy or lactation.

-Blood transfusion within the last 48 hours of sampling.

-Other hereditary diseases.

Methods

Sampling

A volume of 2 ml of venous blood was obtained on potassium ethylene diamine tetra-acetic acid (K2-EDTA) for complete blood count (CBC), preparation of Leishman stained PB smears, reticulocyte count and Hb electrophoresis. Another 1ml of PB on sterile K2-EDTA was collected for flow cytometric studies.

Two ml of venous blood were collected into a sterile dry vacutainer for measuring serum iron, total iron binding capacity (TIBC) and serum ferritin.

Immunophenotype characterization of CEC and AEC by flow cytometry (FCM)

The immunophenotyping staining procedure used was the standard 'whole blood lysis' technique, in which 50 ul of whole blood sample (with leukocyte count adjusted to 5–10 x 10^3 cells/ml) were simultaneously stained with 10 ul of fluorescein isothiocyanate conjugated (FITC)-labeled (anti CD146) and phycoerthrin (PE)-conjugated (anti CD106) anti-human monoclonal antibody (MoAb) (R&D Minneapolis, MN), or with their corresponding isotype-matched controls (FITC mouse IgG_1, PE-mouse IgG_{2A}). After 30 minutes of incubation in the dark, at room temperature, samples were washed twice in 4 ml phosphate buffered saline (PBS), then 2 ml of laboratory-prepared 0.83% ammonium chloride-based lysing solution were added and RBC lysis was allowed for 5 minutes at room temperature. Samples were washed once and re-suspended in 0.5ml of PBS. Staining with isotype controls was performed to distinguish positive staining from auto-fluorescence and nonspecific antibody binding. Cells were analyzed on a Coulter EPICS XL flow cytometer using System II software (Beckman Coulter). The flow cytometer acquired a minimum of 10000 cellular events.

Interpretation of results

A protocol for dual color analysis was constructed. CECs ($CD146^+$ cells) were gated then the percent of AECs ($CD\ 106^+$ cells) within this compartment were quantified along with measuring the mean intensity of CD106 surface expression. The absolute count of each of CECs and AECs was calculated according to the total leucocyte count (TLC).

Statistical analysis

Data were analyzed using IBM SPSS (Statistical Package for Social Sciences) (V. 21.0, IBM Corp., USA, 2012). Graphic presentation of data was done by using EXCEL 2007 software. Qualitative (categorical) data were described in the form of number and percentage, quantitative (numerical parametric) data were described in the form of mean, standard deviation (x ± SD) and quantitative (numerical non parametric) data were described in the form of 25th and 75th interquartile range and median. Student t test (t) was used to compare between two independent mean groups for parametric data. Wilcoxon Rank Sum test (Z) was used to compare between two independent mean groups for non-parametric data.

Ranked spearman correlation test (r) was used to study the possible association between each two variables among each group for non-parametric data. The probability of error (p) at 0.05 was considered significant, while at 0.01 and 0.001 are highly significant and >0.05 is insignificant. Receiver operating characteristics curve (ROC) was done to establish clinically relevant cutoff point of CD106 which allows the most significant separation and differentiation between thalassemic cases with and without vascular complications.

Results

Demographic and clinical data of patients' group are shown in Table 1.

CECs and AECs were higher in patients' group compared to control group (p=0.001) (Figure 1), while no difference was found between thalassemia major and intermedia regarding proportion of CECs and AECs compartment (Table 2). Proportion of AECs, serum ferritin level, TLC and platelet (PLT) count were higher in patients suffering from vascular complications than those without and in splenectomized compared to non-splenectomized patients (p=0.001) (Tables 3 and 4). A positive correlation was found between percent and absolute count of AECs as well as MFI of CD106 expression and each of TLC, PLT count and serum ferritin level (p=0.001). Serum iron was positively correlated with only the percent of AECs (p=0.001) (Table 5). No correlation was found between AECs (percent/absolute count) or MFI of CD106 and serum ferritin neither in group with vascular complications (r=0.15, p=0.26, r=0.001, p=0.99, r=0.02, p=0.85 respectively) nor in splenectomized group (r=0.15, p=0.27, r=0.02, p=0.85, r=-0.008, p=0.95 respectively) (Figure 2). Also, no correlation was found between RBC and PLT counts neither in group with vascular complication and who underwent splenectomy (n:51) (r=0.158, p=0.19), nor in those without vascular complications and did not undergo splenectomy (n:25) (r=0.333, p=0.10), or those who underwent splenectomy but has no vascular complications (n:4) (r=0.062, p=0.93) (Figure 3).

