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To link to this article: https://doi.org/10.1080/09537104.2018.1513473

Published online: 04 Oct 2018.
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Abstract

The ability of platelets to carry out their hemostatic function can be impaired in a wide range of inherited and acquired conditions: trauma, surgery, inflammation, pre-term birth, sepsis, hematological malignancies, solid tumors, chemotherapy, autoimmune disorders, and many others. Evaluation of this impairment is vitally important for research and clinical purposes. This problem is particularly pronounced in pediatric patients, where these conditions occur frequently, while blood volume and the choice of blood collection methods could be limited. Here we describe a simple flow cytometry-based screening method of comprehensive whole blood platelet function testing that was validated for a range of pediatric and adult samples (n = 31) in the hematological hospital setting including but not limited to: classic inherited platelet function disorders (Glanzmann’s thrombasthenia; Bernard-Soulier, Wiscott-Aldrich, and Hermansky-Pudlak syndromes, MYH9-dependent thrombocytopenia), healthy and pre-term newborns, acute and chronic immune thrombocytopenia, chronic lympholeukemia, effects of therapy on platelet function, etc. The method output includes levels of forward and side scatter, levels of major adhesion and aggregation glycoproteins Ib and Ib-ilia, active integrins’ level based on PAC-1 binding, major alpha-granule component P-selectin, dense granule function based on mepacrine uptake and release, and procoagulant activity quantified as a percentage of annexin V-positive platelets. This analysis is performed for both resting and dual-agonist-stimulated platelets. Preanalytical and analytical variables are provided and discussed. Parameter distribution within the healthy donor population for adults (n = 72) and children (n = 17) is analyzed.

Keywords

Flow cytometry, immune thrombocytopenia, pediatric platelets, platelet function, thrombocytopenia

Introduction

Bleeding and thrombotic complications resulting from inherited or acquired disorders of platelet-dependent hemostasis are frequently encountered in clinical practice, and are responsible for a major fraction of mortality and morbidity in the modern world. Although specific causes of their complications for each specific case might be related to changes in the platelet number, or platelet functional activity, or the state of the vessel wall, or some other conditions, there is no doubt that the critically important factor needed for physiological hemostatic response is platelet functional activity. Even when the immediate cause of thrombosis or bleeding is due to some other reason than platelet function (e.g. atherosclerosis), its treatment might target platelets, and assessment of platelet functional activity affected by the drugs and by the disease itself could be needed in any case. Finally, determination of the state of platelets is vitally important as an exclusion criterion, e.g. as a part of the diagnostic tree when bleeding turns out to be due to coagulation system defects.

A critical problem in the contemporary field of platelet-dependent hemostasis is reliable and informative evaluation of platelet function [1]. The classical approach in the field is aggregation assays, but they have poor sensitivity and specificity, and are not applicable to low platelet concentrations in thrombocytopenia. This seems to be a critical shortcoming, as the most common and complex conditions are those where platelet dysfunction is combined with decreased platelet count. Platelets seem to be so much in excess of what is needed for hemostasis that it is extremely difficult to have bleeding at high platelet count unless the functional defect
is something vital like glycoprotein IIb-IIIa deficiency. Flow perfusion chambers have been increasingly useful for research and diagnostics in the field [2], but they are far from being in the acceptably standardized state, and are all after integral assays: they could detect shifts in the hemostatic balance but not the exact cause unless specifically adjusted for the task. Flow cytometry is a very attractive option for detecting molecular changes in platelet function at the single-cell level [3,4], but is not standardized, and has been scarcely used in clinics except for routine glycoprotein level determination, mostly in the field of inherited platelet disorders [5]. There were few attempts to carry out extensive trials with different flow cytometry protocols (like [6]) in order to find optimal conditions and aim to create a thorough system of efficient, clinically validated platelet function testing with inter-laboratory and inter-device agreement.

