

# Impact of activated monocyte and endothelial dysfunction on coagulopathy in Egyptian adult beta thalassemic patients

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## Abstract

The mechanism of the well observed hypercoagulability and high incidence of Thromboembolic Events (TE) in  $\beta$ -thalassemia patients has not been fully elucidated. This study aimed to evaluate endothelial dysfunction and monocyte activation among adult Egyptian  $\beta$ -thalassemic patients and assess their role in the hypercoagulability and development of TE. A total of 40 adults patients with  $\beta$ -thalassemics and 20 healthy age and sex-matched controls were assessed for endothelial dysfunction using serum Von Willebrand Factor Antigen (VWF:Ag) and for monocytic activation using flow cytometric assessment of CD14 monocyte microparticles and CD11b activated monocytes. The VWF:Ag level was significantly higher among thalassemic patients ( $P < 0.001$ ) and was positively correlated to development of TE ( $P < 0.05$ ). There was no significance difference for CD14 between patients and controls ( $P > 0.5$ ) and CD11b was higher in controls ( $P = 0.004$ ) with no significant correlation between both and TE development ( $P > 0.05$ ). VWF:Ag is increased in thalassemic patients and could be used as a risk factor for thrombosis in these patients, while no identified role of activated monocytes in thrombotic tendency in such patients.

## Introduction

Beta-thalassaemia is a monogenic inherited hemoglobin disorder leading to reduced or absent synthesis of the beta-globin subunit of adult haemoglobin.<sup>1</sup> It is characterized by ineffective erythropoiesis, haemolytic anaemia, and subsequent clinical complications that require Life long disease management.<sup>2</sup>

The survival and quality of life of patients with beta-thalassaemia in developed

countries have improved markedly in recent decades as a result of regular blood transfusions and compliance with tight iron chelation therapy. However,  $\beta$ -thalassemia patients, still suffer from a series of serious complications of their chronic disease, including hypercoagulability and thromboembolic events which lead to significant morbidity and mortality in  $\beta$ -thalassaemia.<sup>3,4</sup>

The molecular and cellular mechanisms contributing to hypercoagulable states in thalassaemia are diverse and have not been fully elucidated. It is considered multifactorial with numerous factors have been considered to be involved in, such as endothelial cell dysfunction, abnormal RBCs due to changes in membrane phospholipid asymmetry of RBCs and activation of other blood cells, including monocytes, granulocytes and chronic platelet activation.<sup>5</sup>

The endothelium is a key regulator of vascular homeostasis. Activated endothelial cells tend to prothrombotic properties through the imbalance in expression of anticoagulant and procoagulant factors, adhesion molecules and proinflammatory cytokines.<sup>6,7</sup> It is hypothesized that monocytes are activated and plays a role in coagulopathy in  $\beta$ -thalassemia by activating endothelium.<sup>8</sup>

Multiple pathways for monocyte activation in  $\beta$ -thalassemia have been suggested such as the presence of increased number of RBC microparticles, RBC-derived hemoglobin and heme-derived oxidants and may be *in vivo* activated endothelium that primes the monocyte.<sup>9</sup>

Microparticles (MP) are intact vesicles derived from cell membranes which arise mainly through cell membrane activation processes and from apoptosis.<sup>10</sup> Monocyte Microparticles (MMPs) have vital role in inflammation, blood coagulation, and endothelial cell functions. Identification of monocytic Microparticles (mMPs) is based on phosphatidylserine and CD14 expression on their surface.<sup>11</sup> When activated monocytes conjugate with platelets, the latter activates expression of CD11b/CD18 (macrophage-1 antigen, Mac-1) on monocytes which amplifies its interactions with platelets via fibrinogen bivalent linking Mac-1 with platelet glycoprotein IIb/IIIa. Expression of Mac-1 mediate the adhesive properties of leukocytes and accelerate their shedding of thrombogenic microparticles.<sup>12</sup> The objective of this study was to evaluate the endothelial dysfunction and monocyte activation among adult Egyptian  $\beta$ -thalassemic patients and assess their role in the hypercoagulability and development of Thrombotic events in such patients by assessment of Von Willebrand Factor

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Contributions: HA and NN designed the research, collected the clinical data, carried out the data analysis and wrote the manuscript. NB performed the laboratory work and immunophenotyping. All authors revised the manuscript, approved the final version of the article for submission and agree to be accountable for all aspects of the work.

