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Mechanisms of increased mitochondria-dependent necrosis in Wiskott-Aldrich syndrome platelets

Short title: Programmed necrosis of platelets in WAS

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**Key points**

- WAS platelets rapidly undergo mitochondrially mediated necrosis upon surface attachment or thapsigargin treatment
- This programmed death is due to their decreased size and reduced number of mitochondria, and can contribute to thrombocytopenia in WAS

**Abstract**

Wiskott-Aldrich syndrome is associated with thrombocytopenia of unclear origin. We investigated real-time cytosolic calcium dynamics, mitochondrial membrane potential and PS exposure in single fibrinogen-bound platelets using confocal microscopy. The Wiskott-Aldrich syndrome platelets had higher resting calcium levels, more frequent spikes, and their mitochondria more frequently lost membrane potential followed by the PS exposure (in 22.9% of platelets vs 3.9% in controls, P<0.001) after the collapse of the last mitochondria. This phenomenon was inhibited by mitochondrial permeability transition pore inhibitor cyclosporin A, as well by xestospongin C and lack of extracellular calcium. Thapsigargin by itself caused accelerated cell death in the Wiskott-Aldrich syndrome platelets. The number of mitochondria was predictive for PS exposure: 33% of platelets from Wiskott-Aldrich syndrome patients with less than 5 mitochondria exposed PS, while only 12% did among those who had 5 and more. Interestingly, healthy donor platelets with fewer mitochondria also more readily became procoagulant upon PAR1/PAR4 stimulation. Collapse of single mitochondria led to greater cytosolic calcium increase in Wiskott-Aldrich syndrome platelets if they had 1-3 mitochondria compared with platelets containing higher numbers. Computer systems biology model of platelet calcium homeostasis showed that smaller platelets with fewer mitochondria could have impaired calcium homeostasis due to higher surface-to-volume ratio and greater metabolic load, respectively. There was correlation (C=0.81,p<0.02) between the mean platelet size and platelet count in the Wiskott-Aldrich syndrome patients. We conclude that Wiskott-Aldrich syndrome platelets readily expose PS via mitochondria-dependent necrotic mechanism caused by their smaller size, which could contribute to the development of thrombocytopenia.
Introduction

Wiskott-Aldrich syndrome (WAS) is an X-linked disorder classically characterized by a thrombocytopenia, immunodeficiency and eczema\(^1\). Its pathophysiological mechanisms relate to the defective actin polymerization and abnormal signal-mediated cytoskeleton rearrangements in hematopoietic cells as a result of deficient or dysregulated activity of the WAS protein (WASP) that belongs to a distinct family of proteins involved in the transduction of signals from the cell surface to the actin cytoskeleton\(^2\). The severity of immunodeficiency varies between WAS patients, whereas platelet defect (reduced number and size) is the universal feature of the disease, and thrombocytopenia-related bleeding contributed greatly to the mortality in the untransplanted patients\(^3\). Although major platelet functions in WAS platelets are retained, there is evidence of defects that could potentially additionally contribute to bleeding\(^4\).

The specific mechanisms of thrombocytopenia in WAS remain elusive. Studies of the patient megakaryocytes produced evidence both in favor of defects in platelet production\(^5\) and against it\(^6\). On the other hand, platelets of WAS patients and murine WASP knockouts had shortened lifespan and were subject to increased phagocytosis\(^7\)-\(^9\). In particular, it was shown previously that WAS platelets have increased phosphatidylserine (PS) exposure upon storage and activation\(^10, 11\), which could be one of the mechanisms for their accelerated clearance by splenic macrophages and possibly contribute to thrombocytopenia. Indeed, recent evidence from diverse eukaryotic systems suggests that the actin cytoskeleton has a role in regulating apoptosis via interactions with the mitochondria\(^12\). Changes to the dynamics of the actin cytoskeleton were implicated in the release of reactive oxygen species (ROS) from mitochondria and subsequent cell death\(^13\). Interestingly, recent studies discovered that platelets from patients with deficiency of actin filament branching regulator Arp2/3\(^14, 15\) have major phenotype features similar to those observed in WAS: microthrombocytopenia, deficiency of dense granules and spreading.

Here we investigate the mechanism behind the cell death phenomena in platelet samples from a cohort of 35 WAS patients. The main conclusion is that they are prone to PS exposure upon minor stimulation, which occurs as a result of mitochondrial permeability transition pore opening. We provide evidence for the two major mechanisms responsible for this: a) an increased surface-to-volume ratio of these micro-platelets leading to dysregulation of calcium homeostasis; b) a decreased number of mitochondria per platelet that results in dramatic cytosolic calcium increase upon mitochondrial permeability transition pore opening in a
single mitochondrion. Platelet size correlated with platelet count in the untreated WAS patients.

Methods

Full description of methods and reagents is available in Supplemental materials.

Patients and healthy donors. A total of 35 patients with WAS were included in the study (Table 1). The diagnosis was made according to European Society for Immunodeficiencies diagnostic criteria and genetically confirmed by WAS mutations identification. Romiplostim was administered off-label according to the institutional protocol at 9 µg/kg weekly. 12 of 35 patients had a Zhu score of 1 and 2. Control samples included blood from children and healthy adults as indicated in the experimental descriptions.

Blood collection and platelet isolation. Investigations were performed in accordance with the Declaration of Helsinki under approval of the Children’s for Hematology Ethical Committee, and written informed consent was obtained from all patients (or their parents) and donors. Washed platelets were prepared essentially as described17.

Confocal microscopy experiments: general design. Glass coverslips were cleaned and coated with 1 mg mL⁻¹ fibrinogen or monafram in PBS. Washed platelets were attached to the protein-coated surface by incubating them at 1.3·10⁵ µl⁻¹ (or the maximal concentration attainable for WAS under conditions of thrombocytopenia) for 20 min and rinsing with buffer A with 1.5 mM CaCl₂. PS+ fraction was counted after 30 min of additional incubation.