Using the ROC curve, a cutoff value of 58.3% for percent of AECs, 7.92 for MFI of CD106 with a sensitivity of 100%, specificity of 86.2% and efficacy of 95% and cutoff value of 0.059 x 10^3 /µl for absolute count of ACEs with a sensitivity of 98%, specificity of 79.3% and efficacy of 91.3% were calculated to identify thalassemic patients with risk for vascular complications (Table 6). Median of ACEs % out of total white blood cells (WBCs) was 0.11 in control group and 2.0 in patients' group.

Thalassemic patients (n=80)		
Parameter	**n**	**%**
Sex		
Male	40/80	50%
Female	40/80	50%
Thalassemia		
Major	60/80	75%
Intermediate	20/80	25%
Splenectomy		
Present	55/80	68.80%
Absent	25/80	31.30%
Vascular complication		
Present	51/80	63.80%
	Thalassemia major :38/80	47.50%
	Thalassemia intermedia:13/80	16.25%
Absent	29/80	36.30%
n: number		

Table 1: Demographic and clinical data of studied patients.

Parameter	**Thalassemia major n=60**	**Thalassemia intermedia n=20**	**t**	**p**
Percent of CECs Mean ± SD	2.454 ± 0.801	2.468 ± 0.864	-0.039	0.96
Absolute count of CECs Mean ± SD	0.0751 ± 0.015	0.0748 ± 0.016	0.06	0.95
Percent of AECs Mean ± SD	72.448 ±18.795	69.235 ±19.464	0.645	0.52
Absolute count of AECs Mean ± SD	0.069 ± 0.036	0.106 ± 0.182	-0.267	0.79
MFI of CD106 Mean ± SD	8.602 ± 1.691	8.419 ± 1.968	-0.222	0.82

Table 2: Comparison between thalassemia major and intermedia groups regarding FCM analysis of ECs.

Parameter	**Vascular complications n=51**	**No vascular complications n=29**	**t/z***	**p**
Hb (g/dl) Mean ± SD	7.061 ± 1.025	6.824 ± 0.988	-1.015	0.31
TLC (x10³/µL) Mean ± SD	7.573 ± 1.281	5.297 ± 1.449	-7.037	0.001
PLT (x10³/µL) Interquartile range Median	580-798 670	363.5-434 392	-6.906*	0.001
Serum Iron (µg/dl) Mean ± SD	200.06 ± 27.996	202.14 ± 26.542	0.33	0.74
TIBC (µg/dl) Mean ± SD	162.45 ± 27.531	170.14 ± 29.855	1.138	0.26
Serum ferritin (ng/mL) Interquartile range Median	1250-2400 1700	755-1150 900	-5.614*	0.001
Percent of AECs Mean ± SD	82.786 ± 7.309	52.052 ± 16.968	-9.277	0.001
Absolute count of AECs Mean ± SD	0.089 ± 0.113	0.059 ±0.051	-5.631	0.001
MFI of CD106 Mean ± SD	9.453 ± 0.996	6.979 ± 1.692	-5.765	0.001
TIBC: total iron binding capacity, Hb: hemoglobin				

Table 3: Comparison between patients with & without vascular complications regarding laboratory data and FCM analysis of AECs

Parameter	**Non-splenectomized n=25**	**Splenectomized n=55**	**t/z***	**p**
Hb (g/dl) Mean ± SD	6.932 ± 0.852	6.995 ± 1.084	-0.278	0.78
TLC (x10³/µL) Mean ± SD	4.764 ± 0.465	7.649 ± 1.279	-14.715	0.001

