



Full Length Article

The hemostasis system in children with hereditary spherocytosis

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ABSTRACT

Introduction: Patients with hereditary spherocytosis (HS) are characterized by having an increased risk for thrombosis. An early manifestation of thrombotic complications can occur even in childhood, especially after surgery. Hypercoagulability can be associated with hemolytic crises.

Aim: The aim of this study was to investigate the hemostatic state in children with HS using global hemostasis assays.

Methods: The hemostatic status of 62 children (38 boys and 24 girls; age range: 0.5 to 17 years) with HS during and without hemolytic crisis was assessed using clotting times (APTT, TT, and PR), fibrinogen and D-dimer levels, and global hemostasis, thromboelastography (TEG) and thrombodynamics (TD) assays. One hundred and two healthy children undergoing annual medical examination were enrolled as a control group.

Results: TEG and TD parameters were increased in the children with HS compared to the control group (60 ± 5 mm vs. 53 ± 4 mm, $p < 0.05$ for TEG maximum amplitude; 28 ± 3 $\mu\text{m}/\text{min}$ vs. 24 ± 2 $\mu\text{m}/\text{min}$, $p < 0.05$ for TD clot growth rate), while APTT, TT and PR were not significantly different between the two groups. Patients with HS were divided into 2 groups: those during hemolytic crisis (28 patients) and those without hemolytic crisis (34 patients). TEG and TD parameters were increased in those during hemolytic crisis compared to the steady state HS group (62 ± 5 mm vs. 57 ± 4 mm, $p < 0.05$ for TEG maximum amplitude; 31 ± 4 $\mu\text{m}/\text{min}$ vs. 26 ± 3 $\mu\text{m}/\text{min}$, $p < 0.05$ for TD clot growth rate). The D-dimer levels were increased in 4 HS patients, for whom the activation of blood clotting was noted. Fibrinogen levels were decreased in patients with HS compared to the control group (2.1 ± 0.4 mg/ml vs. 2.6 ± 0.4 mg/ml, $p < 0.05$). Other tests were within the reference ranges for both groups.

Conclusions: The global hemostasis tests TEG and TD revealed hypercoagulability in patients with HS. More dramatic changes were observed in patients experiencing a hemolytic crisis.

1. Introduction

Hereditary spherocytosis (HS) is a congenital disorder associated with increased destruction (hemolysis) of erythrocytes due to a defect in the cell membrane, and it has an incidence of approximately 2–3 per 10,000 [1–5]. Extravascular hemolysis is more common for HS, as the degradation of erythrocytes occurs mainly in the spleen. This disorder is caused by dysfunction of erythrocyte membrane proteins that leads to strength and flexibility disturbance. Due to structural or functional deficiency of spectrin, the erythrocytes become sphere-shaped and are eliminated from the bloodstream by macrophages from the spleen [1–6].

Patients with HS (hereditary spherocytic hemolytic anemia) may have hemolytic crises [1–4,7], which are characterized by the appearance of acute symptoms, fever, abdominal pain, jaundice, weakness, decreased hemoglobin concentration, and increased concentration of unconjugated or indirect bilirubin fraction. In adults, hemolytic crisis is associated with thrombotic complications [2,5,8–10].

Clinically, HS shows heterogeneity, ranging from an asymptomatic condition to fulminant hemolytic anemia with red blood cell transfusion requirements. Hemolysis can be either chronically compensated or subcompensated without crises. Sometimes, in severe cases of HS in children, hemolytic crisis occurs from the first months of life [1–6].

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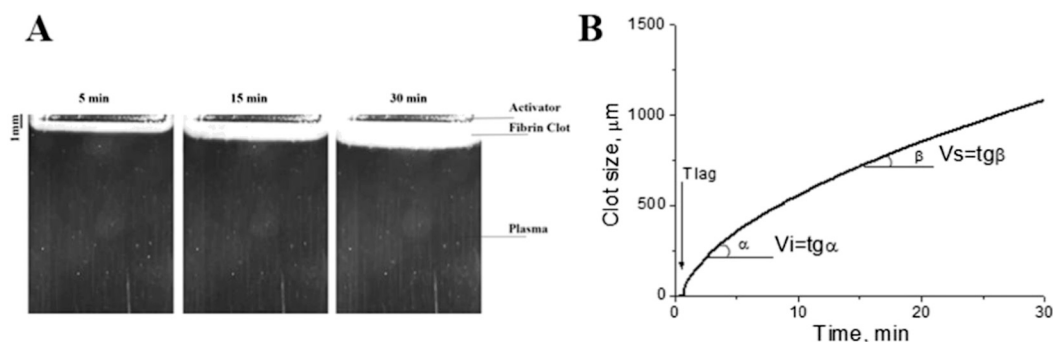


Fig. 1. Thrombodynamics method. Photos of fibrin clot growth (A) and plot of the clot size versus time (B), representing the thrombodynamics parameters.

