Platelet function and blood coagulation system status in childhood essential thrombocythemia

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To cite this article: Dmitrii M. Polokhov, Nikolay M. Ershov, Anastasia A. Ignatova, Evgeniya A. Ponomarenko, Marina V. Gaskova, Pavel A. Zharkov, Daria V. Fedorova, Alexandr V. Poletaev, Elena A. Seregina, Galina A. Novichkova, Nataliya S. Smetanina & Mikhail A. Panteleev (2019): Platelet function and blood coagulation system status in childhood essential thrombocythemia, Platelets, DOI: 10.1080/09537104.2019.1704710

To link to this article: https://doi.org/10.1080/09537104.2019.1704710

Published online: 19 Dec 2019.
Platelet function and blood coagulation system status in childhood essential thrombocythemia

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Abstract
Childhood essential thrombocythemia (ET) is a rare chronic myeloproliferative disorder. The quality of life of ET patients may decrease as a result of ischemic and hemorrhagic complications of unclear origin. Our goal was to characterize the hemostatic system in children with ET. We genotyped and investigated blood samples from 20 children with ET in a prospective case series study using platelet aggregation, functional flow cytometry (FC) assay and standard clotting assays. Three children had a JAK2V617F mutation, 4 had mutations in CALR and 13 were triple-negative. Myelofibrosis in stage 1-2 was detected in 3 children. Three patients had bleeding episodes and seven had ischemic events. Aggregation in response to collagen, adenosine diphosphate, and ristomycin was decreased in all patients. In FC, significant changes in the whole patient group compared to the healthy children control group were decrease in the resting forward scatter and PAC1 binding (activated GPIIb/IIIa) level. For the activated platelets, dense granules release (by mepacrine), PAC1, and GPIIb/IIIa levels were significantly decreased. GPIb/V/IX, P-selectin, and phosphatidylserine levels manifested only moderate differences. Forward and side scatter changes in response to stimulation (representing shape change) and dense granules release were significantly lower in the 3 patients with bleeding than in the 17 patients without hemorrhage. Activated partial thromboplastin time was slightly prolonged, prothrombin index was slightly shortened and thrombin time was normal, while fibrinogen was mildly decreased in the ET patients. It could be concluded that the observed platelet function defects could be related to bleeding in ET, and be potentially used as a marker.

Introduction
Essential thrombocythemia (ET) is a chronic myeloproliferative disorder (CMPD) characterized by an increase in the platelet count of more than 450,000/μL and megakaryocytic hyperplasia in bone marrow (BM). It can be accompanied by leukocytosis, spleno- and hepatomegaly, hemorrhage and thrombosis, and possible transformation to myelofibrosis (MF) or acute leukemia (AL) [1]. The reported incidence of ET in childhood is approximately 1 per 10,000,000, which is 60 times lower than in adults [1,2]. The leading hypothesis regarding the onset of ET is damage to the pluripotent hematopoietic stem cell genome. Emerging mutations in the JAK2, MPL or CALR genes trigger the activation mechanism of the JAK/STAT signaling pathway; however, there are “triple-negative” patients with none of these genes affected [3–10]. There are no reliable data on the prevalence of mutations in children [2–4,8]. Splenomegaly and hepatomegaly in children, individually or in combination, can occur in approximately half of the patients [2]. Clinical manifestations of ET are mainly neurological (e.g., dizziness, headache, syncope, and transient ischemic attack), microcirculatory (e.g., rodonalgia, acroparesthesia, ischemia of the fingers and visual impairment), gastrointestinal (e.g., nausea, vomiting and abdominal pain) and hemorrhagic (e.g., nasal bleeding, petechiae, ecchymosis and other). Thrombotic and hemorrhagic complications are the main causes of mortality among adult patients with ET [1,2,11]. The frequency of thrombotic events in children is 0-17% [2]. It was reported that platelets in adult patients with ET are abnormal. Various changes in the platelet phenotype include the number of CD62p (P-selectin), CD63 and GPIIb/IIIa (PAC1 binding) receptors at rest and after activation of TRAP (thrombin receptor-activating peptide) and CRP (collagen-related peptide) [12]. Immunophenotyping reveals a state of intravascular platelet activation [10]. According to a study of platelet aggregation in 55 adult patients with ET in platelet-rich plasma (PRP), aggregation was impaired in 75% of them. However, these ex vivo tests do not detect platelet hyperaggregability [10]. These changes in the reactivity of platelets have not been definitively identified as the causes

