Platelet haemostatic properties in β-thalassaemia: the effect of blood transfusion

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Background. Patients with thalassaemia may have thromboembolic events and, even without thrombosis, they have a subclinical hypercoagulable state. In this setting, several coagulation laboratory abnormalities have been described, but thus far no studies have explored the contribution of platelet adhesive and procoagulant properties to blood clotting activation. In this study, we dissected the platelet procoagulant effect and influence of blood transfusions on haemostasis and platelet function in thalassaemic patients.

Material and methods. Sixteen subjects with thalassaemia were studied (9 with transfusion-dependent β-thalassaemia, 7 "trait" carriers). Splenectomised and non-splenectomised patients undergoing blood transfusion were compared. All splenectomised patients were then compared to "trait" carriers and to healthy controls (n=9). The following parameters were measured in transfusion-dependent patients before and after monthly transfusions and compared to those of controls: levels of platelet surface activation markers (P-selectin, tissue factor, and fibrinogen), whole blood platelet aggregation, tissue factor or adenosine diphosphate (ADP)-induced platelet thrombin generation (TG) potential, and D-dimer.

Results. Before transfusion, platelets from splenectomised patients showed significantly higher ADP-induced tissue factor expression, ADP- and collagen-induced platelet aggregation and TG potential than those from non-splenectomised patients and controls. Blood transfusion in splenectomised patients reduced platelet activation, aggregation and TG potential.

Discussion. Splenectomised patients with β-thalassaemia had a prothrombotic state, characterised by enhanced platelet reactivity and function, and high platelet-induced TG potential. One hour after blood transfusions platelet and coagulation parameters improved, supporting the hypothesis that transfusion might have a protective role on platelet haemostatic status.

Keywords: β-thalassaemia, platelet aggregation, thrombin generation, blood transfusion, tissue factor.

Introduction

β-thalassaemia is recognised as one of the leading causes of inherited haemolytic anaemia with a global prevalence of mutation carriers of about 1.5%[1-2]. Patients with β-thalassemia are characterised by partial or complete lack of haemoglobin β-chain synthesis, which may lead to anaemia of different degrees of severity[1]. As a consequence, individual requirements of blood transfusions[3], which still represents the main therapeutic strategy in association with iron-chelation therapy[4], vary greatly among subjects. Therapeutic splenectomy may be performed in those individuals who develop β-thalassaemia-associated hypersplenism caused by the increased removal of damaged red blood cells. The improvements of the medical therapy for β-thalassaemia have translated into longer survival of thalassaemic patients over the past decades[5]. However, the increased life expectancy has led to a higher risk of late complications, including venous and arterial thromboembolic events[6-9]. The prevalence of adult thalassaemic patients who have experienced a prior thromboembolic event varies from 4[6] to 29%[8] and these events seem to be associated with the severity of the underlying disease[10-11]. Recent data show that splenectomy significantly increases the relative risk (RR) of thrombosis (RR 6.59, 95% confidence interval [95% CI]: 3.09-14.05), while regular transfusion therapy may exert a risk-reducing effect (RR 0.28, 95% CI: 0.16-0.48)[4,12].

Several pathogenic mechanisms can explain the underlying procoagulant state[13], namely red blood cell membrane damage providing a source of procoagulant phospholipids, thrombocytosis following splenectomy and chronic platelet activation, endothelial injury and activation, impaired hepatic synthesis of coagulation inhibitors (e.g. protein C and protein

S) secondary to haemosiderosis, and high levels of procoagulant microparticles[14,15]. Consequently, increased plasma markers of hypercoagulability[16,17] and enhanced thrombin generation potential[8,18] are characteristics of thalassaemic patients. A case-report by Atichartakarn *et al*.[19] described normalisation of blood hypercoagulability (i.e. plasma thrombin-antithrombin levels) and an improvement in thromboembolic complications (i.e., pulmonary arterial hypertension) in a single asplenic patient with haemoglobin E/β-thalassaemia undergoing chronic blood transfusion. However, to the best of our knowledge, no studies have simultaneously explored the contribution of activation, adhesive and procoagulant properties of platelets or prospectively confirmed the limited evidence of a beneficial role of blood transfusion on the hypercoagulable state.

The aims of our prospective study in a group of patients with β-thalassaemia (β-thalassaemia intermedia or β-thalassaemia major) were to characterise their platelet prothrombotic profile by different assays and to evaluate possible changes of these properties after packed red blood cell transfusion.

