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ARTICLE

Impaired mitochondrial activity explains platelet dysfunction in thrombocytopenic cancer patients undergoing chemotherapy

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ABSTRACT

Severe thrombocytopenia (≤50x10^9 platelets/L) due to hematological malignancy and intensive chemotherapy is associated with an increased risk of clinically significant bleeding. Since the bleeding risk is not linked to the platelet count only, other hemostatic factors must be involved. We studied platelet function in 77 patients with acute leukemia, multiple myeloma or malignant lymphoma, who experienced chemotherapy-induced thrombocytopenia. Platelets from all patients - independent of disease or treatment type - were to a variable extent compromised in Ca^{2+} flux, integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression when stimulated with a panel of agonists. The patients' platelets were also impaired in spreading on fibrinogen. Whereas the Ca^{2+} store content was unaffected, the patients' platelets showed ongoing phosphatidylserine exposure, which was not due to apoptotic caspase activity. Interestingly, mitochondrial function was markedly reduced in platelets from a representative subset of patients, as evidenced by a low mitochondrial membrane potential (P<0.001) and low oxygen consumption (P<0.05), while the mitochondrial content was normal. Moreover, the mitochondrial impairments coincided with elevated levels of reactive oxygen species (Spearman's rho=-0.459, P=0.012). Markedly, the impairment of platelet function only appeared after two days of chemotherapy, suggesting origination in the megakaryocytes. In patients with bone marrow recovery, platelet function improved. In conclusion, our findings disclose defective receptor signaling related to impaired mitochondrial bioenergetics, independent of apoptosis, in platelets from cancer patients treated with chemotherapy, explaining the low hemostatic potential of these patients.

Introduction

Platelets are indispensable for maintaining vascular integrity and accomplishing hemostatic plug formation.[1] A sufficient platelet count as well as an adequate platelet function is required for prevention of bleeding. Patients with hematological malignancies, such as leukemia, multiple myeloma or malignant lymphoma, are commonly treated with combination chemotherapy, frequently followed by bone marrow transplantation. This treatment impairs the proliferation of megakaryocytes and the production of proplatelets. As a consequence, severe thrombocy-

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topenia, i.e., a platelet count of ≤50x10^9/L, develops in virtually all treated patients.[2] These patients are at high risk of bleeding, with up to 43% experiencing clinically significant bleeding (World Health Organization [WHO] grade 2 or higher), and 1% experiencing life-threatening bleeding.[3] Prophylactic transfusion with platelet concentrates for preventing bleeding is given as standard care once the count drops below 10x10^9/L, or in case of active bleeding.[2,4] Randomized clinical trials have indicated that the bleeding risk in this patient group is reduced by platelet transfusion, although it does not completely eliminate hemorrhagic events.[3,5] Since bleeding is relatively infrequent in non-malignant thrombocytopenia,[6,7] it can be considered that a low platelet count is not the sole risk factor for bleeding in chemotherapy-treated patients.

Earlier studies on patients with acute myeloid leukemia, of whom none received chemotherapy, have provided indications for impaired platelet function due to disease, as apparent from low platelet aggregation, reduced granule secretion and weak thromboxane B2 production.[8-10] It was proposed that low expression of the α-granule glycoprotein, P-selectin, can be used as a prognostic marker for hemorrhage.[11] However, bleeding in combination with thrombocytopenia is more frequently observed in cancer patients treated with chemotherapy.[12] The literature thus far only indicates that the anthracycline daunorubicin inhibits integrin $\alpha_{IIb}\beta_3$ activation, aggregation and secretion of platelets upon agonist stimulation.[13,14] Daunorubicin and its analogue idarubicin were found to induce integrin activation and secretion in resting platelets.[15] However, to what extent and by which mechanism myelosuppressive chemotherapy in general affects platelet function has remained largely unclear.

In this study, we evaluated the platelet activation processes and coagulant activity in 77 patients with hematological malignancies treated with chemotherapy. Our results point to multiple functional defects in the patients' platelets which are related to impaired mitochondrial activity, independent of classical apoptosis. In the majority of patients, low platelet activity could be improved by platelet transfusion.

