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Coagulation Activation in Patients with Sickle Cell Disease in Basra, Iraq

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Abstract:

BACKGROUND: Sickle cell disease (SCD) is considered to be a hypercoagulable state that contributes to the morbidity associated with the disease. Numerous mechanisms can attribute to this hemostatic activation among these patients.

OBJECTIVES: The study was designed to evaluate changes in hemostatic tests, coagulation inhibitors, fibrinolysis, and phosphatidylserine exposure on red blood cells (RBCs) among patients with SCD during both a vaso-occlusive crisis (VOC) and a steady state.

MATERIALS AND METHODS: This observational study comprised 61 patients with SCD, 2 to 16 years old, and 65 healthy patients. Thrombophilia evaluation included prothrombin time (PT), activated partial thromboplastin time (aPTT), protein C and S, d-dimer and Annexin V expression. The independent *t* test and one-way analysis of variance test were used for comparison of the mean of different samples.

RESULTS: During steady state, patients with SCD had longer PT (14.36 ± 0.98 and 13.32 ± 0.79 s), longer aPTT (31.48 ± 2.52 and 30.11 ± 2.04 s), lower protein C (90.95 ± 20.11 and 98.18 ± 18.42 U/L), lower protein S (60.18 ± 12.96 and 80.8 ± 12.67 U/L), and higher d-dimer (1.19 ± 1.25 and 0.27 ± 0.23 μg/mL) levels than the control group, respectively, $P < 0.05$.
Furthermore, a longer PT (15.02 ± 2.11 s), lower protein C (69.21 ± 16.32 U/L), lower protein S (46.56 ± 9.47 U/L), and higher d-dimer levels (3.44 ± 2.62 μg/mL) were reported during VOC compared to steady state.
The mean percentage of RBCs expressing Annexin V was assessed in only 10 patients with SCD and eight in the control group. The mean percentage during a VOC (7.66 ± 3.63) was higher than that during steady state (1.57 ± 0.94) and in the control group (0.41 ± 0.15), $P = 0.000$.
Pearson correlation revealed that d-dimer is significantly associated with hemoglobin, indirect bilirubin, and lactate dehydrogenase, $P < 0.05$.

CONCLUSION: Patients with SCD, particularly during VOC, undergo significant hematologic alterations that increase their risk of developing coagulation activation-related complications.

Keywords:

Basra, coagulation activation, Iraq, sickle cell disease

Introduction

Sickle cell disease (SCD) is one of the most common genetic disorders affecting around 30 million people worldwide.[1] It results from a single-point mutation in the 6th codon, leading to substitution of glutamic acid for valine, resulting in an abnormal globin (β^S). Sickle homozygosity for the β^S is responsible for the most common and most severe type of SCD. Other genotypes of SCD result from the interaction of abnormal β genes [like hemoglobin (Hb) C] or with mutations that result in decreased synthesis of β globin genes (β-thalassemia).[2,3]

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The disease is associated with episodes of acute illness and progressive organ damage; therefore, it represents an important public health problem because of its associated complications that adversely affect the life and survival of such patients.[1] Although it is well characterized, still no ideal long-term treatment for this disease is available, apart from allogeneic hematopoietic stem cell (HSC) transplantation which is a potentially curative therapy.[4]

Although hemolytic anemias (both chronic hereditary anemias like SCD and acquired types) are characterized by hypercoagulability, there are multiple mechanisms that can explain the occurrence of the hemostatic activation.[5]

The term thrombophilia is applied for a group of genetic and acquired situations, arising from defects of hemostasis mechanism and generating tendency to thrombosis. Many factors are considered as risk factors for acquired thrombophilia as venous catheters, severe infections, surgery, hyperlipidemia, congestive heart disease, and old age. Although inherited thrombophilia is suspected in cases with spontaneous and recurrent thrombosis occurring at early age, thromboembolic events in other family members, development of massive thrombosis in atypical areas, recurrent abortions, and skin necrosis following the anticoagulant treatment.[6]

Patients with SCD may present with increased thrombin and fibrin generation, increased tissue factor activity, increased platelet activation, depletion of natural anticoagulants, and activation of cellular elements, including white blood cells (WBCs).[5,7-11]

