Journal of the Egyptian Society of Haematology & Research, Vol. 9, No. 2, September: 51-58, 2013

The Potential Role of Annexin V on RBCs and P-Selectin on Activated Platelets in the Hypercoagulable State in Egyptian Patient with Thalassemia

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ABSTRACT

Background: The presence of a high incidence of thromboembolic events has led to the identification of a hypercoagulable state in thalassemia. Several etiologic factors may play a role in the pathogenesis of the hypercoagulable state in thalassemia.

Objectives: The aim of this study was to assess the existence of a chronic hypercoagulable state in Egyptian thalassemic patients and study the potential role of thalassemic RBCs and activated platelets in the hypercoagulable state.

Patients and Methods: Thirty-nine patients with thalassemia (27 with thalassemia major (TM) and 12 with thalassemia intermediate (TI) were used as the study group and 20 healthy volunteers were used as control group. Flowcytemerty was used to study the expression of anionic phospholipids (Annexin V) on the RBCs and CD62p (P-selectin) on the activated platelets.

Results: Annexin V positive RBC in TM and TI patients were significantly over expressed compared to control group ($p<0.001$) with no significant difference between patients with and without splenectomy. The expression of activation-dependent platelets neoantigen, P-selectin, was significantly higher ($p<0.001$) in thalassemic patients compared to the control. There was a strong association between the expression of Annexin V on the RBCs and P-selectin on the activated platelets.

Conclusion: There is a strong association between levels of RBCs expressing Annexin V and levels of platelets expressing P-selectin. The strong association between the expressions of these two cellular markers in the context of the known tendency of the hypercoagulable state observed in patients with thalassemia may help to predict and to avoid the development of this state in patients at risk.

Key Words: *Hypercoagulable state – Thalassemia – Annexin V – P-selectin.*

INTRODUCTION

The thalassemia are a heterogeneous group of genetic disorders of hemoglobin synthesis, all of which result from a reduced rate of production of one or more of the globin chains of hemoglobin. They are divided into α- ß- δ ß-, or γδß - thalassemia, according to which globin chain is produced in reduced amounts **[1]**.

The thalassemia represents the most common monogenetic disorder worldwide **[2]**, there is a particularly high incidence of thalassemia (2.5%-25%) in the Mediterranean basin, the Middle East, the tropical and subtropical regions of Africa, the Asian subcontinent, and Southeast Asia, where milder forms of the disease are most commonly seen. Cases of thalassemia also occur sporadically in virtually every ethnic group and geographic location **[3]**.

Several clinical and laboratory finding suggest the presence of chronic hypercoagulable state in patients of thalassaemia major (TM) and thalassaemia intermedia (TI) including transient ischemic attacks, pulmonary embolism and deep venous thrombosis. Diverse factors contributing to the hypercoagulable state in patients with thalassemia have been identified. In most cases, a combination of these abnormalities leads to clinical thrombosis **[4]**.

Among cellular factors, platelet activation contributes, to a significant extent. Much evidence suggests that patients with thalassemia have activated platelets. First, there is evidence of increased platelet aggregation **[5]**. Moreover,

flow cytometric studies have also confirmed the chronic platelet activation status manifested by an increased proportion of platelets expressing CD62P (P-selectin) and CD63 **[6-7]**, in addition to shortened platelet survival due to enhanced platelet consumption (especially in splenectomized patients) **[8-9]**.

Alteration in RBCs due to oxidation of globin subunits in thalassemia erythroid cells, leading to the formation of red-cell "senescence" antigens such as phosphatidylserine and phosphatidyletholamine causes thalassemic red cells to be rigid and deformed. These changes cause RBCs to aggregate, resulting in premature cell removal **[10]**. So, thalassemic RBCs expressing these negatively charged phospholipids are used as a source of phospholipids, enhancing and eventually increasing thrombin generation in a prothrombinase assay where normal RBC had no effect **[11]**.

