Evaluation of coagulation parameters and impact of transfusion on coagulation in patients with beta thalassemia major

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There have been several studies that have shown that patients with beta thalassemia major are at a higher risk of thrombosis due to the procoagulant activity of thalassemic erythrocytes, decreased liver synthetic function, increased platelet activity and vascular endothelial activation attributed to chronic oxidative stress, although there are no established tests to predict thrombotic risk in TM patients. In this study, we evaluated whether or not the platelet function analyser (PFA-200) and thrombin generation test (TGT) would be useful tools to identify hypercoagulability and risk of thrombosis in thalassemia major patients. The study included 50 patients with thalassemia major and 104 healthy control group. Pretransfusion and posttransfusion PFA-200 and TGT results were compared with control group. We found that median C/ADP and C/EPI values in the thalassemia major group were greater in both the pre and posttransfusion samples than the C/ADP and C/EPI results from the control group. The TGT results showed there was no difference between control group and the results from the thalassemia major group. The TGT and PFA-200 testing did not identify hypercoagulability nor identify clear testing parameters to predict a thalassemia major patient's risk of thrombosis. There may be other mechanisms/causes yet unidentified that could better explain thalassemia major patient's increased risk from thromboembolic events. *Blood Coagul Fibrinolysis* 33:266–271

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Introduction

Improvements in the medical care of patients with thalassemia major have resulted in an increased life expectancy. Most thalassemia major patients are transfusion-dependent placing them at risk of iron accumulation, especially in the heart, liver and endocrine organs, ultimately resulting in a variety of serious health complications [1]. In addition to the long-term complications of iron accumulation, several studies revealed that these patients might also be at a higher risk of thrombosis due to procoagulant activity of thalassemic erythrocytes, decreased synthesis of liver dependent anticoagulant proteins, increased platelet and thrombin activation via negatively charged cell membrane phospholipids in response to chronic hypoxia [2–5].

Although increased coagulability could be the main reason for thrombosis, there is no prevalent test to predict thrombosis risk in patients with thalassemia major.

Recently, more sophisticated coagulation assays such as platelet function analyser (PFA) and thrombin generation test (TGT) have been regularly used to detect abnormalities in the coagulation system [6,7]. In this study, we aimed to evaluate whether the PFA-200 and TGT would reveal a hypercoagulable state in thalassemia major patients that could be used to predict the risk of thrombosis. A secondary aim of this study was to determine the impact that routine transfusions had on thalassemia major patients' coagulation parameters.

Material and methods

Patient selection

The study group comprised of patients with beta-thalassemia major (TG), whereas the control group consisted of healthy volunteers (Control Group Total; CG-T). In order to decrease the amount of blood drawn from volunteers, the CG-T was composed of two subgroups: Control Group-PFA (CG-PFA) and Control Group-TGT (CG-TGT). We stratified the CG-PFA and CG-TGT volunteers according to their age and ABO blood groups. The coagulation profiles (prothrombin time, activated partial thromboplastin time (apt) and fibrinogen levels) and biochemical tests (aspartate aminotransferas, alanine aminotransferease, total and direct bilirubin, total protein, albumin, blood urea nitrogen, creatinine) were studied in the TG before transfusion as part of pretransfusion checklist. Exclusionary criteria for both the TG and CG included a history of

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bleeding diathesis or previous thrombosis, use of anticoagulants, antiplatelet or NSAIDs, or the presence of an infectious disease currently or during the previous month. After completion of this study on 2018, we retrospectively reviewed the patient data for any history of bleeding or thrombosis in the proceeding 3 years. This study was approved by the Ankara Child Health and Pediatric Hematology Oncology Hospital ethical committee (10.02.2016/40).

Eligibility in the study required informed consent from either the patient or the patient's legal guardian for those under 18 years old.

Collection of blood samples and routine testing

After obtaining written informed consent, venous blood samples were collected before and 4 h after the start of scheduled RBC transfusions in thalassemia major patients. The patient blood samples were only obtained once from the CG volunteers. Of note, the CG did not get RBC transfusions. CG-PFA group served as control for the complete blood count, coagulation profile and biochemical tests, and the CG-TGT group results served as the control for the TGT. Complete blood counts were performed on the Beckman Coulter LH780 (Beckman Coulter LH780 System; Beckman Coulter, Inc. Brea, California, USA), biochemical testing on the Beckman Coulter AU580 (Beckman Coulter AU580 System; Beckman Coulter, Inc.) and coagulation testing (aPTT, PT, INR) on the ACL top (ACL top 500 CTS Bedford, Massachusetts, USA). All testing performed on the devices mentioned above was done in accordance with the testing institutions established routine operational protocols.