Methods

Materials and methods

See *Online Supplementary Material.*

Patients and control subjects

The study was approved by the local ethics committee (METC-11-4-097). All participating patients and healthy volunteers gave written informed consent according to the Helsinki declaration. Patients, reporting at the hospital, fulfilling the inclusion criteria and providing informed consent, were consecutively included in the period between November 2014 and April 2018. Eligible patients were ≥18 years of age, received chemotherapy for treatment of a confirmed hematologic malignancy (acute myeloid leukemia, acute lymphocytic leukemia, multiple myeloma or malignant lymphoma), and had, or were expected to have, thrombocytopenia (platelet count ≤50x10^9/L). Morning platelet counts were monitored daily as part of routine clinical care. According to standard practice, when the morning platelet count was <10x10^9/L, patients received prophylactic transfusion with one batch of platelet concentrate (leukocyte-depleted pooled buffy coat from five donors, median storage time: six days, median platelet count: 357x10^9/L). Patient exclusion criteria were: sepsis, splenomegaly, signs of active bleeding at the time of blood withdrawal, previous platelet transfusion within three days (excluding the presence of donor platelets), and/or use of antithrombotic medication during the previous 14 days.

For clinical care, blood samples were collected before and during chemotherapeutic treatment at multiple time points: 1) before the start of chemotherapy, 2) before myelosuppression, 3) during myelosuppression (platelet count ≤50x10^9/L), 4) during myelosuppression: before (platelet count ≤10x10^9/L) and one hour after platelet transfusion, and 5) during bone marrow recovery (platelet count ≤50x10^9/L). Patient blood samples were obtained *via* a central venous catheter, rinsed with 100 mL saline to remove residual traces of heparin (verified by measurement of thrombin time). Blood samples from healthy control subjects were obtained *via* venipuncture of the antecubital vein using a Vacutainer 21-gauge needle (Becton-Dickinson Bioscience, NJ, USA). Blood collection was always into 3.2% (w/v) trisodium citrate (Greiner Bio-One Vacuette, Alphen a/d Rijn, The Netherlands). For clinical care (hematological parameters), separate samples from patients were drawn into vacuette tubes containing K2-ethylenediaminetetraacetic acid (EDTA; Becton-Dickinson Bioscience, NJ, USA).

Experimental setup

Within the limitations of medical ethical permission, a total of 52 blood samples from patients (platelet count ≤50x10^9/L) could be obtained during myelosuppression (study A). In all these samples, platelet responsiveness was assessed using flow cytometry. Due to the limited blood volume and the low platelet counts, a restricted number of additional analyses was carried out per sample. When there remained sufficient sample volume, platelet function was further characterized by measuring the following platelet responses: platelet spreading, intracellular calcium signaling and phosphatidylserine (PS) exposure. To gain a deeper understanding of the underlying mechanisms of platelet dysfunction, subsequent blood samples could be obtained from 25 additional patients (platelet count ≤50x10^9/L) during the myelosuppression phase (study B). The samples were used to investigate apoptotic signaling (caspase activity; western blotting for caspase-mediated protein cleavage), mitochondrial respiration and structure (high-resolution respirometry, citrate synthase activity, transmission electron microscopy) or reactive oxygen species (ROS). The maximum of care was taken that for all measurements patients from the major treatment classes were represented (see Figure Legends).

For 36 of the patients in study A, blood samples could also be obtained at one hour after transfusion with platelet concentrate. Again, platelet responsiveness was determined by flow cytometry.

Statistical analysis

Data are represented as medians with interquartile ranges. Paired data were compared using the Wilcoxon signed-rank test, otherwise the Mann-Whitney U test was used. When comparing more than two groups, the Kruskal Wallis H test was used. *P*-values <0.05 were considered significant. Graphs were made using GraphPad Prism v6 (San Diego, CA, USA). Statistical analysis was performed using the SPSS Statistics 23 package (IBM, Armonk, NY, USA).

Results

Variable impairment of platelet activation in cancer patients with thrombocytopenia after chemotherapy

Blood samples were obtained from a total of 77 patients, who were diagnosed with acute myeloid leukemia or

acute lymphocytic leukemia (AML/ALL, n=37), multiple myeloma (n=21), malignant lymphoma (n=15) or other hematologic malignancies (n=4). All patients experienced severe thrombocytopenia due to chemotherapy, which was stopped at a median of eight days before blood sample analysis (Table 1). The median age of the patient group was 60 years, and 41% was female (Table 1). Leukocyte and platelet counts were below normal, as was the hemoglobin level. Standard coagulation parameters were determined in plasmas from 43 patients following their chemotherapy treatment. For 70% of the patients, values of activated partial thromboplastin time (aPTT), prothrombin time and thrombin time were within reference ranges (*Online Supplementary Table S1*). Fibrinogen and von Willebrand factor (VWF) levels were slightly elevated, while D-dimer levels were substantially increased in patient plasmas. On the other hand, factor VII activity levels were decreased.

