

# Circulating microparticles and the risk of thromboembolic events in Egyptian beta thalassemia patients

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**Abstract** The presence of elevated numbers of circulating microparticles (MPs) has been hypothesized to be responsible for the occurrence of thromboembolic events (TEEs) in thalassemic patients. Our aim is to evaluate the presence and the thrombotic risk of circulating MPs in thalassemia patients and to determine the difference in MPs between  $\beta$ -thalassemia major ( $\beta$ -TM) and thalassemia intermedia (TI). The percentage of the annexin-labeled MPs, platelet-derived MPs (PMPs), erythrocyte-derived MPs (RMPs), and endothelial-derived MPs (EMPs) was measured by flow cytometry, in 87 thalassemia patients (39  $\beta$ -TM and 48 TI). By multiple regression analysis, we then assessed the various independent risk factors for the occurrence of TEE. The thalassemic patients who experienced TEE had a significantly higher platelet count, higher percentage of annexin-labeled MPs, and higher percentage of PMPs (*p* value = 0.014, 0.003, and 0.014, respectively). There was no significant difference between  $\beta$ -TM and TI patients at the level of any of the studied MPs. The predictive risk factors for TEE in thalassemic patients were splenectomy, total and direct bilirubin, the RMPs, and the EMPs (OR = 10.07 (CI = 3.7–27.1), 4.3 (CI = 2.1–8.7), 1.4

(CI = 1.5–6.2), 1.6 (CI = 1.1–2.2), 3.0 (CI = 1.9–4.9), respectively). In conclusion, the elevated numbers of circulating MPs is a risk factor for the TEE in thalassemia patients.

**Keywords** Thalassemia major · Thalassemia intermedia · Microparticles · Hypercoagulable state

## Introduction

Thalassemia is a group of inherited hemoglobinopathies that may lead to many complications such as a hypercoagulable state with a high incidence of TEE. This state has been attributed to a wide variety of hemostatic alterations. The elevated numbers of MPs have been hypothesized to be responsible for the increase in thrombotic risk [1].

Microparticles are submicrometric fragments (0.1 to 2  $\mu$ m) that shed from the remodeling of the plasma membrane in response to cell activation and apoptosis. They express high levels of phosphatidylserine (PS) on their outer leaflet together with other surface markers from their cell of origin [2]. These MPs originate from circulating blood cells, platelets, and endothelial cells (ECs). PMPs constitute 70 to 90% of the total number of MPs found in the blood circulation [3]. Elevated levels of MPs have been reported in many vascular diseases, in thalassemia, sepsis, diabetes, and pre-eclampsia. It has also been postulated that they are associated with an increased risk for both arterial and venous thrombosis [4, 5].

In thalassemia, disruption of the membrane phospholipids in red blood cells (RBCs) facilitates membrane blebbing and the release of MPs with a diameter of  $<1$   $\mu$ m [6]. In addition, evidence suggests that thalassemic patients who undergo splenectomy have significantly increased amounts of MPs originating from activated platelets, making it a possible

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contributing factor for the prothrombotic manifestations in these patients [7].

Thus, we evaluated the presence and the thrombotic risk of circulating MPs for the TEE in thalassemia patients and determined the difference in MPs between  $\beta$ -TM and TI.

