Alexandria Journal of Medicine

ISSN: 2090-5068 (Print) 2090-5076 (Online) Journal homepage: https://www.tandfonline.com/loi/tajm20

Plasma levels of soluble endothelial protein C-receptor in patients with β-thalassemia

Maha Elgammal, Zinab Mourad, Nadia Sadek, Hadeer Abassy & Heba Ibrahim

To cite this article: Maha Elgammal, Zinab Mourad, Nadia Sadek, Hadeer Abassy & Heba Ibrahim (2012) Plasma levels of soluble endothelial protein C-receptor in patients with β-thalassemia, Alexandria Journal of Medicine, 48:4, 283-288, DOI: 10.1016/j.ajme.2011.10.007

To link to this article: https://doi.org/10.1016/j.ajme.2011.10.007

Published online: 17 May 2019.

Alexandria Journal of Medicine (2012) **48**, 283–288

Alexandria University Faculty of Medicine

Alexandria Journal of Medicine

www.sciencedirect.com

ORIGINAL ARTICLE

Plasma levels of soluble endothelial protein C-receptor in patients with β-thalassemia

Maha Elgammal [a,*], Zinab Mourad [b], Nadia Sadek [b], Hadeer Abassy [b], Heba Ibrahim [b]

[a] *Hematology Department, Medical Research Institute, University of Alexandria, Alexandria, Egypt*
[b] *Chemical and Clinical Pathology Department, Medical Research Institute, University of Alexandria, Alexandria, Egypt*

Received 7 September 2011; accepted 23 October 2011
Available online 25 September 2012

KEYWORDS

Soluble endothelial protein C receptors (sEPCR);
Hypercoagulability;
Thalassemia

Abstract *Background:* A hypercoagulable stable has been documented in patients with β-thalassemia. However, the underlying mechanisms are multifactorial. The role of soluble protein C endothelial receptor in hemostatic derangement in these patients has not been investigated.
Design and methods: Plasma soluble EPCR and markers of coagulation have been analyzed in 15 splenectomized patients (group I) and 15 non-splenectomized patients (group II).
Results: We showed significantly higher levels of sEPCR in patients versus the control and in group I more than group II. Levels of sEPCR positively correlated with total leukocytic and platelet counts. No correlation could be established between sEPCR and either age or sex.
Conclusion: The data indicate that high sEPCR levels could be implicated in the hemostatic derangement and endothelial cell dysfunction in patients with β-thalassemia.

1. Introduction

Thalassemia represents the most common hereditary hemolytic anemia which results from partial or complete lack of synthesis of one of the α or β globin chain of Hb.[1] β-Thalassemia is the most common type in Egypt with a carrier rate ranging from 5.3% to >9% and a gene frequency of 0.03%.[2]

Patients with β-thalassemia exhibit thrombotic complications with several published series demonstrating the presence of both arterial and venous thrombosis.[3]

The pathogenesis of this hypercoagulability is multifactorial implicating reactive oxygen species in the oxidized red cell membrane lipids[4] and loss of their organization,[5] alterations in markers of coagulation activation and natural anticoagulant proteins,[6] thrombophilic DNA mutations,[7] endothelial, monocytic and granulocyte activation.[8] Endothelial protein C receptor (EPCR) is a type I transmembrane protein, mainly expressed on the luminal endothelial cell surface of large blood vessels.[9] It plays an anticoagulant role in hemostasis by binding to protein C with high affinity and it increases by more than 10 fold the generation of the activated protein C (APC). It also binds to factor VIIa and inhibits its coagulant activity.[10,11]

* Corresponding author.
E-mail address: mahagammal@yahoo.com (M. Elgammal).
Peer review under responsibility of Alexandria University Faculty of Medicine.

ELSEVIER Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.ajme.2011.10.007

The soluble EPCR (sEPCR) is released from EPCR by thrombin, vascular injury or regulated proteolytic cleavage by metalloproteinases.[12,13]

2. Aim of the work

The aim of the present work was to estimate the plasma levels of sEPCR in patients with β-thalassemia.

3. Design and methods

Thirty adult patients with established β-thalassemia major were enrolled in this study, (15 splenectomized: group I and 15 non-splenectomized: group II) they were recruited from the Hematology Department of the Medical Research Institute. Thirty normal healthy individuals with matched age and sex served as control. They had no any history of venous thrombosis, renal dysfunction or smoking.

All patients and controls gave informed consent for enrollment in accordance with the Declaration of Helsinki and the study was approved by the Medical Research Institute Ethical Committee.