In a way, one of the reasons behind insufficient understanding of the role of platelet dysfunction in bleeding and thrombosis is exactly the lack of efficient (and standardized) tools to study platelet function disorders. Pediatric hematology is in particular need of such tools. All inherited platelet function disorders are detected in childhood, and acquired disorders are quite frequent in many of pediatric diseases [7]. Here we describe a flow cytometry screening method of comprehensive clinical platelet function evaluation for children [8–11], adults [10,12–14], or platelet concentrate samples [15], which was developed based on our previous non-clinical research experience of platelet functions [16–21]. As an output, it provides the basic platelet “mapping” providing information about all major functions (adhesion, aggregation, granule secretion, and procoagulant activity) for resting and dual-agonist-stimulated platelets.

**Method**

**Materials**

Annexin V-Alexa647 and antibodies against P-selectin (CD62P-Alexa647), glycoprotein I (CD42b-PE), integrin αIIbβ3 (CD61-PE) and its activation marker (PAC1-FITC) were from Biolegend (San Diego, CA, USA) or Sony Biotechnology (San Jose, CA, USA).

Cysteine-containing version of cross-linked collagen-related peptide (CRP) was either kindly provided by Prof. R.W. Farndale (University of Cambridge, Cambridge, UK), or custom-synthesized and purified by VCPBIO (Shenzhen, China) and then cross-linked as described [22]. Its activity was determined for each batch and then controlled monthly as described below. All other reagents were from Sigma-Aldrich (St Louis, MO, USA).

**Patients**

Patients and healthy volunteers were recruited in either Rogachev Center of Pediatric Hematology, Kulakov Center for Obstetrics, or Botkin City Clinical Hospital. Investigations were performed in accordance with the Declaration of Helsinki under protocols approved by the respective institutions’ Ethical Committees, and written informed consent was obtained from all donors and patients, or their parents. All diagnoses were performed according to the national and international guidelines based on clinical, genetic and functional assays independently of flow cytometry. The list of conditions included: childhood Glanzmann’s thrombasthenia type I, Bernard-Soulier syndrome, MYH-9 type thrombocytopenia, Wiscott-Aldrich syndrome, Hermansky-Pudlak syndrome, term and pre-term newborns, acute childhood immune thrombocytopenia (ITP), chronic childhood ITP (before treatment and after a month on romiplostim), chronic adult ITP (before treatment and after a month on romiplostim), chronic lympholeukemia (LL, before treatment and after two weeks on ibrutinib).

Patients characteristics are described in Supplementary Table SI.

**Protocol description**

Venous blood was collected by venipuncture into vacuum plastic tubes with sodium citrate, final concentration 3.8%. The first 2 mL of blood was discarded. Blood was processed as described in Table I. Briefly, 20 µL of blood was diluted 1:20 in HEPES-buffered Tyrode buffer (HBT buffer, 150 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl₂, 0.4 mmol/L Na₂HPO₄, 20 mmol/L HEPES, 5 mmol/L glucose, 0.5% bovine serum albumin, pH 7.4). Platelets were either left intact or loaded with mepacrine (10 µM) for 30 min at 37°C. For

**Table I. Flow cytometry protocol.**

<table>
<thead>
<tr>
<th>Step 1: Blood dilution</th>
<th>400 µL</th>
<th>Probes with mepacrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBT buffer</td>
<td>20 µL</td>
<td></td>
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<tr>
<td>Whole blood</td>
<td></td>
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</tbody>
</table>

**Step 2: Mepacrine loading**

<table>
<thead>
<tr>
<th>Diluted blood</th>
<th>Intact probes</th>
<th>Probes with mepacrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>320 µL</td>
<td>100 µL</td>
<td></td>
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<tr>
<td>-</td>
<td>5 µL</td>
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</tbody>
</table>

**Step 3: Platelets activation**

<table>
<thead>
<tr>
<th>Diluted blood</th>
<th>Intact probes</th>
<th>Probes with mepacrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µL</td>
<td>-</td>
<td>-</td>
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</table>

<table>
<thead>
<tr>
<th>Diluted blood with mepacrine</th>
<th>10 µL</th>
<th>10 µL</th>
<th>10 µL</th>
<th>10 µL</th>
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**Step 4: Antibody staining**

<table>
<thead>
<tr>
<th>A mixture of CRP + SFLLRN + CaCl₂</th>
<th>10 µL</th>
<th>10 µL</th>
<th>10 µL</th>
<th>10 µL</th>
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**Step 5: Dilution for flow cytometry**