This was evident by the finding that annexin V, a protein with high affinity and specificity for anionic phospholipids, could block the procoagulant effect of isolated thalassemic RBCs **[12]**. The procoagulant effect of thalassemic RBCs was suggested to contribute to the hypercoagulable state in thalassemia by amplifying thrombin generation and initiating platelet activation in vivo as one aspect of hypercoagulable state **[7]**. These abnormalities have been reduced to normal range after the patients have received a blood transfusion **[11]**.

The finding of elevated levels of endothelial adhesion proteins E-selectin (ELAM-1), intercellular adhesion molecule-1 (ICAM-1) and von Willebrand factor (VWF) and vascular cell adhesion molecule-1 (VCAM-1) in thalassemic patients suggested that endothelial injury or activation may be a feature of this genetic disease which also plays an important role in the recruitment of white blood cells and RBCs and promote thrombosis at vascular inflammation sites, vessel obstruction, tissue hypoxia and death **[13]**. More recently, it was shown that microparticles of red blood cell origins were elevated in patients with TI versus controls; these have a potential to aggravate thrombotic events **[14]**.

Clinical observations have suggested that splenectomy in thalassemia can contribute to an increased susceptibility to thrombosis **[15,16]**. The development of these complications has been ascribed to the presence of high platelet counts following splenectomy and/or to increased number of abnormal RBCs **[6,10,17]**. Also, in splenectomized TI patients, thrombin generation was significantly higher than in control subjects and patients who had not undergone splenectomy **[15,17]**.

The presence of a persistent hypercoagulable state combined with the infrequent occurrence of significant thrombotic events suggests that thrombosis is largely a subclinical process in thalassemia and has been associated with autopsy findings of platelet and fibrin thrombi in the microvasculature in the lungs **[18]** and the brain **[19]**. Management of this hypercoagulable state has two arms: Prevention and treatment. Prevention consists of proper anticoagulation to high-risk patients with thalassemia who are exposed to transient thrombotic risk factors (e.g. surgery, immobilization, pregnancy); treatment entails the adequate use of anticoagulation according to the recommendation for hypercoagulable state **[20-21]**.

The aim of this study is an attempt to confirm the presence of a chronic hypercoagulable state in Egyptian patients with ß-thalassaemia major and intermedia due to the expression of anionic red cell phospholipids leading to platelet activation; and to estimate the magnitude of the problem to be able to plan for prophylactic measures.

SUBJECTS AND METHODS

Ethical approval:

This study was approved by the ethical committee review board of "The General Organization for Teaching Hospitals and Institutes (GOTHI)" in accordance with the Helsinki guidelines for the protection of human subjects. Written informed consent from all participants (above 18 years old) and from the legal guardians (for those below 18 years old) involved in the study were taken.

Subjects:

The present study included 39 patients with Thalassemia (27 with TM, and 12 with TI), presented to the outpatient Clinic of Ahmed Maher Hospital, who were compared to 20 age and gender matched healthy volunteers as a control group.

Patients and control were subjected to:

• *Complete history:* Including age, sex, age of disease onset, height, weight, splenomegaly, hepatomegaly, onset and number of blood units received, splenectomy, history of complications, and treatment with iron chelating agent.

• In all patients the results of complete blood pictures, hemoglobin electrophoresis, hemoglobin A2, hemoglobin F and the clinical course were used to classify our patients as TM or TI. The blood samples required for the study were taken immediately before a blood transfusion (at least 4 weeks after the last transfusion) and samples were obtained by standard venous puncture using a light tourniquet, where the first 2mL of blood were discarded to avoid platelet activation. The blood was drawn into EDTA tubes and the test has been strictly done within 4-6 hours.

• *Annexin V binding to RBCs:* The sample was mixed well and 10µl of EDTA blood were added to 40µl of HEPES buffer, mixed well then a saturating concentrations of specific monoclonal antibodies namely Annexin V (FITC, BD Bioscience Pharmingen, San Diego) and anti-human CD235a (Glycophorin A/RPE, clone JC159, Dako) were added then incubated at room temperature in dark for 20min and the read on a Coulter EPICS XL-MCL flow cytometer system (Coulter Corporation, Hialeah, USA) after re-suspension by isoton without lysis of RBCs. Light scatter fluorescent data were obtained with a gain setting in the logarithmic mode. The results were expressed as percentage (%) of positive cells for the co-expression of Annexin V and Glycophorin on the surface of RBCs and mean fluorescent intensity (MFI) **[22]**.