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Antigen (VWF:Ag) as endothelial activation marker and flow cytometric analysis of CD14 circulating monocytic Microparticles (mMps) and CD11b activated monocytes.

## Materials and Methods

### Subjects

The current case control study was conducted in the Adult Clinical hematology Division, Internal medicine Department, Ain Shams University Hospitals, Cairo, Egypt. 40 adult  $\beta$ -thalassemia patients were recruited from clinical hematology outpatient clinic during their routine follow up between October 2018 and March 2019. Twenty age and sex matched healthy volunteers were enrolled as a control group. All included patients were subjected to detailed medical history and thorough clinical examination with special emphasis on disease duration, history of splenectomy, viral hepatitis, transfusion history (age of starting transfusion, frequency of transfusion with calculation of transfusion index, detailed chelation therapy, compliance to treatment including chelation therapy and history of any recent or past arterial or venous Thromboembolic events. All patients were subjected to the routine diagnostic work-up for hemolytic anemia including (CBC, reticulocytic count, LDH, and hemoglobin electrophoresis), liver function tests and renal function tests. Flow cytometric assessment of CD14 and CD11b as markers of monocytes activation and plasma VWF:Ag assay. Patients who had any of the following conditions were excluded from the study: Thalassaemic patients other than  $\beta$ -thalassaemia, Thalassaemic patients who are on long term anticoagulation or antiplatelets or have end stage kidney or liver disease, and Thalassaemic patients who have other risk factors of thrombosis as pregnancy, diabetes and smoking. All participants were informed about the objectives and procedures of the study and written informed consents were obtained prior to enrollment and the procedures applied in this study were approved by the Ethical Committee of the Ain Shams University hospitals and are in accordance with the Helsinki Declaration of 1975.

### Methods

A volume of 6mL of Peripheral venous blood was collected and divided. The first 2mL was collected on a tube Ethylene diamine tetra-acetate (EDTA) (2.2mg/mL), for for complete blood count (CBC), preparation of Leishman stained PB smears, reticulocyte count and Hb electrophoresis.

Another 2mL of PB on sterile K2-EDTA was collected for CD 14 and CD11b analysis using Beckman Coulter flowcytometry (Beckman Coulter, Brea, CA) at flow cytometry laboratory in faculty of medicine Ain Shams University and 2mL of blood was collected with 3.8% sodium citrate as anticoagulant to measure VWF:Ag level. Quantitative VWF:Ag assay was performed on a Sysmex CA-1500 coagulation analyzer (Siemens Healthcare Diagnostics, Marburg, Germany) with a normal range between 50% and 150% at immunology laboratory in faculty of medicine Ain Shams University.

### Statistical analysis

Data were analyzed using Stata® version 14.2 (StataCorp LLC, College Station, TX, USA). Normality of numerical data distribution was examined using the Shapiro-Wilk test. Non-Normally distributed numerical data were presented as median and interquartile and intergroup differences were compared using the Wilcoxon rank sum test (for two-group comparison) or the Jonckheere-Terpstra trend test (for comparison of multiple tanked grouped). Categorical data were presented as ratio or number and percentage and intergroup differences were compared using Fisher's exact test (for nominal data) or the chi-squared test for trend (for ordinal data). Correlations among numerical variables were tested using the Spearman rank correlation. 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.

## Results

Forty  $\beta$ -thalassaemic patients, including 23 (57.5%) males and 17 (42.5%) females with a male to female ratio of 1.2:1 and an age range of 20-26 years, with a mean age of (23 $\pm$ 4) years. Thirteen patients presented with thalassaemia major (32.5%), twenty two with thalassaemia intermediate patients (55.0%) and five patients with thalassaemia minor (12.5%). Splenectomy was done to eleven (27.5%) of these patients, only fifteen (37.5%) of patients were compliant to Iron chelation therapy and seven patients (17.5%) gives history of thromboembolic events. While the healthy control group, they were 11(56.7%) male and 9 (43.3%) female with a male to female ratio of 1.2:1. Their ages ranged from 21-28 years, with a mean of age 23.35 $\pm$ 4.61 years. Demographic and clinicolaboratory data of patients' group are shown in Table 1. On comparing the studied adult beta thalassaemia patients and the healthy controls, they were age and sex

matched while their laboratory results revealed that thalassaemia patients have statistically significant high increase in the level of platelets and WBCs than control with p-value  $<0.001$  and  $<0.001$  respectively also Liver enzymes SGPT&SGOT were significantly higher in thalassaemia patients as p-value =0.008 and 0.004 (Table 2).