The mechanism underlying coagulation activation is likely to be multifactorial.[12] In SCD, repeated cycles of sickling and unsickling, resulting from polymerization and depolymerization of sickle Hb, play a part in the abnormal phosphatidylserine (PS) exposure.[13] The abnormal exposure of PS enhances removal of nucleated cells and functions as a docking site for proteins complexes important in the process of coagulation, including Factor X and prothrombinase complex.[13-16]

The procoagulant state has been linked with acute painful episodes that characterize SCD, the increased frequency of these episodes, and a shortened time interval to the next pain episode among patients with SCD.[17] The thrombotic vascular occlusion is responsible for the increased risk of development of stroke, pulmonary hypertension, and avascular bone necrosis.[18] There is also evidence that thrombi are frequently found the pulmonary arteries in patients with acute chest syndrome (ACS).[19]

This study aimed to evaluate patients with SCD [during both a steady-state period and a vaso-occlusive crisis (VOC)] for changes in selected hemostatic tests and evidence of PS exposure and to compare these parameters with those in healthy children and adolescents.

Materials and Methods

Patients

This case–control study involved 61 patients with SCD registered at the Basra center for hereditary blood diseases from the first of November 2013 through June 2014, their age ranged from 2 to 16 years.

All patients had a history of admission to the hereditary blood diseases ward for the management of a VOC. In fact, these patients were assessed initially during a VOC, and subsequently during a steady-state period.

Patients were also evaluated for site and frequency of VOCs per year, frequency of blood transfusions (BTs) per year, previous history of stroke, ACS, hydroxyurea (HU) intake, and history of splenectomy.

Patients were considered to be in a steady state if they had no fever, had no history of hospitalization or BT for 8 weeks, and had no VOC during the previous 14 days with no recent drop in Hb level.[7,20] These data were obtained by direct interview of the patient and the caregiver, and also by reviewing the medical records.

Severe disease was defined as frequent VOCs, requiring hospitalization ≥3/year, BT ≥ 3/year, frequent hospitalizations ≥3/year, an episode of ACS, acute splenic sequestration crisis, or avascular necrosis of the bone.[21]

Exclusion Criteria

Patients were excluded if they had fever, ACS, or heart failure[17,18,22] and were taking HU, because HU alters markers of thrombin generation and affects hemostatic activation.[23-25]

Patients, who had undergone a splenectomy, were also excluded because splenectomy may lead to a hypercoagulable state.[12]

Control group

The control group included 65 age-matched children with no hemoglobinopathy, no history of

fever, infection, coagulopathy, or thromboembolic events.[7,9]

Exclusion criteria: participants in the control group were excluded if there was a family history of hemoglobinopathy, a history of fever, or current infection.[7,9]

The objectives of the study as well as the method of specimen collection were explained to at least one of the parents/caregiver and all gave an informed consent before enrollment in the study. This work was carried out following the approval of the Ethical Committee of the College of Medicine, University of Basra and Basra Health Directorate, Center of hereditary blood diseases.

Methods

Blood samples have been collected from patients (during painful episodes and during a steady-state period) and control groups and send for laboratory as follows:

1. EDTA tube for:
   (a) Complete blood count using hematology analyzer Mindray-BC 5300 (Shenzhen, China) within 30 min of collection.
   (b) High performance liquid chromatography (VARIANT™; Bio-Rad Laboratories, Hercules, California, USA). The sample may be stored for 1 to 3 days in the refrigerator.
   (c) Annexin V, a marker for PS expression on red blood cells (RBCs) by flow cytometry BD Accuri C6 (Accuri cytomters, Inc., Ann Arbor 21, MI 48103, USA) within 24 h of sample collection.
2. Clot-activator-gel tube for total serum bilirubin (TSB), aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase (LDH) levels using automated chemistry analyzer Cobas111c (Roche Cobas, Roch Diagnostic, USA) related kits as per manufacturer instructions.
3. Sodium citrate tube presented within 30 min to the laboratory for coagulation studies, using fully automated coagulation analyzer (DIAGNOSTIC STAGO, Asnières-sur-Seine, France). The sample was divided into two parts:
   (a) Immediately processed for prothrombin time (PT) and activated partial thromboplastin time (aPTT).
   (b) Stored sample frozen at −30° centigrade for 30 days to be thawed immediately before analysis for protein C, protein S, and d-dimer.