## Patients and methods

In this cross-sectional study, 87 thalassemia patients (39 patients with  $\beta$ -TM and 48 patients with TI) were randomly enrolled while attending their regular follow-up visits in the Hematology Clinic of the New Cairo University Children Hospital. Patients were enrolled regardless of age and gender, and they were not transfused for at least 1 month before blood sample. None of the patients were diabetic, and none had evidence of concurrent infection, cardiac, inflammatory, and pulmonary diseases. Glucose-6-phosphate dehydrogenase (G6PD)-deficient patients were excluded because G6PD-deficient cells are extremely sensitive to oxidative damage which may lead to the formation of MPs [8]. The diagnosis of thalassemia was established through the assessment of complete blood count (CBC), Hb-electrophoresis, high performance liquid chromatography (HPLC), and clinical status. By HPLC, the HbF% ranged from 10 to 93.6% among the TM patients and from 6.4 to 88.8% among the TI patients. Confirmation of diagnosis was further established by studying the  $\beta$ -globin gene mutations. This revealed that the most common mutations among the studied 39 TM patients were IVS I-110(G>A) in 19 cases (49%), IVS I-6(T>C) in 15 cases (38%), and IVS I-1(G>A) in 5 cases (13%). On the other hand, the most common mutations among the studied 48 TI patients were IVS I-6 (T>C) in 22 cases (46%), IVS I-110(G>A) in 19 cases (40%), and IVS I-1(G>A) in 7 cases (15%).

Patients and/or their guardians gave their informed consent, and the Institutional Review Board (IRB) approved the study.

The studied patients were 42 (48.3%) females and 45 (51.7%) males with a mean age of  $11.93 \pm 7.81$  years (range 1.5–38). A full history of each of the patient was taken, and it included the age, sex, medication history, and age of onset of blood transfusion with its frequency and volume. A complete physical examination was performed and included cardiac, chest, neurological assessment, and assessment of the splenic status. Laboratory tests were done including CBC, serum ferritin level, coagulation profile, and hemolytic profile. A flow cytometer (Beckman Coulter Cytomics FC 500, BD Bioscience, France) and dual expression of annexin and CD 41 (platelet-derived MPs (PMPs), annexin and CD235a (RMPs), and annexin and CD146 (EMPs) were all used to determine the percentage of MPs present.

## Microparticle isolation and characterization by flow cytometry

Samples were prepared following procedures previously reported in the literature [9]. Samples were collected in an atraumatic fashion using larger needles in order to avoid sheer stress and endothelial activation. Two milliliters of venous blood from each patient was withdrawn on 3.2% sodium citrate in vacutainer tubes. Samples were then processed within 15 min of collection. Platelet-poor plasma (PPP) was prepared immediately after venipuncture by centrifuging whole blood at  $5000 \times g$  for 5 min. The PPP was separated in another tube while discarding the last amount of plasma above the cell pellet. A microparticle pellet was obtained from the PPP by a second ultracentrifugation step at  $17,000 \times g$  for 2 min at  $4^\circ\text{C}$ . Subsequently, the supernatant was discarded and the microparticle pellet was vortexed for assay of MPs by flow cytometry.

The sample was stained following the previously reported procedures by Elsayh et al. [10]. Five microliters of the sample were diluted in 35  $\mu\text{L}$  of diluted  $1 \times$  annexin binding buffer. The samples were then incubated for 20 min at  $4^\circ\text{C}$  in the dark with 1  $\mu\text{L}$  of fluoresoithiocyanate-conjugated annexin V (purchased from Beckman Coulter, Cat No. IM03546) and 10  $\mu\text{L}$  of PE CD41 anti-human monoclonal antibody (purchased from Beckman Coulter, Cat No. A07781) or CD235a (purchased from Beckman Coulter, Cat No. A89314) or CD146 (purchased from Beckman Coulter, Cat No. A22364). After incubation, sample was diluted in 500  $\mu\text{L}$  of filtered diluted  $1 \times$  annexin binding buffer (purchased from Beckman Coulter, Cat No. IM03546). Anti-human immunoglobulin G (IgG) was used as an isotype-matched negative control for each sample. One-micrometer latex beads were used to calibrate the size range of MPs (latex beads, amine-modified polystyrene, fluorescent red aqueous suspension, 1.0- $\mu\text{m}$  mean particle size, Sigma-Aldrich Chemie GmbH, Munich, Germany)