Treatment regimens in accordance with national guidelines varied with disease type.[16-18] Since these regimens consisted of multiple chemotherapeutic compounds, the distribution of the drugs was evaluated among patients with different diagnoses. Therefore, the various drugs were assigned to one of five pharmacological classes: A, antitumor antibiotics & topoisomerase II inhibitors; B, antimetabolites; C, alkylating agents; D, mitotic inhibitors; E, other (*Online Supplementary Table S2*).[19] Most patients were treated with anti-tumor antibiotics/topo-isomerase inhibitors, antimetabolites and/or alkylating agents (*Online Suplementary Table S3*). The patients diagnosed with AML/ALL and lymphoma usually received drugs from one or more of these three classes, while the patients diagnosed with multiple myeloma only received alkylating agents. Of all 77 patients, 50 had undergone hematopoietic stem cell transplantation before inclusion, of which 39 patients received an autologous transplant and 11 an allogenic transplant (Table 1). Blood samples were obtained at eight days (median) after the last administration of chemotherapy or at eight days (median) after stem cell transplantation.

Responsiveness of washed platelets was determined by flow cytometry, using a platelet count of $10x10^9$/L, for 52 patients and 27 healthy control subjects. In the absence of agonists, surface activation markers were low for both patient and control platelets. After stimulation with adenosine diphosphate ([ADP]; P2Y1/12 agonist), collagen-related peptide (CRP-XL; Glycoprotein VI (GPVI) agonist) or thrombin (PAR1/4 agonist) at maximal doses, integrin $\alpha_{IIb}\beta_3$ activation (Figure 1A) and P-selectin expression (Figure 1B) of the patients' platelets were reduced to a variable extent, when compared to the controls, irrespective of the agonist used.

Detailed analysis indicated that the overall platelet responsiveness (median=36.8% interquartile range [IQR]=29.7- 46.7%), defined as the average fraction of platelets positive for integrin activation and P-selectin expression for the three agonists: (i) was not different between diagnoses, i.e., AML/ALL, multiple myeloma, lymphoma and other hematological malignancies (Kruskal Wallis H test, P=0.192); (ii) was not affected by stem cell transplantation, i.e., no transplant, autologous or allogenic stem cell transplantation (Kruskal Wallis H test, P=0.640); (iii) was similar for the four major treatment classes, i.e., A+B, A+B+C, B+C, C (Kruskal Wallis H test, P=0.512; *Online Supplementary Figure S1*); and (iv) did not correlate with the whole blood platelet count (Spearman's rho=0.175, P=0.239). Together, this suggested that the variability in platelet responsiveness among patients was not directly linked to the disorder, treatment type or number of (residual) circulating platelets. Additional functional analyses were performed with platelets, invariably from patients in the major treatment classes.

Table 1. Characteristics and hematological parameters of patients during myelosuppression.

Patients characteristics	Number / Value	
Age (years)	60 (60)	
Female/male (n)	32/45 (20/32)	
Diagnosis (n)		
AML/ALL	37 (25)	
Multiple myeloma	21 (12)	
Lymphoma	15 (13)	
Other	4 (2)	
Stem cell transplantation (n)		
Autologous	39 (26)	
Allogeneic	11 (8)	
Time since chemotherapy (days)	8 (9)	
Time since stem cell transplantation (days)	8 (8)	
Blood parameters	**Value**	**Reference range**
Leukocyte count (x 10^9/L)	0.15 (0.22)	3.5 - 11.0
Hemoglobin (mM)	5.7 (5.7)	7.5 - 11.0
Platelet count (x 10^9/L)	8 (7)	150 - 400
Absolute immature platelet number (x 10^9/L)	0.31 (0.26)	
Immature platelet fraction (%)	3.9 (3.6)	1.1 -6.1[47]

Data are for total number of patients (n=77). Patient information for study A (n=52) is indicated between brackets. Median values are given. AML/ALL: acute myeloid leukemia or acute lymphocytic leukemia.