Keywords
Blood coagulation tests, essential thrombocythemia, flow cytometry, platelet aggregation

History
Received 19 July 2019
Revised 15 November 2019
Accepted 8 December 2019
Published online 21 December 2019
of thrombotic or hemorrhagic complications, with the exception of erythromelalgia and acquired von Willebrand syndrome [1]. In the present study, we attempted to gain insight into the mechanism of hemostasis abnormalities in children with ET by carrying out laboratory analysis of their hemostasis parameters.

Materials and Methods

Study Design and Recruitment of Patients

Study design is a prospective, case series investigation. During the period from February 2015 to April 2019, we examined 20 children with ET (Table I). The diagnosis of ET was established according to the diagnostic criteria of WHO [13]. The study was approved by the Independent Ethics Committee of the Rogachev National Medical Research Center of Pediatric Hematology, Oncology, and Immunology (protocol #8/2016 from 18.10.2016). Written informed consent was obtained from all healthy volunteers and patients, or from their official representatives.

Clinical and Laboratory Examination

The patients underwent clinical and laboratory examination, including routine studies, genetic and morphological studies of bone marrow and platelets, examination of the liver and spleen, and functional tests of the hemostasis system. Genetic studies were conducted by the Sanger sequencing method. The dimensions of the liver and spleen were assessed by ultrasound and/or multislice computed tomography (MSCT)/magnetic resonance imaging (MRI). For hemostasis analysis, blood was collected by venipuncture into 3 ml vacuum citrate tubes (S-Monovette), which were delivered to the laboratory within 15 min. Sample preparation and analysis required 1 h.

Blood Test

The peripheral blood test was performed on a hematological analyzer Sysmex XS-1000i (Sysmex Corporation, Japan).

Genetic Studies

Genetic studies were conducted by Sanger’s sequencing method on a genetic analyzer Applied Biosystem 3130xl (Applied Biosystems, LLC, USA).

Morphology of the Bone Marrow

The trepanobioplate was stained with hematoxylin and eosin, followed by impregnation with silver. The sample was examined using an ECLIPSE 80i microscope (Nikon Instruments Europe B.V., Netherlands).

Radiation Methods

Visualization of the liver and spleen was performed using a magnetic resonance imager Achieva 3.0T, (Philips Medical Systems, Nederland B.V., Netherlands), and a completely digital universal ultrasound diagnostic apparatus of the expert class ACUSON S2000 ultrasound system (Siemens Medical Solution, USA).

Functional Activity of Platelets: Flow Cytometry

In a cytometric study, we analyzed the marker composition of platelets at rest and after activation in diluted blood with a mixture of TRAP (PAR-1 receptor agonist) and CRP (a glycoprotein VI agonist) as described previously [14–16]. Annexin V-Alexa647 and antibodies against P-selectin (CD62p-Alexa647), GPIIb/IIa (CD61-PE), its activation (PAC1-FITC), and glycoprotein I (CD42b-PE) were from Biolegend (San Diego, CA, USA). CRP was kindly provided by Prof. R.W. Farndale (University of Cambridge, Cambridge, UK). All other reagents were from Sigma-Aldrich (St Louis, MO, USA). Platelets were either left intact or loaded with mepacrine (1 mM) for 30 min at 37°C. Subsequently, they were either left unstimulated or stimulated with CRP at 20 μg/μl and SFLLRN at 12.5 μM for 10 min in the presence of 2.5 mM calcium chloride. Both resting and activated samples were incubated with antibodies against CD61, CD42b, CD62p, as well as PAC1 and annexin V for 10 min. Then, they were diluted 10-fold with buffer

Table I. Clinical and instrumental characteristics of patients.