Materials and methods

Study subjects

All patients followed at the Centre of Immunohaematology and Transfusion Medicine of "Papa Giovanni XXIII" Hospital (Bergamo, Italy) were screened for the purpose of this study. Inclusion criteria were: (i) a diagnosis of β-thalassaemia intermedia based on accepted clinical criteria and globin genotype[20] or a diagnosis of β-thalassaemia major, defined as severe anaemia within the first year of age, higher foetal haemoglobin levels, and thalassaemia trait in both parents; and (ii) requirement of regular blood transfusions. The main exclusion criterion was ongoing therapy with anticoagulant or antiplatelet drugs. Two control populations were selected: (i) thalassaemic "trait" subjects (thalassaemia carriers with microcytosis or mild microcytic anaemia), who were otherwise healthy, and (ii) healthy volunteers. All adult subjects, as well as parents or legal tutors of minors, were required to provide written informed consent to participation in the study. The study was performed according to the latest Declaration of Helsinki and the principles of good clinical practice, with no variations on the international management protocols. The study protocol was approved by the Ethical Committee of our Institution.

Blood samples and withdrawal

Blood samples were drawn early in the morning with a 21-gauge butterfly needle. After applying a light tourniquet, venous blood was collected into sterile siliconised tubes containing ethylenediaminetetraacetic acid or trisodium citrate (BD Vacutainer® 5.4 mg and 0.129 M 1:9 vol:vol, respectively; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). In thalassaemic patients only, blood samples were collected before (T0) and within 1 hour after (T1) the monthly packed blood red cell transfusion.

Haemocromocytometric analysis

The haemocromocytometric study was performed using a fully automated haematology analyser, Sysmex XE-2100 (TOA Medical Electronics, Kobe, Japan).

Flow cytometric analysis of platelets

Flow cytometric analysis of platelet surface antigens was performed with a FACS Canto™ II (Becton, Dickinson and Company) cytofluorimeter in citrated whole blood samples after labelling with monoclonal antibodies for P-selectin (anti-CD62p Biolegend, London, UK), tissue factor (TF) (anti-CD142/FITC Space Import Expert srl, Milan, Italy) and fibrinogen (anti-fibrinogen antibody Stago, Milan, Italy), as described elsewhere[21]. An anti-IgG1/FITC (Stago) antibody was used as an isotype control. To evaluate platelet activation in response to exogenous stimuli, the cytoflurimetric analysis was performed on an aliquot of whole blood collected from the ADP/collagen (equine type I collagen 2 μg, ADP 50 μg) cartridge of a Platelet Function Analyzer (PFA)-100® System (Dade®, Siemens, Milan, Italy). Data were analysed by FACSDiva software (Becton, Dickinson and Company). The percentage increase of positive platelets after ADP/collagen stimulation was calculated as follows:

$$\frac{(\%\ \text{positive PLTs after ADP} - \%\ \text{positive PLTs before ADP})}{\%\ \text{positive PLTs before ADP}} \times 100$$

(PLTs: platelets).

Whole blood platelet aggregation

Platelet function was evaluated by the multiple electrode aggregometry (MEA, Multiplate® analyser, Roche, Milan, Italy), as described previously[22]. Multiplate (short for "multiple platelet function analyser") is a multichannel analyser of platelet reactivity, which performs the analysis on samples of whole blood. This method involves the measurement of the change in electric resistance occurring during platelet adhesion and aggregation to two electrodes, which is recorded by the analyser over a period of 6 consecutive minutes. Briefly, 300 μL saline and 300 μL of whole blood were added to the test cell, and, after 3 minutes of incubation at 37 °C, samples were activated

with ADP, collagen, thrombin receptor-activating peptide 6 (TRAP), or arachidonic acid (ASPI). Platelet aggregation results are expressed as area under curve in arbitrary units (AU).

Platelet thrombin generation potential

To evaluate the global procoagulant potential of the platelets, we performed the thrombin generation (TG) assay in platelet-rich plasma (PRP) using the calibrated automated thrombography (CAT assay, Thrombinoscope™, Stago)[23]. PRP was isolated from citrated whole blood by centrifugation at 200 *g* for 10 minutes at room temperature and then diluted with homologous platelet-poor plasma in order to obtain a fixed concentration of 150,000 platelets/μL. The TG assay was performed in the presence of 1 pM TF, or ADP at 1.6 and 8.3 μM, as described previously[24]. TG curves were described in terms of lag-time, peak height (i.e. the maximum concentration of thrombin formed), time-to-peak and area under the curve (i.e.: the endogenous thrombin potential, ETP). Furthermore, an additional parameter called mean rate index (MRI) or slope, which reflects the speed of TG, was calculated as follows:

$$\frac{\text{peak height (nM)}}{[\text{time-to-peak (min)} - \text{lag-time (min)}]}.$$

The TG studies were performed in thalassaemic patients and healthy subjects, but not in thalassaemic "trait" carriers.