• *Circulating activated platelets:* Blood was immediately centrifuged at 750g for 5 minutes at 22ºC. The supernatant was separated as Platelet Rich Plasma (PRP) and diluted 1:10 in HEPES buffer saline. Fifty-µL aliquot from each diluted PRP sample was added to a tube containing saturating concentrations of specific monoclonal antibodies, the platelets activation marker P-Selectin (CD62p) FITC (clone CLB Thromb/6, Dako) and the normal platelet marker CD41 RPE (clone 5B12, Dako). Samples were incubated in the dark at room temperature for 20 minutes. After immunolabelling, the samples were diluted 1:10 HEPES buffer and analysed on a Coulter EPICS XL-MCL flow cytometer system (Coulter Corporation, Hialeah, USA). Platelets are distinguished by the characteristic light scatter; results were expressed as percentage (%) of positive cells for the co-expression of CD41 and CD62p on the activated platelets and MFI **[23]**.

Statistical analysis:

A statistical analysis was performed using SPSS version 14. Nonparametric Kruskal-Wallis test was used to compare between studied markers in each group. ANOVA test was used to compare between variance of each marker level among two different groups of TM and TI and the control **[24]**.

RESULTS

This study included 39 patients with thalassemia (27 TM and 12 TI) in addition to 20 healthy age and sex matched control. The age of the patients with TM ranged from 4-19 with a median of 14; for TI, it ranged from 5-18 with a median of 15 and for the control group, it ranged from 6-16 with a median of 13 years. Male to female ratio was 1:1 in TM and 1.2:1 in TI.

Patients with TM showed an early onset of the disease at age 0.9±0.2 months (within the first year of life); while those with TI showed a late onset of disease at age 5.3±1.7 years (p<0.001). Also, patients with TM showed growth retardation; their height and weight were 128.6±21.1cm and 27.8±8.5kg respectively, compared to both patient with TI (148.3±23.3cm and 42.9±14.9kg) and the control group (149.7±15.9cm and 44.5±14.6kg) (p<0.001).

Splenomegaly was reported in all cases (100%) of TM and in 8/12 (66.7%) of patients with TI, while splenectomy was done for 5/27 (18.5%) of TM and none of the TI patients.

All patients with both TM and TI needed to receive blood transfusion and most of patients with TM (97%) received at least one unit of blood or packed RBCs every month, while patients with TI needed to receive one unit of blood or packed RBCs every four to six months. A significant early onset of transfusion was encountered in patients with TM, within the first year of life (0.98±0.27 year) as compared

to those with TI who needed to receive blood later on (5.79±2.13 year), (p<0.001). Also, a significant difference regarding the need to receive iron chelating agent as 25/30 (83%) of TM and none of TI patients received Desferol.

The results showed a significant reduction in the Hb level in patients with TM and TI compared to the control group, as the Hb was 6.1±0.5gm/dl for TM, 7.4±0.3 for TI and 12.3±0.6 for healthy controls (p<0.001). Ferritin level was markedly higher in patients with TM (2626±1094) compared to (636±172) for TI and (220±59) for control group, (p<0.001).

Expression of platelets activation markers:

The percentage expression of activation-dependent platelets neoantigen CD62p (P-Selectin) was 50.1±20.1% in thalassemic patients compared to 10.9±7.9% in the control (p<0.001) (Fig. 1). There was higher co- expression of CD 41 and CD62p on the activated platelets in patients with spleenectomy (58.1± 15.8%) compared to (44.6±21.4%) on patient without Splenectomy but this difference was not statically significant (p>0.05).

The Mean Florescent Intensity (MFI) for the co-expression of CD 41 and CD62p (P-Selectin) on the platelets was 19.0±12.7 in all thalassemic patients compared to 6.1±1.1 in the control (p<0.001), but the MFI was higher in our patients with TI (19.3±9.7) compared to those with TM (18.9±8.5); the difference was in (p>0.05).