Platelet function analyser test

The PFA-200 device (Innovance PFA-200 system; Siemens Healthcare Diagnostics, Marburg, Germany) with reference ranges for collagen/epinephrine aperture-closing time was 84–160 s, and for collagen/ADP was 68–121 s, was used for analysis.

Thrombin generation test

Standardization of TGT has been performed according to the protocol by Dargaud *et al.* [8]. Patient samples were moved to the laboratory in an upright position in a rack, without shaking, within 1 h of collection. Samples were subjected to a two-step centrifugal process at room temperature, in order to obtain platelet-rich and platelet-poor plasma, respectively. The samples were frozen at -80 C° until time of testing, prior to testing, samples were water bath thawed at 37 C°. A calibrated automated thrombogram thrombinoscope device was used for analysing thrombin generation. Twenty microliter thrombin calibrator was added to 80 μl plasma. After 15 min of incubation at 37°C, 20 μl buffer containing a fluorogenic substrate was added to 20 μl of mixture and reaction was monitored with flourometer. Thrombogram curve was drawn by thrombinoscope programme; lag time, endogenous thrombin potential (ETP) peak height (PEAK) and time to PEAK height (ttPEAK) were calculated. In this test, ETP is a measure of the amount of thrombin generated after in-vitro activation with tissue factor and phospholipid and denotes thrombin concentration over time. Lag time is the amount of time that it takes 10 nmol/l thrombin to generate a curve.

Data analysis

Statistical analysis was performed by using the Statistical Package for Social Sciences (SPSS) programme (by IBM SPSS Inc., Chicago, Illinois, USA). Normal distribution of variables was analysed with Kolmogorov–Smirnov test. Parametric tests were used for normally distributed variables, and all results were expressed as means ± standard deviations. If variables were not normally distributed, in keeping with the nonparametric tests, median values were reported with confidence intervals. Median scores of all the unpaired groups were compared with the nonparametric Mann–Whitney *U*-test. The Student's *t*-test was used for unpaired data for parametric variables, and the Chi-square and Fisher exact test were used for frequencies. Before and after transfusion comparisons were performed with mixed model for repeated measures analysis. The association between numeric variables was analysed with Spearman correlation test. Diagnostic performance of TGT and PFA-200 was determined with ROC curve analysis. Cut-points were identified with Youden index. For statistical analysis, *P* value less than 0.05 was accepted as significant.

Results

A total of 50 patients with thalassemia major were included in the TG [median age 12.3 (2.2–19.9) years with a male/female ratio of 1/1.6]. One hundred and four CG-T volunteers [median age 11.1 (1–23) years with male/female ratio of 1/1] were further subdivided into CG-PFA and CG-TGT groups with 54 and 50 healthy children, respectively. Demographic data demonstrated no statistical difference between TG and the CG. PFA-200 testing was carried out in 49 of the 50 enrolled TG patients and TGT was carried out in 29 of the 50 enrolled TG patients.

Complete blood count, biochemical and routine coagulation assays

In the thalassemic patients, the pretransfusion CBC results were compared to the posttransfusion results. The comparison revealed that haemoglobin (Hb), haematocrit (Hct), red blood cell (RBC), white blood cell (WBC), mean corpuscular volume (MCV) and red blood cell distribution width (RDW) values all significantly improved with transfusion, whereas posttransfusion platelet counts were often significantly lower than the pretransfusion levels. (Table 1). The comparison between the posttransfusion TG data with the nontransfused CG-PFA group revealed that the transfused thalassemia major patients' Hb, Hct and RBC counts were significantly lower with conversely