The flow cytometry study was done in 10 patients with SCD (during a VOC and a steady-state period) and in eight children in the control group. It was difficult to present samples from all patients to flow cytometry department as samples need to be analyzed without storage for more than 24 h.

The BD Accuri C6 software version 1.0.264.21 was used to calculate the percentage of annexin-positive cells, the mean fluorescence of these cells, and the heterogeneity of fluorescence intensity of positive cells [Figure 1].

Statistical analysis

Statistical Packages for the Social Sciences (SPSS) software, version 18, (Chicago: SPSS Inc., USA) was used for data analysis. Data were expressed as the mean ± standard deviation. The independent *t* test was utilized for quantitative comparison and for comparison between two means of different samples. One-way analysis of variance test used for quantitative comparison more than two means of different samples.

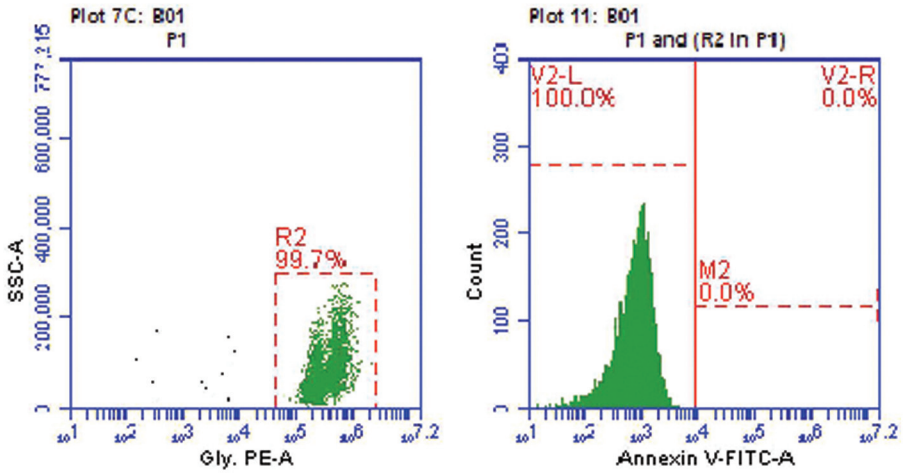

Figure 1: A histogram for glycophorin gating red blood cells. (a) Scattergram for red blood cells gating using antiglycophorin. (b) Annexin V-negative events threshold

The Pearson or Spearman coefficient evaluated correlations between variables.

Mann–Whitney test was used to overcome the underlying assumption of normality in parametric tests (the test does not assume that there is normal distribution in the difference between two samples). *P* values of less than 0.05 were considered as statistically significant.

Results

This study comprised 126 children and adolescents, 61 patients with SCD and 65 patients in the control group. The age ranged from 2 to 16 years. The mean age of the patients with SCD was 8.131 ± 3.319, and that of the control group was 8.120 ± 3.289, $P = 0.985$. Among the patients with SCD, there were 35 (57.4%) males and 26 (42.6%) females compared with 36 (55.4%) males and 29 (44.6%) females in the control group, $P = 0.889$.

Of 61 patients with SCD, 47 were diagnosed with sickle cell anemia (SCA) and 14 with S/β thalassemia (11 patients with S/β° thalassemia and three with S/β^+ thalassemia).

In comparison with the healthy controls, patients with SCD had significantly lower values of Hb, and a higher total WBC count, neutrophil and platelet counts, bilirubin, liver enzymes, and LDH, $P < 0.05$.

The coagulation inhibitor proteins (protein C and S) were significantly lower, whereas PT, aPTT, and d-dimer values were higher among patients with SCD ($P < 0.05$; Table 1).

All patients with SCD who were evaluated initially during a VOC were followed up and evaluated again when they were in a steady-state period. The Hb level and platelet count were significantly lower during VOC compared with a steady-state period and the d-dimer, total and indirect serum bilirubin, and LDH were significantly higher ($P < 0.05$; Table 2).

The aPTT values were comparable among patients during a VOC and a steady-state period, $P > 0.05$. However, PT was more prolonged during a VOC, $P < 0.05$. In addition, protein C and S values were significantly lower, whereas d-dimer was significantly higher among patients during a VOC compared with a steady-state period ($P < 0.05$; Table 2).