D-dimer test

D-dimer, a fibrin degradation product of cross-linked fibrin, was quantified in plasma samples by an automated latex enhanced immunoassay (HemosIL D-dimer HS, Werfen, Milan, Italy) on an ACL TOP500 coagulometer (Werfen, Milan, Italy). Results are expressed in ng/mL.

Statistical analysis

The results are described in terms of mean ± standard deviation (SD). Baseline clinical characteristics and platelet/haemostatic tests of included subjects were compared with variance analysis (ANOVA) followed by post-hoc exploratory analyses of differences between subgroups: Fisher's least significant difference and Dunnett's test (splenectomised patients served as the reference for comparisons). All reported p values were two-sided with a type I error rate of 5%. Linear regression analysis was used to test the association between continuous variables. The differences were considered statistically significant at $p<0.05$. Statistical analysis was performed with the Graph Pad Prism 5 (Graph Pad Software, San Diego, CA, USA) and with SPSS v.21 (IBM, Armonk, NY, USA).

Results

Characteristics of the study population

Twelve consecutive β-thalassaemic patients on chronic red blood cells transfusions were screened between July and October 2012. Two patients were excluded because they were receiving anticoagulant or antiplatelet therapy at the time of the study; one patient did not consent to participate in the study. Nine patients were included, of whom six had β-thalassaemia major and three had β-thalassemia intermedia.

All patients needed monthly transfusions with leucocyte-reduced red blood cells with a mean monthly requirement of 1.9 International Units (IU). Four patients had been splenectomised at least 10 years before inclusion in the present study, and two of them had had a prior thromboembolic event (1 portal vein thrombosis, 2 superficial vein thromboses). As control groups, we enrolled seven female subjects with β-thalassaemia "trait" and no co-morbidities and nine healthy subjects. Table I shows the study subjects' baseline characteristics, as well as the results of haemocromocytometric analysis before (T0) and after (T1) blood transfusion. Thalassaemic patients and "trait" carriers had lower levels of haemoglobin and haematocrit compared to healthy controls, while splenectomised patients had higher platelet counts compared to both thalassaemic "trait" subjects and healthy controls.

Flow cytometric analysis of platelets

Before transfusion (T0) and in the absence of ADP/collagen stimulation, no differences were observed between patients, "trait" carriers, and healthy subjects in the percentages of platelets positive for TF, fibrinogen and P-selectin (data not shown). Similarly, no differences were found between splenectomised and non-splenectomised patients. Interestingly, the measurements of the surface activation markers after ADP/collagen stimulation revealed different platelet reactivity among the study subjects. In particular, as shown in Figure 1, ADP/collagen stimulation induced a higher increment in the percentage of TF-positive platelets in splenectomised patients (127.3±38.1% increase: $p<0.05$) and non-splenectomised patients (95±58% increase; p=ns) compared to healthy subjects (40.7±15.7% increase) (left panel). The increase in fibrinogen binding to platelets in response to ADP/collagen stimulus was also higher in patients with thalassaemia than in healthy controls, although the difference did not reach statistical significance (right panel). After transfusion (T1), however, splenectomised patients had a lesser response to stimulation in terms of surface TF (14.4±10.3% increase) and fibrinogen (5.9±11.3% increase)-positive platelets compared

Table I - Baseline clinical characteristics of the study subjects.

	Patients		Controls	
	Splenectomised (n=4)	*Non-splenectomised (n=5)*	*Trait (n=7)*	*Healthy (n=9)*
Male/female (n)	0/4	2/3	0/7	5/4
Age, years	38 (26-49)	21 (4-41)	41 (19-58)	38 (20-56)
β-thalassaemia major, n	3	3	-	-
β-thalassaemia intermedia, n	1	2	-	-
Mean IU monthly RBC, n	2	1.8	0	0
Chronic iron-chelation therapy, n	3	5	-	-
Haemoglobin (g/L)				
T0	83 (10)	89 (13)	110 (15)*^	141 (10)*^
T1	102 (9)	106 (9)	-	-
Haematocrit (%)				
T0	24.7 (2.3)	25.4 (3.7)	35.1 (3.5)*^	41.6 (2.2) *^
T1	30.6 (2.9)	30.5 (2.8)	-	-
RBC ($\times 10^{12}$/L)				
T0	3.4 (0.6)	3.3 (0.5)	5.6 (0.3)*^	4.9 (0.2)*^
T1	4.1 (0.7)	3.9 (0.3)	-	-
Platelets ($\times 10^{9}$/L)				
T0	399 (157)	261 (92)*^	211 (29)*^	231 (48)*^
T1	349 (133)	209 (50)$^{+}$	-	-