Table 1 Comparison of complete blood count results of patient and control group

	Pretransfusion, patient ($n=49$)	P^1 (Pre vs. posttransfusion)	Posttransfusion, patient ($n=49$)	P^2 Posttransfusion vs. control	Control group ($n=54$)	P^3 Pretransfusion vs. control
Hb (g/dl)	9.5 ± 0.8 (6.7–10.7)	<0.001	12.4 ± 1 (10.7–14.8)	<0.001	13.4 ± 1.4 (11.5–17.2)	<0.001
Htc (%)	28 ± 3.0 (19.1–34)	<0.001	36.6 ± 2.9 (31.5–44.4)	<0.001	40.5 ± 4.2 (33.1–51.1)	<0.001
MCHC (g/dl)	34 ± 0.8 (32.2–36.4)	0.571	33.9 ± 0.7 (31.9–34.9)	<0,001	33.1 ± 0.6 (31.6–35)	<0.001
MCV (fL)	79.6 ± 2.8 (73.1–84.6)	<0.001	81.7 ± 2.6 (75.4–88.4)	0.626	81.3 ± 4.9 (70.5–91.9)	0.034
EC ($10^6/\mu l$)	3.5 ± 0.4 (2.61–4.2)	<0.001	4.9 ± 0.4 (3.82–5.26)	<0.001	5 ± 0.5 (4.27–6.17)	<0.001
RDW (%)	15.1 ± 2.9 (0.4–26)	0.186	15.5 ± 1.5 (13.3–22.6)	<0.001	14.2 ± 1.4 (11.4–18.9)	<0.001
WBC (10^3 /μl)	9.3 (3.6–23.4)	0.010	10.1 (2.4–25.7)	<0.001	7.5 (4.1–14,9)	0.002
PLT ($10^3/\mu l$)	375 (163–872)	<0.001	316 (125–641)	0.900	300 (165–549)	0.007
PDW (%)	16.9 ± 1.1 (14.9–18.9)	0.388	17 ± 1.2 (14.4–19.3)	<0.001	16.2 ± 0.6 (13.5–18.1)	0.002
MPV (fL)	9.1 ± 1.1 (7.1–11.9)	0.413	9.2 ± 1.4 (7.2–14.2)	<0.001	8.1 ± 0.7 (6.8–9.9)	<0.001

Normally distributed variables were shown with mean ± standard deviation, nonnormally distributed variables were shown with median (minimum – maximum). EC, erythrocyte count; Hb, haemoglobin; Htc, haematocrit; MCHC, mean erythrocyte haemoglobin concentration; MCV, mean erythrocyte volume; MPV, mean platelet volume; PDW, platelet distribution width; PLT, platelet; RDW, red cell distribution width; WBC, white blood cell. $P<0.05$ reveals statistical significance.

significantly higher MCHC, RDW, PDW, MPV values and WBC counts.

Biochemical studies revealed significantly higher aspartate aminotransferas, alanine aminotransferease, total and direct bilirubin, blood urea nitrogen, ferritin and fasting glucose levels and significantly lower total protein levels in the pretransfusion TG than in the control group.

Comparison of PT, INR, aPTT values and fibrinogen levels between the TG and the CG-PFA group revealed that the TG group had PT, INR and aPTT values that were significantly longer and significantly lower fibrinogens levels.

PFA-200 test results

PFA-200 was studied in 49 of the thalassemia major patients who gave informed consent. The TG ($n=49$) PFA-200 results revealed that the pretransfusion C/ADP and C/EPI values were not significantly different from the posttransfusion results. Of note, both the pre and posttransfusion C-ADP and C-EPI values were significantly longer than the those in C-PFA group (Table 2). There was a higher frequency of patients with prolonged C/ADP or C/EPI values in the pre and posttransfusion TG group than the C-PFA group (Table 2).

When we compared pretransfusion and posttransfusion C/ADP and C/EPI values of the group O-blood TG patients with the nongroup O-blood TG patients, all closure times were longer in the group O-blood patients. In addition, the differences between C/ADP and C/EPI aperture-closing times were consistently longer in the group O-blood control group than the nongroup O-blood control group.

There were no significant differences in the total number of group O-blood individuals between the TG and C-PFA groups (27/49 in the TG and 22/54 in the CG-PFA, $P>0.05$).

Thrombin generation test results

Thrombin generation testing was performed on 29 out of 49 thalassemia major patients who gave informed consent. The comparison of the thalassemia major patients' pre and posttransfusion TGT results (lag time, ETP, PEAK and ttPEAK) did not reveal any differences between the platelet-poor or platelet-rich plasma samples. There were also no discernible differences between the pre and posttransfusion TGT results of the thalassemia major patients and the CG-TGT group, with either platelet-poor or platelet-rich plasma (Table 3).