4. Methods

All patients were thoroughly examined. Laboratory investigations included CBC using automated cell counter[14] (Sysmex XT-1800i), PT and INR by quick one stage method using calcium thromboplastin, PTT with Kaolin (KCCT) using pathrombin,[15] D-dimer by semi-quantitative latex agglutination kit (DiALAB, Austria)[15] and estimation of sEPCR by ELISA (Stago diagnostica France).[16] Venous samples were withdrawn after 8–10 h fasting and prior to receiving blood transfusion. Plasma was separated immediately, INR, PTT and D-dimer were done at once and the remaining plasma was kept at −40 °C until processed for sEPCR assay.

4.1. Statistical analysis

Results were statistically analyzed using SPSS version 16A. Microsoft excel was used for graphing. All tests were two tailed and a 0.05 level of significance was used. Data were analyzed using the Kolmogorov. Smirnov test and Mann-Whitney test.

5. Results

Group I included six males (40%) and nine females (60%) with their age ranging from 19 to 59 years with a mean of 29.3 ± 12.2 years, while group II included four males (26.67%) and 11 females (73.33%) with their age ranging from 18 to 38 years with a mean of 23.4 ± 5.6 years. History of epistaxis was found in eight patients (26.7%) and history of thrombotic attacks (DVT and pulmonary embolism) in only two patients (6.7%). Clinical examination revealed hepato-

Table 1 Hematological parameters in patients compared to control.

		Cases	Control	Z	$P^{\#}$
Hb (g/dl)	Mean ± SD	7.6 ± 1.1	13.2 ± 0.8	−6.657	0.000*
	Min–Max	5.7–10.8	11.9–14.6		
	Median	7.5	13.0		
RBC ($\times 10^{12}$ L^{-1})	Mean ± SD	2.9 ± 0.6	4.2 ± 0.6	−4.411	0.000*
	Min–Max	1.9–4.6	4.0–5.1		
	Median	2.9	4.8		
MCV (Fl)	Mean ± SD	67.9 ± 6.9	84.6 ± 3.0	−6.567	0.000*
	Min–Max	50.3–79.2	78.2–88.9		
	Median	69.9	85.2		
WBC ($\times 10^{9}$ L^{-1})	Mean ± SD	9.8 ± 2.9	6.9 ± 1.5	−4.217	0.000*
	Min–Max	5.3–19.2	4.3–9.1		
	Median	9.4	6.9		
Platelets ($\times 10^{9}$ L^{-1})	Mean ± SD	428.1 ± 193.9	318.8 ± 70.6	−2.329	0.020*
	Min–Max	141.0–793.0	198.0–423.0		
	Median	450.0	321.0		
MPV (Fl)	Mean ± SD	9.2 ± 1.2	9.1 ± 0.8	−0.415	0.679
	Min–Max	7.1–11.4	7.8–10.4		
	Median	9.4	9.1		
Normoblasts/100WBCs	Mean ± SD	21.9 ± 38.6	0.0 ± 0.0	−7.114	0.000*
	Min–Max	4.0–222.0	0.0 ± 0.0		
	Median	12.5	0.0		
Absolute reticulocytic count	Mean ± SD	130,500 ± 11.2	42,000 ± 01.3	−6.663	0.000*
	Min–Max	47,500–345,000	24,000–81,600		
	Median	130,500	48,000		

P value based on Mann–Whitney test.
* $P < 0.05$ (significant).

Table 2 Hematological parameters in group I compared to group II.

		Group I	Group II	Z	$P^{\#}$
Hb (g/dl)	Mean ± SD	7.8 ± 0.8	7.5 ± 1.3	−1.268	0.217
	Min–Max	6.3–9.5	5.7–10.8		
	Median	7.5	7.1		
RBC ($\times 10^{12}$ L^{-1})	Mean ± SD	3.1 ± 0.6	2.8 ± 0.7	−6.628	0.351
	Min–Max	2.3–4.6	1.9–3.9		
	Median	3.2	2.6		
MCV (Fl)	Mean ± SD	67.1 ± 7.1	68.9 ± 6.9	−0.954	0.340
	Min–Max	50.3–78.3	55.5–79.2		
	Median	68.9	70.8		
WBC ($\times 10^{9}$ L^{-1})	Mean ± SD	9.8 ± 2.1	9.9 ± 3.7	−0.394	0.693
	Min–Max	6.4–13.4	5.3–19.2		
	Median	9.6	9.2		
Platelets ($\times 10^{9}$ L^{-1})	Mean ± SD	561.5 ± 150.7	294.6 ± 130.4	−3.859	0.000*
	Min–Max	252.0–793.0	141.0–607.0		
	Median	532.0	298.0		
MPV (Fl)	Mean ± SD	9.6 ± 1.1	8.9 ± 1.2	−1.619	0.106
	Min–Max	7.5–11.1	7.1–11.4		
	Median	9.6	8.7		
Normoblasts/100WBCs	Mean ± SD	34.0 ± 52.5	9.8 ± 3.5	−3.636	0.000*
	Min–Max	5.0–222.0	4.0–16.0		
	Median	24.0	10.0		
Absolute reticulocytic count	Mean ± SD	142,600 ± 11.4	120,400 ± 01.9	−0.851	0.412
	Min–Max	62,100–345,000	47,500–237,900		
	Median	156,800	106,600		