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*Patients on therapy; **Platelets count, thousand cells/μL.
A containing 2.5 mM calcium and analyzed using a Novocyte flow cytometer (Acea Bioscience, San Diego, CA, USA). As control groups for functional platelet activity assay, blood samples were collected from 72 healthy adults aged 18–50 years and 58 healthy children (27 boys and 31 girls) with a median age of 10 years (2–17 years).

Aggregometry

The study of platelet aggregation function in PRP was performed on the Biola aggregometer (Biola, Moscow, Russia) in accordance with the Recommendations for the Standardization of Light Transmission Aggregometry (LTA): A Consensus of the Working Party from the Platelet Physiology Subcommittee of SSC/ISTH [17]. PRP was prepared by centrifuging blood samples at 200 x g for 10 min. The volume of plasma PRP for the study was 300 μL. The agonists used were: adenosine diphosphate (ADP), 5 μM (Sigma-Aldrich, St Louis, MO, USA), collagen, 0.06 mg (NPO Renam, Russia), ristomycin, 0.45 mg (NPO Renam, Russia). Aggregation curves were recorded for 10 min. As control blood samples were collected from 14 healthy adults with a median age of 26.5 years (22–44 years) and 14 healthy children with a median age of 10.5 years (5–17 years) were used.

Routine Coagulation Assays

The activated partial thromboplastin time (APTT) (normal range 25.1–36.5 s), prothrombin index (PI) (normal range 9.4–13.7 s), thrombin time (TT) (normal range 15.8–24.9 s) and fibrinogen level (normal range 2–3.93 g/L) were determined using the ACL TOP 700 coagulation analyzer (Instrumentation Laboratory, Bedford, MA, USA) with assay kits provided by the manufacturer (Instrumentation Laboratory). To interpret the results, the reference values of the manufacturer and the data obtained for the study were used. The activity of vWF (vWF: Rco) was determined only for patients (normal range 48.2–201.9%).

Statistical Analysis

The analyses of significant differences were performed using a nonparametric Mann-Whitney test with a significance level of p < .05. For the correlation analysis, the Pearson correlation coefficient was calculated.

Results

Patient Demographics and Characterization

The median age of the patients at the time of the laboratory examination was 12.5 years (from 1.5 to 17 years). The median age of disease onset was 10 years (range 1.5–16 years). The gender composition was 1.5:1 (12 boys and 8 girls, respectively). In 10 (50%) patients, the disease progressed without clinical symptoms (group 1). Seven (35%) had ischemic symptoms, including erythromelalgia, chest pain, headaches, sometimes with nausea and vomiting (group 2). Thrombosis was not observed. In 3 (15%), ET manifested according to the hemorrhagic type, including ecchymosis and/or nosebleeds of different severity (group 3). Instrumentally identified splenomegaly occurred in 10 (50%), hepatomegaly in 9 (45%) and splenohepatomegaly in 6 cases (30%) (Table I). Out of 20 children with ET examined, clonal markers were not revealed in 13 (65%); these were marked as triple-negative ET. Three children (15%) had a JAK2V617F mutation, and four (20%) had a mutation in the CALR gene. Mutations in the MPL gene did not occur (Table II). ET in children has a low frequency of transformation into post-ET MF or other malignant tumors [2]. Among the 20 children observed, MF-0-1 was detected histologically in 17 and MF-1-2 in 3 children with clinical symptoms (Table I). In trepanobiopate, a free reticulin network was observed with many crossings, especially in perivascular regions.