Table 2 Comparison of PFA levels between patients with thalassemia major and control group

		Thalassemia major $n=49$					
PFA-200		Pretransfusion	Posttransfusion	Control group $n=54$	P^1	P^2	P^3
C/ADP (s)	median (min-max)	114 (58–300)	106 (52–300)	92 (55–266)	**<0.001***	0.303	**0.003***
Low	*n* (%)	1(2)	1(2)	4(7.4)	**0.007***	0.808	**0.014***
Normal	*n* (%)	31(63.3)	32(65.3)	44(81.5)			
High	*n* (%)	17(34.7)	16(32.7)	6(11.1)			
C/EPI (s)	median (min-max)	148(86–300)	139(50–300)	126(59–182)	**0.003***	0.159	**0.019***
Low	*n* (%)	–	2(4)	3(5.6)	**0.018***	0.251	**0.048***
Normal	*n* (%)	31(63.3)	32(65.4)	42(77.8)			
High	*n* (%)	18(36.7)	15(30.6)	9(16.7)			

PFA-200 test were not normally distributed. C/ADP, collagen/adenosin-5'-diphosphate; C/EPI, collagen/epinephrine. P^1: Pretransfusion vs. control group (Mann–Whitney *U*-test), P^2: Pretransfusion vs. posttransfusion (mixed model for repeated measures analysis), P^3: Posttransfusion vs. control group (Mann–Whitney *U*-test). *$P<0.05$ reveals statistical significance.

Table 3 Thrombin generation test results

Thrombin generation test		Control ($n=50$)	Thalassemia major ($n=29$) Pretransfusion	Thalassemia major ($n=29$) Posttransfusion	P^1	P^2	P^3
Poor plasma							
Lagtime	mean ± SD	3.3 ± 0.6	3.7 ± 1.1	3.5 ± 0.6	0.060	0.118	0.342
	median (min-max)	3.3 (2–4.7)	3.5 (2.6–7.6)	3.5 (2.5–5.2)			
ETP	mean ± SD	1260.2 ± 267.8	1289.4 ± 314.6	1345.1 ± 503.9	0.996	0.607	0.897
	median (min-max)	1233 (772–2043)	1170 (842–2184)	1287 (605–3191)			
PEAK	mean ± SD	237.5 ± 64.8	282.5 ± 106.9	252 ± 79.3	0.154	0.654	0.230
	median (min-max)	236.8 (109.6–466.8)	249 (133.7–599.5)	227.6 (142.8–496)			
ttPEAK	mean ± SD	6.6 ± 1.1	6.5 ± 1.5	6.9 ± 1.2	0.579	0.321	0.154
	median (min-max)	6.3 (4.3–9.7)	6.3 (4.3–12)	6.9 (5.2–9.9)			
Rich Plasma							
Lagtime	mean ± SD	-	4.7 ± 1.2	4.2 ± 0.9	-	-	0.138
	median (min-max)	-	4.3 (3.3–9)	4 (3–7)	-	-	
ETP	mean ± SD	-	1504.4 ± 618.3	1405.4 ± 517.6	-	-	0.482
	median (min-max)	-	1402 (616–2986)	1264 (761–3086)	-	-	
PEAK	mean ± SD	-	261.3 ± 129.5	264.3 ± 106.6	-	-	0.770
	median (min-max)	-	252.4 (60.2–647.8)	239.4 (80.6–512.1)	-	-	
ttPEAK	mean ± SD	-	8.8 ± 2.4	80 ± 2.1	-	-	0.139
	median (min-max)	-	8 (6–15.6)	7.3 (5–12.3)	-	-	

P^1: Pretransfusion vs. control group (Student test or Mann–Whitney *U*-test), P^2: Posttransfusion vs. control group (Student test or Mann–Whitney *U*-test), P^3: Pretransfusion vs. posttransfusion (repeated measures analysis). SD, standard deviation.

Correlations

There appears to be a positive correlation between ferritin and aPTT level in the TG ($r=0.441$, $P=0.001$). However, there was no significant correlation between ferritin level and the PFA200 or the TGT results. There was also no clear association between the PFA200 test and TGT results. Pretransfusion C/ADP and C/EPI values had a negative correlation with Hb, Hct, RBC values and fibrinogen levels and a positive correlation with MCHC value, and total bilirubin, direct bilirubin, platelet count, albumin and ferritin levels.