$^{\#}$ *P*-value based on Mann–Whitney test.
* $P < 0.05$ (significant).

Table 3 Coagulation parameters in patients compared to control.

		Cases	Control	Z	P
INR	Mean ± SD	1.2 ± 0.2	1.04 ± 0.01	−6.927	0.000*
	Min–Max	1.0–1.8	1.0–1.1		
	Median	1.3	1.09		
PTT (seconds)	Mean ± SD	41.2 ± 6.6	35.1 ± 2.0	−3.942	0.000*
	Min–Max	32.1–61.2	32.1–38.2		
	Median	40.2	35.2		

* $P < 0.05$ (significant).

Table 4 Coagulation parameters in group 1 compared to group 2.

	Group 1	Group 2	Z	P
INR				
Mean ± SD	1.3 ± 0.2	1.2 ± 0.2	−0.664	0.507
Min–Max	1.1–1.8	1.0–1.5		
Median	1.3	1.3		
PTT (seconds)				
Mean ± SD	42.1 ± 7.2	40.3 ± 6.1	−0.602	0.547
Min–Max	32.1–61.2	33.0–52.0		
Median	41.9	38.0		

megaly in all patients, while splenomegaly was evident in group II. In the other group (group I) none of them had spleen detected clinically due to previous splenectomy.

Table 1 shows the hematological data of patients compared to the controls where the mean Hb concentration in patients was 7.6 ± 1.1g/dl while in the control group it was 13.2 ± 0.8 g/dl. The mean WBC count was $9.8 \pm 2.9 \times 10^{9}$ L^{-1} in the cases whereas it was $6.9 \pm 1.5 \times 10^{9}$ L^{-1} in the control group. As regard the platelet counts, it had a mean of $428.1 \pm 193.9 \times 10^{9}$ L^{-1} in the patients group and a mean of $318.8 \pm 70.6 \times 10^{9}$ L^{-1} in the control group. Nucleated RBCs were found in the peripheral blood of all the cases with a mean of 21.9 ± 38.6/100WBCs while they were absent in the peripheral blood of the control group. The absolute reticulocytic count ranged from 47,500 to 345,000 in the patients with a mean of 130,500 ± 11.2 while it ranged from 24,000-81,000 in the controls with a mean of 42,000 ± 1.3. Statistical analysis showed that there was statistical significant difference between patients and controls as regard Hb values, RBC counts, MCV, WBC counts($P = 0.000$), platelets counts ($P = 0.020$) as well as normoblasts and reticulocytic counts ($P = 0.000$). On comparing the hematological parameters of group I and group II, platelet counts had a mean of $561.5 \pm 150.7 \times 10^{9}$ L^{-1} in group I which was significantly higher than its mean values in group II ($294{,}6 \pm 130.4 \times 10^{9}$ L^{-1}) ($P = 0.000$) as well as the normoblasts count which ranged from 5.0 -222.0/100WBCs with a mean of 34.0 ± 52.5 /100WBCs in group I whereas it ranged from 4.0 – 16.0/100WBCs with a mean of 9.8 ± 3.5/100WBCs in group II and the difference was statis-

tically significant ($P = 0.000$) as shown in Table 2. Analysis of variance showed statistically significant difference between all thalassemic patients and normal control ($P = 0.000$) as regard the coagulation parameters as the mean INR was 1.2 ± 0.2 in patients and was 1.04 ± 0.01 in controls. On comparing both groups, the mean INR values in group I are higher than that of group II but with no statistical significant difference (Tables 3 and 4).

