Journal of the Egyptian Society of Haematology & Research, Vol. 13, No. 1, March: 1-6, 2017

Circulating Erythrocyte Derived Microparticles in Pediatric Thalassemia Intermedia Patients

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ABSTRACT

Background: Elevated levels of erythrocyte-derived microparticles (EMP) are present in the circulation in medical conditions affecting the red blood cells as in β thalassemia. Patients with elevated levels of circulating EMPs have increased susceptibility for thrombotic disorders.

Objective: To determine the levels of circulating EMP in β thalassemia intermedia (TI) patients and to detect their procoagulant activity.

Patients and Method: Identification and quantification of EMP was performed by flow cytometry using combination of carboxy fluorescein diacetae succinimidyl ester (CFSE) and glycophorin A (CD235) in 20 TI patients and 20 apparently healthy controls.

Results: This study showed a highly statistically significant increase in EMP in thalassemia intermedia patients as compared to control group (p=0.00). There was statistically significant increase in EMP in cases with thrombotic complications compared to patients with no thrombotic complications (p=0.04).

Conclusion: Elevated levels of circulating EMPs were related to occurrence of thrombotic complications in TI patients.

Key Words: *EMP – TI – Thrombotic complications.*

INTRODUCTION

Thalassemia are heterogeneous autosomal recessive hereditary anemia characterized by reduced or absent β-globin chain synthesis. Approximately 68,000 children are born with various thalassemia syndromes each year. β-thalassemia is highly prevalent, with 80 to 90 million people reported to be carriers across the world (1.5% of the global population). It includes three main forms: β-thalassemia major (TM), also referred to as "Cooley's anemia" and "Mediterranean anemia"; β-thalassemia intermedia (TI); and thalassemia minor, called "β-thalassemia carrier", "β-thalassemia trait", or "heterozygous β-thalassemia". Apart from the rare dominant forms, subjects with TM are homozygotes or compound heterozygotes for β0 or β+ genes, subjects with TI are mostly homozygotes or compound heterozygotes, and subjects with thalassemia minor are mostly heterozygotes **[1]**.

β-Thalassemia represents a major public health problem in Egypt. The carrier rate varies between 5.5% to ≥9%; it is estimated that there are 1000/1.5 million per year live births born with β-thalassemia **[2]**.

β-thalassemia intermedia (TI) is caused by a marked imbalance between α and β globin chains leading to accumulation of α globin and damage to the RBCs membrane which causes anemia and that needs intermittent blood transfusion. TI may result from defective production of β globin chains due to β globin gene defect or from the increased production of α globin chains. The excess free α chains will precipitate within erythroid precursors, as hemichromes (HMC), forming large inclusion bodies. In turn HMC alter the membrane clustering band 3 and enhance the deposition of opsonin autologus Igs and C3 fragments **[3]**.

Splenectomy, performed to alleviate anemia in TI patients, may result in severe thrombotic episodes and may cause a rise of pro-thrombotic circulating microparticles (MP) **[4]**.

Microparticles (MPs) are defined as membrane-derived vesicles smaller than 1mm that are shed from any cell type in response to cell

activation, cell stress or apoptosis. The cellular origin of the MPs can be identified by the presence of surface molecules from their parent cells. In blood circulation, MPs originating fromplatelets, erythrocytes, leukocytes, and endothelial cells can be identified. The most abundant MPs arise from platelets followed by MPs from endothelial cells, granulocytes and erythrocytes (EMPs) **[5]**.

Increased concentrations of circulating EMPs have been found in patients with diseases affecting the red blood cells, such as sickle cell anemia, paroxysmal nocturnal hemoglobinemia (PNH) and β-thalassemia **[6]**.

Presence of EMPs is specifically correlated to in vivo markers of increased coagulation and several studies have shown that EMPs have the ability to support blood coagulation in vitro **[7,8]**.

In this study we determined the levels of circulating EMP in β thalassemia intermedia (TI) patients to detect their pro-coagulant activity.

SUBJECTS AND METHODS

This study was carried out on twenty patients with thalassemia intermedia selected from Al-Zahraa University Hospital, Pediatric Outpatients Clinic. Their ages ranged from 5 to 15 with a mean of 9.5±3.0 and a median of 9.25 years, they were 17 males and 3 females. Twenty samples were collected from age and gender matched apparently healthy children, to be used as control. The study was conducted according to the rules of Helsinki declaration for studies on human subjects. It was approved by the Institutional Review (IRB) Board of Al-Azhar University and a written informed consent was obtained from children's guardians.