Annexin V binding to RBCs:

The percentage expression of Annexin V on RBCs of thalassemic patients was 68.4±8.0% compared to 9.9±3.6% on normal RBCs (p< 0.001) (Fig. 2). The values were comparable on RBCs of patient with splenectomy and without splenectomy (69±8.3% vs. 66.9±8.6%, p> 0.05). Also, the MFI was 2.1±0.5 in all thalassemic patients compared to 1.6±0.1 in the control group (p=0.03), with comparable MFI of Annexin V on the RBCs of patients with TM and TI (1.9±0.4 and 2.1±0.8 respectively, p>0.05). Patients who receive regular blood transfusion showed a lower percentage of Annexin V expression on RBCs (55.4±7.3%) compared to (68.7±9.6%) in poorly transfused patients (p<0.05).

In this study a significant positive correlation between the expression of the platelet activation marker, CD62p, and the Annexin V binding to thalassemic RBCs were observed (r=0.42, p< 0.05).

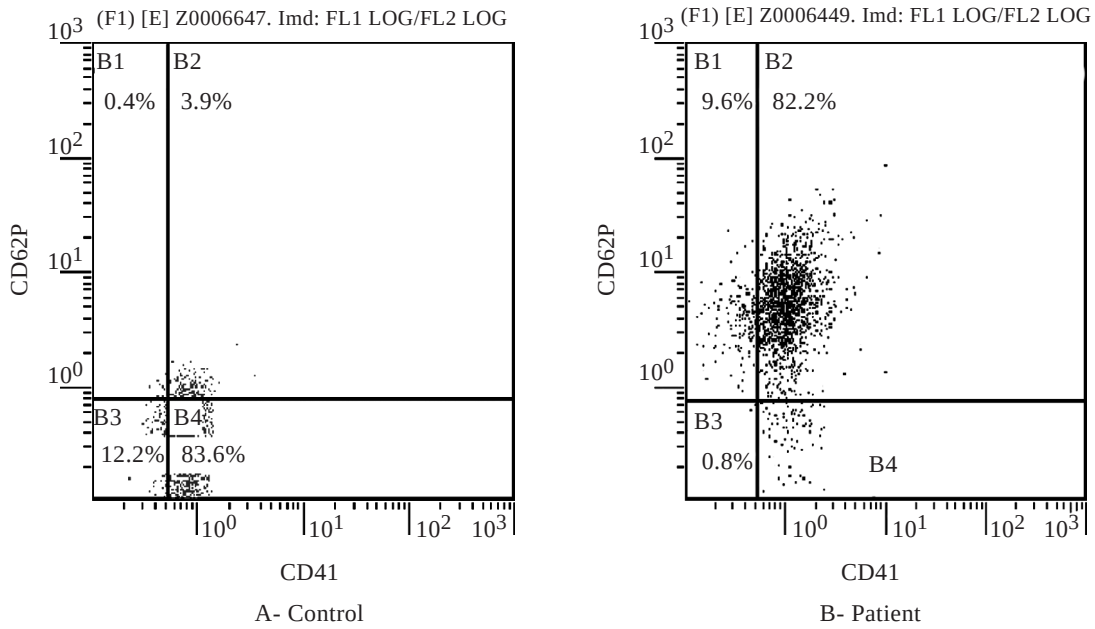

Fig. (1): The expression of CD41 and CD62p on the activated platelets of the control (A) and thalassemic patient (B).