As regard sEPCR level, it was significantly higher in patients than control ($P = 0.008$) (Table 5). Although group I had a higher mean level of plasma sEPCR compared to group II, yet the difference was not statistically significant ($P = 0.709$) (Table 6). But still there was a significant difference when each group was separately compared to the control ($P = 0.021$ and 0.048, respectively) (Tables 7 and 8).

D-dimer test was negative in all the studied groups (data not shown).

Table 5 sEPCR level in patients compared to control.

sEPCR (ng/ml)	Cases	Control	Z	P
Mean ± SD	265.6 ± 255.1	106.3 ± 46.1	−2.632	0.008*
Min–Max	38.7–1073.1	20.0–151.3		
Median	157.4	129.6		

* $P < 0.05$ (significant).

Table 6 sEPCR level in group 1 compared to group 2.

sEPCR (ng/ml)	Group 1	Group 2	Z	P
Mean ± SD	309.9 ± 314.4	221.1 ± 177.9	−0.373	0.709
Min–Max	38.7–1073.1	40.0–600.0		
Median	160.0	144.7		

Table 7 sEPCR level in group 1 compared to control.

sEPCR (ng/ml)	Cases	Control	Z	P
Mean ± SD	309.9 ± 314.5	106.3 ± 46.1	−2.312	0.021*
Min–Max	38.7–1073.1	20.0–151.3		
Median	160.0	129.6		

* $P < 0.05$ (significant).

Table 8 sEPCR level in group 2 compared to control.

sEPCR (ng/ml)	Group 2	Control	Z	P
Mean ± SD	221.1 ± 177.9	106.3 ± 46.1	−1.975	0.048*
Min–Max	40.0–600.0	20.0–151.3		
Median	144.7	129.6		

* $P < 0.05$ (significant).

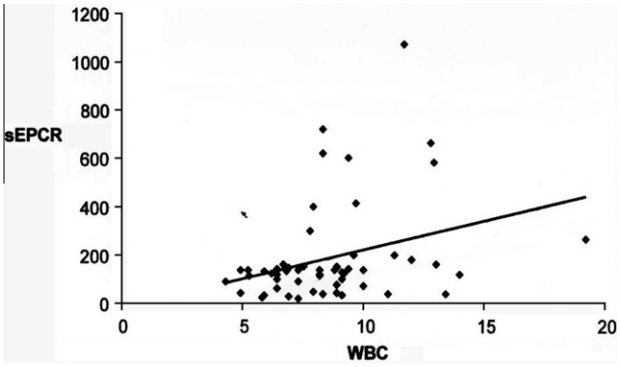

Figure 1 Correlation between sEPCR and WBC count.

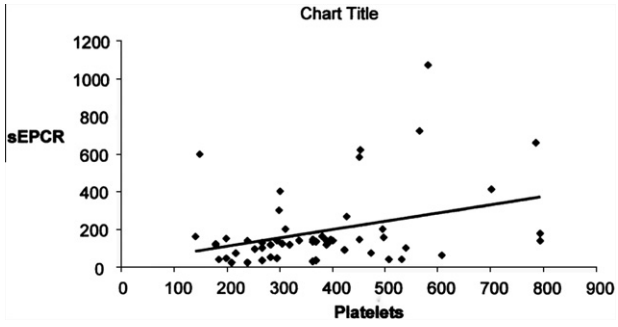

Figure 2 Correlation between sEPCR and platelet counts.

5.1. Correlation studies

A significant positive correlation was observed between sEPCR and total leukocytic count ($P = 0.015$) as well as platelet count ($P = 0.020$) (Figs. 1 and 2), respectively. But no correlation was found between sEPCR and age or sex.

6. Discussion

Profound hemostatic derangements are observed in patients with β-thalassemia. The chronic hypercoagulable state in these patients is multifactorial, and endothelial dysfunction has emerged as a causal factor in this hypercoagulability.[17,18]

In the present study, sEPCR was significantly higher in thalassemic patients than the control ($P = 0.008$). This may reflect a state of hypercoagulability, possibly induced by endothelial injury secondary to oxidative stress or platelet activation with subsequent release of procoagulant proteins. This is evidenced by the positive correlation with platelet count. A higher platelet count was observed in patients compared to the control ($P = 0.02$) especially in splenectomized patients ($P = 0.000$).