<table>
<thead>
<tr>
<th>HBT buffer with 2.5 mM CaCl₂</th>
<th>360 µL</th>
<th>360 µL</th>
<th>360 µL</th>
<th>360 µL</th>
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</table>
platelet activation a mixture of CRP (originally 20 µg/mL, the specific concentration for each lot was adjusted by titration as described below), PAR-1 activating peptide SFLLRN (25 µM) and 5 mM CaCl$_2$ was added to diluted blood in proportion 1:1 (10 + 10 µL) and incubated for 10 min. The appropriate amount of HBT buffer with 2.5 mM CaCl$_2$ was added to the samples without activation. Annexin V-Alexa647 for evaluation of procoagulant platelets and antibodies against P-selectin (CD62P-Alexa647), CD61-PE and PAC1-FITC, CD42b-PE were used. A mixture of antibodies was added to the activated and non-activated samples for 10 min. The samples were then diluted by adding of 180 µL HBT buffer with CaCl$_2$ and analyzed with flow cytometry.

**Platelet flow cytometry protocols: brief review and choices**

There are several essential variables in the flow cytometry protocols that are briefly reviewed here in order to substantiate choices used in our methodology. Although there are protocols for platelet-rich plasma (PRP) and washed platelets, clinical flow cytometry analysis is usually performed in diluted whole blood [23]. We followed this choice to minimize sample handling time and avoid platelet damage. Blood is usually diluted with buffer such as HEPES-buffered saline to prevent aggregation (and clotting if activation is done in the presence of calcium). We used Tyrode-HEPES buffer as a more physiological medium for platelet activation studies and to keep continuity with our previous studies [17,19,24,25]. Monoclonal antibodies are added to the mixture prior to platelet activation in the lowest saturating concentration determined by titration. Generally, one antibody is used to identify platelets, while another antibody serves as platelet phenotypic marker. In guidance from the SSC of the ISTH it is recommended to use antibodies against major surface glycoproteins (GPIb/IIa, GPIIIa, GPIb, GPIb/IX, and GPIb/IIa activation epitope) as part of the first-step tests and expanded flow cytometry with additional antibodies (against GPIa/IIa, GPVI and procoagulant activity by annexin V binding) as one of second-step tests in platelet function disorder diagnosis [5,6]; these steps were combined in our protocol aimed at developing a test for all-purpose screening. Various agonists are added to the mixture to induce platelet activation and degranulation. The commonly used ones are adenosine diphosphate, arachidonic acid, collagen, epinephrine, thrombin, thrombin receptor-activating peptide (TRAP) and thromboxane A$_2$ analogs (e.g., U46619). The concentration and type of agonist depend on the type of assay being performed. We chose dual-stimulation of CRP and TRAP to allow robust and potent stimulation activation employing two major pathways. The activation is stopped by adding large volume of buffer. A fixative can be added to the stopping solution, but it could affect platelet membrane properties and may be wrongly interpreted as part of activation response [23]; we avoided this part. Flow cytometry analysis results can be expressed as percentage of cells that are marker positive or mean fluorescence intensity of whole platelet population; the latter solution was adopted in our approach to obtain values that could be interpreted as number of receptors.

**Reagent standardization and transfer of the method between cytometers**

Antibodies from different manufacturers (or different lots of the same manufacturer) were calibrated by comparing with the previous one on the same blood sample, and conversion coefficients were determined as described in the online supplement. Transfer between the cytometers was performed by running the same labeled and unlabeled samples on different machines and calculating conversion coefficients; our experience currently includes transfer of the method to BD Accuri, FACS Canto (BD Biosciences), and Navios (Beckman Coulter, Indianapolis, IN, United States). Ability of CRP from different manufacturers or different batches to activate platelets was tested alone and in combination with SFLLRN to adjust working concentration. For monthly control of the reagents and upon reagent change control experiments were performed with blood of four healthy volunteers.