Data are mean (SD); *post-hoc analysis (Fisher's LSD) significant (splenectomised patients: comparison group); ^post-hoc analysis (Dunnett's test) significant (splenectomised patients: comparison group); $^{+}$*t*-test significant.
n: number; IU RBC: international units of packed red blood cells; SD: standard deviation. T0: before transfusion; T1: after transfusion.

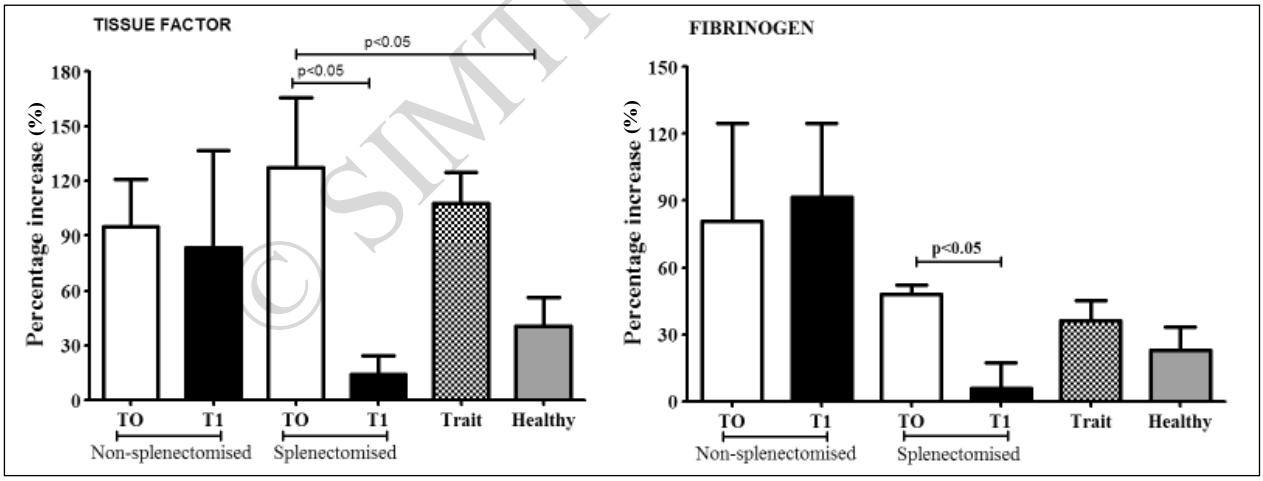

Figure 1 - ADP/collagen-induced expression of tissue factor (TF) and fibrinogen binding on the platelet surface.
The figure shows the results of platelet flow cytometry analysis of blood samples stimulated with ADP/collagen in thalassaemic patients, "trait" carriers, and healthy subjects. Data are expressed as percentage increase= [(% positive platelets after ADP − % positive platelets before ADP)/% positive platelets before ADP ×100]. For thalassaemic patients, data are shown before (T0) and after blood transfusion (T1). Before transfusion the splenectomised patients had a significantly ($p<0.05$) greater ADP-induced increase in TF-positive platelets compared to healthy subjects (left panel). In addition, after transfusion, splenectomised patients had a significantly greater reduction ($p<0.05$) of both TF and fibrinogen expression on platelets compared to the respective pre-transfusion values (left and right panels).

to pre-transfusion values. This was not observed in non-splenectomised patients, who maintained a similar percent increase to that prior to the transfusion.

After ADP/collagen stimulation the percentages of platelets expressing P-selectin increased by 6- to 10-fold in the various study subjects, but differences were not statistically significant.

Platelet aggregation study

As shown in Figure 2, splenectomised patients had higher platelet aggregation values than had either healthy subjects or thalassaemic "trait" carriers. Comparison within the group of patients, showed significantly higher ADP- and collagen-induced platelet aggregation in splenectomised patients than in non-splenectomised ones. After transfusion, a trend to a reduced platelet aggregation response to all agonists was observed in splenectomised patients. No significant correlations were found between platelet aggregation values and platelet count, red blood cell count, or haematocrit.