Cytosolic calcium signaling and mitochondrial membrane potential change. The methodology was essentially as described18. Calibrations for ratiometric measurements were made separately for healthy and WAS platelets. Calcium concentrations were calculated using an equation for ratiometric indicators19. Tetramethylrhodamine methyl ester (TMRM) was used to detect mitochondrial potential dynamics.

Characterization of platelet response to TRAP-6 in platelet-rich plasma (PRP). Samples of PRP were diluted with PPP to a final concentration of 20,000 µL⁻¹ and buffered with HEPES at pH 7.4 (100 mM final concentration). The irreversible thrombin inhibitor Phe-Pro-Arg chloromethyl ketone (PPACK) was added to the final concentration of 100 µM in order to block spontaneous thrombin generation upon recalcification. Platelets were recalcified by addition of calcium chloride (final concentration 20 mM, which corresponds to 2 mM of free calcium²⁰) and activated by 25 µM TRAP-6 for 5 min at room temperature.

ATP measurement in platelets. In brief, platelets of the samples were lysed (addition 90 µl DMSO to 10 µl platelet suspension) and analyzed by luciferase-luciferin assay as described²¹.
while the other part was stained with CD61-FITC and annexin V-Alexa Fluor 647 and analyzed by flow cytometry.

**Flow cytometry characterization of platelet functional activity.** The experiments were performed essentially as described\textsuperscript{23, 24} with minor modifications.

**Transmission electron microscopy.** The protocol was essentially as described\textsuperscript{25}.

**Statistics.** Data are presented as means ± standard deviations. The statistical significance of the differences between groups was determined with the non-parametric Mann–Whitney U-test (p) or Wilcoxon signed-rank test (p*) for paired samples. Differences were considered to be significant when the P-value was < 0.05.

**Computational modeling of platelet calcium homeostasis.** A systems biology model of platelet calcium signalling was based on the one developed previously\textsuperscript{18}. In contrast to its predecessor pure calcium signalling/homeostasis models\textsuperscript{26, 27}, it had several mitochondrial compartments and included equations describing dependence of ATP production and calcium pumps activity on mitochondrial inner membrane potential.

**Results**

**PS exposure by the fibrinogen-immobilized WAS platelets.** In order to gain insight into the mechanisms of increased/accelerated PS exposure of the WAS platelets, we investigated dynamics of status change by the single fibrinogen-bound cells (Fig. 1A). Unexpectedly, this relatively mild method of platelet immobilization produced massive spontaneous PS exposure in the WAS platelets during 30 min without any additional stimulation compared with either healthy adults (n=18) or children without WAS (n=6, aged 0-7 years with a median of 2.5) (Fig. 1B). The smaller number of the adherent platelets in some of the WAS patients could lead to some underestimation of their PS-positive platelets, so that the effect could be even greater. Taking into account that some of the patients received romiplostim previously shown to potentially affect platelet function\textsuperscript{28, 29}, it was reasonable to separately evaluate its effect on platelets\textsuperscript{30, 31}. However, there was no statistically significant difference between PS externalization by platelets from the untreated WAS patients and from those on romiplostim (Fig. 1C): in both groups, approximately 20% of platelets on the average exposed PS. The phenomenon was not fibrinogen-specific, as platelet attached to the \(\alpha_{\text{IIb}}\beta_3\) antagonist monafram produced the same results (Fig. 1D).

**Functional activity of the WAS platelets.** To thoroughly characterize the status of the WAS platelets involved in the study, we analyzed them by the diluted whole blood flow cytometry using a comprehensive set of functional markers (Fig. 2 and Fig. S1). Platelets were either left
in the resting state or subjected to a potent dual-stimulation with TRAP-6 and CRP. As a control, we used a group of n=21 healthy children (9 boys and 11 girls, aged 0-13 years with a median of 5). The WAS platelets had significantly decreased FSC and levels of major surface glycoproteins (Fig. 2A-C) reflecting their decreased size. There were two interesting exceptions: patients 5 and 18 had normal FSC. Patient 5 was splenectomized, which could be a plausible explanation of his larger platelets, while patient 18 had an exceptionally mild WAS phenotype. The size-independent parameter of shape change evaluated as the light scattering ratios for the resting/stimulated platelets (Fig. S1A,B) was significantly decreased in WAS. In order to take into account the difference in the platelet size and surface area, we evaluated integrin $\alpha_{IIb}\beta_3$ activation and alpha-granule release by either the percentage of the PAC1-positive (Fig. 2D) or CD62P-positive (Fig. 2E) platelets, or by normalizing the data on CD61 fluorescence intensity as described$^{31}$ (Fig. S1). In both cases, there was somewhat decreased response of the WAS platelets to activation compared with the healthy ones. However, there was a relative increase in baseline platelet activation in the WAS patients compared with the controls, as judged by integrin (Fig. S1D) and P-selectin (Fig. S1F) surface expression of the resting platelets. Dense granule release in WAS was essentially lower than in healthy children (Fig. 2F). Interestingly, the completely size-independent response of procoagulant platelet formation evaluated as a percentage of annexin V-positive cells was also clearly decreased (Fig. 2G). Neither of these phenomena changed in the romiplostim-treated patients compared with untreated ones. Analysis with TRAP stimulation in diluted platelet-rich plasma revealed minor increase in the PS-positive platelets under resting conditions and normal PS expression upon stimulation (Fig. S2). In summary, WAS platelets demonstrated decreased size and some preactivation features in rest state but they did not reveal any drastic functional differences from the normal ones in activated state.

**Signalling events in single fibrinogen-attached WAS platelets.** To identify the mechanisms of the PS externalization of the WAS platelets, we simultaneously examined dynamics of calcium in cytosol, mitochondrial membrane potential and PS exposure in WAS and control platelets (Fig. 3). The mean intracellular cytosolic calcium level in the WAS platelets was 4-fold greater than in the control ones at the beginning, and the average difference was increasing with time (Fig. 3A). While healthy unstimulated platelets in line with previous reports$^{18,32}$ had only occasional calcium spikes when bound to fibrinogen (Fig. 3B), those of the patients had frequent oscillations with longer spike duration (Fig. 3C, D). The mitochondria in the WAS platelets lost their membrane potential one after another and, if all of them became TMRM-negative, the cell began to bind annexin V within ten seconds (Fig.
3D), exactly as reported before for the PS-positive platelet formation induced in healthy donors with TRAP-6 or thrombin\textsuperscript{18, 27}. This is in agreement with the mitochondrial-calcium-overloading-induced necrosis scenario of procoagulant platelet formation\textsuperscript{18, 33, 34}.