**Preanalytical and analytical variables**

The results of the assay were not sensitive to the blood/citrate ratio (the results did not differ when citrate concentration was increased three-fold in order to mimic a severe blood collection shortage), and did not differ significantly for freely flowing blood or that collected using citrate tubes of different manufacturers. They remained reproducible within time interval of 15–90 minutes after blood collection if stored at room temperature without disturbance. It should be noted that this is not in line with previous reports on the instability of platelet activation-dependent markers over the course of the first hour [27]; additional research is required to clarify the nature of these differences. Transportation within the city by courier using car or train was acceptable if carried out within an hour, at 20–25°C, with the tubes fixed in a vertical position and without excessive perturbation.

For whole blood platelet count $> 20$ 000/µL, including abnormally high platelet count (patients with thrombocytosis), standard protocol could be used. For lower platelet count, collection time has to be increased. In the case of a very low platelet count (patients with severe thrombocytopenia, PLT $< 20$ 000/µL) PRP should be...
obtained by centrifugation for 3 min at 100 g (or by blood settling within 20–30 min). To further increase the platelet number in the analyzed sample a lower dilution of whole blood or PRP can be used. The results of the assay were reproducible when whole blood was diluted with buffer 1:15 (instead of 1:21 in standard protocol), but not at 1:10. The assay is not sensitive to the incubation time with the mixture of SFLLRN and CRP (10–20 min).

**Method validation with blood of healthy subjects**

Blood samples from \( n = 72 \) healthy subjects claiming not to have used medication for a week prior to analysis were used to investigate the assay properties. In order to evaluate the parameter ranges for pediatric platelets, we collected blood of \( n = 17 \) children (aged 0–16, median 5 years) who were either healthy or had diagnoses that could be assumed not to affect essentially the platelet parameters (such as leukemia in long-term remission). The assay parameter distribution for healthy adult donors and children is shown in Figure 1b–i and Supplement Figure S1 and Table S2.

Both forward and side scatter in the adults followed Gaussian distribution (at the 95% confidence levels as evaluated with the Shapiro–Wilk and Kolmogorov–Smirnov normality tests) centered at 100 ± 12 units (mean± S.D.). FSC was clearly decreased by 37 ± 7 units upon platelet stimulation, while the effects for SSC were much less clear (11 ± 11 units). Although pediatric platelets had slightly slower FSC (90 ± 12 units), the difference did not reach statistical significance. In contrast, SSC of pediatric platelets was significantly smaller both before (74 ± 9 units versus 100 ± 12 in adults) and after stimulation.

The main adhesion glycoprotein GP Ibβ (CD42b) of the adults followed exactly the same pattern as FSC, with Gaussian distribution of 100 ± 13 units, and almost two-fold decrease on stimulation. The main aggregation receptor integrin αIIbβ3 (CD61) had slightly larger variability (S.D. of 17 units) and was increased 2.5-fold upon stimulation. Distribution of both these receptors in the samples of the children was similar to that of the adults (Figure 1d,e).

The next four parameters described four major activation responses of platelets (Figure 1f–i). PAC1 binding was used as a measure of integrin αIIbβ3 activation. Its binding in the resting platelets was low and did not follow normal distribution (Supplement Figure S1a), so its normal reference range was set as that below 6 units (Supplement Table S2). PAC1 in the stimulated adult platelets was relatively variable among donors at 100 ± 22 units. Likewise, the normal range of P-selectin (CD62P, used as a measure of alpha-granule release) in the intact platelets was set as less than 6.4 units, while its distribution in the activated samples was 24 ± 12 units. Under fluorescence both in the resting platelets (100 ± 12 units) and after stimulation.

Flow cytometry for patients with acquired platelet dysfunction

The typical outcomes of the assay for the patients with acquired (or at least non-hereditary) disorders of platelet function are shown in Figure 3. The pattern of assay results for healthy newborns is consistent with previous reports [28]: mostly normal initial parameters, but three out of four responses to stimulation (PAC1, CD62P, and dense granule release) are clearly below the lower boundary of the normal range for adults. The platelet activation responses of the moderately pre-term twins (both with skin bleeding) were only a bit inferior to the term ones. Both acute and chronic childhood ITP (with bleeding) was associated with slight increase of platelet size; however, their increase of FSC, SSC, and CD42b was much more than that in BSS or MYH9. Both of them had platelet pre-activation judging by PAC-1, CD62p, or procoagulant platelets. In the same chronic ITP treated