All patients were subjected to detailed medical history including history of thrombotic complications, history of splenectomy, history of blood transfusion and history of chelation therapy.

Diagnostic chriteria:

The diagnosis of TI was established through the assessment of complete blood count (CBC), Hb analysis using high performance liquid chromatography (HPLC) and clinical status.

Exclusion criteria:

- Children with acute or chronic infection.
- Children with other chronic illness (as diabetes, cardiac complications and G6PD dificiency).
- Children with primary coagulation disorders.

Laboratory investigations included:

I- Complete blood count (CBC): Using SYSMEX KX 21-N Cell Counter.

II- Hemoglobin analysis by high performance liquid chromatography (HPLC) D-10 (Bio-Rad, France).

III- Fibrinogen concentration from platelet poor citrated plasma samples using (fibrinogen Diagnostica, Stago, France).

IV- Prothrombine time (PT): Usingfully automated coagulation analyzer (Stago, France).

V- Biochemical analysis: Using Cobasc 311 (Germany) and Kits of Roche (Germany) forferritin and lactate dehydrogenase (LDH).

VI- Microparticle preparation and labeling:

EDTA blood samples were processed within one hour of collection. Platelet free plasma (PFP) was prepared by serial centrifugations at 1,500g for 20 minutes twice to remove cells and apoptotic debris ≥1μm. The supernatant plasma became MP-enriched plasma. The microparticles were pelleted from MP-enriched plasma by another centrifugation at 21,000g for 30 minutes at 4°C. The majority of exosomes <100nm should remain in the supernatant while the microparticles will form a pellet; the pellets were stored at –80ºC until analysis **[9]**.

For each analysis 50μL of freshly thawed PFP was transferred to a true count tube (BD Biosciences, San jose, CA, USA, LOT 57221) containing standardized number of fluorescent beads (=50433) to quantify microparticles, then EMP were labeled by adding 5μL phycoerythrin (PE)-conjugated anti-human CD235 (Immunotech, Beckman Coulter, Marsellia, France, LOT 22), 2μL of carboxyfluoresceinsuccinimidyl ester (CFSE) (Biolegend, San Diego, USA, LOT B219508) and 5μ of peridin chlorophyll protein complex (PerCP)-conjugatedanti-human CD45 (Immunotech, Beckman Coulter, Marsellia, France, LOT71126). The optimal concentration was determined for each antibody or dye

by titration experiment. All dyes were applied gently to the Vortex before using to avoid any clumping.

After 15 minutes of incubation in the dark, 500µL of filtered phosphate buffered saline (PBS) was added to each labeled sample. Filtered solutions were used to avoid any noise signals. Acquisition was discontinued after 5000 events in relevant region.

CFSE/glycophorinA staining allows proper quantification of EMP. CFSE is a fluorescent dye used for the detection of microparticles (Fig. 1). CFSE definitively distinguishes microparticles with intact membrane structures from cellular debris. CFSE passively diffuses into microparticles and once within the microparticles can be hydrolysed by intracellular esterase, becoming fluorescent and easily distinguishing the microparticles from cellular fragments **[10]**.