Setiabudy et al.[19] found higher platelet count in splenectomized versus non-splenectomized group. The hazards of thrombosis especially following splenectomy may range from portal vein thrombosis to pulmonary embolism and deep vein thrombosis.[19–21] As platelets play an important role in the pathogenesis of thrombosis, autopsy based studies by Fucharoen and his colleagues[22] in a large number of thalassemic patients documented striking pulmonary artery occlusions as well as another study done by Taher et al. had estimated the

prevalence of thrombotic events in β-thalassemia in 9% of the patients.[23]

In agreement with our findings, Mohren et al.[24] and Van't Riet et al.[25] also reported thromboembolic complications in splenectomized patients.

On the other hand, a higher mean platelet volume (MPV) was found in group I compared to group II reflecting a state of platelet hyperactivity. The enhanced platelet function added to the higher platelet number constitutes a double risk of hypercoagulability in splenectomized patients. These findings coincide with those of Setiabudy et al.[19] and they highlight the role of the platelets in inducing endothelial damage which is followed by enhanced production of sEPCR.

In the present study, D-dimer test was negative in all studied groups even in patients with the highest plasma levels of s EPCR which confirmed the study of Setiabudy et al.[19] who reported normal D-dimer in all their studied thalassemic patients.

In addition, we demonstrated a positive correlation between sEPCR and total leukocytic count (TLC). This was attributed to the fact that the activated neutrophils and monocytes in thalassemic patients release their proteolytic enzymes with production of free oxygen radicals as well as inflammatory cytokines that damage the endothelium which eventually produce EPCR degradation into its soluble form. This agrees with Boossma et al.[26] who studied plasma levels of sEPCR in cases of Wagener granulomatosis and found a positive correlation between their levels and disease activity.

As regards the INR and PTT, they were found prolonged in 86.7% and 83.3% of the patients and were statistically significant compared to the control ($P = 0.000$, $P = 0.000$), respectively. Similarly, Naithani et al.[27] found prolongation of INR in 40.7% and PTT in 46.3% of their patients.

Epistaxis was found in 26% of our patients in agreement with the findings of Naithani et al.[27] study, in which sixteen out of the studied 54 thalassemic patients had bleeding manifestations. Altered liver functions, and poor chelation might be risk factors for altered hemostasis in these patients. In their study, they reported low protein C, protein S and antithrombin III as well as Eldor et al.[17] and Sivgar et al.[28] who found significantly decreased levels of these anticoagulant proteins which were explained by combined consumption and liver dysfunction.

In the present study, no correlation was found between sEPCR and age as our patients were adults. Recently, Orhon et al.[29] reported higher levels in healthy children compared to healthy adults. Similarly, Kuraswa et al.[28] found a negative correlation between sEPCE level and age of the pediatric group, but they failed to establish this negative correlation in the adult group. The higher level of sEPCR in healthy children suggests a regulatory mechanism for the protein C system over the first years of life, as the highest level of sEPCR in their study, was found in the first year of life.[28]

As regards gender, we could not establish a relation between gender and sEPCR. On the contrary, Orhon et al.[29] found higher levels in males and were attributed to environmental factors including smoking.

In conclusion, our study documented increased sEPCR in patients with β-thalassemia which could be implicated in the hemostatic derangement in these patients. Hence, close monitoring of the hemostatic system in thalassemia is highly recommended especially with increasing age and in splenectomized patients to avoid the morbid complications of hypercoagulability .