Importantly, time lapse imaging revealed that mitochondrial collapse in WAS platelets with few mitochondria in turn led to rapid cytosolic calcium increase (Fig. S3A, time point 116 sec). If it was reversed, calcium concentration also decreased (Fig S3A, time point 146 sec). This is drastically different from healthy donors’ activated platelets, where calcium was not so sensitive to single mitochondrion’s collapse\textsuperscript{18}. Interestingly, WAS platelets with large number of mitochondria had increased background cytosolic calcium and frequent oscillations, but were not sensitive to the collapse of single mitochondria either (Fig. S3B).

\textit{PS exposure of the WAS platelets is mediated by mitochondrial permeability transition pore opening.} The critical element of the mitochondrial cell death-regulating signalling pathways during their incubation on fibrinogen (CsA, necrostatin-1, Z-VAD-FMK) or spreading (calpeptin). The mitochondrial permeability transition pore inhibitor CsA (5 μM) significantly diminished PS+ fraction formed by the WAS platelets on fibrinogen (Fig. 4A). Other inhibitors including necroptosis inhibitor necrostatin-1, calpain inhibitor calpeptin and pan-caspase inhibitor Z-VAD-FMK had no significant effect on PS exposure (Fig. 4B). These data strongly suggest that it is indeed the necrotic mechanisms of mitochondrial permeability transition pore opening that is responsible for the increased PS exposure in the WAS platelets.

\textit{Role of calcium homeostasis, cellular energetics, and ROS in the PS exposure by the WAS platelets.} In order to get further insight into the necrosis of the surface-attached WAS platelets, they were treated with xestospongin C (inositol trisphosphate receptor blocker), or thapsigargin (TG, a sarco-endoplasmic reticulum Ca\textsuperscript{2+} ATPase inhibitor), or in buffer A without addition of calcium chloride (Fig. 4C). Xestospongin C inhibited PS exposure suggesting involvement of inositol trisphosphate signalling, while TG potently boosted platelet necrosis. In contrast, the effects were drastically decreased in the absence of extracellular calcium.

Importantly, TG caused accelerated cell death in the WAS platelets compared with healthy controls in suspension as well without any surface attachment (Fig. 4D), which suggested that their propensity towards necrosis is caused by disregulation of their calcium homeostasis. Same experiment with lactadherin and without addition of extracellular calcium did not show increased PS+ fraction of WAS platelets (Fig. 4E). To additionally check the effect of
outside-in signaling on TG-induced PS-exposure in this design, we pre-treated platelets with integrin $\alpha_{IIb}\beta_3$ antagonist monafram which did not affect the TG-induced PS-exposure (Fig. S4). Pre-incubation of the WAS platelets with mitochondrial ATPase inhibitor oligomycin or with mitochondrial uncoupler CCCP increased the formation of PS-positive platelets at TG treatment in case of WAS platelets, while mitochondrial respiratory chain complex I inhibitor rotenone had less affect the TG-induced PS-exposure (Fig 4F); neither of these three drugs caused platelet necrosis by themselves. These data indicate that deficiency of energy could be a contributing factor to the platelet necrosis but not the defining one. In line with this, although the levels of ATP in cells were decreased in parallel with the increase of the PS-positive platelets upon TG treatment, the same decrease of ATP was caused by CCCP without PS exposure indicating that the observed phenomenon is not purely energetical collapse (Fig. 4G,H). ROS production in the WAS platelets was not essentially different from that in healthy donors, and was only mildly increased upon stimulation with CRP (Fig. S5). The morphology of the mitochondria in WAS platelets was not apparently different from the normal ones as judged by transmission electron microscopy (Fig. S6).

*Platelet necrosis directly correlates with the number of mitochondria.* During examination of the images, it became apparent that the WAS platelets undergoing PS exposure and mitochondrial membrane potential loss rarely have more than two mitochondria per cell. Therefore, we performed the experiments to count the number of mitochondria for each platelet and correlated this with the outcome (i.e. PS exposure) in Fig. 5. For both WAS patients and healthy donors, the number of mitochondria was significantly smaller in the platelets that became PS-positive (Fig. 5A). This number affected the fate of platelets in a dose-dependent manner: about 33% of the WAS platelets exposed PS if they had 1-4 mitochondria per platelet, and only about 11% if they had more than 5 mitochondria (Fig. 5B). A similar dependence was observed for healthy donors (Fig. 5B), although they had more rare PS exposure. The histogram in Fig. 5C shows the distributions of mitochondria number for WAS and healthy donors side by side. Importantly, although the mean number of mitochondria in WAS platelets was not much less than in the control platelets, there was significant skewing to the left of the curve: a total of 27±12% of WAS platelets had less than 3 mitochondria, compared to only 8.7±4.4% of healthy platelets. In order to check if the number of mitochondria has a wider significance in platelet necrosis, we performed experiments with fibrinogen-attached healthy platelets stimulated with TRAP-6 or thrombin revealing the same pattern (Fig. 5D,E).
Systems biology simulations reveal critical roles of mitochondrial number and surface-to-volume ratio in the programmed cell death in WAS. In order to carefully dissect the mechanisms of mitochondria-dependent necrosis in WAS, we developed a computational systems biology model of calcium signalling (Fig. 6). In the model, which had all compartments and major calcium signalling mechanisms, we investigated dependence of platelet calcium response on two major variables that differ for the WAS platelets, the number of mitochondria and platelet size.