Discussion

It is important to consider beta-thalassemia's proclivity towards abnormal coagulation when following these patients [5]. The most common haemostatic challenge and frequent complication for these patients is recurrent epistaxis. Although relatively rare, thalassemia major patients have a higher frequency of arterial and venous thrombosis when compared with the normal population [3,4,9]. Thrombotic complications are more common among patients with thalassemia intermedia, especially after splenectomy. There is also an increased risk for thrombosis in thalassemia major patients who are transfused irregularly [4,10]. A meta-analysis of 720 thalassemia major patients revealed a thrombotic complication rate of 1.1% [11]. Another survey of 8860 patients with β-thalassemia in Iran and the Mediterranean area identified a 4.38 times higher risk of having a thromboembolic event in thalassemia intermedia patients compared with patients with thalassemia major [12]. The study by Taher *et al.* [12] also revealed that patients with thalassemia intermedia generally had venous thrombosis, whereas thalassemia major patients were more prone to arterial thrombosis [12]. Postmortem examination of β-thalassemia patients who had no clinical findings of VTE revealed frequent thromboemboli, indicating that many of these events may have had a subclinical process [13]. However, the underlying mechanisms of hypercoagulation have yet to be elucidated. In general, only conventional coagulation tests have been used to evaluate clotting risk in patients with thalassemia major. Given the dynamic nature of haemostasis and clotting, conventional coagulation testing does not appear to be a sufficient or effective means to detect in-vivo defects in coagulation in thalassemia patients. In this study, we ventured to use dynamic coagulation tests in conjunction with more conventional coagulation assays to see if the combination would yield a clearer understanding of why thalassemia patients have a higher risk of thrombosis. In the past, bleeding time testing was used to screen for platelet-related bleeding disorders. However, its use has largely fallen out of favour due to its invasive nature, applicator-dependence, poor reproducibility, limiting its ability to predict bleeding risk [14]. In its place, many laboratories have moved towards the bench-top automated platelet function analyser 100 (PFA-100) to assess primary haemostasis under shear stress. The PFA-100 (and the PFA-200) can detect moderate/severe bleeding disorders despite having some problems catching mild bleeding disorders [6]. Therefore, we used platelet function analysis by PFA-200 to evaluate primary haemostasis *in vitro* in our study [6,14]. There are known prominent factors that can affect the results of this test, including HB/Hct, leukocyte and platelet counts, and von Willebrand factor (vWF) levels. It is well known that single or multilineage cytopaenias may also cause prolonged PFA-200 test results [15]. Our study found that aperture closure times in the pre and posttransfusion thalassemia groups were longer than that of controls. However, there was no difference in PFA values when comparing the pre and posttransfusion TG results to one another, suggesting

the difference in the overall number of RBCs between the two groups had a little impact. Laboratory results showed that the TG's pretransfusion platelet values were higher than posttransfusion values and that even the lower posttransfusion platelet count from the TG was still higher than CG platelet count overall. We theorize that the increased number of platelets in the TG may be offsetting the effect that a decreased number of RBC has on PFA testing in thalassemia major patients. The control group had the lowest platelet count and approximately the same number of RBCs as the TG posttransfusion group, with closure times shorter than those obtained from the TG pre or posttransfusion samples. This result suggests that other factors may affect the PFA results other than the cell counts. Elevated von Willebrand factor has been known to shorten closure times on the PFA-200 [6]. However, low vWF levels (which we did not evaluate for in our study) might be another factor to explain why the closure times were longer in preand posttransfusion samples. A study in 40 adult patients with thalassemia major from Egypt disclosed that vWF levels were significantly higher in the study group than those in the control group [16]. As low levels of vWF are expected to explain longer PFA closure times in patients with thalassemia major, these studies may indicate that this was not the reason. Over time, elevated total body iron, transfusion-related infections and iron chelators' side effects may cause impaired liver function and damage in thalassemia major patients, which may result in liver insufficiency and cirrhosis [17]. Platelet function is another variable that affects PFA closure times. It is well known that patients with significant liver disease often have prolonged C/ADP and C/EPI closure times due to impaired platelet function [18]. A study disclosed that thalassemic erythrocytes were a source of negatively charged phospholipids and could independently cause thrombin generation and platelet activation [4]. As they are not included in testing, the effect of negatively charged erythrocytes on coagulation could not be excluded. Tripodi *et al.* [19] revealed hypercoagulability in adult thalassemia major patients with thromboelastometry by using whole blood; however, they found hypocoagulability when it was performed using platelet-poor plasma. Their results concluded that cellular components of blood may affect thrombin generation and the likelihood of thromboembolic events. Our study compared thrombin generation between TG and C-TGT groups revealed the same results with platelet-rich plasma and platelet-poor plasma. In a recent study, we found results indicating hypocoagulability via thromboelastography in patients with thalassemia major [20]. However, a 5-year follow-up of these patients revealed neither bleeding nor thrombosis.

In conclusion, although TGT and PFA200 results from thalassemia major patients did not show a clear tendency or association to being hypercoagulable, clinical studies have established that these patients are at an increased risk for thromboembolic events. Other factors, such as endothelial impairment, may better explain thalassemia major patients' tendency to thrombose. Future studies with more patients and dynamic testing that may better simulate in-vivo coagulation could help us to explain these contradictory findings.

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Conflicts of interest

Authors have no conflicts of interest to disclose.

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