Annexin V positive MPs was reported as the percentage of total MPs sized 1  $\mu\text{m}$  and annexin V labeled. We defined MPs as particles measuring approximately 1.0  $\mu\text{m}$  in diameter and had dual positive staining for annexin V and CD41 (PMPs), annexin and CD235a (RMPs), and annexin and CD146 (EMPs)

## Statistical methods

Data were collected and analyzed using Statistical Package for Social Science (IBM SPSS) version 20, and the following were done: qualitative data were presented as number and percentages while quantitative data were presented as mean, standard deviations, and ranges. Comparison between the two groups with qualitative data was done by using *chi-squared*

test and/or Fisher exact test. Comparison between the two independent groups with quantitative data and parametric distribution was done by using independent *t* test. On the other hand, the comparison between the two independent groups with quantitative data and nonparametric distribution was done by using Mann-Whitney test. The spearman correlation coefficient was used to assess the significant relationship between the two parameters with quantitative data in the same group. A logistic regression analysis was used to assess the predictors for the TEE. The confidence interval was set to 95%, and the margin of error accepted was set to 5%.  $P < 0.05$  was considered statistically significant.

## Results

TEE was encountered in 41 patients (47.1%), including avascular necrosis (AVN) of the head femur in 17 patients, superficial thrombophlebitis in 13 patients, deep vein thrombosis (DVT) in 8 patients, portal vein thrombosis in 2 patients, and right basal ganglia infarction in one patient. In this study, splenectomy was found at a higher incidence among patients with TEE than patients without TEE (78.0 vs. 22.0%, respectively,  $p$  value = 0.000). Moreover, patients with TEE had older age than those without TEE with mean age  $15.8 \pm 6.74$  vs.  $08.45 \pm 7.07$  years, respectively,  $p$  value = 0.000. Thalassemic patients who experienced TEE had a significantly higher platelet count, higher percentage of annexin-labeled MPs, and a higher percentage of PMPs ( $p$  value = 0.014, 0.003, and 0.014, respectively). They also had higher red cell-derived MPs but did not reach significant values (Table 1 and Fig. 1).

By multivariate analysis, the predictive risk factors for TEE in thalassemic patients were splenectomy with 10 times increase in the risk of TEE, total bilirubin with 4 times increase in the risk of TEE, the endothelial derived MPs with nearly 3 times increase in the risk of TEE, the red cell-derived MPs with 1.5 times increase in the risk of TEE, the direct bilirubin with 1.3 times increase the risk of TEE, and the older the age with nearly 1.2 times increase in the risk of TEE (Table 2).

TEE was significantly higher in the studied TI patients than in the  $\beta$ -TM patients (58.3 vs. 33.3%, respectively,  $p$  value 0.02). Among TI patients, 41.67% of them were on hydroxyurea therapy with mean age at the start of therapy of  $8.67 \pm 3.82$  years, at a mean dose of  $18.67 \pm 4.6$  mg/kg/day. There was no significant difference in the volume of blood transfusion among TI patient with and without TEE ( $88.24 \pm 68$  vs.  $115.71 \pm 69.98$  cm<sup>3</sup>/kg/year,  $p$  value = 0.23). Moreover, 43.8% of TI patients and 59.0% of  $\beta$ -TM patients were on salicylate therapy,  $p$  value = 0.158. However, there was no significant difference between TI and  $\beta$ -TM patients at the level of any of the studied MPs and platelet count (Table 3). Also, the number of splenectomy cases showed no significant difference between TI and  $\beta$ -TM patients (43.8 vs. 59.0%,  $p$  value = 0.16).