PLT(x10³/µL) Interquartile range Median	360-405 383	563-750 666	-7.137*	0.001
Serum Iron (µg/dl) Mean ± SD	198.68 ± 25.98	201.78 ± 28.097	-0.482	0.63
TIBC (µg/dl) Mean ± SD	168.72 ± 31.49	163.65 ± 27.116	0.696	0.49
Serum ferritin (ng/mL) Interquartile range Median	745-1050 900	1250-2400 1700	-6.124*	0.001
Percent of ACEs Mean ± SD	46.42 ± 9.722	83.111 ± 7.187	-16.888	0.001
Absolute count of ACEs Mean ± SD	0.057 ± 0.055	0.088 ± 0.109	-6.375	0.001
MFI of CD106 Mean ± SD	6.402 ± 0.865	9.535 ± 1.021	-7.137	0.001

Table 4: Comparison between splenectomized and non-splenectomized patients regarding laboratory data and FCM analysis of AECs.

	% of ACEs		Absolute count of ACEs		MFI of CD106	
	r	p	r	p	r	p
Hb (g/dl)	-0.02	0.81	0.2	0.07	-0.02	0.8
TLC (x10³/µL)	0.62	0.001	0.63	0.001	0.72	0.001
PLT (x10³/µL)	0.64	0.001	0.6	0.001	0.55	0.001
Serum Iron (µg/dl)	0.23	0.03	-0.03	0.75	0.06	0.55
TIBC (µg/dl)	-0.012	0.918	-0.154	0.172	0.003	0.97
Serum ferritin (ng/mL)	0.59	0.001	0.48	0.001	0.55	0.001

Table 5: Correlation between percent, absolute count of AECs and MFI of CD106 and different parameters.

Parameter	Cutoff	TP	FN	FP	TN	SP	SN	P-	P+	Eff.
Percent of AECs	58.8	51	0	4	25	86.2	100	100	92.7	95
Absolute count of AECs	0.059	50	1	6	23	79.3	98	95.8	89.3	91.3
MFI of CD106	7.92	51	0	4	25	86.2	100	100	92.7	95

TP: true positive, FN: false negative, FP: false positive, TN: true negative, SP: specificity, SN: sensitivity, P-: negative prediction, P+: positive prediction, Eff.: efficacy

Table 6: ROC curve analysis showing the performance characteristics for FCM assessment of AECs in thalassemic patients.

A color tracking was performed for the population of ACEs ($CD146^+$, 106^+ cells) to locate its scatter among PB WBCs and a diffuse scatter of cells was seen among all cases and was located with higher side and forward scatter than the granulocytic series with a small fraction of cells lodged within the granulocytic population (Figure 4).

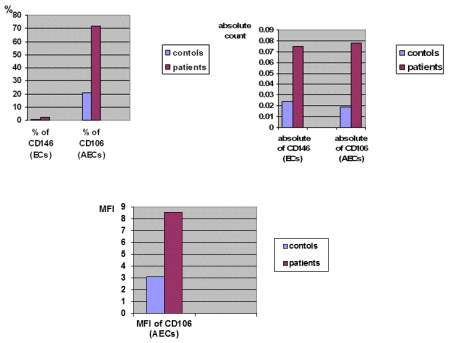

Figure 1: Comparison between patients and controls regarding percent, absolute count of CECs, AECs and MFI of CD106.

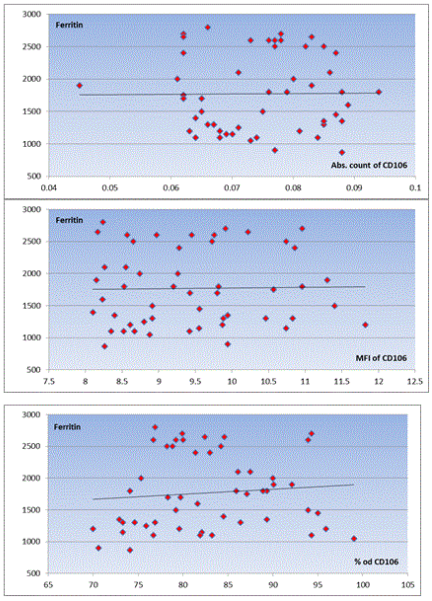

Figure 2: Correlation between serum ferritin and absolute count, MFI and % of CD106 in group with vascular complications and in splenectomized group.