Platelet procoagulant properties

CAT assay in PRP was used to characterise the platelet TG potential. Examples of TG curves from representative TG experiments performed in the presence of TF (left panel) or ADP (right panel) are shown in Figure 3. The splenectomised patient showed an increased TG potential compared to both the non-splenectomised patient and healthy control subject, as demonstrated by the shorter lag-time necessary for the start of the TG burst, the higher peak of maximum thrombin generated, and the shorter time to reach this peak. TG analysis in all study subjects paralleled this profile. Figure 4 summarises data regarding the kinetic parameters of TG in the presence of TF (upper panels) or 1.6 μM ADP (lower panels). TG induced by 6.3 μM ADP gave similar profiles to those obtained with 1.6 μM ADP (data not shown).

Comparing TG of PRP samples from splenectomised patients with that of healthy control subjects, before transfusion the former group had statistically significant ($p<0.05$) shorter lag-times in the presence of TF

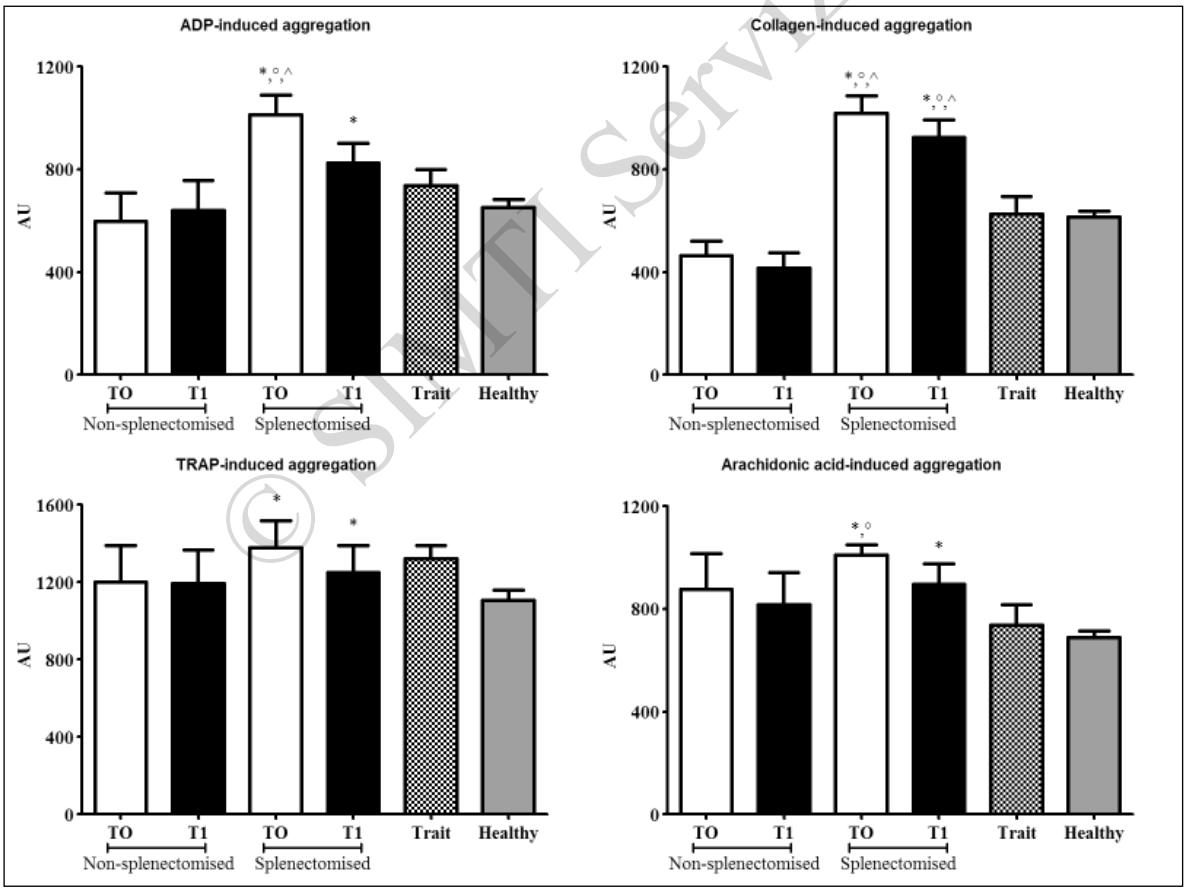

Figure 2 - Platelet aggregation studies using the Multiplate analyser.
The figure represents platelet aggregation in whole blood samples collected from thalassaemic patients before (T0) and after (T1) red blood cell transfusion, "trait" carriers and healthy control subjects.
ADP: adenosine diphosphate; AU: area under the curve; TRAP: thrombin receptor-activating peptide 6.
*p<0.05 *vs* healthy controls; °p<0.05 *vs* trait; ^p<0.05 *vs* non-splenectomised.