For 36 of the patients, blood samples could be obtained before and one hour after platelet transfusion. As expected, platelet count increased after transfusion (*Online Supplementary Table S4*). The clinical efficacy of transfusion was evaluated from the corrected count increment (CCI: [platelet count increment x body surface area]/[number of transfused platelets x 10^{11}]).[20] This was adequate for 96% of the patients, as indicated by a CCI value of >7.5 (median: 14.8, IQR: 11.3-18.0).

Flow cytometric analysis of integrin activation and P-selectin expression demonstrated that at one hour after transfusion, platelet responsiveness was improved for most patients (*Online Supplementary Figure S2*). Whenever possible, platelets were also isolated from the remainder of the transfusion concentrates. It appeared that the activity of the circulating platelets after transfusion approached that of the platelets of the concentrates when triggered with thrombin or CRP-XL. However, the responsiveness to ADP of the circulating platelets after transfusion was higher than in concentrates (integrin $\alpha_{IIb}\beta_3$ activation, P=0.002). The improved platelet responses after transfusion underlined the low responsiveness of the autologous platelets after chemotherapy.

Impaired platelet responsiveness during myelosuppression

To determine whether the reduced platelet responsiveness was linked to the treatment phase, flow cytometric

analysis of platelet responsiveness was performed during the decreasing period of platelet count (50-11 x10^9/L and ≤10 x10^9/L), and the recovery of platelet count (11-50 x10^9/L). The latter was defined as a sustained increase in the platelet count (observed for patient care), independent of platelet transfusion. Of the eight patients included in this category, three had received an autologous transplant and one patient an allogeneic stem cell transplant, prior to recovery. In the decreasing period, integrin activation and P-selectin expression following stimulation with thrombin or CRP-XL were comparable in patients with platelet counts in the range of 50-11 x10^9/L and ≤10 x10^9/L (Figure 2). In contrast, platelet responsiveness to thrombin and CRP-XL significantly improved in the case of count recovery (P<0.001). For stimulation with ADP, these differences were less pronounced, with only P-selectin expression increased during count recovery. These results indicated that platelet count alone is not a good marker of platelet activity.

For five patients (one AML, three multiple myeloma, one lymphoma), blood samples could also be analyzed at an earlier time point, i.e., after the stop of chemotherapy, but before severe thrombocytopenia occurred. Remarkably, in all these samples, platelet function was within the normal range for the three agonists (integrin activation 69-86%, P-selectin expression 49-85%). Furthermore, *in vitro* treatment of control blood with clinically relevant concentrations of cytarabine and/or melphalan did not affect platelet reactivity (*Online Supplementary Figure S3A,B*). These results argue against a direct effect of chemotherapeutics on the platelet activation properties.

Impaired platelet spreading and Ca^{2+} signaling of platelets after chemotherapy treatment

To further characterize the patient platelets, they were allowed to adhere and spread for ten minutes on a fibrinogen surface, interacting with platelet integrin $\alpha_{IIb}\beta_3$. The observed morphology of the cells was divided into three stages: 1) formation of filopodia, 2) formation of lamellipodia, and 3) full spreading. Most of the platelets from control subjects were in stages 2-3, while the patient platelets predominantly stayed in stage 1 (forming filopodia only), with few platelets being fully spread (Figure 3A). The patients' platelets displayed a slightly decreased expression of glycoprotein (GP)Ibα and GPVI, but not in integrin β_3 expression (*data not shown*). This suggested a diminished integrin activity and outside-in signaling in the patient platelets.

We further examined agonist-induced Ca^{2+} signaling after loading the platelets with Fluo-4. Stimulation with thrombin or CRP-XL induced only a small rise in $[Ca^{2+}]_i$ in patient platelets when compared to control platelets (Figure 3B,C). On the other hand, the $[Ca^{2+}]_i$ rise induced by thapsigargin (an inhibitor of endoplasmic reticulum Ca^{2+}-ATPases), as a measure of Ca^{2+} store content,[21] was similar for patient and control platelets. Together, this pointed to a defective agonist-induced Ca^{2+} signaling machinery, independently of receptor type (i.e., PAR1/4 or GPVI receptors).