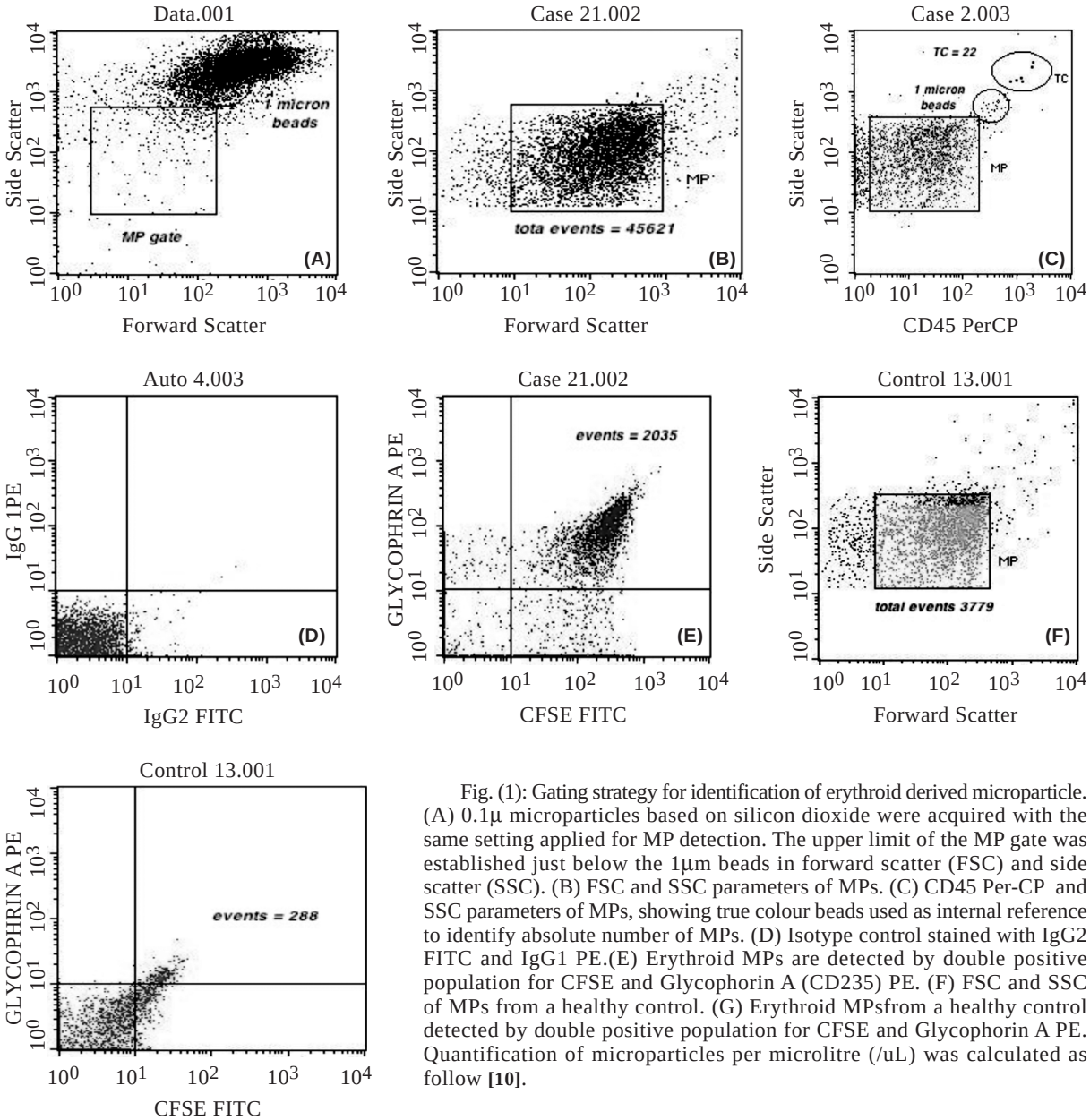

Fig. (1): Gating strategy for identification of erythroid derived microparticle. (A) 0.1µ microparticles based on silicon dioxide were acquired with the same setting applied for MP detection. The upper limit of the MP gate was established just below the 1µm beads in forward scatter (FSC) and side scatter (SSC). (B) FSC and SSC parameters of MPs. (C) CD45 Per-CP and SSC parameters of MPs, showing true colour beads used as internal reference to identify absolute number of MPs. (D) Isotype control stained with IgG2 FITC and IgG1 PE.(E) Erythroid MPs are detected by double positive population for CFSE and Glycophorin A (CD235) PE. (F) FSC and SSC of MPs from a healthy control. (G) Erythroid MPsfrom a healthy control detected by double positive population for CFSE and Glycophorin A PE. Quantification of microparticles per microlitre (/uL) was calculated as follow **[10]**.

Flow cytometry:

The MPs were detected by multicolourFACSCalibur (BD Biosciences, San jose, USA). CellQuest Pro software (BD Biosciences, San jose, USA) was used for data analysis. Compensation setting was established before acquiring the samples using color calibrite beads (BD, Biosciences, San jose, USA, LOT 5093879). Forward scatter and side scatter channels (FSC and SSC) were used on a logarithmic scale. For SSC the assigned voltage was 407 Volts and the threshold was 200. For FSC photomultiplier tube (PMT), the assigned voltage was E 01 Volts and the threshold was zero.

A MP gate was established using size calibrated fluorescent beads 1µm in diameter based on silicon dioxide (sigma Aldrich, Germany, LOT BCBR 605 V). The upper limit of the MP gate was established just below the 1µm beads in FSC and SSC.