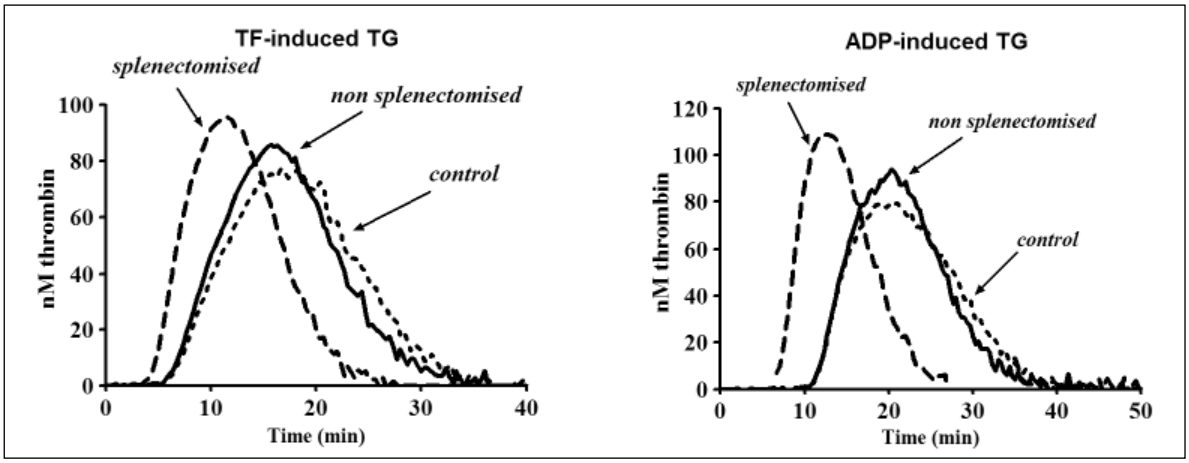

Figure 3 - Sample curves of thrombin generation (TG) induced by tissue factor (TF) and ADP.
The figure illustrates the results of assays of 1 pM TF-induced (left panel) and 1.6 μM ADP-induced (right panel) TG in representative study subjects: a splenectomised patient, a non-splenectomised patient and a healthy subject. The splenectomised patient had greater TG potential, with shorter lag-time and time-to-peak compared to the other subjects.