Table 1

Hematological data of the studied patients.

	During hemolytic crisis			Without hemolytic crisis			Healthy children		
	Mean	SD	Min-Max	Mean	SD	Min-Max	Mean	SD	Min-Max
Hb, g/l	100	18	58–131	111	20	69–153	130	17	111–140
Ferritin, µg/l	216	225	39–929	271	452	10–1561	28	19	10–60
Reticulocyte, %	7.6	6.8	1.23–31.7	4.6	3.5	0.34–12.26	0.8	0.4	0.3–1.2
Total bilirubin, µM/l	68	67	7–279	33	27	5–120	8	4	3–17
PLT, 10 ⁹ cells/l	341	98	169–500	286	86	153–482	275	68	185–370
Fibrinogen, g/l	2.1	0.5	1.47–4.08	2.2	0.4	1.5–3	2.6	0.4	1.8–4.3
ALT level	17	11	7–60	15	7	7–47	14	4	8–22
AST level	35	16	17–83	29	13	17–70	25	5	14–32
TEG, R, min	12	4	4–21	19	5	7–30	19	5	13–32
TEG, k, min	4	2	1–9	6	2	2–9	6	2	4–14
TEG, α, grad	50	13	25–73	37	7	24–65	34	7	20–44
TEG, MA, mm	62	5	53–66	57	5	46–63	53	4	42–60
TD, Vi, µm/min	60	5	52–68	52	6	40–62	50	3	41–56
TD, Vs, µm/min	31	4	23–40	26	3	20–32	24	2	21–29

Hypercoagulation in HS is associated with several causes, including plasma coagulation disturbances [11–13], the presence of procoagulant microparticles released during erythrocyte lysis [10,14,15], platelet activation [8,10,16], endothelial dysfunction and peripheral circulatory disorders [11,12,17]. All of these can lead to various disorders, including pulmonary insufficiency, thrombosis, and cardiac disturbances [8–13].

The risk for thrombosis in children with hemolytic anemia is nearly 5% [12,16,17]. This is several times higher than the risk for thrombosis in children of the general population (0.0007%) [18].

The risk of thromboembolic complications appears to be higher following splenectomy in general [19,20]. Thrombosis in children are rare enough, however, the risk for thrombosis in children with HS is mostly associated with surgery, particularly with splenectomy [12,13,17,20,21]. A retrospective study of 246 children with HS showed that 94% of children who had elevated platelet levels and decreased cholesterol levels before and after splenectomy suffered thrombotic complications after surgery [16]. Analysis of the hematological state of 231 children with hemolytic anemia (111 of them with HS) before and after splenectomy showed that most of these patients had thrombocytosis, which requires increased attention and can be associated with thrombotic complications. However, thrombosis occurred in only 1 child diagnosed with sickle cell anemia who had one of the highest platelets levels. Such a low percentage of thrombosis is associated with the fact that thrombotic complications are extremely rare in the age group of children with HS (5–6 years), and they are rather more typical for a teenage group [21]. Gelas et al. reported portal vein thrombosis in a 17-year-old teenager after splenectomy. Eleven children with HS after surgery were included in this study [17].

The risk for ischemic stroke and thrombosis in hemolytic anemia, both after surgery and during hemolytic crisis, starts to increase in the teenage years (after 11 years) and gradually becomes nearly equal to that for adult patients [17,18].

Some patients with HS develop trophic ulcers on the lower limbs, which disappear only after spleen removal is performed. One mechanism for the pathogenesis of trophic ulcers involves microcirculatory disorders, diminished local fibrinolytic activity and microthrombosis [1–3].