Table 1: Selected hematological and biochemical parameters of patients with sickle cell disease during the steady state and control group

Variable	Patients (total no. 61) (mean ± SD)	Control (total no. 65) (mean ± SD)	*P* value*
Hb (g/dL)	8.8 ± 1.094	12.2 ± 0.79	<0.001
Total WBC × 10^9	10.66 ± 4.159	7.4 ± 2.36	<0.001
Neutrophil × 10^9	6.9 ± 12.1	3.2 ± 1.42	0.018
Lymphocyte × 10^9	4.02 ± 1.91	3.6 ± 3.02	0.39
Monocyte × 10^9	0.6 ± 0.81	0.55 ± 0.22	0.6
Platelet × 109	355 ± 156.4	307.3 ± 68.68	0.027
PT (s)	14.3 ± 0.98	13.3 ± 0.79	<0.001
aPTT (s)	31.4 ± 2.5	30.1 ± 2.04	0.001
Protein C (U/L)	90.95 ± 20.11	98.18 ± 18.42	0.042
Protein S (U/L)	60.1 ± 12.96	80.8 ± 12.67	<0.001
d-dimer (μg/mL)	1.19 ± 1.25	0.27 ± 0.23	<0.001
LFT TSB (μmol/L)	21.7 ± 9.07	11.95 ± 1.64	<0.001
Indirect bilirubin (μmol/L)	17.02 ± 6.84	9 ± 1.26	<0.001
Direct Bilirubin (μmol/L)	5.9 ± 6.7	3.02 ± 0.78	0.001
AST (IU/L)	12.2 ± 6.77	7.6 ± 1.68	<0.001
ALT (IU/L)	11.03 ± 5.37	7.29 ± 1.9	<0.001
LDH (U/L)	367.2 ± 105.8	167.5 ± 69.9	<0.001

ALT = alanine aminotransferase, aPTT = activated partial thromboplastin time, AST = aspartate aminotransferase, Hb = hemoglobin, LFT = liver function tests, PT = prothrombin time, SD = standard deviation, TSB = total serum bilirubin, WBC = white blood cell. *P value is assessed by using independent *t* test.

Table 2: Selected hematological and biochemical parameters of patients with sickle cell disease during vaso-occlusive crisis and steady state

Variable	Patients (no. 61)		*P* value*
	VOC (mean ± SD)	Steady state (mean ± SD)	
Hb (g/dL)	8.2 ± 1.06	8.8 ± 1.09	0.005
Total WBC × 10^9	13.07 ± 7.3	10.66 ± 4.15	0.028
Neutrophil × 10^9	7.94 ± 4.6	6.91 ± 12.1	0.53
Lymphocyte × 10^9	4.21 ± 3.2	4.02 ± 1.9	0.68
Monocyte × 10^9	0.73 ± 0.47	0.60 ± 0.81	0.26
Platelet × 109	267 ± 123.01	355 ± 156.42	0.001
PT (s)	15 ± 2.1	14.35 ± 0.98	0.028
aPTT (s)	32.3 ± 2.7	31.4 ± 2.5	0.079
Protein C (U/L)	69.21 ± 16.32	90.95 ± 20.11	<0.001
Protein S (U/L)	46.56 ± 9.47	60.1 ± 12.96	<0.001
d-dimer (μg/mL)	3.44 ± 2.62	1.19 ± 1.25	<0.001
LFT TSB (μmol/L)	41.3 ± 46.3	21.95 ± 9.07	0.002
Indirect bilirubin (μmol/L)	31.4 ± 28.1	17.02 ± 6.84	0.001
Direct bilirubin (μmol/L)	6.9 ± 4.7	5.9 ± 6.7	0.34
AST (IU/L)	15.7 ± 12.5	12.2 ± 6.77	0.05
ALT (IU/L)	14.4 ± 15.4	11.03 ± 5.3	0.10
LDH (U/L)	574.4 ± 119.02	400.2 ± 116.7	<0.001

ALT = alanine aminotransferase, aPTT = activated partial thromboplastin time, AST = aspartate aminotransferase, Hb = hemoglobin, LFT = liver function tests, PT = prothrombin time, SD = standard deviation, TSB = total serum bilirubin, VOC = vaso-occlusive crisis, WBC = white blood cell. *P value is assessed by using independent *t* test.