The model demonstrated that decrease in the number of mitochondria should make platelets more sensitive to mitochondrial collapse and result in higher calcium increase because the remaining mitochondria could not bear the ATP production load (Fig. 6A, B), which agrees well with the experimental observations. We also simulated platelets of different sizes; when scaling them, the ratio between surface and volume molecules was naturally changed (Fig. 6), as volume is proportional to the size to the third degree, while surface is proportional to the size to the second degree. Upon stimulation, the virtual platelets with smaller size had comparable active phospholipase C per volume (Fig. 6C), but more inositol trisphosphate and ultimately much more calcium (Fig. 6E) because they had more inositol trisphosphate receptors per volume (as they were assumed to be proportional to the surface). This is again in line with experimental data above that show increase calcium levels in WAS even prior to mitochondrial permeability transition pore opening, and with the sensitivity of the phenomenon to xestospongin C.

The model prediction was, therefore, that the size of the untreated WAS patients' platelets should negatively affect their ability to spontaneously expose PS and (if this is the mechanism behind thrombocytopenia) positively affect their platelet count. Interestingly, there was a significant positive correlation between platelet size and platelet count of the untreated WAS patients (Supplement Fig. S7A). Although we did not observe significant correlations with the PS exposure probably as a result of the limited number of samples (Fig. S7B,C), it is interesting that patient #18 (indicated with red arrow), who had normal-sized platelets and mild phenotype, also had the least PS exposure upon immobilization and incidentally the highest platelet count.

**Discussion**

In this study, we show that cell death of the WAS platelets upon minor stimulations such as fibrinogen attachment or low-dose thapsigargin treatment follows the mitochondrial necrosis pathway. It is a rapid process associated with mitochondrial permeability transition...
pore opening that actually precedes phosphatidylserine exposure at the single platelet level, extracellular calcium-dependent, and it is downregulated by cyclophilin D and inositol trisphosphate receptor antagonists, but not by apoptosis or necroptosis inhibitors. It is associated with decrease of platelet ATP levels, and downregulation of the energy metabolism with CCCP, rotenone and oligomycin promoted necrosis, but did not cause it by itself. This cell death phenomenon predominantly occurs in the platelet fraction having less than 4 mitochondria per platelets. Importantly, beyond the scope of this WAS study, the low number of mitochondria turned out to be predictive for the agonist-induced formation of procoagulant platelets by healthy donors. The immediate cause of this necrosis are: a) increased calcium concentration and spiking frequency of WAS platelets upon spreading; b) increased sensitivity of calcium homeostasis to collapse of single mitochondria in platelets with fewer mitochondria. Computational systems biology analysis confirms that both increased surface-to-volume ratio (leading to impaired calcium homeostasis) and the smaller number of mitochondria (resulting in increased sensitivity of calcium to mitochondrial collapse) contribute to the tendency of the WAS platelets to undergo necrosis upon minor stimulation. Although the clinical consequences of this phenomenon are beyond the scope of this paper, this mechanism is supported by the observation of correlation between platelet size and platelet count in WAS. In contrast to the observations for immune thrombocytopenia, there were no statistically significant changes in the WAS platelet functionality upon romiplostim treatment.

The phenomena investigated in the present study agree well with previous observations of Shcherbina et al. who reported increased, accelerated or spontaneous PS exposure by the platelets of WAS patients or WAS knockout mice associated with increased calcium levels at rest, and provide a molecular basis for these previous reports. The mechanism of this massive PS exposure in WAS platelets appears to be essentially similar to the one that determines agonist-induced procoagulant platelet formation in physiological potent platelet activation: increase of cytosolic calcium followed by mitochondrial calcium overload and collapse, ultimately leading to necrotic cell death. The difference was that PS exposure in WAS was triggered by weak stimuli like fibrinogen attachment, which, although recognized as activating, produced negligible PS exposure in healthy donor platelets by itself. High percentage of PS-positive platelets seems to be a universal feature of the disease, irrespective of its severity and absence/presence of other WAS features.

The similarity between these phenomena (PS exposure by normal platelets via thrombin and/or collagen receptors and PS exposure by WAS platelets induced by fibrinogen
(attachment) goes so far that the phenomenon of predominant necrosis by platelets with fewer mitochondria was observed here for TRAP-6 or thrombin stimulated healthy platelet activation as well. This is interesting in itself and might have implications beyond the scope of the present study: although several previous studies attempted to identify properties of platelets that pre-dispose them to procoagulant formation such as age or resting calcium concentration\textsuperscript{18, 33, 39}, the effects were much less than those of the number of mitochondria, and it has been generally assumed in the field that it is unclear which platelets become necrotic and which do not.

Although the data of the present study clearly characterize the immediate molecular cause and the sequence of events leading to the spontaneous PS exposure in the WAS platelets, they are more limited with regard to linking this phenomenon to the genetic cause of the disease or clinical consequences. While decreased number of mitochondria in the WAS platelets (natural due to their decreased size) is likely to contribute to their tendency to undergo necrosis, this difference by itself is not great enough to have such drastic consequences. Non-stimulated WAS platelets undergo necrosis more efficiently than non-stimulated healthy platelets having the same number of mitochondria (Fig. 5), so there should be additional mechanisms. The most promising one is disruption of cytosolic calcium balance (even without regard to mitochondria) simply due to the greater surface-to-volume ratio in WAS: computer systems biology simulations indicated that this mechanism alone is sufficient to explain their necrosis. This hypothesis is supported by the sensitivity of PS+ fraction to xestospongin C and extracellular calcium decrease, as well as by the ability of calcium pump inhibitor thapsigargin to promote WAS platelet necrosis much rapidly than that of healthy donor platelets. Inability of mitochondrial function antagonists to cause necrosis by itself also in line with the proposed picture of events. However, we do not show a causal relationship between size and calcium in direct experiments, and cannot exclude participation of other contributing factors. Furthermore, although it is tempting to speculate than increased PS exposure may promote platelet clearance by macrophages\textsuperscript{10, 11}, statistics of the present study is not sufficient to confirm or disprove relationship between clinical severity and the tendency of platelets to expose PS, although we do show relationship between platelet count and platelet size. Involvement of impaired actin cytoskeleton dynamics due to WASP mutations in the programmed cell death of WASP platelets cannot be excluded either, and requires additional research.
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Author contributions

S.I.O. performed fluorescence and electron microscopy experiments, and analyzed the data. E.O.A. and A.A.I. performed flow cytometry experiments. A.N.S. developed the computational model and performed simulations. T.V.V. performed genetic analysis. S.G. analyzed the data and edited the paper. G.Y.L. and N.N.U. designed and performed metabolic experiments. I.I.K. designed electron microscopy experiments and analyzed the data. F.I.A. designed experiments and analyzed the data. G.A.N. and A.A.M. suggested the idea of the study and analyzed the data. A.S. planned the study, recruited and characterized patients, and analyzed the data. M.A.P. planned the study and wrote the paper with contributions from all authors.