## Discussion

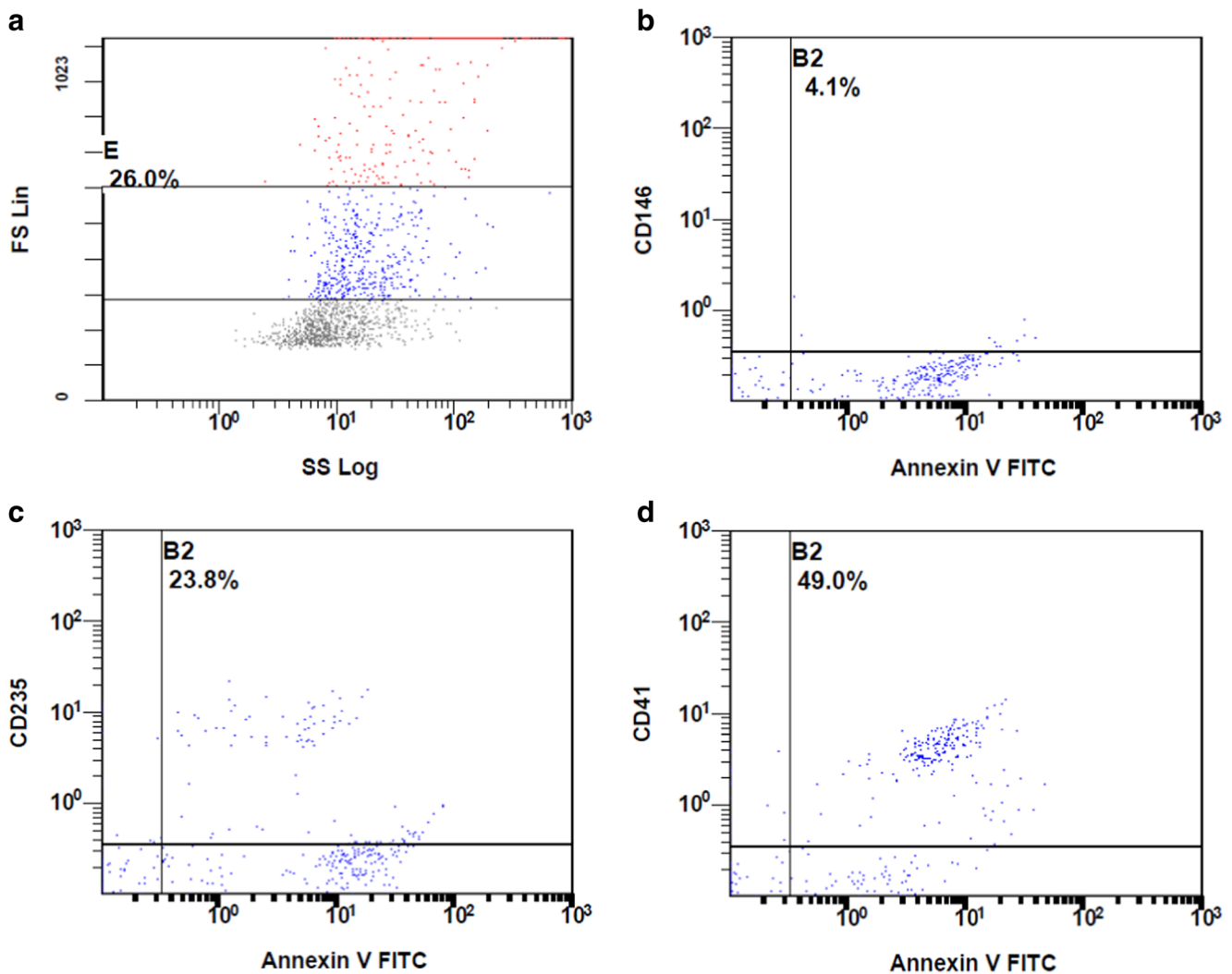
Several studies that have looked at coagulation proteins have provided strong evidence for the existence of a chronic hypercoagulable state in thalassemia [11]. The pathophysiology of hypercoagulability is a multifactorial process.