### Comparison of plasma VWF Ag, CD14 and CD11b among patients with $\beta$ thalassaemia and healthy control

Patients with  $\beta$  thalassaemia had significantly higher VWF:Ag levels than that of controls ( $P<0.001$ ), while CD11b level was significantly lower in patients than controls ( $P=0.004$ ) however no statistically significant difference was found in CD14 level between patients and healthy controls ( $P>0.5$ ) (Table 2, Figure1).

While upon comparing VWF:Ag, CD14,CD11b levels between the different types of  $\beta$  thalassaemia there was no statistically significant difference between the different types ( $P= 0.45, 0.80, 0.56$  respectively).

### Relation between VWF:Ag, CD14 and CD11b and different laboratory characteristics of patients with thalassaemia

No significant correlations were found between VWF:Ag and CD14 or CD11b while a significant positive correlation was found between CD14 level and CD11b level ( $\rho=0.443, p=0.004$ ) and a significant

**Table 1. Demographic and clinical data of studied thalassaemia patients.**

Variable	Value
Age (years)	23 $\pm$ 4
Gender	
M	23 (57.5%)
F	17 (42.5%)
Type of thalassaemia	
Thalassaemia minor	5 (12.5%)
Thalassaemia intermedia	22 (55.0%)
Thalassaemia major	13 (32.5%)
Iron chelation therapy	15 (37.5%)
Splenectomy	11 (27.5%)
SGPT (IU/l)	52 (31–76)
SGOT (IU/l)	51 (31–73)
Urea (mg/dl)	25.5 $\pm$ 4.2
Creatinine (mg/ml)	0.7 $\pm$ 0.2
Hemoglobin (g/dl)	6.7 $\pm$ 1.6
WBCs (x1000/mm <sup>3</sup> )	9.1 (6.6–13.7)
Platelets (x1000/mm <sup>3</sup> )	449 (303–720)
vWFA (IU/dl)	3.98 (1.65–6.00)

Data are mean  $\pm$  SD, number (%) or median (interquartile range).

negative correlation was observed between CD11b and both platelets count ( $\rho=0.399$ ,  $P=0.01$ ) and White blood cell count ( $\rho=0.368$ ,  $P=0.02$ ).

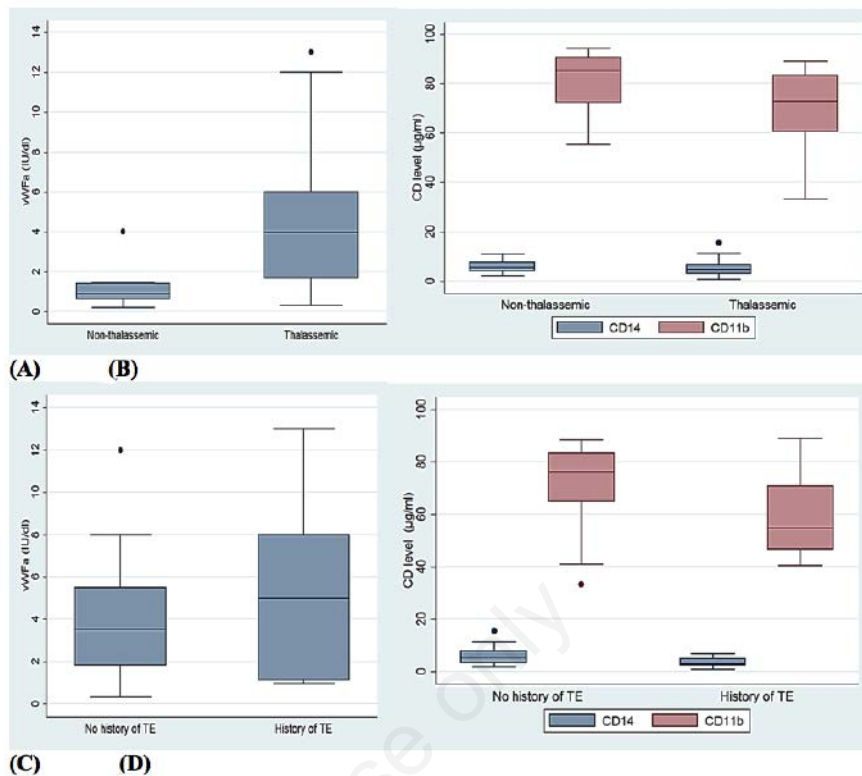
### Relation between different clinico-pathological characteristics of thalassemia patients and the development of thrombotic events