Conflict-of-interest disclosure

The authors declare no conflict of interest.
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### Table 1. Characteristics of WAS patients

| Patient # | Age | WAS gene mutation | Disease severity score* | Romiplostim treatment | platelet count 10^9/l |
|-----------|-----|-------------------|-------------------------|-----------------------|----------------------|
| 1         | 12  | c.777+1G>A        | 5                       | After                 | 114                  |
| 2         | 13  | c.1453G>A, p.D485N | 4                       | Before                | 52                   |
| 3         | 0.9 | c.929_931+9del (c.929_931+9delAGGgtgagaccc) | 5      | Before                | 30                   |
| 4         | 2   | c.223G>A, p.V75M  | 3                       | After                 | 82                   |
| 5         | 11  | c.1201_1205dupCCACC, p.P403HfsTer44 | 5            | After                | 34                   |
| 6         | 0.8 | c.777+1G>A        | 3                       | After                 | 27                   |
| 7         | 4   | c.4A>T, p.S2C     | 1                       | After                 | 145                  |
| 8         | 12  | c.560-1G>A        | 5                       | After                 | 53                   |
| 9         | 0.6 | c.631C>T, p.R211Ter | 4                   | After                | 12                   |
| 10        | 10  | c.116T>C, p.L39P  | 2                       | Before                | 7                    |
| 11        | 17  | c.223G>A, p.V75M  | 2                       | After                 | 34                   |
| 12        | 12  | c.134C>T, p.T45M  | 2                       | Before                | 20                   |
| 13        | 14  | c.4A>T, p.S2C     | 2                       | Before                | 31                   |
| 14        | 10  | c.4A>T, p.S2C     | 2                       | Before                | 32                   |
| 15        | 0.5 | c.4A>T, p.S2C     | 2                       | Before                | 77                   |
| 16        | 1   | c.961C>T, p.R321Ter | 3                   | After                | 170                  |
| 17        | 0.6 | c.107_108delTT, p.F36Ter | 5            | After                | 42                   |
| 18        | 5   | c.413G>A, p.R138Q | 1                       | Before                | 54                   |
| 19        | 0.9 | c.37C>T, p.R13Ter | 3                       | Before                | 19                   |
| 20        | 7   | c.961C>T, p.R321Ter | 3                   | Before                | 11                   |
| 21        | 2   | c.559+5G>A        | 5                       | Before                | 55                   |
| 22        | 1   | c.631C>T, p.R211Ter | 5                   | After                | 192                  |
| 23        | 1   | c.273+2T>C        | 5                       | Before                | 20                   |
| 24        | 2   | c.281G>C, p.R94P  | 1                       | After                 | 81                   |
| 25        | 2   | c.777+2del4(GAGT) | 5                       | After                 | 73                   |
| 26        | 2   | c.2T>C, p.M1R     | 4                       | Before                | 16                   |
| 27        | 7   | c.961C→T, p.R321Ter | 3                   | After                | 20                   |
| 28        | 11  | c.559+5G>A        | 4                       | Before                | 17                   |
| 29        | 7   | c.107_108delTT, p. F36Ter | 5            | After                | 16                   |
| 30        | 0.5 | c.314T>C, p.L105P | 1                       | Before                | 36                   |
| 31        | 9   | c.143C>T, p.471>T | 3                       | After                 | 72                   |
| 32        | 8   | c.223G>A, p.V75M  | 3                       | After                 | 37                   |
| 33        | 3   | c.1430G>A, p.R477K | 2                       | After                 | 147                  |
| 34        | 17  | c.559+5G>A        | 5                       | Splenectomy           | 170                  |
| 35        | 2   | c.267G>A, p.R86H  | 2                       | After                 | 30                   |

* Scoring as suggested in [40](#)
Figure Legends

Fig 1. Exposure of PS by the WAS platelets upon fibrinogen binding. (A) Confocal microscopy images of the healthy (left) and WAS (right) platelets after spreading for 30 min on the fibrinogen surface in presence of 1.5 mM Ca\(^{2+}\). Labeling was with CD61 (green) and annexin V (red), scale bar 10 µm. (B) PS+ fraction of the WAS patients (27 patients, >7500 cells), adult healthy (18 donors, >6500+N cells) and 0-7 years old donors without WAS (6 donors, age: 0, 0, 2, 3, 4, 7 years, 2300 platelets) on the fibrinogen surface. (C) PS+ fraction for the WAS platelets on the fibrinogen surface, comparison for romiplostim-treated or untreated WAS patients, p=0.94. (D) Monafram coated coverslips did not change PS+ fraction, p=0.86, n=4, 2500 platelets.

Fig 2. Functional response of the WAS and healthy platelets. (A-G) Whole blood platelets were stimulated (designated by A) or not (designated N/A) with TRAP-6 plus CRP and analyzed by flow cytometry. Parameters for healthy children (n=21, age 0-13 years, median 5.0) and patients (17 treated with romiplostim, 11 non-treated): platelet size by FSC, MFI (A); CD42b level, MFI (B); CD61 level, MFI (C); PAC1-positive platelets, % (D); CD62p-positive platelets, % (E); dense granule release by mepacrine level, MFI (F); PS+ platelet fraction, % (G). p – Mann–Whitney U-test, p* – Wilcoxon signed-rank test.