Platelet Count and Status of Other Cells

The number of platelets in our study varied from 603 to 2744 (normal range 204–356), with a median of 1468 thousand cells/μL. In group 1, there were 951 to 2303, with a median of 1468 thousand cells/μL. In group 2, there were 770 to 2620, with a median of 1562 thousand cells/μL. In group 3, there were 603 to 2744, with a median of 780 thousand cells/μL. Differences between patient groups are not significant (p > .05) (Table I, Figure 1A). The mean platelet volume (MPV) ranged from 8.2 to 11.8 fL, with a median of 9.15 (normal range 9–13 fL). The leukocytes (WBC) ranged from 4.8 to 16.11 thousand cells/μL, with a median of 8.325 (normal range 4.05–9.85). WBC were increased in 7 (9.99 to 16.11 thousand cells/μL, median 12.07) and decreased in 2 independently of their symptoms. WBC in patients without symptoms was 4.8–13.27 thousand cells/μL, with a median 8.825. In those with clinical symptoms (ischemic and hemorrhagic), it was ranged from 5.85 to 16.11 thousand cells/μL, with a median of 7.84. Median for all patients was 8.325. Differences between groups were not significant (p > .05) (Figure 1B). The red blood cells (RBC) count ranged from 3.91 to 5.45 million cells/μL with a median of 4.615 (normal range 4.2–4.6) (data not shown).

Seventeen patients did not receive cytoreductive therapy for at least 2 weeks before blood collection for the assays of hemostasis function. Three patients received cytoreductive therapy with anagrelide or peg-interferon with effect (Table I).

Platelet Functional Activity: Flow Cytometry

Two groups of healthy volunteers were studied, healthy adults and healthy children (fig. 2, 3). Adult control was used to distinguish between the age-dependent and age-independent changes in the patient platelet functionality. At rest, the healthy children platelets had significantly smaller side light scattering (SSC) (p < .001),
volume of dense granules (evaluated based on mepacrine fluorescence) \( (p < .001) \), density of the CD62p receptor \( (p < .001) \), and phosphatidylserine (PS) \( (p = .002) \). The PAC1 binding was significantly increased in healthy children compared with adults \( (p < .001) \) (Figure 2A, C, E, G and I). No significant differences between healthy adults and children were observed in forward scattering (FSC), CD61 receptor density (GPIIb/IIIa) and CD42b (GPIb/V/IX) of the resting platelets (Figure 3A, C and E). After activation, healthy children had significantly reduced SSC \( (p < .001) \), the volume of dense granule release \( (p < .001) \), CD62p \( (p < .001) \), and PAC1 binding \( (p < .001) \) (Figure 2B, D, F, H and J). No significant differences were observed in FSC platelet size, CD61, CD42b (Figure 3B, D and F) and PS density (Figure 2H). The overall phenotype of healthy pediatric platelets in our study could be described as similar to that of the adults in size and expression of major glycoproteins, with decreased volume and release of

Figure 1. Platelet and white blood cell counts in the patient groups. (A) Panels show platelet counts and (B) leukocyte counts determined in the whole investigated cohort of 20 pediatric patients and individually in the groups: without clinical manifestation, with ischemic manifestations, and with bleeding symptoms. The data are shown as box charts with individual data points overlap. There is no statistically significant difference between the groups.

Figure 2. Age-sensitive functional markers of platelets in the patients and healthy volunteers. Panels show platelet function parameters determined in diluted whole blood by flow cytometry before and after stimulation with TRAP plus CRP in the presence of calcium as described in the Methods: (A) SSC1 at rest, and (B) SSC upon activation; (C) mepacrine fluorescence (volume of dense granules) at rest, and (D) mepacrine release volume after activation; (E) CD62p at rest, and (F) CD62p after activation; (G) percentage of the phosphatidylserine-positive platelets at rest, and (H) phosphatidylserine positive platelets after activation; (I) PAC1 binding at rest, and (J) PAC1 binding after activation. The data format is as in Figure 1.
alpha- and dense granules, and with decreased level of procoagulant platelets and increased level of activated integrins at rest (these two differences disappear upon activation).