The current study results suggest that splenectomy could be considered a significant risk factor for thrombosis as 71.4% of patients who developed TE, had been splenectomized with  $p$ -value=0.01, there is a statistically significant relation between splenectomy and Thromboembolism (TE) with an odds ratio of 11.25 (95% CI=1.75 to 72.50,  $p$ -value=0.011). On the other hand compliance to iron chelator therapy had no statistically significant impact on the development of thrombotic event ( $P=0.6$ ) (Table 3). The data of the present study confirmed that Platelet count is a significant risk factor for TE development as ( $p$ -value<0.001) (Table 4).

Receiver-Operating Characteristic (ROC) curve analysis was used to determine the cut off value of platelet count, vWFAg, CD14 or CD11b for development of thrombotic events. When assessing the best cut off value of platelet count in discriminating thalassemic patients who are at high risk for development of thrombotic events the area under the curve (AUC) was.924, suggesting that Patients with platelets count  $>770,000/\text{mm}^3$  are at high risk for TE development with a sensitivity of 85.7% and specificity of 90.9%

### Relation between vascular dysfunction marker, activated monocytes and monocyte microparticles and the development of thrombotic events of thalassemia patients

The present study revealed that VWF:Ag level which is a marker of vascular endothelial dysfunction, is a good marker for TE development in the studied thalassemia patients as it has a statistically significant positive correlation with development of TE ( $P<0.05$ ) and the discriminative value of vWF level was good (AUC=0.871). Patients with vWFA level  $>7$  IU/dL are considered at high risk for TE development with a sensitivity of 42.9% and specificity of 93.9%. On the other hand this study revealed that both CD11b level as a marker of activated monocytes and CD14 level (expressed on monocyte microparticles) are not considered as risk factor for development of TE in thalassemic patients as no significant correlation was found between both of them and development of TE ( $p$ -



**Figure 1.** Box plot A showing the vWFA level in thalassemic patients and non-thalassemic controls, B showing the CD14 and CD11b level in thalassemic patients and non-thalassemic controls, C showing the vWFA level in thalassemic patients with or without history of (TE), D showing the CD14 and CD11b level in thalassemic patients with or without history of (TE).

**Table 2.** Comparison between studied thalassemia patients and healthy control.

Variable	Control (n=20)	Thalassemia (n=40)	p-value
Gender (M/F)	11/9	23/17	1.000 <sup>¶</sup>
Age (years)	25 (21–28)	21 (20–26)	.0800 <sup>§</sup>
SGPT (IU/l)	32 (26–40)	52 (31–76)	.008 <sup>§</sup>
SGOT (IU/l)	30 (23–38)	51 (31–73)	.004 <sup>§</sup>
Urea (mg/dL)	25.5 (20.5–29.0)	26.0 (22.0–29.0)	.620 <sup>§</sup>
Creatinine (mg/mL)	0.7 (0.5–0.8)	0.7 (0.6–0.8)	.173 <sup>§</sup>
Hemoglobin (g/dl)	13.1 (12.0–13.9)	6.7 (5.5–7.9)	<.001 <sup>§</sup>
WBCs (x1000/mm <sup>3</sup> )	5.69 (5.35–7.80)	9.10 (6.60–13.65)	.002 <sup>§</sup>
Platelets (x1000/ml <sup>3</sup> )	266 (213–298)	449 (303–720)	<.001 <sup>¶</sup>
vWFA (IU/dL)	0.89 (0.61–1.40)	3.98 (1.65–6.00)	<.001 <sup>¶</sup>
CD14 (µg/mL)	5.43 (4.04–7.58)	4.80 (2.82–6.77)	.204 <sup>¶</sup>

value>.05) (Table 4, Figure 1).