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**Figure 1. Design of the method and reference ranges.** Typical dot plots with data gating (a) used to generate mean values of fluorescence intensity for each parameter, and assay parameter distributions (b–i) for healthy adult donors (\( n = 72 \)) and children (\( n = 17 \)). The time from blood collection to processing was within 30 min. The data points in the box charts are diamond-shapes, square symbols show mean values, horizontal lines are medians, boxes show 25–75 percentiles, error bars show 5–95% intervals, and X symbols show maximal and minimal values. Statistic significance: *, \( p < 0.05; ***, p < 0.01; ***, p < 0.005.**
Figure 2. Inherited disorders of platelet function. Panels (a–p) show distribution of the indicated parameters for typical patients with classic inherited thrombocytopenias. The patterned regions indicate reference ranges. Abbreviations: GT1, Glanzmann’s thrombasthenia type 1; BSS, Bernard-Soulier syndrome; MYH9, thrombocytopenia associated with MYH9 mutations; WAS, Wiscott-Aldrich syndrome; HPS, Hermansky-Pudlak syndrome. The time from blood collection to processing was within 30 min.
Figure 3. Acquired disorders of platelet function. Panels show distribution of the indicated parameters for typical patients with acquired disorders of platelet function. Dotted lines indicated references ranges. The time from blood collection to processing was within 30 min.

1 – preterm newborns (twins); 2 – term newborns; 3 – acute itp, children; 4 – chronic itp, children; 5 – chronic itp, children, on romiplostim; 6 – chronic itp, adults; 7 – chronic itp, adults, on romiplostim; 8 – CLL; 9 – CLL on ibrutinib;
with romiplostim, there was not pre-activation, and the size was normal. Adult chronic ITP and its response to romiplostim followed the same pattern. In CLL, platelet response appeared similar to the normal one, but PAC-1 and procoagulant platelets were consistently at the bottom end of the reference range. On ibrutinib, they went further down outside of the normal range.

Discussion

The flow cytometry method of platelet function testing described here was focused on the following goals: comprehensive characterization of all major platelet functions (adhesion and aggregation glycoproteins, integrin activation, release of dense and alpha-granules, procoagulant activity) in the resting and stimulated state, small volume of blood required, minimal preanalytical processing, possibility of using reagents from different manufacturers and almost any two-laser flow cytometer, robust response, and above all sensitivity to function disorders most often encountered in pediatric platelets and being in need of evaluation. Our experience with this method suggests that it is capable of reaching these goals; it is sufficiently robust and reproducible, and it is sensitive to both classic inherited disorders and various acquired conditions.

The data generated by this method with regard to inherited disorders are generally in agreement with previous reports [3,4,6,29]. The levels of glycoproteins absent in the respective disorders (CD61 and PAC1 in GT, CD42b in BSS) were negligibly low, antibody binding and mepacrine loading in all cases correlated with platelet size (giant for BSS and MYH9, small for WAS) except for HPS that did not have dense granules. Although we were not able to reveal any correlations of PAC1 and CD61 within each of the groups, the large-scale differences between healthy donors, BSS, MYH9, and WAS reveal that PAC1/CD61 is maintained at the same ratio suggesting that differences between these disorders are associated with platelet size. One unexpected observation was that giant platelet in BSS had reduced probability of becoming procoagulant under our conditions; this is different from what was reported for BSS before [30].

The results for acquired conditions also agree with some of the published data: some of the main observation reported by other methods before and observed here in our assay include enlargement [31] and pre-activation [32] of platelets in ITP, hyperactivation of neonatal platelets [33], impaired platelet function in CLL [34], inhibitory action of ibrutinib [35]. However, these changes are more difficult to judge, as complexities of these conditions cause controversies in almost all of these facts (as discussed below for neonatal and pediatric platelets). Additional research is required to clarify these matters. In addition to the data presented here, our preliminary data suggest that for other cases, like pediatric bleeding of unclear origin [11] and quality of platelet concentrates [15], it could also provide clinically relevant outcomes. Impaired PAC1 and PS exposure in HPS and in CLL indicate that the assay is sensitive to the feedback ADP secretion from dense granules.