The aim of present study was to assess the state of the hemostatic system in children with HS. Conventional clotting times are not useful for diagnosing the state of the coagulation system in patients with HS, with the exception of the fibrinogen concentration [11]. None of the clotting tests, such as APTT or TT, have sufficient sensitivity to characterize the mechanisms of hypercoagulation in hemolytic anemia. Global hemostatic assays, such as thromboelastography (TEG) and thrombodynamics (TD), are known to be highly sensitive to hypercoagulable changes and have already been shown to be sensitive to coagulation activation in adult patients with hemolytic anemia [22,23].

2. Materials and methods

2.1. Patients

Sixty-two patients with HS (38 boys and 24 girls, age range: 0.5 to 17 years, median age: 5.5 years) were enrolled in this study. All patients were analyzed before undergoing splenectomy. The ultrasound investigation and of the abdomen showed the enlarged spleen in all patients. All patients were suffered from hereditary hemolytic anemia

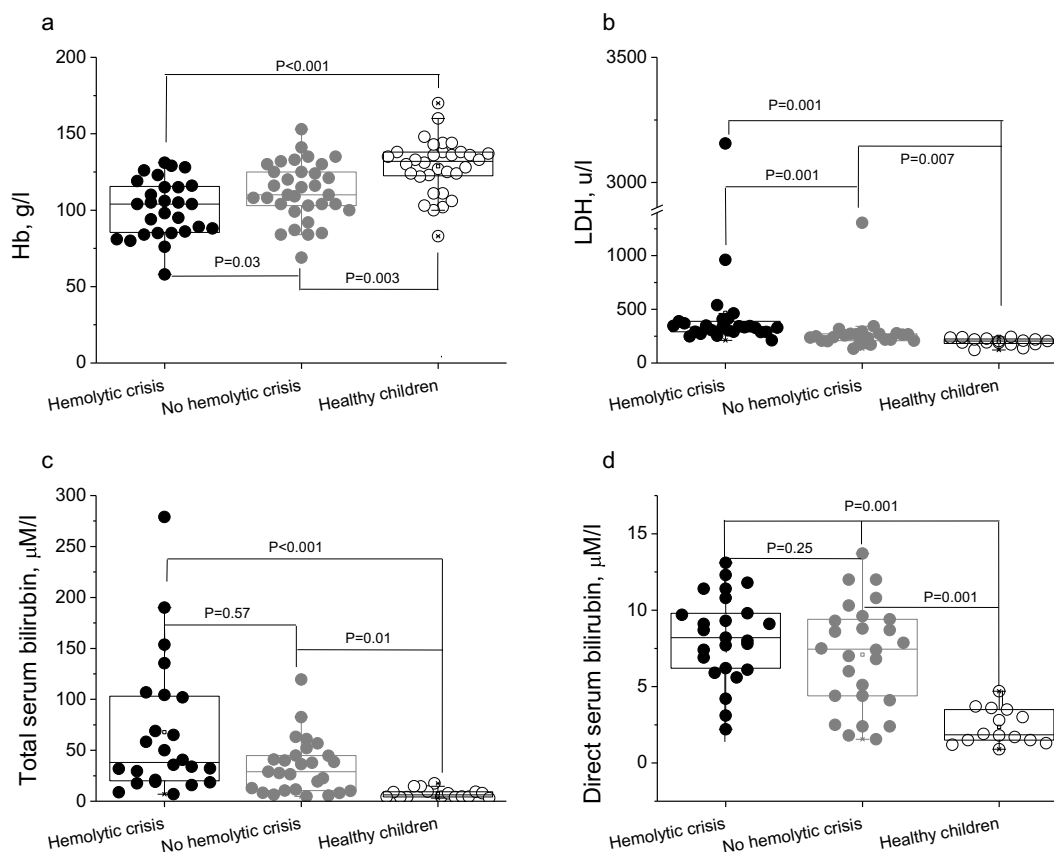


Fig. 2. The laboratory results in children with HS during hemolytic crisis and in a stable state in comparison to the healthy children group (a – hemoglobin level, b – LDH activity, c – total bilirubin level, d – direct bilirubin level).

since birth and had transfusion-dependent hemolysis. The last transfusion was at least 1.5 months ago in all patients. No patients had attack of thrombotic event previously. The diagnosis of HS in all patients was established in accordance with international clinical guidelines [24]. Patients with hemolytic anemia were divided into 2 groups: those during hemolytic crisis (28 patients, 18 boys and 10 girls, age 0.5 to 17 years) and those without hemolytic crisis (34 patients, 20 boys and 14 girls, age 1 to 17 years).