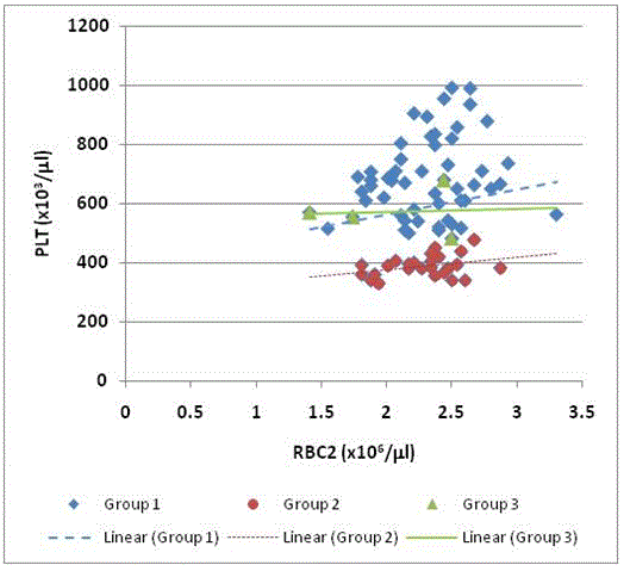

Figure 3: Correlation between RBC and PLT count in different groups. Group 1: patients with vascular complications and underwent splenectomy; Group 2: patients with no vascular complications nor underwent splenectomy; Group 3: patients with no vascular complications and underwent splenectomy

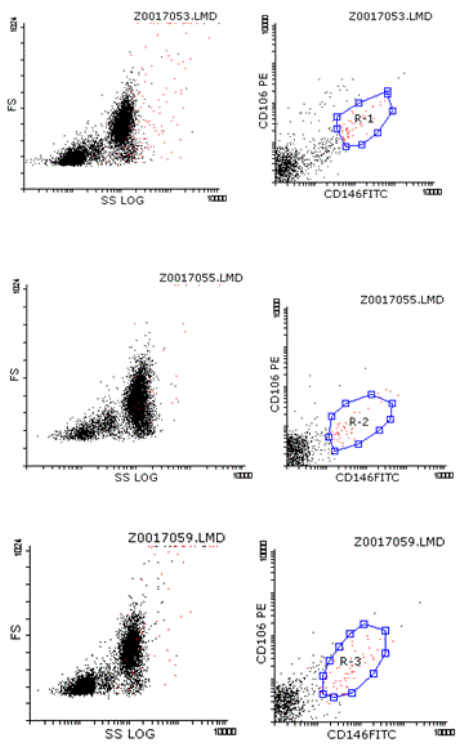

Figure 4: Color tracking for AECs in three different cases.

Discussion

Arterial and venous thromboembolic episodes in ß-thalassemia major patients have been reported. Endothelial cell activation and impaired flow-mediated dilation in the brachial arteries of ß-thalassemic patients, as shown in previous in vivo studies, implicate endothelial dysfunction in the pathogenesis of vascular complications. Endothelial dysfunction generally leads to vascular remodeling and potential changes in mechanical properties [10].

CECs elevation in the blood of patients has become a useful marker for severe vascular dysfunction. The presence of CECs in significant numbers denotes a high degree of vascular damage [11]. The expression of endothelial adhesion molecules on the surface of microvascular activated CECs has long been reported to play significant roles in the recruitment of WBCs and RBCs [12], and to promote thrombosis at sites of vascular inflammation [13].

In this study, we aimed to quantify CECs particularly their activated fraction in the PB of β- thalassemic patients. We found a significantly higher fraction of CECs ($CD146^+$ cells) particularly AECs ($CD146^+$ cells/($CD106^+$ cells) in thalassemic patients compared to controls (Figure 1). Butthep and colleagues, in 2002 [14], reported similar results.