In the cohort of patients compared with healthy children, there was a significant decrease in platelet size in FSC in the resting state ($p < .001$) (Figure 3A), and a decrease in PAC1 binding ($p = .005$) (Figure 2I). Significant differences were not observed in the SSC, volume of dense granules, the level of CD62p, PS (Figure 2A, C, E and G) and CD61, CD42b (Figure 3C, E). After activation, the patients had significantly reduced FSC ($p = .008$) (Figure 3B), volume of dense granules release ($p = .035$) (Figure 2D), the density of CD61 ($p < .001$) (Figure 3D) and the binding of PAC1 ($p = .03$) (Figure 2J). No significant differences were observed in SSC granularity, CD62p (Figure 2B, F) and CD42b receptor density (Figure 3F). One can summarize that the overall phenotype of the ET patient platelets is that of a smaller size, decreased dense granule release and integrin expression/activation.

Flow cytometry parameters of the platelets in 3 patients with hemorrhagic symptoms did not differ significantly from 17 patients without hemorrhages at rest. However, after activation, they did not demonstrate a characteristic decrease in FSC ($p = .008$) (Figure 3B) and SSC ($p = .008$) (Figure 2B) suggesting shape change defects. When comparing the ratio of FSC and SSC before and after activation, this difference remained (Figure 3F, H). The volume of dense granule release was significantly reduced ($p = .03$) (Figure 2D) and the density of CD42b receptor was increased ($p = .02$) (Figure 3F) in the patients with hemorrhage, but no significant differences were observed in the CD62p alpha granules, PS, binding of PAC1 (Figure 2F, H and J) and CD61 (Figure 3D).
Patients with ischemic symptoms did not significantly differ from patients with asymptomatic ET.

We examined differences in platelet phenotype depending on the genetic markers of the disease. Patients with a triple-negative form of the disease (n = 13) and having a mutation in the JAK2 or CALR gene (n = 7) were compared. In patients with mutations, platelet size was significantly increased (Figure 6A, B, p = .03), CD62p was significantly increased after activation (Figure 6C, p = .03) and the volume of dense granules release was reduced (Figure 6D, p = .047).

Platelet Aggregation
Platelet aggregation in response to ADP, collagen, and ristomycin in patients revealed a significant impairment (p < .05) compared...
with the adult and pediatric control groups. Interestingly, no significant differences were observed between healthy children and adults. Likewise, there were no significant differences in aggregation between groups of patients with different clinical manifestations (Figure 4A–C). However, the mean values of aggregation degree were several-fold smaller for patients with bleeding suggesting that the difference could become significant with more patients.

Blood Coagulation Assays

In the cohort of patients compared with healthy children, fibrinogen level was decreased \((p = .005)\), while TT did not differ significantly in patients compared with the control group. APTT was prolonged \((p < .001)\), and PI was decreased \((p = .016)\). The vWF:Rco was measured in 19 patients, and it was decreased in 14 of them. There were no significant differences between patient groups in these tests \((p > .05)\) (Figure 5A-E). The activity of von Willebrand factor was reduced to 0% in 2 children with clinical symptoms and a platelet count more than 2600 thousand cells/μL. In the 10 children without clinical symptoms median of vWF Rco was 34%. In the nine children with clinical manifestations, the median of vWF Rco was 14.3%.

The number of platelets and leukocytes, the results of LTA and clotting assays did not significantly differ between patients with a triple-negative form of ET and having mutations in the CALR and JAK2 genes (data not shown).

A marked negative correlation \((C_{\text{pearson}} = -0.64)\) between the platelet count and von Willebrand factor activity was revealed in the study of the Pearson correlations between the tests (Figure 5F). A weak negative correlation \((C_{\text{pearson}} = -0.3, p = .2)\) was observed between the activity of von Willebrand factor and aggregation with ristomycin (Figure 4D). We did not observe a correlation between CD42b density and aggregation with ristomycin \((C_{\text{pearson}} = -0.06)\), correlation between PAC1 binding after activation and aggregation with ADP or collagen was also weak \((C_{\text{pearson}} = -0.16\) and \(-0.13\), respectively) (data not shown).