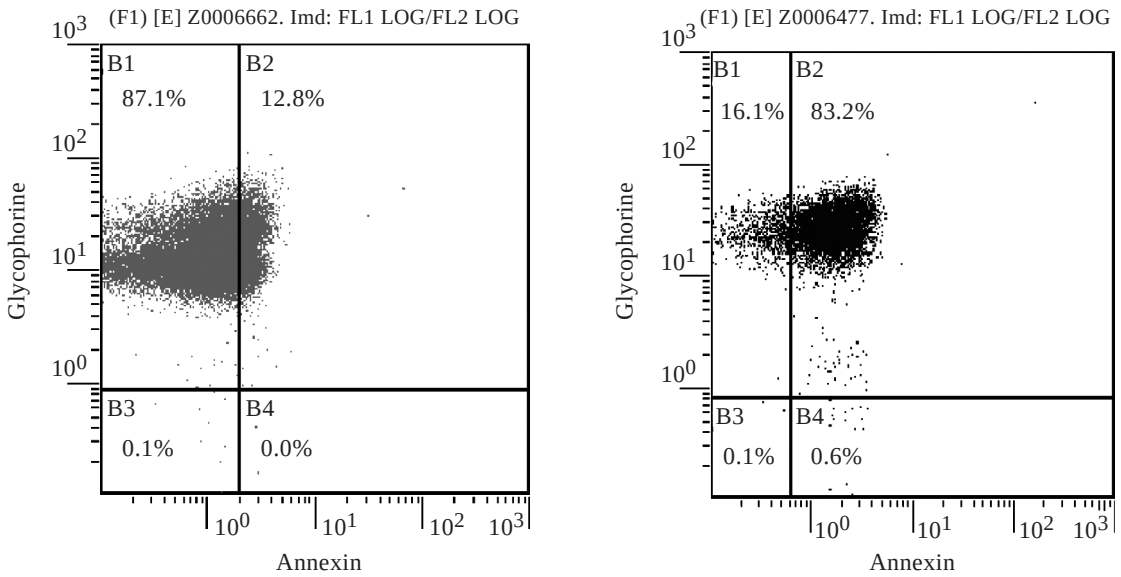

Fig. (2): The expression of Annexin V and Glycophorin on the RBCs of the control (A) and thalassemic patient (B).

DISCUSSION

There is increasing evidence that chronic hemolytic anemia such as sickle cell disease (SCD), thalassemia, paroxysmal nocturnal hemoglobinuria, autoimmune hemolytic anemia and unstable hemoglobinopathies, are characterized by a hypercoagulable state **[25-28]**. In addition to increased thrombin and fibrin generation, increased tissue factor activity, and increased platelet activation, patients with hemolytic anemias manifest thrombotic complications, including venous thromboembolism **[5]**, in situ pulmonary thrombosis **[20]** and stroke **[29]**. Furthermore, the risk of thromboembolic complications appears to be higher following splenectomy **[18,30]**.

The mechanism of coagulation activation in hemolytic anemia is likely multifactorial. Both SCD and thalassemia are characterized by red blood cell membrane abnormalities, with abnormal exposure of phosphatidylserine **[25]**. Abnormal phosphatidylserine exposure functions as both a recognition signal for cell removal during apoptosis of nucleated cells **[11]** and as a docking site for enzymatic complexes involved in coagulation and anticoagulation pathways **[31]**. External exposure of phosphatidylserine alters the adhesive properties of RBC **[32-33]** and appears to be involved in the hemostatic changes observed in hemolytic anemia **[34-36]**.

In this study there was a significantly increased fraction of Annexin V labelled RBC in TM and TI patients compared to RBC from healthy individuals. These findings are consistent with an abnormal membrane phospholipids asymmetry and exposure of phosphatidylserine (PS) in thalassemic patients, which may increase thrombin generation and may enhance the hypercoagulable state in thalassemic patients.

These findings are in line with those reported by many authors **[5,8,32,33]** who stated that the hypercoagulable state in TM and TI may result from procoagulant effect of abnormal RBCs of thalassemic patients, by amplifying thrombin generation and initiating platelet activation. Furthermore it was found that Annexin V, a protein with high affinity and specificity for anionic phospholipids, could block the procoagulant effect of isolated thalassemic RBCs and that these abnormalities have been reduced to normal range after the patients have received a blood transfusion **[13]**.

Similar results were reported by Capellini who found that thalassemic RBCs expressing these negatively charged phospholipids may be used as a source of phospholipids, enhancing and eventually increase thrombin generation in a prothrombinase assay where normal RBC had no effect **[12]**. The persistent hypercoagulable state in thalassemic patients was explained by

the abnormal exposure of some phospholipids, especially Annexin V, on the surface of these RBCs **[11,15,25,34,35]**.