Fig 3. Dynamics of cytoplasmic calcium, mitochondrial potentials and PS exposure of single platelets. Plots show dynamics of intracellular calcium concentration and annexin V binding to single platelets during incubation on fibrinogen in presence of 1.5 mM of extracellular calcium. (A) Averaged calcium dynamics +/- SD for WAS N/A patients (n=4, 34 platelets), HD activated with 10 µM TRAP (n=3, 30 platelets) and N/A (n=3, 26 platelets). (B) Dynamics for a single healthy PS- platelet; (C) the same for a WAS PS- platelet; (D) the same for a WAS PS+ platelet. TMRM signal is represented as a number of TMRM-positive mitochondria in the platelet (B-D). Intracellular events leading to PS exposure induced with mitochondria collapse with following cytoplasmic calcium increase and PS exposure. All three processes were almost simultaneous with duration of decades of seconds. Both WAS (E) and normal (not shown) PS+ platelets lost their mitochondrial potentials. Scale bar is 1 micron for all microscopic images.
**Fig 4. Prevention of mitochondrial permeability transition pore opening and mitochondrial count affect spontaneous PS+ exposure of the WAS platelets.** (A) Fibrinogen-spread platelets were incubated in the absence/presence of 5 μM CsA (3900 platelets from 11 patients and 4000 platelets from 6 healthy donors were observed); (B) DMSO or programmed cell death inhibitors calpeptin (200 μM, 20 min incubation), Nec-1 (50 μM, 50 min incubation) and Z-VAD-FMK (50 μM, 50 min incubation) (100-300 platelets were observed for each dot). (C) Modulation of intracellular calcium signalling in spread platelets with Xestospongin C (3 μM, 50 min); thapsigargin (TG, 1 μM, 30 min); with lactadherin and without addition of 1.5 mM CaCl₂ (n=5, 6800 platelets). (D) Flow cytometry analysis of WAS platelet PS exposure in suspension. Incubation of platelets in suspension with 1 μM TG in presence of 1.5 mM CaCl₂ during 10 minutes induced PS+ platelet fraction comparable with fibrinogen-spreading both for WAS patients (n=7, mean 19.7%±11.8% SD) and HD (n=11, 6.6%±8.0%). (E) Flow cytometry analysis of WAS platelet PS exposure in suspension without addition of CaCl₂ (n=3); (F) Analysis of the mitochondrial inhibitors in suspension at TG treatment (n=3); (G, H) ATP levels (G) versus the number of PS-positive platelets (H): in healthy donors and WAS at TG and CCCP treatment (n=3).

**Fig 5. Dependence of PS exposure on mitochondria count.** Platelets that exposed PS during incubation on fibrinogen had significantly less mean mitochondria count than PS-cells. (A) Mean mitochondria number in platelet subpopulations per WAS patient or HD for non-activated fibrinogen-bound platelets. Each dot represents one WAS patient (7 patients, 381 platelets) or HD (n=4, 567 platelets). (B) Averaged PS+ fraction ± SD of the same WAS and HD platelets with different mitochondria amount. (C) Averaged distribution of mitochondria per platelet (both subpopulations) for 7 WAS patients and 11 HD (WAS n=7, 381 platelets; HD n=11, 1179 platelets). (D-E) Healthy activated platelets, overall 613 cells from 7 HD activated with TRAP-6 (n=5, 306 cells) or thrombin (n=4, 307 cells). Platelets most likely to expose PS had less mitochondria. Mitochondria were counted by TMRM fluorescence using microscope after 20 minutes spreading (before activation in experiments with activated Healthy donors), subpopulation were determined after additional 30 minutes incubation. Each dot represents the mean of mitochondria count in a patient or HD (A,C). p – Mann–Whitney U-test.
Fig 6. Increased cytosolic calcium as a result of downsizing: computer systems biology simulation of calcium signalling in normal and WAS platelets. WAS platelets were assumed to have the same content of signalling proteins scaled to the respective volume of compartments. (A, B) Stochastic simulation of the activation of normal platelet with 2 (A) or 4 (B) mitochondria with thrombin at 10 nM. With the collapse of one mitochondrion the average cytosolic calcium increases 1.5-fold (A) in case of 2 mitochondria or does not change (B) in case of 4 mitochondria. (C-E) Stochastic and deterministic simulations of normal and WAS platelet stimulated with 1 nM thrombin.
Figure 1

A  

Normal  |  WAS
---|---
Merge

Annexin V

CD61

B  

PS+ fraction, %

WAS  |  Healthy donors  |  Children

C  

PS+ fraction, %

WAS w/o treatment  |  Treated WAS

D  

PS+ fraction, %

WAS FG  |  WAS Monafram

p < 0.001
p < 0.01
p = 0.44
p = 0.94
p = 0.86
Figure 4

A. PS+ fraction, %

B. WAS

C. WAS

D. PS+ fraction, %

E. PS+ fraction, %

F. PS+ fraction, %

G. Contrast of ATP, x 10^11 mol/platelet

H. PS+ fraction, %
Fig. S1. Additional panels for Fig. 2. Comparison of healthy children (n=21), WAS patients with (n=17) or without treatment (n=11) by SSC/FSC/CD42b ratios upon activation (A-C). D-G normalization of the expression of activation markers PAC1 and CD62p (MFI) on the CD61 MFI. Mann Whitney U test was used. "act", activated platelets, "rest", resting platelets.
Fig. S2. The PS+ platelet fraction of WAS and healthy donors with/without 25 µM TRAP-6 activation. In order to investigate the activation status of WAS platelet in plasma (with minimal mechanical action at platelet isolation), we analyzed the PS-positive platelet subpopulation formation at low platelet activation by TRAP-6. (A-B) We used two different approach to obtain the data of platelet activation in plasma: in the absence of thrombin inhibitor PPACK and in the presence of high concentration of PPACK. We observed that the activation status of WAS platelets in plasma is increased, as compared with normal platelets. In the presence of PPACK is inhibited the activation-induced formation of PS-positive platelet subpopulation in WAS and HD, that suggest that the formation PS-positive platelets in plasma partly is due to additional thrombin generation at platelet activation. p – Mann–Whitney U-test, p* –Wilcoxon signed-rank test. (C) - Dependence of PS+ fraction on TRAP concentration for HD and WAS (n=2).
Fig. S3. Additional panel for Fig. 3. Calcium and mitochondrial membrane potentials dynamics of WAS PS+ platelet with 2 mitochondria (A) and PS- platelets with 6 mitochondria (B).
Fig S4. Effects of monafram on TG-induced necrosis in suspension (n=3).
Fig S5. Reactive oxygen species detection. 2’7’-dichlorodihydrofluorescein diacetate (DCFDA) was used for analysis of ROS production upon platelet activation. ROS production was observed only at stimulation of platelets with CRP or dual agonist stimulation CRP+TRAP (n=3). At the same time ROS production was not detected at stimulation of platelets with TRAP or spreading on fibrinogen (data not shown). This suggest that ROS production is not involved in PS exposure at spreading on fibrinogen.
Fig. S5. Electron microscopy of platelets. (A) – healthy donor platelets, (B-C) – platelets from 2 WAS donors. Arrows show mitochondria. Bar 1 μM.
Fig. S7. Correlation of platelet size, platelet count and tendency to expose PS in the untreated WAS patients' platelets. The analysis is based on the PS exposure results in Fig. 1B, FSC results of Fig. 2A, and platelet counts in Table 1.
Supplemental methods