One hundred and two healthy children undergoing annual clinical examination were enrolled in the study as a control group (62 boys and 40 girls, age range: 1 to 17 years, median age: 8 years).

All patients and children from the control group were admitted to Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Oncology and Immunology. The clinical protocol was approved by the Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Oncology and Immunology ethics committee.

2.2. Reagents

SynthASil, RecombiPlasTin 2G, QFA-Fibrinogen, Thrombin Time, and D-dimer HS kits were obtained from HemosIL, Instrumentation Laboratory, Massachusetts, USA, and Thrombodynamics kits were obtained from HemaCore LLC, Russia.

2.3. Blood collection and plasma preparation

Blood samples were drawn into 3-ml vacuum tubes (Monovette,

Sarstedt, Germany) with 0.106 M sodium citrate buffer. The blood samples were processed by centrifugation at $1500 \times g$ for 15 min to obtain platelet-poor plasma (ppp), and part of the plasma was subsequently subjected to centrifugation at $10,000 \times g$ for 5 min to obtain platelet-free plasma (pfp).

2.4. Clotting time tests, Fibrinogen and D-dimer assays

The following tests were performed using frozen ppp samples. All tests were performed using an ACL TOP-700 (Instrumentation Laboratory, Massachusetts, USA) automated analyzer according to the respective manufacturer's instructions.

2.5. Thromboelastography

Citrated native TEG was performed using a TEG 5000 hemostasis analyzer system and disposable cups (Haemonetics Corporation, MA, USA). The assays were performed 10 to 30 min after blood collection using citrated blood samples (340 μ l) that had been recalcified with 20 μ l of 0.2 M CaCl_2 .

2.6. Thrombodynamics

The TD assay was performed using a TromboImager device and thrombodynamics kit (HemaCore LLC, Moscow, Russia), as described earlier [22,23,25]. Briefly, this method was based on registering serial photos of fibrin clot growth in a thin layer of plasma (Fig. 1A). Plasma