**Table 1** The laboratory variable of the studied thalassemia patients with and without thromboembolic events (TEEs)

Variables	No TEE ( $n = 46$ )		TEE ( $n = 41$ )		$p$ value
	Mean	SD	Mean	SD	
Plt. ( $10^3/\text{mm}^3$ )	492.15	288.23	692.41	445.27	0.014*
LDH level (U/L)	406.35	188.93	540.24	261.06	0.007
Bil. T (mg/dl)	1.39	0.65	2.37	1.07	0.000*
Bil. D (mg/dl)	0.29	0.15	0.51	0.26	0.000*
Serum ferritin (ng/ml)	1212.25	931.83	1657.25	1072.91	0.041*
PT (s)	13.67	1.43	14.40	1.68	0.033*
Annexin-labeled MPs (%)	14.80	4.30–58	20.80	7.10–65.70	0.003*
PMPs (%)	6.63	1–10.24	15.29	7.31–49	0.014*
RMPs (%)	6.95	1.1–33.3	8.30	1.7–30.9	0.174
EMP (%)	0.55	0–12.20	0.60	0–9.9	0.624

Plt platelets, LDH lactate dehydrogenase, Bil T total bilirubin, Bil D direct bilirubin, PT prothrombine time, MPs microparticles, PMPs platelet-derived microparticles (dual +ve annexin, CD41), RMPs red cell-derived microparticles (dual +ve annexin, CD235a), EMPs endothelial derived microparticles (dual +ve annexin, CD146), g/dl gram/deciliter, % percentage,  $\text{mm}^3$  cubic millimeter, U/L units per liter, ng/ml nanograms per milliliter, s seconds, TEE thromboemboli event

\*Statistically significant ( $p$  value < 0.05)



**Fig. 1** Flow cytometric analysis of MPs. **a** The forward scatter cutoff was set using 1.0- $\mu$ m standard beads to define the upper limit of the MPs population. **b** Endothelial-derived MPs were identified by gating on CD146 and annexin dual positive events (B2 = 4.1%). **c** Erythrocyte-

derived MPs were identified by gating on CD235 and annexin dual positive events (B2 = 23.8%). **d** Platelet-derived MPs were identified by gating on CD41 and annexin dual positive events (B2 = 49%). FITC fluorescein isothiocyanate, CD cluster of differentiation

One study suggested that thalassemic RBCs may provide a source of anionic phospholipids like phosphatidylethanolamine (PE) and PS, which can increase thrombin generation [11]. The procoagulant effect of these thalassemic RBCs is due to the increased surface expression of PE and PS and itself contributes to the hypercoagulable state in thalassemia through the amplification of thrombin generation and initiation of platelet activation. So, as a result of lipid membrane peroxidation in both RBCs and platelets, there is a loss of the normal asymmetrical distribution of the membrane phospholipids of the RBC and exposure of PE and PS by a flip-flop mechanism [12]. These changes depict the enhanced aggregation of PS- and PE-exposing

RBCs, their increased adherence to ECs, and their capacity to enhance thrombin generation via the assembly of the prothrombinase complex which generates thrombin which leads to platelet activation [12]. Because symptomatic TI patients tend to have to have more pathological RBCs and more activated platelets, they have a higher incidence of TEE.

Recent investigations have shown that there exists a high incidence of silent cerebral infarcts (SCIs) in the brain of thalassemia patients [13]. Several risk factors are involved in the pathophysiology of SCI in thalassemia. Among them is mean age, where the number of SCIs was more frequent in older patients [14]. Splenectomy is another risk factor or contributor

**Table 2** Risk factors for TEE in the studied thalassemia patients ( $n = 87$ )

Variable	Odds ratio	95% CI	<i>p</i> value
Age	1.164	1.081 to 1.253	0.001
Sex	0.550	0.235 to 1.290	0.169
Age of onset	1.035	1.012 to 1.059	0.001
Splenectomy	10.074	3.743 to 27.110	<0.001
Salicylate	0.406	0.126 to 1.163	0.601
Bil D	1.363	1.453 to 6.231	0.005
Bil T	4.266	2.099 to 8.670	0.001
Chelation therapy	0.422	0.141 to 2.030	0.327
Hydroxyurea	0.548	0.189 to 1.385	0.428
Annexin-labeled MPs (%)	1.002	0.300 to 1.904	0.003
PMPs (%)	1.240	1.01 to 1.587	0.014
RMPs (%)	1.572	1.14–2.234	0.01
EMP (%)	2.99	1.896–4.934	0.001