*Patients and healthy donors.* A total of 35 patients with WAS were included in the study (Table 1). The diagnosis was made according to European Society for Immunodeficiencies diagnostic criteria and genetically confirmed by WAS mutations identification. Samples from 12 patients were collected only when not on romiplostim, 17 patients were studied after at least 1 month of romiplostim treatment, and 5 patients were investigated both before treatment and on treatment. Romiplostim was administered off-label according to the institutional protocol at 9 µg/kg weekly. 12 of 35 patients had a Zhu score of 1 and 2. Control samples included blood from children and healthy adults as indicated in the experimental descriptions.

*Reagents.* The following materials were used: prostaglandin E1 (MP Biochemicals, Irvine, CA, USA); Fura Red AM, (Molecular Probes, Eugene, OR, USA); Alexa Fluor 647-conjugated annexin V, CD61-fluorescein isothiocyanate (FITC), CD61-phycoerythrin (PE) (Biolegend, San Diego, CA, USA); tetramethylrhodamine methyl ester (TMRM), nonyl acridine orange (NAO), 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) (Life Technologies, Grand Island, NY, USA); cyclosporine A, calcium ionophore A23187, calpeptin; Z-VAD-FMK, thapsigargin, CCCP, rotenone, oligomycin A, Xestospongin C (Tocris Biosciences, Bristol, UK); Phe-Pro-Arg chloromethyl ketone (PPACK) (Merck KGaA, Darmstadt, Germany), lactadherin-FITC and human thrombin (Haemotologic Technologies, VT, USA); osmium tetroxide (Ted Pella, CA, USA), luciferin-firefly luciferase reagent, dimetylsulphoxide, ATP-control (BCM ST, Russia). All other reagents were from Sigma-Aldrich (San Diego, CA, USA). Integrin αIIbβ3 antagonist monafram was a kind gift of Prof. A.V. Mazurov. Cysteine-containing version of cross-linked collagen-related peptide (CRP) was kindly provided by Prof. R.W. Farndale (University of Cambridge, Cambridge, UK).

*Blood collection and platelet isolation.* Investigations were performed in accordance with the Declaration of Helsinki under approval of the Children's for Hematology Ethical Committee, and written informed consent was obtained from all patients (or their parents) and donors. Washed platelets were prepared essentially as described. Blood was collected into sodium citrate, and supplemented with prostaglandin E1 (1 µmol L⁻¹). Platelet-rich plasma was obtained by centrifugation at 100 × g for 8 min. Three parts of platelet-rich plasma were diluted with one part of 3.8% sodium citrate (pH 5.5), and centrifuged at 400 × g for 5 min (for WAS platelets, 700 g); platelets were then resuspended in buffer A (150 mmol L⁻¹ NaCl, 2.7 mmol L⁻¹ KCl, 1 mmol L⁻¹ MgCl₂, 0.4 mmol L⁻¹ NaH₂PO₄, 20 mmol L⁻¹ HEPES, 5 mmol L⁻¹ glucose, 0.5% bovine serum albumin, pH 7.4).
Confocal microscopy experiments: general design. Glass coverslips (24 × 24 mm; Heinz Herenz, Hamburg, Germany) were cleaned by plasma cleaner (Harrick Plasma, Ithaca, NY, USA). They were coated with 1 mg mL\(^{-1}\) fibrinogen or monafram in PBS for 45 min at room temperature, rinsed, and used as the substrate for washed platelets. Washed platelets were attached to the protein-coated surface by incubating them at 1.3·10\(^5\) µl\(^{-1}\) (or the maximal concentration attainable for WAS under conditions of thrombocytopenia) for 20 min and rinsing with buffer A with 1.5 mM CaCl\(_2\). For the experiments on PS exposure, the fields were imaged once, and then again after 30 min of additional incubation. For experiments with intracellular dyes loading, continuous video-imaging was performed. Confocal images were acquired using an Axio Observer Z1 microscope (Carl Zeiss, Jena, Germany) equipped with a Yokogawa spinning disc confocal device (CSU-X1; Yokogawa Corporation of America, Sugar Land, TX) and a 1.3 numerical aperture × 100 objective. Optical filters (Semrock, Rochester, NY, USA) for fluorophores were used: Alexa Fluor 647– annexin V and Fura Red (647 nm long-pass filter); (FITC)–CD61 (520/35 nm); and TMRM (587/35 nm). The excitation wavelengths were 405 nm and 488 nm for Fura Red, 488 nm for FITC; 561 nm for TMRM; and 635 nm for Alexa Fluor 647. Analysis of the obtained images was carried out with ImageJ (http://imagej.nih.gov/ij/) software.