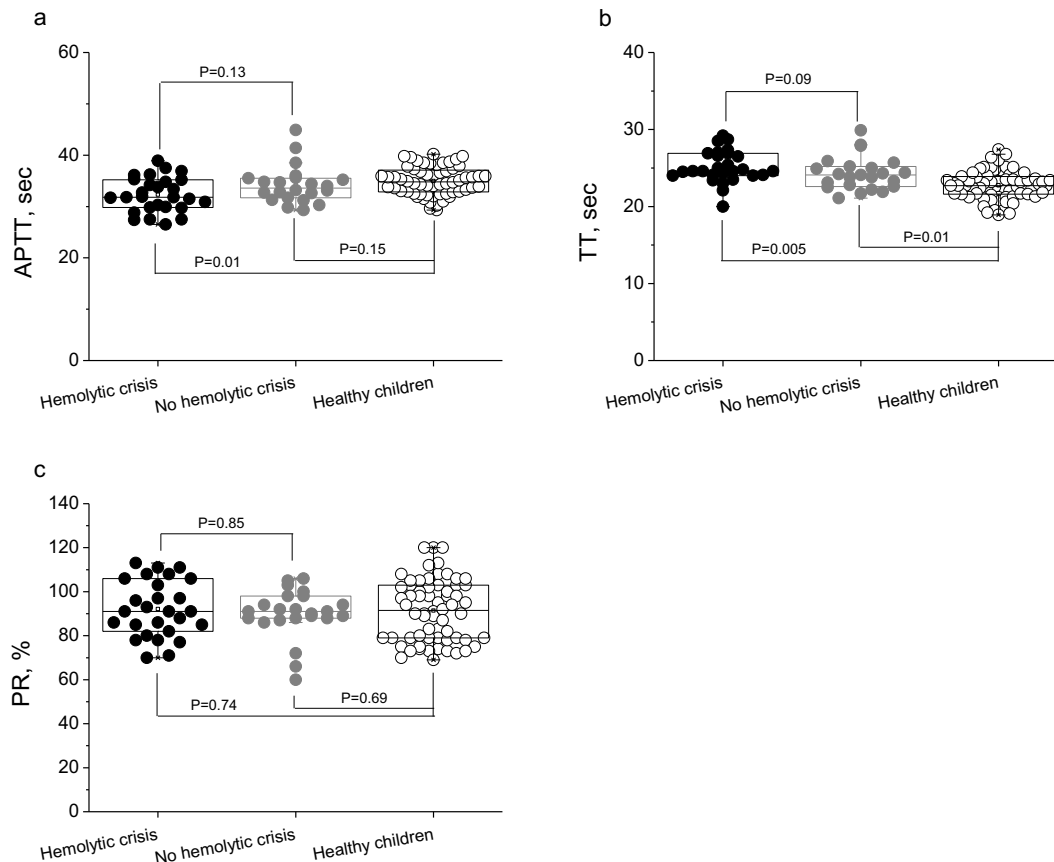


Fig. 3. Standard clotting times in children with HS during hemolytic crisis and in a stable state in comparison to the healthy children group (a – APTT, b – TT, c – PR).

clotting was activated by a surface with immobilized tissue factor. Based on the photos, a plot of clot growth versus time was obtained (Fig. 1B). The following TD parameters were calculated: lag time (Tlag- the delay between the test start and onset of clot formation); the initial velocity of clot growth (Vi- calculated as the mean clot growth velocity over Tlag + 2 min to Tlag + 6); the stationary velocity of clot growth (Vst- calculated as the mean clot growth velocity over the Tlag + 15 min to Tlag + 25 min interval); the density of the clot (D- the light scattering intensity of the clot).

2.7. Statistical analysis

Statistical analyses of differences between the datasets were performed using the Mann-Whitney *U* test for statistical significance, with $p < 0.05$ indicating significance. The Pearson correlation test was used for correlation calculations, with statistical significance set to $p < 0.05$. For all calculations, the OriginPro 8.0 program (OriginLab Corporation, MA, USA) was used.

3. Results

On admission, the HS patient's condition was assessed in accordance with the clinical symptoms and results of the laboratory tests. The laboratory data are shown in Table 1.

Active hemolysis was suggested by results of the laboratory analyses that revealed a significantly ($p < 0.05$) decreased hemoglobin level and increased levels of bilirubin and LDH activity for patients with HS in comparison to a group of healthy children (Fig. 2). The changes in blood parameters in the group with hemolytic crisis were more

dramatic than those in the group with the stable state.

Standard clotting tests (activated partial thromboplastin time [APTT], thrombin time [TT] and prothrombin rate [PR]) were not significantly different for both groups of the HS patients and in comparison with the control group (Fig. 3). Patients with hemolytic crisis had a shorter APTT and longer TT in comparison with the control group ($p < 0.05$). TT directly depends on the fibrinogen level which has been reduced in patients with HS compared to normal group (Fig. 6a); however, the parameters were within the normal ranges. Only several APTTs of the HS patients were slightly above the normal range. The values of PR did not significantly differ among all three groups.

A global hemostasis test, thromboelastography (TEG), allows testing of both clotting pathways, plasma and platelets, to evaluate the state of the hemostasis system. TEG has shown a tendency towards activated blood coagulation in all patients with HS. All parameters of thromboelastography, R, k, angle α and MA, were shifted to the region of hypercoagulation in HS patients in comparison with the control group (Fig. 4). A statistically significant difference was observed between the groups of patients and in comparison to the healthy children. The changes in the TEG parameters in the group with hemolytic crisis were more dramatic than those in the group with a stable state.

Another global hemostasis test, thrombodynamics (TD), allows the evaluation of the spatial dynamics of clot growth. TD also indicated the presence of hypercoagulation with the parameters of clot growth rates (initial clot growth rate, Vi, and stationary clot growth rate, Vs) in patients with HS in a state of hemolytic crisis, which is correlated with data in the literature regarding the increased risk for thrombosis precisely in the moment of crisis [6,8]. Furthermore, the Vi and Vs TD