Despite of the fact that none of our TM or TI patients in this study had an overt thrombotic event, a chronic hypercoagulable state was evident by the increased fraction of circulating platelets expressing activation dependent neoantigen, P-Selectin (CD62p). In consistence with the current results several authors demonstrated that overt thromboembolic events (TEE) occur only rarely in thalassaemia patients; however, laboratory tests have provided evidence for a chronic hypercoagulable state which already exists in early childhood **[7-9,12,37]**.

On the other side higher, incidence of thrombotic events were observed in 4% of 683 patients with TM and in 9.6% of 52 patients with TI presented with TEE **[37]**. The same group showed six years later lower incidence as only 1.1% of 720 patients from seven Italian centers with TM, had thrombosis **[38]**. In a large clinical study among 8860 thalassemia patients (6670 TM and 2190 TI), the cumulative prevalence of thromboemolic events was estimated at 1.65%, with thrombosis occurring 4.38 times more frequently in TI than TM **[39]**.

In the current work the lower chance for developing overt thrombosis may be due to the young age as all patients were below 20 years old and spleenectomy was done for only 18.5% of thalassemic patients. The main risk factors for developing thrombosis were described as: Age beyond 20 years, spleenectomy, family history of TEE and previous TEE **[39]**.

In this work we found a strong association between the expression of the platelet activation marker, CD62p, and the Annexin V binding to thalassemic RBCs and the two cellular anomalies are highly correlated and the two cellular anomalies are linked together. In consistence with our results Rulf et at., **[8]** reported a strong correlation between the cellular anomalies and assumed that the abnormal RBCs might enhance thrombin generation in vivo and thus trigger platelet activation in thalassemia.

In parallel with the results of this study, it was hypothesized that there is a causal relationship and a significant association between RBCs membrane anomaly and the degree of in vivo platelet activation **[40]**. More recently, Monnucci et al., **[41]** stated that the RBCs from thalassemic patients are an important player for the activation of platelets in patients with TM.

In this study we found that patients who received regular blood transfusion had lower percentage of Annexin V expression as markers for hypercoagulable state than poorly transfused patients.

The same results were found by Taher et al., **[17]**, who stated that a positive history of transfusion and hemoglobin level ≥9g/dl were found to be protective against thrombosis in patients with TI, while splenectomy, age above 35 years and serum ferrtin ≥1,000ug/l were associated with a higher risk of thrombosis.

In conclusion, we found a significantly higher number of circulating activated platelets expressing CD62p (P-Selectin) and increased fraction of RBCs expressing Annexin V in both TM and TI patients with a strong association between the expression of these two cellular markers. Although there are diverse factors contributing to the hypercoagulable state observed in patients with thalassemia, the strong association between the expression of these two cellular markers and the tendency of the hypercoagulable state observed in patients with thalassemia may help to predict and probably avoid the development of thromboembolic events in those patients.

REFERENCES

1- Steinberg MH, forget BG, Higgs DR, et al. Disorders of Hemoglobin. Cambridge University Press, Cambridge. 2001.

2- Weatherall DJ, Clegg JB. Inherited haemoglobin disorders, an increasing global health problem. Bulletin of the World Health Organization. 2001; 79: 704-1.

3- Weatherall DJ. Genetic variation and susceptibility to infection: the red cell and malaria. Br J Haematol. 2008; 141 (3): 276-286.

4- Weatherall DJ. The inherited diseases of hemoglobin are an emerging global health burden. Blood. 2010; 115 (22): 4331-4336.

5- Taher AT, Otrock ZK, Uthman I, Cappellini MD. Thalassemia and hypercoagulability. Blood Rev. 2008; 22: 283-292.

6- Atichartakarn V, Angchaisuksiri P, Aryurachai K, Chuncharunee S, Thakkinstian A. In vivo platelet activation and hyperaggregation in hemoglobin E/beta-thalassemia: A consequence of splenectomy. Int J Hematol. 2003; 77: 299-303.

7- Del Principe D, Menichelli A, Di Giulio S, De Matteis W, Cianciulli P, Papa G. PADGEM/GMP-140 expression on platelet membranes from homozygous beta thalassaemic patients. Br J Haematol. 1993; 84: 111-117.