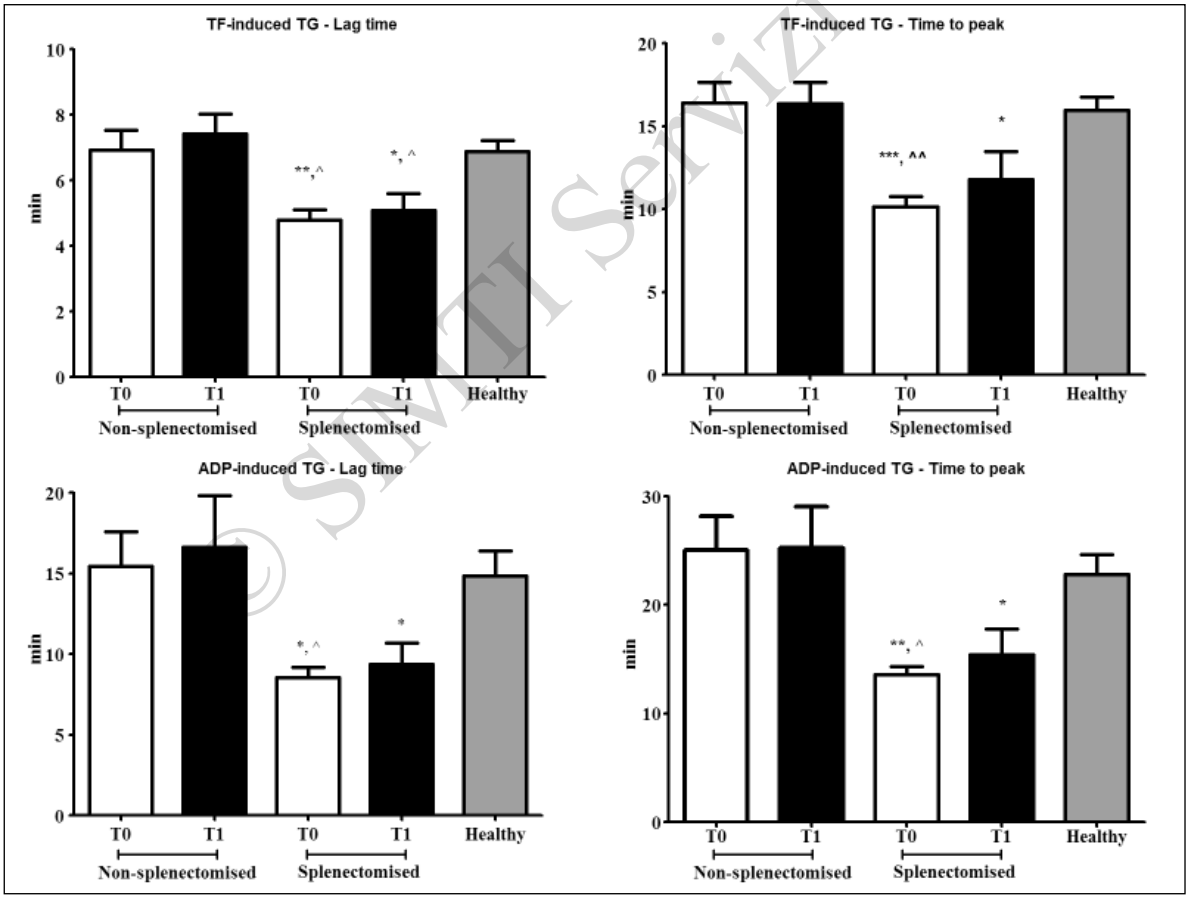

Figure 4 - Kinetic parameters of tissue factor (TF)- and ADP-induced thrombin generation (TG).
Pre-transfusion (T0) lag-time and time-to-peak of TG induced by both TF (upper panels) and ADP (lower panels) are significantly shorter in splenectomised patients than in healthy control subjects. After blood transfusion (T1), lag-time and time-to-peak were slightly prolonged in splenectomised patients, but still remained significantly shorter ($p<0.05$) than in healthy controls.
*$p<0.05$, **$p<0.01$; ***$p<0.001$ *vs* healthy controls; ^$p<0.05$, ^^$p<0.01$ *vs* non-splenectomised patients; °$p<0.05$ *vs* trait.

(4.79±0.32 *vs* 6.89±0.33 min) or ADP (8.55±0.66 *vs* 13.3±4.9 min). Similarly, the same group of patients showed significantly ($p<0.05$) shorter time-to-peak with both TF (10.1±0.61 *vs* 15.9±0.77 min) and ADP (13.6±0.75 *vs* 22.8±1.84 min) compared to healthy controls. The slope of TF and ADP-induced TG was significantly ($p<0.05$) higher in splenectomised patients (19.5±9.2 nM/min, 22.0±9.6 nM/min, respectively) than in healthy controls (10.6±2.8 nM/min, 12.6±3 nM/min, respectively) and non-splenectomised patients (6.81±2 nM/min, 7.27±3.6 nM/min, respectively).

After transfusion, lag-time and time-to-peak were slightly prolonged in splenectomised patients (lag-time TF: 5.09±0.52 min; lag-time ADP: 9.35±1.34 min; time-to-peak TF: 11.7±1.73 min; time-to-peak ADP: 15.4±2.35 min), compared to the pre-transfusion values, still remaining significantly shorter ($p<0.05$) than those of healthy controls. The slope increased slightly, although not statistically significantly (TF: 21.4±14.9 nM/min; ADP: 30.0±25.5 nM/min). No differences were observed between patients and controls in ETP or peak height values of TG either at T0 or T1 (data not shown).

D-dimer

Mean D-dimer values were within the normal reference range in all groups. Thalassaemic patients had higher mean D-dimer values (90±90 ng/mL) than both "trait" carriers (46±30 ng/mL) and healthy controls (30±46 ng/mL). Splenectomised patients showed the highest mean values (125 ng/mL). D-dimer values were not modified by blood transfusions.

Discussion

This study was designed to evaluate different platelet haemostatic functions in relation to the administration of blood transfusions in a group of patients with β-thalassaemia attending our outpatient clinics. In detail, the following platelet haemostatic properties were investigated: (i) platelet activation through the evaluation of surface activation marker expression (i.e. P-selectin and TF) or the binding of fibrinogen to activated GPIIb-IIIa, both in basal conditions and after ADP/collagen stimulation; (ii) the aggregation capacity in response to several agonists; and (iii) the contribution to TG induced by TF and ADP.