Discussion

The main goal of our work was to study the characteristics of the hemostasis system in pediatric patients with ET and their potential associations with the relevant symptoms of the disease. The main conclusions are:

1. Patients’ platelets are smaller in size compared with healthy volunteers; the volume of the release of dense granules, the level of CD61 and PAC1 binding are reduced after activation;
2. In the three patients with bleeding, the platelets did not show a reduction in FSC size and SSC granularity after activation that was characteristic of others; these morphological abnormalities were combined with a decrease in dense granule release and decreased CD42b loss;
3. Despite the pronounced thrombocytosis and clinical manifestations, no significant increase in markers of intravascular activation (PAC1 binding, CD62p, and PS) was detected on non-stimulated platelets;
4. The marker composition of platelets in healthy adults is somewhat different from healthy children, which makes it inappropriate to use references for adults to pediatric patients.

An important point for the interpretation of these results is the problem of “healthy childhood hemostasis”. The reference ranges...
for platelet-dependent and blood coagulation assays in children of different ages are sorely lacking. In particular, there are reports that platelet function could be either significantly decreased \[16,18,19\] or increased \[20\] in children compared to adults. Therefore, we included not only healthy adult, but also matched children control groups for all hemostasis assays of the present study. This turned out not to be critical for aggregation, where we observed no difference between healthy adults and children, but an impaired response for all children patients compared with healthy children. However, this had a critical impact on the conclusions from the flow cytometry part of the study, where healthy children had less reactive platelets (thus more in line with \[16,18,19\]). Like in aggregation, patients as a group differed from healthy children by decreased integrin activation and dense granule release. However, cytometry was able to observe differences between bleeding and non-bleeding patients to the best of our knowledge, there were no previous reports on this possibility. Interestingly, hemorrhage were mostly related to light scatter changes and impaired mobilization of dense granules. These results could be important for a better understanding of the nature of platelet function in children and its relationship to bleeding, although additional research and larger age-dependent cohorts are needed for definitive conclusions.

According to Soyer N, among 708 adult patients with CMPD, ET patients accounted for more than half (390 patients). When diagnosed, thrombosis was observed in 15.12% of patients and bleeding occurred in 9% \[11\]. For comparison, among our 20 pediatric patients, ischemic symptoms were observed in 35%, and

Figure 5. Coagulogram results in healthy volunteers and patients. Panels show coagulation assays with (A) fibrinogen concentration, (B) thrombin time, (C) APTT, (D) prothrombin index, (E) activity of von Willebrand factor for healthy donors and patient groups. Panel (F) shows a correlation between platelet count and the activity of von Willebrand factor.
hemorrhagic in 15%. It is worth noting that we did not find significant differences in any of the tests for patients with ischemic symptoms compared with asymptomatic ET and to the best of our knowledge, there were no previous reports about these differences. This could mean that, even if these symptoms are related to hemostasis, the mechanisms could be beyond the platelet functionality.

The general decrease of activated integrins on platelets of the patients may be important as a mechanism of their decreased aggregation. Still, correlation analysis showed a poor correlation of the degree of aggregation of platelets with ADP and collagen with PAC1 binding after platelet activation by a mixture of CRP + TRAP in patients and healthy children (data not shown) and to the best of our knowledge, there were no previous reports on this possibility. This result clearly illustrates the need to understand the characteristics of the nature of platelet activation in response to various agonists and their combinations to interpret the results.

The platelet differences in size and characteristics of dense and alpha granules observed between patients with mutations and triple-negative forms of ET require further study. We have not seen such observations in the literature. Likewise, a larger group of patients is needed to ascertain whether increased P-selectin is associated with an increase in the number of leukocyte-platelet aggregates, and a decrease in the release of dense granules is associated with the increased manifestation of bleeding.