**Table 3.** Correlation between demographic and clinical variables and the development of thrombotic events in thalassemic patients.

Variable	No history of TE (n=33)	History of TE (n=7)	p-value
Gender (M/F)	19/14	4/3	1.000 <sup>¶</sup>
Age (years)	22 (20–26)	20 (19–21)	.217 <sup>§</sup>
Type of thalassemia			.095 <sup>#</sup>
Thalassemia minor	5 (15.2%)	0 (0.0%)	
Thalassemia intermedia	19 (57.6%)	3 (42.9%)	
Thalassemia major	9 (27.3%)	4 (57.1%)	
Splenectomy	6 (18.2%)	5 (71.4%)	.011 <sup>¶</sup>
Iron chelation therapy	13 (39.4%)	2 (28.6%)	.691 <sup>¶</sup>

Data are ratio, median (interquartile range) or number (%). <sup>¶</sup>Fisher's exact test. <sup>§</sup>Wilcoxon rank sum test.,<sup>#</sup>Chi-squared test for trend test.



## Discussion

Currently hypercoagulable state is a well described complication in  $\beta$ -thalassemia and is associated with increased risk for thromboembolic complications in such patients however, the underlying mechanisms has not been fully elucidated.<sup>13</sup>

$\beta$ -thalassemia is a common hematologic disorder in the Mediterranean basin, parts of North and West Africa, the Middle East, India, the Southern Far East, and Southeast Asia; these areas make up the so-called thalassemia belt.<sup>14</sup> Particularly in Egypt,  $\beta$ -thalassemia has been the most common type of hereditary anemia, with a carrier rate of approximately 10%, and it has been estimated that per 1.5 million annual live births, approximately 1000 babies are born with  $\beta$ -thalassemia major however little is known about the clinical associations of markers of hypercoagulability in the Egyptian thalassemia patients.<sup>15</sup>

In the present study, 40 thalassemic patients were evaluated for the presence of vascular endothelial activation using (VWF:Ag) level and for monocyte activation with microparticle formation by detection of CD11b, CD14 expression by flow cytometry analysis and to clarify their role in the hypercoagulability and development of thrombotic event in adult  $\beta$ -thalassemia patients.

The current study revealed significantly higher serum level of VWF:Ag in all thalassemics compared with the control group ( $P<.001$ ) confirming endothelial activation. This goes in agreement with both Adely *et al.*, who have reported higher plasma VWF:Ag levels in patients with  $\beta$ -thalassaemia, particularly those with evident heart disease,<sup>16</sup> and Al-Harbi *et al.*, who showed that VWF:Ag level is increased in most of thalassemic patients even if they

were not splenectomized.<sup>17</sup>

However in the present study, there is no statistically significant difference was found in CD14 level between patients and healthy controls ( $P>.05$ ). Moreover CD11b level, which is a marker of monocytic activation, was significantly lower in patients than controls ( $P=0.004$ ), these results were in agreement with both. Kheansaard *et al.*, who have studied the origin and the effect of circulating microparticles in thalassemic and reported that the increased microparticles in thalassemia are not monocytic derived and confirmed that other types of MPs play an important role on thrombosis and vascular dysfunction in  $\beta$ -thalassaemia,<sup>18</sup> and Elsayeh *et al.*, who reported an increase in the total MPs in thalassemic patients who had previous history of thrombosis or pulmonary hypertension, however monocytic microparticles did not increase.<sup>19</sup> This finding was explained by Slater *et al.*, who reported that iron overload in thalassemic patients can modulate monocytic functions and activation through inhibition of TLR4 (toll-like receptor 4) which is responsible for monocytes activation by TLR4 -endothelial signaling.<sup>20,21</sup> The results of another studies were in contrast to this finding, as a recent study by Ghozali *et al.*, who reported increased CD14<sup>+</sup> monocytes in  $\beta$ -thalassaemia and highlighted its major role in the state of chronic inflammation in such patients.<sup>22</sup> This discrepancy in the results of different studies dealing with  $\beta$ -thalassemia patients, may be because of the heterogeneity of the studied  $\beta$ -thalassemia patients in their genotype, age, frequency of transfusions, and iron overload.