TEE thromboembolic events, 95% CI 95% confidence interval, Bil T total bilirubin, Bil D direct bilirubin, MPs microparticles, PMPs platelet-derived microparticles (dual +ve annexin, CD41), RMPs red cell-derived microparticles (dual +ve annexin, CD235a), EMPs endothelial-derived microparticles (dual +ve annexin, CD146)

to the occurrence of SCI [13]. Most studies have been done on TI patients. Less data is available regarding the incidence of SCI in  $\beta$ -TM patients. However, counter the expectations, it was found that there was no difference in the incidence of SCI

between transfused and nontransfused patients with  $\beta$ -TM and TI, suggesting that activated platelets may have a major role mainly in symptomatic patients [13, 14].

The studied thalassemic patients with TEE had a significantly higher percentage of annexin-labeled MPs and higher percentage of PMPs ( $p$  value = 0.003 and 0.014, respectively). Also, they had higher percentage of RMPs but did not reach a statistical significant value (Table 1 and Fig. 1). These findings may reflect enhanced platelet activation among patients with thrombotic events. By multivariate analysis (Table 2), the endothelial-derived MPs increased the risk of TEE by 3 times and the red cell-derived MPs increased in the risk of TEE by 1.5 times. Similarly, our findings were confirmed by previous flow cytometric studies that demonstrated increased levels of circulating MPs derived from blood cells in patients with TEE, including myocardial infarction, idiopathic thrombocytopenic purpura (ITP), and disseminated intravascular coagulation [16]. Moreover, a previous study that was performed on 60 young  $\beta$ -TM patients revealed that both PMPs and RMPs were significantly elevated in  $\beta$ -TM patients compared with controls [17]. It was also found that TI patients have higher levels of procoagulant red cell-derived MPs, leukocytic-derived MPs, platelet-derived MPs, and endothelial-derived MPs compared with controls, but the contribution of these fragments to TEE was still under investigation because of the small number of patients with TEE in their study [18].

By bivariate analysis, splenectomy was found significantly higher among the studied thalassemic patients who have

**Table 3** Laboratory data of the studied TM and TI patients

Variables	TI ( $n = 48$ )		TM ( $n = 39$ )		<i>p</i> value
	Mean	SD	Mean	SD	
Age (years)	12.26	7.84	11.52	7.86	0.66
Volume of blood (cc/kg/year)	105.33	74.42	167.31	34.16	0.000*
frequency of blood transfusion/year	7.0	4.98	11.2	2.3	*0.000
Plt. ( $10^3/\text{mm}^3$ )	657.40	466.89	499.31	214.92	0.382
LDH level (U/l)	518.90	251.55	408.59	197.69	0.039*
Bil. T (mg/dl)	1.93	1.13	1.75	0.82	0.403
Bil. D (mg/dl)	0.38	0.24	0.41	0.22	0.611
Serum ferritin (ng/ml)	1146.94	810.28	1760.46	1152.29	0.009*
	Median	Range	Median	Range	<i>p</i> value
Annexin-labeled MPs (%)	19.01	7.5–65.07	15.10	8.97	0.104
PMPs (%)	8.357	7.03–49	6.72	1–36.4	0.058
RMPs (%)	8.10	1.1–33.3	6.95	1.50–30.90	0.303
EMP (%)	0.60	0–12.20	0.50	0–10.70	0.029