The indicator markers chosen for the assay are well-characterized in the field [3,6,26] and comprehensive within reasonable limits. While they do not cover the whole range of platelet responses, they cover the major ones. They are subject to the limitations and shortcomings described by others such as potential non-linearity of PAC-1 (compared with fibrinogen binding), non-hemostatic nature of P-selectin (select with alpha-granular fibrinogen or factor V(a)), or additional preanalytical complications, non-specificity and cytotoxicity of mepacrine. However, these do not seem to be essential under conditions of this assay, and these markers can be easily substituted for alternatives or supplemented with others when needed.

Although the described method was able to identify reproducible platelet function changes for a wide range of pediatric hematology disorders associated with bleeding, the evidence base for its predictive ability with regard to bleeding is very limited. Prospective studies are needed to evaluate its applicability for this purpose. Likewise, its standardization, variability, possibility of reagent modification, characterization of preanalytical requirement are acceptable for a single-laboratory research use, but have not been tested and validated at the multi-center level. Its correlation with other laboratory assays of platelet function is still poorly investigated. In the early screening stage it can be used as a part of diagnostic strategy aimed at the elucidation of the disorder.

This study provides preliminary data on the normal parameter ranges in our assay not only for adults, but also for children aged 1–17 years. The sample size for children is relatively small and does not allow formation of age groups; and additional caution should be exercised because some of the children included were actually patients assumed to have normal platelet function at the time of analysis. Despite these limitations, these results allow deeper interpretation of the results with patients and could be a useful supplement for assay usage. While some of the pediatric parameter ranges are similar to the adult ones, their SSC and mepacrine uptake in the resting state as well as PAC1 and CD62 expression upon activation are decreased. Even greater hyporeactivity was observed in the few neonatal samples of this study. This hyporeactivity of neonates and older children agrees well with some of the previous reports [36–38] but not with others reporting hyper reactivity or no difference related to adults [33,39,40]. Although detailed analysis of these differences is beyond the scope of the present study, it could be speculated that they are due to the use of high agonist concentrations in this study and in [36], while previous reports of hyper-reactivity employed low agonist concentrations [40]. It is worth noting that use of a super-maximal stimulation condition is not capable of demonstrating hyper-reactivity (activation cannot be increased above 100%) and can only measure hypo-responsiveness.

Our results with adult hematology patients seem to be in line with the children. This method however has not been extensively tested outside of the hematology field, so its applicability to many of the problems of adult hemostasis (e.g. arterial thrombosis and most importantly monitoring of antithrombotic drugs) has not been tested. It can be expected, however, that strong stimulation used in this method together with potent blood dilution might make it difficult to sense subtle changes caused by modern antiplatelet drugs (that would be additionally diluted unless they act covalently). Some of these problems might be resolved by modifying assay conditions (less potent activation, different calcium), but it is quite possible that the price to be paid could be loss of robustness.

Conclusions

Here we describe a flow cytometry-based method of comprehensive platelet function testing that validated for a range of pediatric and adult samples in the oncologyhematology hospital setting. The method output includes levels of forward and side scatter, CD42b, CD61, CD62P, dense granule level and release, annexin V and PAC-1 binding for both resting and dual-agonist-stimulated platelets. Preanalytical and analytical variables are provided and discussed, and parameter distribution within the healthy donor population is analyzed.
Acknowledgements

The authors were supported by the grant from the endowment foundation «Doctors, innovations, science for children», by the Russian Federation President grant for young scientists MD-229.2017.4, and by the Russian Foundation for Basic Research [Grants 17-00-00139/17-00-00140 and 17-04-01309].

Declaration of interest

The authors report no declarations of interest.

Funding

This work was supported by the Russian Federation President Grant for Young Scientists [MD-229.2017.4]; Endowment foundation «Doctors, innovations, science for children» [Not applicable]; Russian Foundation for Basic Research [17-00-00139/17-00-00140, 17-04-01309].

Supplementary material

Supplementary data can be accessed here.

References