8- Rulf A, Pick M, Deutsch V, Patscheke H, Goldfarb A, Rachmilewitz EA, Guillin MC, Eldor A. In-vivo platelet activation correlates with red cell anionic phospholipid exposure in patients with beta-thalassaemia major. Br J Haematol. 1997; 98: 51-56.

9- Eldor A, Lellouche F, Goldfarb A, Rachmilewitz EA, Maclouf J. In vivo platelet activation in beta-thalassemia major reflected by icreased platelet-thromboxane urinary metabolites. Blood. 1991; 77: 1749-1753.

10- Cappellini MD, Grespi E, Cassinerio E, Bignamini D, Fiorelli G. Coagulation and splenectomy: An overview. Ann N Y Acad Sci. 2005; 1054: 317-324.

11- Kuypers FA, De Jong K. The role of phosphatidylserine in recognition and removal of erythrocytes. Cell Mol Biol (Noisy-le-grand). 2004; 50: 147-158.

12- Cappellini MD, Motta I, Musallam KM, Taher AT. Redefining thalassemia as a hypercoagulable state. Ann NY Acad Sci. 2010; 1202: 231-236.

13- Helley D, Rachmilewitz EA, Mosseri M, et al. Comparison of the procoagulant activity of red blood cells from patients with homozygous sickle cell disease and ß-thalassaemia. Thrombosis and Haemostasis. 1996; 76: 322-327.

14- Butthep P, Rummavas S, Wisedpanichkij R, Jindadamrongwech S, Fucharoen S, Bunyaratvej A. Increased circulating activated endothelial cells, vascular endothelial growth factor, and tumor necrosis factor in thalassemia. Am J Hematol. 2002; 70: 100-106.

15- Habib A, Kunzelmann C, Shamseddeen W, Zobairi F, Freyssinet JM, Taher A. Elevated levels of circulating procoagulant microparticles in patients with b-thalassemia intermedia. Haematologica. 2008; 93: 941-942.

16- Iolascon A, Giordano P, Storelli S, Li HH, Coppola B, Piga A, Fantola E, Forni G, Cianciulli P, Perrotta S, Magnano C, Maggio A, Mangiagli A, Devoto M. Thrombophilia in thalassemia major patients: Analysis of genetic predisposing factors. Haematologica. 2001; 86: 1112-1113.

17- Taher AT, Musallam KM, Karimi M, et al. Splenectomy and thrombosis: The case of thalassemia intermedia. J Thromb Haemost. 2010; 8: 2152-2158.

18- Cappellini MD, Robbiolo L, Bottasso BM, Coppola R, Fiorelli G, Mannucci AP. Venous thromboembolism and hypercoagulability in splenectomized patients with thalassaemia intermedia. Br J Haematol. 2000; 111: 467-473.

19- Taher AT, Musallam KM, Nasreddine W, Hourani R, Inati A, Beydoun A. Asymptomatic brain magnetic resonance imaging abnormalities in splenectomized adults with thalassemia intermedia. J Thromb Haemost Jan. 2010; 8 (1): 54-9.

20- Gillis S, Cappellini MD, Goldfarb A, Ciceri L, Fiorelli G, Rachmilewitz EA. Pulmonary thromboembolism in thalassemia intermedia patients. Haematologica. 1999; 84: 959-960.

21- Taher AT, Mussallam KM, Karimi M, et al. Overview on practices in thalassemia intermedia management aiming for lowering complication rates across a region of endemicity: The optimal care study. Blood. 2010; 115 (10): 1886-1892.

22- Freikman J, Amer I, Ringel E, Fibach A. Flow cytometry approach for quantitative analysis of cellular phosphatidylserine distribution and shedding, Anal Biochem. 2009; 393: 111-116.

23- Villagra J, Shiva S, Hunter LA, Roberto F, Machado MT, Kato GJ. Platelet activation in patients with sickle disease, hemolysis-associated pulmonary hypertension, and nitric oxide scavenging by cell-free haemoglobin. Blood. 2007; 110 (6): 2166-2172.