Among both types of SCD, protein S was significantly lower in SCA than sickle/β thalassemia ($P < 0.05$; Table 3).

We assessed the mean Annexin-V-FITC positive cells in eight healthy children and adolescents and in 10 patients during a VOC and during a steady-state period. The mean value of Annexin-V-FITC fluorescence and the mean percentage of positive cells were significantly higher during a VOC than during a steady-state period in patients with SCD, and both were significantly higher in patients with SCD during a steady-state period than in healthy children and adolescents ($P < 0.05$; Table 4).

The study also revealed that d-dimer is significantly associated with Hb level, indirect serum bilirubin, and LDH ($P < 0.05$), although no significant association was found with indicators of disease severity, hematological indices, and HbF.

Discussion

As a part of coagulation activation evaluation, this study found significant differences between patients with SCD and control group regarding most hematological parameters. These results are in agreement with the findings of other studies.[9,26]

Consistent with results reported by Ataga *et al.*,[18] Chinawa *et al.*,[27] and Liesner *et al.*,[28] WBCs and platelets were higher in patients with SCD during a steady-state period than in control group. These findings can be explained by the inflammatory state and autosplenectomy in SCD. The higher total WBCs, neutrophils, and a lower Hb concentration and platelet count compared to the steady-state period can be explained by the turnover of a large number of WBCs in SCD patients and redistribution of the WBCs between the marginal and circulating pools.[29]

In patients with SCD, hepatic dysfunction is commonly caused by viral hepatitis (acute and chronic), iron overload, hepatic crises related to intrahepatic cholestasis, and ischemic necrosis.[11] In addition, the TSB and indirect fraction were significantly elevated during a VOC in comparison with a steady-state period. These changes may be due to a transient hepatic functional derangement during a VOC.[30]

LDH, a marker of hemolysis and pain, was significantly higher in patients with SCD during a steady-state period than in control group, a finding that is in agreement with a study by Colombatti *et al.*[31] in Italy. Consistent with the findings by Najim and Hassan[32] in Basra, we found that the level of LDH was markedly elevated during a VOC.

During the steady-state period, PT and PTT were significantly prolonged compared with control group. This result is similar to that of Chinawa *et al.*,[27] whereas Wright *et al.*[11] reported a prolonged PT. These results can be explained by several factors, such as hepatocyte dysfunction and hepatic injury that decrease the synthesis of clotting factors as well as synthesis of dysfunctional clotting factors or consumption of

Table 3: Selected hematological and biochemical variables of patients with sickle cell anemia and sickle/β thalassemia

Variables	SCA total (47) (mean ± SD)	S/β thalassemia total (14) (mean ± SD)	*P* value
Hb (g/ dL)	8.8 ± 1.18	8.7 ± 0.76	0.881*
Total WBC × 10^9	10.5 ± 4.01	11.0 ± 4.75	0.678*
Neutrophil × 10^9	5.35 ± 2.483	12.14 ± 24.830	0.065†
Lymphocyte × 10^9	4.14 ± 1.93	3.59 ± 1.80	0.349*
Monocyte × 10^9	0.48 ± 0.373	0.99 ± 1.543	0.041†
Platelets × 10^9	334.45 ± 135.55	424.36 ± 233.01	0.058*
Hb F (%)	20.26 ± 8.08	14.39 ± 6.80	0.017*
LDH (U/L)	426 ± 128.49	392.93 ± 71.30	0.790*
TSB (μmol/L)	21.34 ± 9.21	23.29 ± 8.72	0.486*
Indirect bilirubin (μmol/L)	16.81 ± 7.37	17.71 ± 4.82	0.668*
PT (s)	14.23 ± 1.002	14.57 ± 0.840	0.084*
aPTT (s)	32.29 ± 2.664	32.48 ± 3.246	0.827*
Protein C (U/L)	89.66 ± 20.667	95.26 ± 18.176	0.363*
Protein S (U/L)	58.02 ± 10.767	67.43 ± 17.100	0.016*
d-dimer (μg/mL)	1.06 ± 0.764	1. 63 ± 2.219	0.137*

aPTT = activated partial thromboplastin time, Hb = hemoglobin, LDH = lactate dehydrogenase, PT = prothrombin time, SCA = sickle cell anemia, SD = standard deviation, TSB = total serum bilirubin, WBC = white blood cell. *P value is assessed by independent *t* test; † *P* value is assessed by Mann–Whitney test.