In the present study, the history of thrombotic event was found in 17.5% of the studied adult patients with  $\beta$ -thalassemia, which is in line with many previous studies confirming the presence of hypercoagulable state of  $\beta$ -thalassemia.<sup>23,24</sup>

The current study suggest that both Splenectomy and platelet count could be considered as risk factors for thrombosis p-value=0.01 and <0.001 respectively and 71.4% of patients who developed TE had been splenectomized. Karimi *et al.*, were in agreement with the current study, they found that older age and splenectomized multi transfused  $\beta$ -thalassemic patients even with normal platelet count have a higher incidence of Silent Cerebral Ischemia and the effect of splenectomy is more significant in statistical analysis.<sup>25</sup> Merchant *et al.*, explained that splenectomy increases the hypercoagulability, by permitting the circulation of greater numbers of altered membranes cells, and by increasing the number of Platelet.<sup>26</sup> The results of this study suggest that thalassemic patients with platelets count >770,000/mm<sup>3</sup> are at higher risk for TE development than other patients with less platelets count AUC=0.924 with a sensitivity of 85.7% and specificity of 90.9%. Further more the present data revealed that VWF:Ag level, which is a marker of vascular endothelial dysfunction, is a good marker for TE development ( $P<.05$ ) and suggested that patients with vWFA level >7IU/dL are considered at higher risk for TE development than other thalassemia patients with a sensitivity of 42.9% and specificity of 93.9%. This findings were in agreement with many previous studies which confirm that the contributing role of vascular endothelial dysfunction in the hypercoagulable state and thrombotic complication, the most recent one by Poredos *et al.*, who reported that endothelial dysfunction was a particular characteristic of patients with unprovoked VTE and that impairment of endothelial function in patients with spontaneous VTE was significantly associated with higher levels of markers of endothelial activation in the plasma (eg, vWF and soluble P-

**Table 4. Relation between laboratory variables, vWFa, CD14 and CD11b and the development of TE in thalassemic patients.**

Variable	No history of TE (n=33)		History of TE (n=7)		p-value <sup>¶</sup>
	Median	interquartile range	Median	Interquartile range	
SGPT (IU/L)	40	31–7070	51–90	0.226	
SGOT (IU/L)	50	31–6557	48–110	0.200	
Urea (mg/dL)	26.0	21.0–29.0	25.0	25.0–27.0	0.900
Creatinine (mg/mL)	0.7	0.6–0.80.7	0.6–0.8	0.704	
Hemoglobin (g/dL)	6.7	5.8–0.85.6	5.2–7.0	0.219	
WBCs (x1000/mm <sup>3</sup> )	8.70	6.40–12.00	15.00	8.00–38.00	0.139
Platelets (x1000/mm <sup>3</sup> )	400	300–520	949	800–1000	<0.001
vWFa (IU/dL)	3.50	1.80–5.50	5.00	1.10–8.00	<0.05
CD14 ( $\mu$ g/ml)	5.15	3.10–7.86	2.80	2.33–4.91	0.067
CD11b ( $\mu$ g/mL)	76.20	64.80–83.50	54.90	46.50–70.90	0.075

Data are median and interquartile range (interquartile range), <sup>¶</sup>Wilcoxon rank sum test.

selectin).<sup>27</sup> On the other hand the present study revealed that both CD11b level as a marker of activated monocytes and CD14 level (expressed on monocyte microparticles) are not considered as risk factor for development of TE in thalassemia patients as no significant correlation was found between both of them and development of TE (p-value>0.05) which may be attributed to iron overload state.

## Conclusions

In conclusion, our results indicated that VWF:Ag is increased in Egyptian  $\beta$ -thalassemic patients and could be used as a surrogate marker for thrombotic tendency in these patients. It also seems reasonable to add assessment of VWF:Ag to the follow up laboratory investigations for thalassemic patients while no identified role of activated monocytes in hypercoagulability and thrombotic tendency in thalassemic patients. Establishment of thrombosis risk assessment model specific for thalassemics, including platelets count, splenectomy, VWF:Ag and other biological parameters to identify high risk thalassemic patients for thrombosis, is highly warranted for establishment of specific proper prophylactic antithrombotic plane for such patients.

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