Differently from previous studies conducted in this disease[25,26], in the absence of stimulation platelets from our patients did not show either higher platelet expression of P-selectin or higher surface expression of TF or fibrinogen binding, compared to those from healthy subjects. However, compared to healthy controls, splenectomised patients had greater platelet reactivity to ADP/collagen in terms of increase in TF-positive ($p<0.05$) and fibrinogen-positive ($p>0.05$) platelets. This finding is in line with published observations that splenectomy is characterised by platelet hyper-reactivity, rather than activation[27,28]. Interestingly, after blood transfusion statistically significant reductions in the percentages of TF- and fibrinogen-positive platelets occurred, suggesting that blood transfusion improves platelet hyper-reactivity.

Before blood transfusion, platelet aggregation capacity in response to all agonists used (ADP, collagen, thrombin, and arachidonic acid) was significantly higher in splenectomised patients than in healthy controls. Platelet aggregation in the same patients decreased after transfusion, albeit not significantly so ($p>0.05$). To the best of our best knowledge, whole blood aggregation was assessed with a Multiplate® platelet function analyser in thalassaemic patients for the first time. Our data agree with those from previous studies, which evaluated platelet aggregation in thalassaemic patients by light transmission aggregometry in PRP[29,30] or in whole blood[31], and found higher platelet aggregation in splenectomised patients than in non-splenectomised ones and in patients with β-thalassaemia intermedia than in healthy controls. According to these studies, our results confirmed that platelet hyper-aggregability still exists, even in chronically transfused splenectomised patients. Furthermore, our data suggest that this trend is only slightly corrected by blood transfusion.

In addition, β-thalassaemia patients, especially those who were splenectomised, showed higher D-dimer levels than controls, confirming an ongoing blood clotting activation.

To explore the procoagulant contribution of platelets, we performed the TG assay in PRP, given the growing evidence that the parameters of the thrombogram are useful in assessing bleeding or thrombotic risk and that several conditions (congenital and acquired) that increase TG contemporaneously cause a thrombotic tendency[23]. In our study, conducted with PRP samples, the splenectomised patients had a higher platelet TG potential (i.e. shorter lag-time and time-to-peak, and higher slope) compared to healthy controls and non-splenectomised patients, with the highest values being reached in the presence of TF. No significant differences in static TG parameters such as ETP and peak height were found between groups, suggesting that splenectomised patients generate thrombin to the same extent as controls but more rapidly. The kinetic parameters of the TG curve, particularly lag-time, are strictly dependent on TF expressed by cell membranes, while the parameters of the propagation phase of TG (the peak height representing the maximum quantity of thrombin produced) are mostly dependent on tenase

and prothrombinase complexes[32-34]. In our experimental system, platelets might contribute to accelerating TG by providing not only phospholipids but also TF. This hypothesis is also supported by our finding of a high ADP-induced platelet surface TF expression in splenectomised patients. The differences in TG potential between patients and controls were attenuated after blood transfusion.

TG in PRP has been studied in several clinical conditions[22,35,36] but never in β-thalassaemia. Indeed, in this condition, TG has been characterised in plasma samples. In one study, in which a chromogenic-based TG assay was used, a higher TG potential was demonstrated in β-thalassaemia intermedia splenectomised patients than in non-thalassaemic splenectomised controls[8]. In another study, by Tripodi *et al.*[18], paired measurements for the same patients were acquired by means of both thromboelastometry in whole blood and the CAT assay in plasma. Data from this study showed a hypercoagulable state in splenectomised thalassaemic patients only with the thromboelastometry analysis, but not with the TG assay. As suggested by the authors, these findings indicate that blood cell and/or platelet abnormalities rather than plasma abnormalities are the most important determinants of the prothrombotic profile observed in thalassaemic patients who had been splenectomised. Our data on TG in PRP are in agreement with the conclusions of the study by Tripodi *et al.*[18], suggesting a contribution of platelets to hypercoagulability in this disease. The small sample size of our study does not allow firm conclusions to be drawn and, clearly, the contribution of other factors in the hypercoagulable state of patients with β-thalassaemia, such as low levels of haemoglobin, cannot be excluded[16].

Conclusions

In conclusion, splenectomised patients with β-thalassaemia showed a prothrombotic state, characterised by enhanced platelet reactivity and function, and high platelet-induced TG potential. One hour after blood transfusions, platelet and coagulation parameters improved. It would be worth conducting further, larger studies, also taking into account the long-term effects of transfusions, in these patients.

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Authorship contributions

AT, MM and AF designed the study and provided the study material; AT, CG, CJT and LR performed the measurements. AT and MM analysed the results and performed the statistical analysis; AT, MM and AF wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

The Authors declare no conflicts of interest.

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