24- Snedecor G W, Cochran W. Statistical Methods, Eighth Edition, Iowa State University Press. 1989.

25- Ataga KI, Cappellini MD, Rachmilewitz EA. B-thalassemia and sickle cell anaemia as paradigms of hypercoagulability. Br J Haematol. 2007; 139: 3-13.

26- Stein PD, Beemath A, Meyers FA, Skaf E, Olson RE. Deep venous thrombosis and pulmonary embolism in hospitalized patients with sickle cell disease. Am J Med. 2006; 119: 7-11.

27- Ziakas PD, Poulou LS, Rokas GI, Bartzoudis D, Voulgarelis MJ. Thrombosis in paroxysmal nocturnal hemoglobinuria: sites, risks, outcome. Thromb Haemost. 2007; 5: 642-5.

28. Hendrick AM. Auto-immune haemolytic anaemia-a highrisk disorder for thromboembolism. Hematology. 2003; 8: 53-6.

29- Karimi M, Bagheri H, Rastgu F, Rachmilewitz EA. Magnetic resonance imaging to determine the incidence of brain ischaemia in patients with beta-thalassaemia intermedia. Thromb Haemos. 2010; 103 (5): 989-993.

30- Franchini M, Mannucci PM. Hypercoagulability in congenital haemolytic anaemias. Blood Transfusion. 2012; 10: 423-427.

31- Zwaal RF, Schroit AJ. Pathophysiologic implications of membrane phospholipids asymmetry in blood cells. Blood.1997; 89: 1121-1132.

32- Borenstain-Ben Yashar V, Barenholz Y, Hy-Am E, Rachmilewitz EA, Eldor A. Phosphatidylserine in the outer leaflet of red blood cells from b-thalassemia patients may explain the chronic hypercoagulable state and thrombotic episodes. Am J Hematol. 1993; 44: 63-65.

33- Chen S, Eldor A, Barshtein G, Zhang S, Goldfarb A, Rachmilewitz E, Yedgar S. Enhanced aggregability of red blood cells of beta-thalassemia major patients. Am J Physiol. 1996; 270: 1951-1956.

34- Setty BN, Kulkani S, Stuart MJ. Role of erythrocyte phosphatidylserine in sickle red cell-endothelial adhesion. Blood. 2002; 99: 1564-71.

35- Leventis PA, Grinstein S. The distribution and function of phosphatidylserine in cellular membranes, Annu Rev Biophys. 2010; 39: 407-427.

36- Eldor A, Rachmilewitz EA. The hypercoagulable state in thalassemia. Blood. 2002; 99: 36-43.

37- Borgna Pignatti C, Carnelli V, Caruso V, Dore F, De Mattia D, Di Palma A, Di Gregorio F, Romeo MA, Longhi R, Mangiagli A, Melevendi C, Pizzarelli G, Musumeci S. Thromboembolic events in beta thalassemia major: An Italian multicenter study. Acta Haematol. 1998; 76-79.

38- Borgna-Pignatti C, Rugolotto S, De Stefano P, Zhao H, Cappellini MD, Del Vecchio GC, Romeo MA, Forni GL, Gamberini MR, Ghilardi R, Piga A, Cnaan A. Survival and complications in patients with thalassemia major treated with transfusion and deferoxamine. Haematologica. Oct. 2004; 89 (10): 1187-93.

39- Taher A, Isma'eel H, Mehio G, Bignamini D, Kattamis A, Rachmilewitz EA, Cappellini MD. Prevalence of thromboembolic events among 8,860 patients with thalassaemia major and intermedia in the Mediterranean area and Iran. Thromb Haemost. 2006; 96: 488-91.

40- Atichartakarn V, Angchaisuksiri P, Aryurachai K, Onpun S, Chuncharunee S, Thakkinstian A, Atamasirikul K. Relationship between hypercoagulable state and erythrocyte phosphatidylserine exposure in splenectomized haemoglobin E/beta-thalassaemic patients. Br J Haematol. 2002; 118: 893-898.

41- Mannucci PM. Red cells playing as activated platelets in thalassemia intermedia. J Thromb Haemost. 2010; 8: 2149-2151.