References

1. Thein SL, Rees D. Hemoglobin and the inherited diseases of globin synthesis. In: Hoffbrand Av, Catovsky D, Tuddenham EG, Green AR, editors. Postgraduate haematology, 6th ed., vol. 6, 2011. p. 83–107.
2. Elbeshlawy A, Yossry I. Prevention of hemoglobinopathies in Egypt. *Hemoglogin* 2009;**33**:4–20.
3. Ataga KI, Cappellini MD, Rachmileuritz EA. β-Thalassemia and sickle cell anaemia as paradigms of hypercoagulability. *BJH* 2007;**139**:3–13.
4. Tavazzi D, Ducal, Graziadei G, Comino A, Fiorelli G, Capallini MD. Membrane-bound iron contributes to oxidative damage of beta-thalassemia intermedia erythrocytes. *BJH* 2001;**112**:48–50.
5. Gandrille S. Endothelial cell protein C receptor and the risk of venous thrombosis. *Haematologica* 2008;**93**:812–6.
6. Riewald M, Petrovan RJ, Donnr A, Ruf M. Activated protein C signals through the thrombin receptor PAR1 in endothelial cells. *J Endotoxin Res* 2003;**9**:317–21.
7. Zalloua PA, Shbaklott, Mourad YA, Koussa S, Tahert. Incidence of thromboembolic events in Lebanese thalassemia intermedia patients. *Thrombosis Haemostaisis* 2003;**89**:767–88.
8. Buthep P, Rummavas S, Wisedpanichkij R, Fucharoen S. Increased circulating activated endothelial cells, vascular endothelial growth factor, and tumour necrosis factor in thalassemia. *Am J Hematol* 2002;**70**:100–6.
9. Ghosh S, Pendurthi UR, Steinoe A, Esmon CT, Mohan V, Rao L. Endothelial cell protein C receptor acts as a cellular receptor for factor VIIa on endothelium. *J Biol Chem* 2007;**282**:11849–57.
10. Lopez-Sagaseta J, Montes R, Puy C, Diez N, Fukudome K, Hermida J. Binding of factor VIIa to the endothelial cell protein C receptor reduces its coagulant activity. *J Thromb Haemost* 2007;**5**:1817–24.
11. Xu J, Qu D, Esmon NL, Esmon CT. Metalloproteolytic release of endothelial cell protein C receptor. *J Biol Chem* 2000;**275**:6038–344.
12. Gu JM, Katsura Y, Ferrell GL, Grammas P, Esmon CT. Endotoxin and thrombin elevate rodent endothelial cell protein C receptor mRNA levels and increase receptor shedding in vivo. *Blood* 2000;**95**:1687–93.
13. Qu D, Wang Y, Esmon NL, Esmon CT. Regulated endothelial protein C receptor shedding is mediated by tumor necrosis factor-converting enzyme/ADAM17. *J Thromb Haemost* 2007;**5**:395–402.
14. Bain BJ, Lewis SM, Bates I. Basic hematological techniques. In: Bain BJ, Lewis SM, Bates I, editors. Dacie and Lewis practical hematology, 10th ed., vol. 3, 2006. p. 25–7.
15. Laffan M, Manning R. Investigations of haemostasis. In: Bain BJ, Lewis SM, Bates I, editors. Dacie and Lewis practical hematology, 10th ed., vol. 16, 2006. p. 379–440.
16. Lequin R. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELIZA). *Clin Chem* 2005;**51**:2411–5.
17. Eldor A, Rachmilewitz EA. The hypercoagulable state in thalassemia. *Blood* 2002;**99**:36–43.
18. Hassn AM, Fallouzi MA. Portal vein thrombosis following splenectomy. *Br J Surg* 2000;**87**:362–73.
19. Setiabudy R, Wahidiyat PA, Setiawan L. Platelet aggregation and activation in thalassemia major patients in Indonesia. *Clin Appl Thromb/Hemost* 2008;**14**:346–51.
20. Fujita FS, Lyass S. Portal vein thrombosis following splenectomy: identification of risk factors. *Am J Surg* 2003;**69**:951–6.
21. Ikada M, Sekimoto M. High incidence of thrombosis of the portal vein system after laparoscopic splenectomy: a prospective study with contrast enhanced CT scan. *Ann Surg* 2005;**241**:205–16.

22. Fucharoen S, Winichagon P. Thalassemia and abnormal hemoglobin. *Int J Hematol* 2002;**76**:83–9.
23. Taher A, Isma'eel H, Mehio G, Bignamini D, Kattamis A, Rachmilewitz EA, et al. 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.
24. Mohran M, Markmann, Dworochak V. Thromboembolic complications after splenectomy for hemolytic diseases. *Am J Hematol* 2004;**76**:143–6.
25. Vant Riet M, Burger JW. Diagnosis and treatment of portal vein thrombosis following splenectomy. *Br J Surg* 2000;**87**:1229–33.
26. Boomsma MM, Stearns-Kurosawa DJ, Stegman CA, Raschi E, Meroni PL, Kurosawa S, et al. Plasma levels of soluble endothelial cell protein C receptor in patients with Wegener's granulomatosis. *Clin Exp Immunol* 2002;**128**:187–94.
27. Naithani R, Chandsa J, Narayan S. Thalassemia major on the verge of bleeding or thrombosis. *Hematology* 2006;**11**:57–61.
28. Sivger ST, Kuypers FA, Styles L. Pulmonary hypertension in thalassemia: association with platelet activation and hypercoagulable state. *Am J Hematol* 2006;**81**:670–5.
29. Orhon FS, Frgun H, Egin Y, Ulukol B, Baskan S, Akar N. Soluble endothelial protein C receptor levels in healthy population. *J Thromb Thrombol* 2010;**29**:46–51.