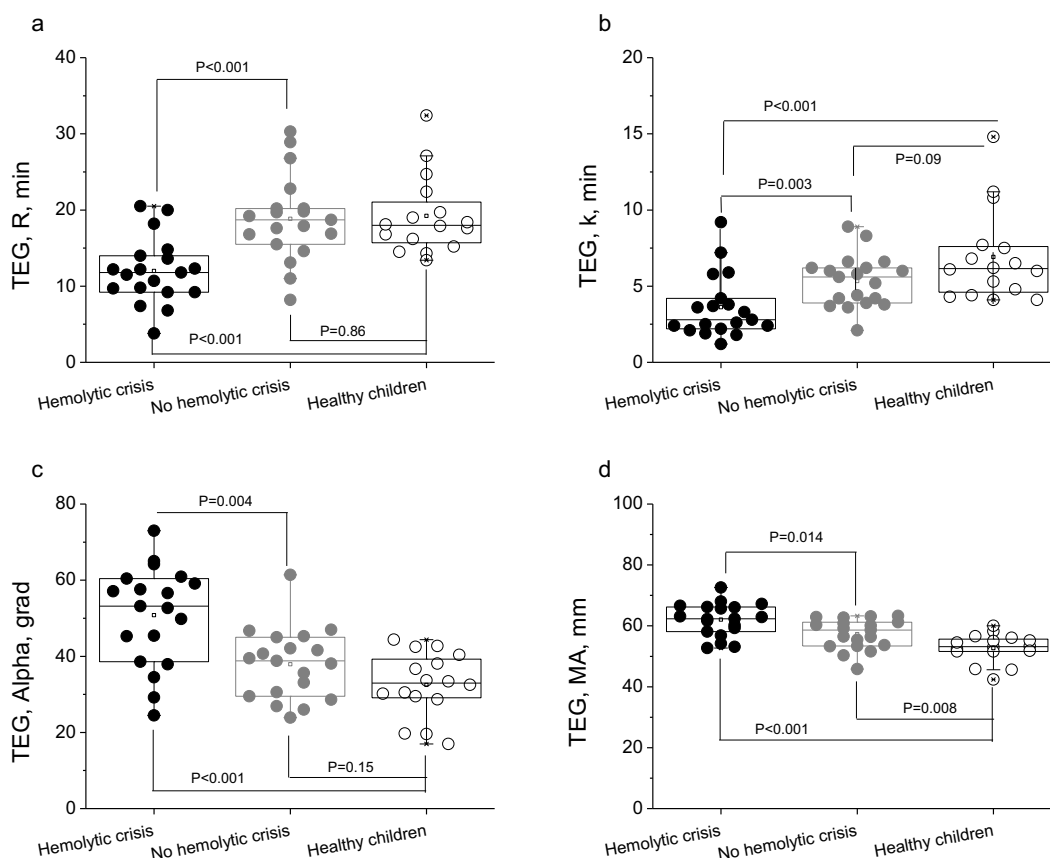


Fig. 4. TEG parameters in children with HS during hemolytic crisis and in a stable state in comparison to the healthy children group (a – reaction time R, b – kinetics k, c – angle α , d – maximum amplitude MA). *TEG was not performed for all patients or children from control group.

parameters show a statistically significant tendency for activated clotting in the group of patients during crisis compared with the group of patients without hemolytic crisis (Fig. 5). The clot density, D, was decreased in HS patients compared to the healthy children group ($p < 0.05$).

Interestingly, the concentration of fibrinogen was decreased in HS patients compared to the healthy children group ($p < 0.05$). The concentration of fibrinogen is more often associated with the consumption of this protein in the processes of pathological coagulation during hemolysis [26]. Additionally, in some patients with HS, there was an increase in D-dimer level and fibrin degradation markers, which also confirms the presence of pathological coagulation during hemolysis (Fig. 6).

The correlations between the hemoglobin, LDH, bilirubin, reticulocytes level and global hemostatic tests were statistically non-significant ($p > 0.05$, data not presented). It is known that laboratory parameters of hemolysis do not always directly correlate with the condition of the patient. However, comparing the HS patient groups with and without hemolytic crisis, global hemostasis assays indicated hypercoagulation on crisis. Furthermore, biochemical blood parameters indicated increased hemolysis in HS patients in crisis. There was a correlation observed between the reaction time, R, which is more associated with plasma clotting and the stationary clot growth rate, Vs (Fig. 7a). This result confirmed the adequate sensitivity of global hemostasis assays for the activation of coagulation in patients with HS.

Additionally, there was a correlation observed between clot density in TD and fibrinogen concentration (Fig. 7b) in patients with HS. The

clot density, D, in TD is dependent on the concentration of fibrinogen, as described previously [27] in in vitro data.