Cytosolic calcium signaling and mitochondrial membrane potential change. The methodology was essentially as described\(^2\). For experiments with calcium measurements washed platelets were incubated with 10 µmol L\(^{-1}\) Fura Red AM for 45 min at room temperature followed by centrifugation and resuspension in buffer A. Intracellular calcium levels of non-activated platelets were determined with confocal microscopy. Calibrations for ratiometric measurements were made using 10 mM calcium ionophore A23187 and 4 mM EGTA for minimal and maximal fluorescence ratios, separately for healthy and WAS platelets. Background signals were substractive from images and calcium concentrations were calculated using a formula for ratiometric indicators\(^3\). TMRM was used to detect mitochondrial potential dynamics. Washed platelets were spread on the fibrinogen-coated coverslip for 20 min in the presence of 200 nM TMRM at room temperature. Unattached cells were then removed with buffer A with 1.5 mM CaCl\(_2\), and imaging was started as described above.

Characterization of platelet response to TRAP-6 in platelet-rich plasma (PRP). Samples of PRP were diluted with PPP to a final concentration of 20,000 µL\(^{-1}\) and buffered with HEPES at pH 7.4 (100 mM final concentration). The irreversible thrombin inhibitor Phe-Pro-Arg chloromethyl ketone (PPACK) was added to the final concentration of 100 µM in order to block spontaneous thrombin generation upon recalcification (Fig. S1). Platelets were recalcified by addition of calcium chloride (final concentration 20 mM, which corresponds to 2 mM of free calcium\(^4\)) and activated by 25 µM TRAP-6 for 5 min at room temperature. They were stained with
CD61-FITC (antibody to membrane integrin αIIbβ3) and annexin V-Alexa Fluor 647 (marker of PS-positive platelets) for 5 min, diluted with buffer A containing 1.5 mM calcium chloride and immediately analyzed using flow cytometry.

Determination of ROS. Washed platelets at 20000 uL-1 were incubated with 10 µM CM H2-DCFDA for 30 min at 37°C in buffer A and activated for 10 min at RT with the indicated concentrations of agonists in the presence of 2.5 mM calcium chloride. Platelets were stained with CD61-PE and annexin V-Alexa Fluor 647 for 5 min at RT, diluted with buffer A containing 2.5 mM calcium chloride and immediately analyzed by flow cytometry. Flow cytometry analysis was performed using Accuri Flow Cytometer (BD Biosciences, San Jose, CA, USA).

Modulation of calcium signalling and mitochondrial function. Platelets in buffer A at 20,000 µL-1 were incubated with 1 µM TG in the presence of 1.5 mM calcium chloride for 10 min. At the same time platelets were stained with CD61-FITC and annexin V-Alexa Fluor 647. TG-treated stained platelets were diluted with buffer A containing 1.5 mM calcium chloride and immediately analyzed using flow cytometry. In the case of experiments with integrin αIIbβ3 antagonist, the platelets in buffer A at 20,000 µL-1 were preincubated with 200 µg/ml monafram for 20 min at RT before treatment with TG. For calcium-free experiments, platelets at 20,000 µL-1 were incubated with 1 µM TG in buffer A without addition of calcium chloride and stained with CD61-PE and lactadherin-FITC, then diluted with buffer A without addition of calcium chloride and immediately analyzed using flow cytometry. The mitochondrial inhibitors oligomycin (2.5 µM), rotenone (10 µM) and uncoupler CCCP (10 µM) were incubated with platelets at 20,000 µL-1 in buffer A for 15 min at RT before addition of TG.

ATP measurement in platelets. Platelets in buffer A at 100,000 µL-1 (for donors) and 30,000 µL-1 (for patients) were incubated with 1 or 5 µM TG in the presence of 1.5 mM calcium chloride for 5, 20 or 40 min or with 10 µM CCCP for 10 min. The samples were taken at indicated time points to determine ATP concentration and percentage of PS-positive platelets. In brief, part of the sample was lysed (addition 90 µl DMSO to 10 µl platelet suspension) and analyzed by luciferase-luciferin assay as described5, 6 while the other part was stained with CD61-FITC and annexin V-Alexa Fluor 647 and analyzed by flow cytometry.

Flow cytometry characterization of platelet functional activity. The experiments were performed essentially as described7, 8 with minor modifications. Citrated blood samples were diluted 1:20 with buffer A. Platelets were either left intact or loaded with mepacrine (10 µM final concentration) for 30 min at 37°C. Subsequently, they were either left unstimulated or stimulated with CRP at 20 µg/µl plus TRAP-6 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 in the presence of 2.5 mM calcium
chloride. Subsequently, they were diluted 10-fold with buffer A containing 2.5 mM calcium, and analyzed using Novocyte (Acea Bioscience, San Diego, CA, USA) flow cytometer. Blood samples from healthy children claiming not to have used medication for a week prior to analysis were used for control.

Transmission electron microscopy. The protocol was essentially as described\(^9\). PRP was fixed for 1 hour with freshly prepared 2.5% glutaraldehyde in PBS (pH 7.4). After centrifugation and rinsing with PBS (pH 7.4), cells were postfixed in 1% osmium tetroxide for 1 hour. Then each sample was dehydrated in a graded acetone series and embedded in Epon 812. Ultrathin sections were produced using Ultracut E (Reichert, Vienna, Austria). The sections were stained with lead citrate followed by uranyl acetate and observed with the transmission electron microscope JEM-1400 (JEOL Tokyo, Japan).

Statistics. Data are presented as means ± standard deviations. The statistical significance of the differences between groups was determined with the non-parametric Mann–Whitney U-test (p). The effects in the paired samples were evaluated using Wilcoxon signed-rank test (p*). Differences were considered to be significant when the P-value was < 0.05.

Computational modeling of platelet calcium homeostasis. A systems biology model of platelet calcium signalling was described previously\(^18\). Briefly, it was a multicompartmental stochastic computational model of platelet calcium signaling resulting from stimulation of PAR1 receptor. In contrast to its predecessor pure calcium signalling/homeostasis models\(^10,\ 11\), it had several mitochondrial compartments and included equations describing dependence of ATP production and calcium pumps activity on mitochondrial inner membrane potential. The model consisted of 35 ordinary differential equations. The set was solved using COPASI software (www.copasi.org) and stochastic adaptive SSA/tau-leap method.

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