Table 4: Mean Annexin-V-FITC of positive cells and percentage of positive cells among patients with sickle cell disease and control group

Variables		Mean ± SD	*P* value*
Mean Annexin-V-FITC	Patients-steady state	1170.21 ± 491.96	0.024
	Control group	786.12 ± 114.46	
	Patients-VOC	6277.9O ± 7905.28	0.024
	Control group	786.12 ± 114.46	
	Patients-VOC	6277.9O ± 7905.28	0.022
	Patient-steady state	1170.21 ± 491.96	
Percentage of positive cells	Patients-steady state	1.57 ± 0.94	<0.001
	Control group	0.41 ± 0.15	
	Patients-VOC	7.66 ± 3.63	<0.001
	Control group	0.41 ± 0.15	
	Patients-VOC	7.66 ± 3.63	0.001
	Patients-steady state	1.57 ± 0.94	

coagulation factors, leading to prolongation of PT and aPTT.[33,34] Vitamin K deficiency caused by decreased dietary intake, intrahepatic cholestasis, or chronic antibiotics that are frequently used by patients with SCD are alternative explanations.[34]

PT and PTT were further prolonged during a VOC; however, the difference was significant for PT only. In addition, subclinical hepatic dysfunction can prolong PT.[30]

With regard to protein C and protein S, patients with SCD during the steady-state period had significantly lower values of protein C and S than control group. This finding is in agreement with a study by Bayazit and Kilinc[9] in Turkey. Another study by Piccin *et al.*[35] in Ireland reported that levels of protein C and S were further decreased during a VOC, when compared to a steady-state period. This finding is similar to our result.

In agreement with Tantawy *et al.*[36] in Egypt and Hagger *et al.*[37] in United Kingdom, the levels of proteins C and S in children with SCA in steady state are in the range observed in heterozygous congenital deficiency, where they are associated with increased thrombotic risk. Hepatic dysfunction in patients with SCD can decrease synthesis of protein C and S, in addition consumptive coagulopathy.[11]

This study found a significantly higher d-dimer level among patients during a steady-state period than in control group. In addition, the level of d-dimer was further elevated during a VOC. These findings are similar to that reported by Piccin *et al.*[35] in Ireland, Hagger *et al.*[37] in United Kingdom, and Fakunle *et al.*[38] in Nigeria and confirm that the coagulation and fibrinolytic systems were activated in patients with SCD in the steady-state, which are further intensified during a VOC.

Similar to findings by Setty *et al.*[7] in the United States, flow cytometric analysis of PS among patients with SCD in a steady-state and control group showed a significant difference in the exposure of PS on the surface of RBCs. However, the mean level of PS-positive cells in RBCs in controls and the mean percentage of positive cells in SCD are lower during steady-state than those reported by Setty *et al.*[7] These findings illustrate the pivotal role of erythrocytes in SCD as being directly responsible for PS exposure and further acceleration of coagulation activation.

The activation of the coagulation system will result in fibrin formation. Its production is followed by activation of the fibrinolytic system, resulting in plasmin generation, and subsequent fibrin lysis.[39] The d-dimer values (reflect the overall activity of fibrin clot formation and lysis) were found to be correlated with Hb, indirect bilirubin, and LDH levels in our SCD patients. This is because coagulation activation in patients with SCD was found to be associated with hemolysis as reflected by Hb, indirect bilirubin, and LDH levels. These findings are consistent with those reported by Ataga *et al.*[18]

Our study has many limitations: these include the relatively small sample size, the correlation of the hypercoagulability with other vasculopathies (lung or cerebral) was not evaluated, and other potential markers like those involved in neutrophil and platelets activation, which were not included due to shortage in materials and diagnostic procedures.

Conclusion

The results from our study, therefore, support the view that patients with SCD, especially during a VOC, require increased monitoring, including the routine transcranial Doppler, echocardiography, and coagulation activation markers testing as they undergo significant hematologic alterations that increase their risk of developing thrombophilia-related complications.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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