TI thalassemia intermedia, TM thalassemia major, Plt platelets, LDH lactate dehydrogenase, Bil T total bilirubin, Bil D direct bilirubin, MPs microparticles, PMPs platelet-derived microparticles (dual +ve annexin, CD41), RMPs red cell-derived microparticles (dual +ve annexin, CD235a), EMPs endothelial-derived microparticles (dual +ve annexin, CD146), g/dl gram/deciliter, % percentage,  $\text{mm}^3$  cubic millimeter, U/L units per liter, ng/ml nanograms per milliliter

\* $p$  value < 0.05 is significant

experienced TEE. By multivariate analysis (Table 2), splenectomy increased the risk of TEE by 10 times in our studied thalassemic cases. Similarly, it was found previously that absence of the spleen was a strong causative factor to the hypercoagulable state in thalassemia [19]. It was further proved that significantly higher levels of MPs as a strong predisposing factor of thrombosis were significantly higher in splenectomised thalassemic patients versus non-splenectomised controls [18]. Additionally, it was endorsed that TEE after splenectomy attributed to the presence of high platelet counts, aggregation, and/or to increased number of damaged RBCs [20, 21].

We found that platelet count was significantly higher in thalassemic patients who experienced TEE (Table 1). Similarly, it was reported that thalassemic patients with a hypercoagulable state have higher platelet counts, platelet aggregation, and activation [22].

Our thalassemic patients with TEE had significantly higher levels of lactate dehydrogenase (LDH) and bilirubin (total and direct) compared to those who did not have TEE (Table 1). By multivariate analysis, the total bilirubin increased the risk of TEE among our studied patients by 4 times and the direct bilirubin increased the risk of TEE by 1.4 times (Table 2). These results can be explained using previous findings in the literature which similarly noted that the increased levels of hemolytic indicators in thalassemic patients, particularly LDH activity, reflected a shift towards anaerobic metabolism and increased glycolysis in the cytoplasm of thalassemia cell accompanied by a high turnover rate [23].

In this study, the serum ferritin level was significantly higher among thalassemic patients with TEE (Table 1). This is supported by a previous study which showed that a serum ferritin more than 1000 ng/dl was associated with an increased risk of thrombosis in thalassemic patients [19]. Moreover, iron-derived reactive oxygen species are implicated in the pathogenesis of several vascular disorders including atherosclerosis, microangiopathic hemolytic anemia, vasculitis, and reperfusion injury [24, 25].

By multivariate analysis, the salicylate, chelation, and hydroxyurea therapy in the studied thalassemic patients showed no effect on the risk of TEE (Table 2). Unlike our findings, previous studies have showed that fetal hemoglobin inducing agents like hydroxyurea can modulate hypercoagulability in thalassemic patients in several ways. It may reduce phospholipid expression on the surface of the RBCs and platelets, decrease RBC adhesion to thrombospondin, lower the plasma markers of thrombin generation, decrease leucocyte count, and decrease the monocyte-expressing transcription factor [22].

In this study, there was a difference in the incidence of the TEE between the studied  $\beta$ -TM and TI despite the absence of any significant difference in the level of any of the studied MPs and the splenectomy between the two groups. However, this difference can be explained by the significant higher frequency of blood transfusion/year in the studied  $\beta$ -

TM patients if compared to the TI patients,  $11.2 \pm 2.3$  vs.  $7.0 \pm 4.98$  times/year,  $p$  value = 0.000 (Table 3). Similarly, it was stated previously that TI patients seem to be at greater risk of TEE than TM due to increased frequency of blood transfusion in  $\beta$ -TM which suppresses the inherent ineffective erythropoiesis and hence the erythrocyte [15].

In conclusion, the elevated levels of circulating MPs derived from the ECs, the red cells, and platelets is a risk factor for the TEE in thalassemia patients.

**Compliance with ethical standards** Patients and/or their guardians gave their informed consent, and the Institutional Review Board (IRB) approved the study.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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