The presence of these cellular markers of endothelial adhesion in blood of thalassemic patients have previously been attributed to the cascade of events that starts with the oxidation of globin subunits in thalassaemic erythroid cells and leads to the formation of haemichromes [15], which precipitate, instigating haem disintegration and the eventual release of toxic non-transferrin-bound iron species [16].

The free iron in turn catalyzes the formation of reactive oxygen species, leading to oxidation of membrane proteins and formation of red-cell 'senescence' antigens such as phosphatidylserine [17], which cause the thalassaemic RBCs to become rigid, deformed and to aggregate, resulting in premature cell removal [18]. Thalassaemic RBCs with negatively charged phospholipids increase thrombin generation [19,20].

The membrane changes may partly explain the enhanced aggregation of PS-exposing RBCs, their increased adherence to ECs, and their capacity to enhance thrombin generation via the assembly of the prothrombinase complex. The enhanced thrombin generation leads to activation of PLTs, monocytes, granulocytes, and ECs and expression of tissue factor, which further enhances the thrombotic process. The low levels of the coagulation inhibitors, protein C and protein S, further facilitate the resultant hypercoagulable state [21,22].

Further study into our patients' population revealed no significant differences between β-thalassemic patients of varying clinical severity (major and intermedia) with respect to either percent or absolute values for CECs or AECs (Table 2). Vascular complications were encountered in 47.5% of thalassemia major patients and 16.25% of thalassemia intermedia patients (Table 1). On the other hand, AECs were significantly higher among thalassemic patients with vascular complications compared to patients without vascular complications (Table 3).

Regarding molecules regulating leukocyte endothelial adhesion, previous studies found that half of the CECs isolated from patients with thalassaemia expressed intercellular adhesion molecule-1 (ICAM-1), E-selectin and vascular cell adhesion molecule-1 (VCAM-1) [CD106+ cells] [14]. In addition, they detected elevated

levels of endothelial adhesion proteins [ICAM-1, E-selectin, VCAM-1 ($CD106^+$cells), von Willebrand factor, and thrombomodulin] in serum and plasma of thalassemic patients. They suggested that endothelial activation or injury may be a feature of this genetic disease that plays an important role in the recruitment of WBCs and RBCS, promotes thrombosis at vascular inflammation sites and may be involved in vascular occlusion [23].

Moreover, it was found that RBCs from patients with ß- thalassemia major and intermedia have enhanced adhesion to cultured ECs (10- to 25-fold increase) compared to normal RBCs [24]. Other investigators, described the presence of deep venous thrombosis (DVT), pulmonary embolism and recurrent arterial occlusion, with thrombi in small and large pulmonary vessels in patients with thalassemia major and with thalassemia intermedia as well [25-27].

In our study, AECs were significantly higher among splenectomized thalassemic patients (55/80) compared to non splenectomized patients (25/80) (Table 4). Fifty one out of our fifty five splenectomized patients had vascular complications. Previously, venous thrombosis was found to be more prevalent in β-thalassemia intermedia patients who were not receiving regular transfusions and who had undergone splenectomy. They proposed that these patients may have been more susceptible to thromboembolism because they had more circulating damaged RBCs and increased PLT counts. Thromboembolic manifestations are more frequently recorded in less developed countries with limited transfusion resources. Ex vivo and in vitro experiments show that normal RBCs can eliminate the abnormal aggregation observed with thalassemic RBCs [28]. Patients with thalassemia and previous splenectomy appear to have an increased incidence of venous thromboembolism beyond the portal venous system [29].

Evidence in thalassemia supports the presence of a hypercoagulable state greatly exacerbated by splenectomy, which is the result of platelet activation [30], enhanced red blood cell adherence to the endothelium [31], reduced levels of the natural anticoagulants protein C and protein S, and increased thrombin generation [32].