Unstained samples were acquired to detect the sample auto-fluorescence and isotype controls (Mouse IgG2a FITC and IgG1 PE Controls BD, Biosciences, San jose, USA, LOT 87903) and used as negative controls. Only erythroid derived microparticles showing positivity for CD235a and CFSE were quantified.

$$\frac{\text{N. of eventsingate containing microparticle}}{\text{N. of events in absolute count bead region}} \times \frac{\text{N. of beads counts/test}}{\text{Sample volume (uL)}} \times \text{Dilution factor}$$

N.: Number

Statistical analysis:

Data were analyzed using SPSS version 17.0 (Statistical Package for Social Sciences Inc., Chicago, IL, USA) and Microsoft Excel 2010. Parametric data was expressed as mean ± SD and non-parametric data was expressed as number and percentage. Student's *t*-test was done to compare between two groups. Pearson Correlation Coefficient was done to correlate between different parameters among groups. *p*-value of >0.05 was considered insignificant, *p*-value of ≤0.05 considered significant, *p*-value of <0.01 was considered highly significant.

RESULTS

The study was performed on 20 TI patients including 17 males and 3 females with an age range of 5-15, mean of 9.5±3.0 and a median of 9.25 years. Twenty age and gender matched apparently healthy children were included as control.

Hematological parameters for patients and control are presented in Table (1). As expected, there was significant increase in RBCs count, platelet count, WBCs count, PT, LDH, ferritin and % Hb F in thalasemia intermedia patients compared to control group. While Hb, MCV, MCH and percentage of Hb A were significantly lower in thalasemia intermedia patients compared to control group.

EMP were significantly higher in the Thalassemia intermedia patients compared to control (5.5±1.9 vs. 1.1±0.6, *p*=<0.00).

EMP level in various clinical presentation of TI is presented in Table (2). Increased level of EMP was significantly associated withthrombotic complications (*p*=0.04) and with splenectomy (*p*=0.00).

Correlations between EMP and other lab parameters in thalassemia intermedia group are presented in Table (3). Significant positive correlation was encountered with Hb level, ferritin, and percentage Hb A; significant negative correlation was encountered with percentage of Hb F and fibrinogen; while no correlation was encountered between EMP and other parameters.

Table (1): Comparison of different lab parameters in the studied groups.

Item \ Group	Thalassemia intermedia (N=20)	Control group (N=20)	*p*-value
WBCs (X 10^9/L)	7.89±1.27	6.87±1.71	0.03
HB (g/dl)	8.14±0.66	14.20±1.0	0.000
RBCs (X 10^{12}/L)	5.06±0.31	4.46±0.7	0.01
MCV (fl)	47.25±10.22	86.44±5.67	0.000
MCH (pg)	17.6±1.47	29.45±2.58	0.000
PLT (X 10^9/L)	379.1±47.39	272.35±66.11	0.000
Prothrombin Time (sec.)	13.15±1.57	12.13±0.9	0.02
LDH (U/L)	547.25±96.68	268.25±0.89	0.000
Ferritin (ng/ml)	3737±999.9	152.33±83.04	0.000
HbA%	67.53±11.98	78.05±11.8	0.000
HbF%	30.4±12.41	0.56±0.27	0.000
Fibrinogen (mg/dl)	249.6±67.92	282.65±55.4	0.100

WBCs : White blood cells.
RBCs : Red blood cells.
MCV : Mean corpuscular volume.
MCH : Mean corpuscular hemoglobin.
PLT : Platelet count.
LDH : Lactate dehydrogenase.
HbA : Hemoglobin A.
HbF : Hemoglobin F.

Table (2): Association of erythrocyte microparticles (EMP) with different clinical.

Parameter	Yes		No		*p*
	Number	EMP	Number	EMP	
Splenectomy	7	6.9±1.6	13	4.7±1.5	0.01
Hemolytic attacks	10	5.3±2.2	10	5.6±1.6	0.72
Thrombosis	6	7.2±1.9	14	4.8±1.5	0.04
Medication	9	4.8±1.7	11	6.0±1.9	0.18

Parameters in 20 thalassemia intermedia patients.

Table (3): Correlation between erythroid microparticles and laboratoryparameters in 20 thalassemia intermedia patients.