It was previously reported that, in adult patients, CD42b and CD41 (GPIIb/IIIa) levels at rest were significantly reduced compared with a control group of healthy volunteers [21]. Binding of PAC1 [12], CD63 of dense granules [22] and CD62p [12,21,22] was higher, which is sign of intravascular platelet activation. After stimulation, CD41, CD62p [21] and CD63 [12,21] were decreased compared with the normal values, which agrees with the suggestion of intravascular platelet activation and suggests impaired platelet function. PAC1 after activation was not significantly different from healthy volunteers [12]. Severe intravascular activation in adult patients with ET compared with children resulted from a large number of age-related risk factors, especially the presence of cardiovascular diseases [10,12,21,22]. These data with adult patients differ from those obtained by us with children (we observed impaired PAC1 and not signs of pre-activation at rest), but also point toward the generally impaired platelet function.

In the analysis of 657 cases of ET, with the age of patients from 8 to 93 years, a median of 52 years, it was shown that an increased number of leukocytes (from 3.28 to 35 thousand cells/μL, median 8.73) is associated with a threefold increase in the risk of thrombosis [23]. It has also been reported that, in patients with polycythemia vera and ET, an increased WBC count is associated with an increased incidence of thrombotic or hemorrhagic events [24]. In our study, the median number of leukocytes in patients with clinical symptoms was lower than without symptoms, which differs the previously cited data and requires further study. It is known that ET, in adult and children, with platelet counts in excess of 500–1000 thousand cells/μL is often associated with an acquired von Willebrand syndrome (aVWS). Such patients have reduced vWF activity due to a decrease or absence of large forms of plasma vWF multimers [25–28]. In clonal and reactive thrombocytosis, the basic mechanisms appear to be the same: vWF multimers with a high molecular weight in plasma temporarily bind to an increased mass of circulating platelets and are primarily degraded by ADAMTS13 [29]. Thus, in both cases, aVWS can explain the obvious paradox of why thrombocytosis can be associated with bleeding. Our study demonstrated an aVWS in patients and indicates the possible connection between

Figure 6. Significant differences in the platelet phenotype of patients were observed depending on the genetic characteristics of the disease. Panels show the relationship between the disease genotype and platelet phenotype in patients with triple-negative forms of ET and with gene mutations (JAK2 and CALR): (A) FSC at rest and (B) FSC upon activation, (C) CD62p after activation and (D) mepacrine release volume after activation.
the degree of thrombocytopenia, von Willebrand factor activity and the nature of the clinical manifestations (Table I, Figure 5E, F), in agreement with the existing concepts about aVWS.

Summarizing the results of the study, we show that platelet functions is generally impaired in ET, in particular, adhesion (decreases vWF Rco, disruption of aggregation with ristocytin), integrin activation and dense granule release (as observed by aggregation assay and flow cytometry), while platelet shape change and dense granule release seem to be specifically impaired in the patients with bleeding. These results suggest the possibility of using these tests to evaluate the risks of complications.

Acknowledgements

The authors thank the patients and healthy volunteers who participated in the study and colleges who supported our work: Dmitry S. Abramov for bone marrow morphology, Yuliya V. Olshanskaya for genetic analysis, Svetlana A. Plyasunova for blood tests, Fazooli I. Ataullakhanyan for important discussions. The authors were supported by a grant from the endowment foundation «Doctors, innovations, science for children», and by the Russian Foundation for Basic Research grants 17-04-01309, 17-00-00140 and 18-34-20026. This research was supported by the charitable “Grant life” foundation.

Addendum

Dmitrii M. Polokhov designed the study, performed experiment, analyzed data, and wrote the manuscript. Nicolay M. Ershov, Pavel A. Zharkov and Nataliya S. Smetanina recruited patients and critically revised the manuscript. Daria V. Fedorova recruited patients. Anastasia A. Ignatova, Evgeniya A. Ponomarenco, Alexander V. Polotaev, Elena A. Seregina performed experiments and analyzed data. Marina V. Gaskova performed experiments and edited the manuscript. Galina A. Novichkova and Mikhail A. Panteleev edited the manuscript.

Disclosure of Conflict of Interests

All authors state that they have no conflict of interest.

References