In this work, there was a higher TLC and PLT count in both patients with vascular complications and splenectomized thalassemic patients compared to those without vascular complications and the non-splenectomized ones (Tables 3 and 4). Similarly, it was stated before that splenectomy increases the hypercoagulability, by permitting the circulation of greater numbers of altered membranes, and by increasing the number of PLTs. Thrombocytosis, in fact, develops in 75% of the splenectomized patients, and in 15% it reaches 1.000.000/mm^3 or more, reaching a maximum between one week and 4 months after splenectomy [33]. Recently, a significant increase in TLC [34], and platelet count was observed among splenectomized patients [35].

We found that the mean serum ferritin level was higher in patients with vascular complications and splenectomized patients than those free of vascular manifestations and non splenectomized patients (Tables 3 and 4). Other investigators assumed that presence of higher serum ferritin in splenectomized patients compared with non splenectomized patients may have been related partially to decreased duration of iron chelation therapy in their study. They also considered an aggravating effect of splenectomy on hemosiderosis as a possibility [36]. Also, Aessopos et al. [37], found that complications (including cardiac complications, bone deformities, diabetes, and gall stone) were higher in their splenectomized group when compared to non splenectomized group and attributed this to the higher level of serum ferritin in the splenectomized patients group.

In our study, there was a positive correlation between AECs and TLC, PLT count, serum iron and serum ferritin in thalassemic patients (Table 5), while no correlation was found between AECs (percent/absolute count) or MFI of CD106 and serum ferritin neither in group with vascular complications nor in splenectomized group) (Figure 2). A previous study proved before that a positive correlation was found between serum VCAM-1 ($CD106^+$ cells) and serum ferritin in thalassemic patients. In their study serum level of VCAM-1 was determined by enzyme-linked immunosorbent assay for studied thalassemic groups. They found significantly higher levels of these molecules in the serum of the thalassemic patients (both transfusion dependent and transfusion non-dependent) than in the controls [38].

In this work, no correlation was found between RBC and PLT counts neither in group with vascular complication and who underwent splenectomy, nor in those without vascular complications and did not undergo splenectomy, or those who underwent splenectomy but has no vascular complications (Figure 3). While, it was previously stated that in thalassemic patients, a highly significant correlation (P<.001) was found between the number of RBC-bound annexin V molecules and the fraction of CD62P (P selectin) or $CD63^+$ platelets. This association between annexin V binding and the expression of platelet activation markers was also found in individual thalassemic patients over time and was not dependent on whether the patients had undergone splenectomy. These results support the idea that the procoagulant surface of thalassemic RBCs promotes thrombin generation in vivo leading to platelet activation [39].

Levels of CECs in the blood stream have been shown to significantly correlate with several well-established markers of endothelial function and are technically simple to measure [40]. Accordingly, on studying the performance of these markers among our thalassemic patients a cutoff value was established for AECs at a percent of 58.8% of CECs, absolute count of 0.059x10^3/µl and mean intensity of 7.92 with efficacy of 95%, 91% and 95% respectively for discriminating patients with vascular complications from those without (Table 6). To the best of our knowledge, no previous studies established a clinically relevant cutoff value for AECs in thalassemic patients with respect to predicting the risk of thrombotic episodes.

Conclusion

The measurement of immunologically defined AECs in venous blood represents an important accessible tool for assessment of endothelial injury and risk of vascular complications in thalassemic patients. AECs are proven to be biomarkers of damage, and high levels predict a poor outcome. Our findings incriminate alterations in the compartment of ECs particularly AECs in the pathophysiology of vascular complications with clinically relevant cutoffs. These findings include involvement of mechanisms such as the state of iron excess (due to many causes) exacerbated by splenectomy with subsequent PLTs activation.

An individualized approach incorporating the established cutoff for CECs and AECs is recommended to establish an optimal strategy for preventing the occurrence of vascular complications in thalassemics, particulary splenectomized patients. It is vital to reach a general consensus regarding the most appropriate technique in order to validate the reporting of CECs in large cohorts of patients. This validation has critical implications on future clinical trials in which

CECs might function as a novel biomarker and perhaps as a surrogate endpoint.

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Conflict of Interest

The authors declare no conflict of interest

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