Variable	*r*	*p*	Variable	*r*	*p*
Total leukocytic count	0.072	0.767	LDH	–0.012	0.957
Hemoglobin	–0.759	0.000	Ferritin	0.892	0.000
RBCs	–0.082	0.731	HbA%	0.551	0.022
MCV	0.053	0.828	HbF%	–0.554	0.021
MCH	0.213	0.837	HbA2%	0.007	0.981
Platelets	–0.027	0.910	Fibrinogen	–0.480	0.035
Prothrombin Time	0.128	0.592			

DISCUSSION

Patients with β-thalassemia intermedia (TI) are completely asymptomatic until adult life; they experience only mild anemia, maintain hemoglobin levels between 7 and 10g/dL, and require only occasional blood transfusions **[11]**.

Three main factors are responsible for the clinical sequelae of TI: Ineffective erythropoiesis, chronic hemolytic anemia, and iron overload. The degree of ineffective erythropoiesis is the primary determinant of the severity of anemia, however several specific complications are thought to be more frequent in TI than in thalassemia major, such as thrombosis, pulmonary hypertension, extra-medullary erythropoietic masses, leg ulcers and cholelithiasis **[3]**.

Microparticles (MPs) are shed sub–micrometric plasma membrane fragments (~0.1-1μm) harboring negatively-charged pro-coagulant phosphatidylserine (PS) in their extracellular membrane leaflet. They are mainly derived from apoptotic or activated cells, and generally present a pro-coagulant potential. Increased levels of circulating MPs were described in many vascular disorders as thalassemia and diabetes **[11]**.

Previous studies showed that high counts of circulating microparticles, originated from the membrane of abnormal erythrocytes and have been associated with increased thrombotic risk in hemolytic disorders **[3]**.

The present study showed highly statistically significant increase in EMP in thalassemia intermedia patients as compared to control group ($p<0.001$); this is in agreement with previous studies **[3,12]**.

Habib et al. **[11]**, 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.

The present study showed that 6/20 TI patients suffered from thrombotic complications and the level of EMP in those patients was statistically significantly higher ($p<0.05$) than patients with no thrombotic complications. These results are in accordance with Eldor and Rachmilewitz study **[8]**, which suggested that thalassemic RBCs may provide a source of anionic phospholipids like phosphatidyl ethanolamine (PE) and PS, which can increase thrombin generation and initiation of platelet activation leading to the hyper-coagulable state in thalassemia.

The present study showed that the 7/20 splenectomized TI patients showed statistically significantly higher EMP level than patients without splenectomy ($p<0.05$); This in consistence with a previous study **[12]**.

In contrast, Agouti et al., **[13]** found that EMPs were not related to splenectomy while platelet MP were related to splenectomy; however this study involved intensively transfused patients diluting EMPs.

The current study showed highly statistically significant increase in ferritin in thalassemia intermedia patients compared to control group ($p<0.05$). These results are in agreement with shah, et al. **[14]** who reported thatferritin increased with age in non-transfused thalassemia patients especially when the iron overload is excessive. InTI, iron absorption is 3-10 times normal, which is 17-89% compared to 14.8± 9.5% in normal subjects, and increases after splenectomy.

The present study showed statistically significant negative correlation between EMPs and plasma level of fibrinogen (r=–0.480, p<0.05), suggesting that EMP participates in the pro-coagulant activity. This is in consistence with Keuren et al., **[15]** who stated that MPs have platelet like adhering properties and accelerate thrombin generation. MPs attain the capacity to firmly adhere to collagen, vWF, fibrinogen and surface adherent platelet at low and high shear rate.

In contrast, Agouti et al., 2015 stated that EMP have no correlation with pro-coagulant activity; they explained that difference by the fact that their study included multi-transfused patients leading to dilution of EMP due to their lesser amount in plasma in contrast to platelet MPs which produced pro-coagulant activity despite the dilution caused by their higher amounts **[13]**.

Our study shows no significant correlation between EMPs and level of LDH enzyme (r=–0.012, p>0.05) which is in consistence with Agouti et al. **[13]** who stated that there was no statistically significant correlation between EMP and markers of hemolysis as Hb value, LDH or reticulocyte count.

In the current study, there was significant negative correlation between EMP and percent of Hb F (r=–0.554, p<0.05). This is in concordance with the results obtained by Neufeld et al. **[16]**.

In conclusion, our study demonstrated the presence of elevated levels of EMPs in TI patients compared to control group. Elevated levels of circulating EMPs were related to occurrence of thrombotic complications in TI patients. Thus early detection and quantification of EMPs may provide utility for early detection of thrombotic complications in TI patients.

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