4. Discussion

It is known that patients with hereditary hemolytic anemia have a tendency towards having hypercoagulation that can lead to thrombotic complications. The risk for thrombosis is increased in teenagers. In children with HS, according to the published data, thrombosis observed mainly after surgery and the risk for such complications is approximately 5% [10,12,13,17,20]. It is described that hematological complications can occur not only after splenectomy but also after other surgical interventions, for example, orthodontic operations [28]. Additionally, the risk for thrombosis increases during hemolytic crisis. Disorders of the erythrocyte membrane lead to a loss of flexibility properties and, at the same time, to microcirculatory disturbance. They also lead to increased destruction of erythrocytes and to the appearance of large concentrations of procoagulant microparticles in the bloodstream [14,29]. This can be one of the possible mechanisms of activation of the blood clotting system. However, the overall risk for spontaneous thrombosis in HS patients still remains low. However, in some cases, hypercoagulation can cause severe thrombotic complications.

Screening clotting times (APTT, TT, and PR) were not sensitive to prothrombotic tendencies in children with HS. However, there was the decrease in the concentration of fibrinogen and an increase in D-dimer levels in some HS patients during hemolytic crisis. This may be due to the consumption of fibrinogen for pathological fibrin clot formation

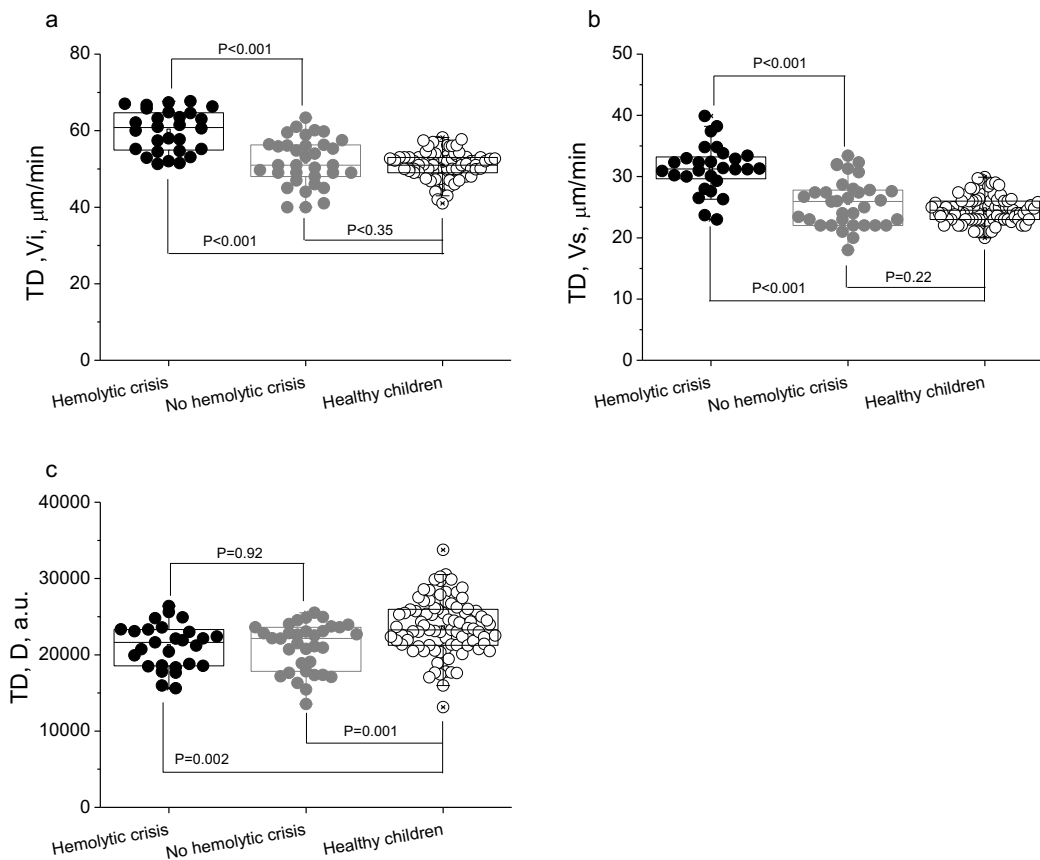


Fig. 5. TD parameters in children with HS during hemolytic crisis and in a stable state in comparison to the healthy children group (a – the initial clot growth rate Vi, b – the stationary clot growth rate Vs, c – clot density D).

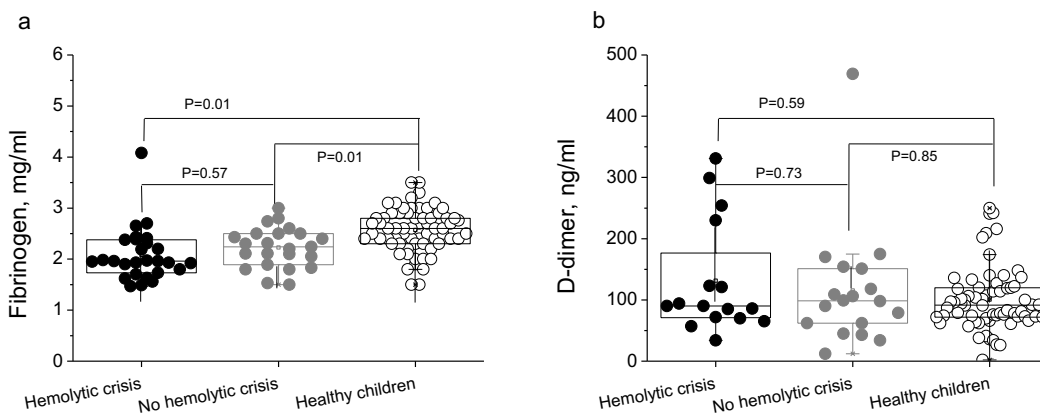


Fig. 6. The concentrations of fibrinogen (a) and D-dimer (b) in children with HS during hemolytic crisis and in a stable state in comparison to the healthy children group.

during acute hemolysis with subsequent lysis that leads to an increase in the D-dimer level. This hypothesis is consistent with the hypercoagulation indicated by TEG and TD. TEG is sensitive to platelet count and to fibrinogen level, TD is sensitive to fibrinogen level. The platelet count was normal (Table 1), and despite the reduced concentration of fibrinogen (Fig. 6a) TEG and TD indicated the increased clotting. Global hemostasis assays indicate high procoagulant activity in patients with acute hemolysis. TEG and TD indicated changes in the state of the blood clotting system in patients with HS, especially in the group of HS

patients during hemolytic crisis. This result agrees well with the existing literature on hypercoagulability revealed with TEG in patients with hemolytic anemia [30].

Patients with hemolytic anemia have an increased risk for thrombotic complications. It is necessary to monitor the hemostasis system in HS patients to predict prothrombotic tendencies, especially before and after surgery. The results of this study demonstrate that the global hemostasis assays TEG and TD may be useful tools for monitoring the condition of patients with HS.

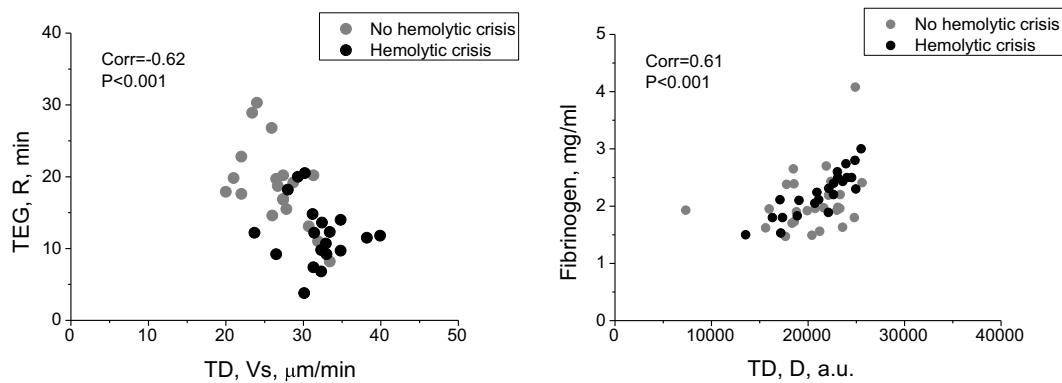


Fig. 7. Correlation plots (for patients with HS). Correlation between the reaction time, R (TEG), and the stationary clot growth rate, Vs (TD) – a; correlation with clot density D (TD) and fibrinogen concentration – b.

Disclosure of interest

FIA is former employee and founder of HemaCore LLC, which holds several patents and patent applications related to the diagnostic use of spatial clot growth and developed an assay under the trade name Thrombodynamics®.

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