Impaired mitochondrial bioenergetics but no apoptosis in platelets after chemotherapy

Given the cytotoxicity of chemotherapeutic compounds, we evaluated if patient platelets showed characteristics of apoptosis, since this process is known to lead to dysfunctional signaling.[22] As a marker of apoptosis, PS exposure was determined by fluorescein isothiocyanate (FITC)-annexin A5 binding. In contrast to control platelets, the patient platelets were prone to expose PS upon short-term storage without external stimuli (Figure 4A). Upon stimulation with the BH3 mimetic ABT-737, triggering the intrinsic pathway of apoptosis,[22] PS exposure was initially accelerated in the patient platelets, when compared to control platelets (Figure 4B). As expected, preincubation with the pan caspase inhibitor quinoline-val-asp-difluo-

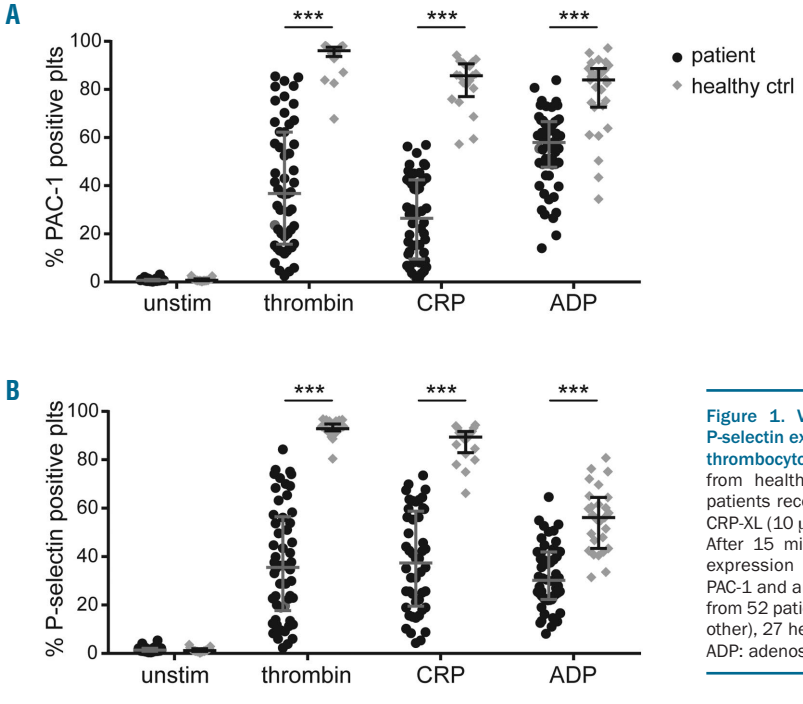

Figure 1. Variable impairment of integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression in stimulated platelets from cancer patients with thrombocytopenia after chemotherapy. Washed platelets (10x10^9/L) from healthy control subjects (healthy ctrl) and thrombocytopenic patients receiving chemotherapy were activated with thrombin (4 nM), CRP-XL (10 μg/mL) or 2MeS-ADP (1 μM) in the presence of 2 mM $CaCl_2$. After 15 min activation, integrin $\alpha_{IIb}\beta_3$ activation (A) and P-selectin expression (B) were measured by flow cytometry using PAC-1 and anti-P-selectin antibody, respectively. Medians with IQR; data from 52 patients (25 AML/ALL, 12 multiple myeloma, 13 lymphoma, two other), 27 healthy controls, ***P<0.001. CRP: collagen-related peptide; ADP: adenosine diphosphate.

rophenoxymethyl ketone (Q-VD-OPh) fully inhibited the PS exposure triggered by ABT-737. However, Q-VD-OPh failed to affect the storage-dependent PS exposure (Figure 4C). Furthermore, whereas ABT-737 stimulation resulted in high caspase-3 activity, no such activity could be detected during storage (Figure 4D). Additional confirmation for the absence of apoptotic signaling was obtained by assessing the caspase-dependent cleavage of the integrin-binding protein, kindlin-3.[23] Western blot analysis indicated that, in platelets from control subjects, ABT-737 treatment induced full cleavage of kindlin-3, which was prevented by Q-VD-OPh (Figure 4E). In the patient platelets (with confirmed functional impairment of integrin activation and P-selectin expression), however, no kindlin-3 cleavage could be detected in the absence of ABT-737.

Platelet activation is known to rely on mitochondrial activity for sufficient ATP production.[24] Given that mitochondrial impairment can lead to PS exposure,[25,26] we assessed the activity of mitochondria in several ways. As part of the initial characterization of the patient platelets, the mitochondrial membrane potential was assessed by staining with TMRE. Whereas control platelets displayed high TMRE fluorescence, the patient platelets showed much less fluorescence intensity (Figure 5A). This suggest-

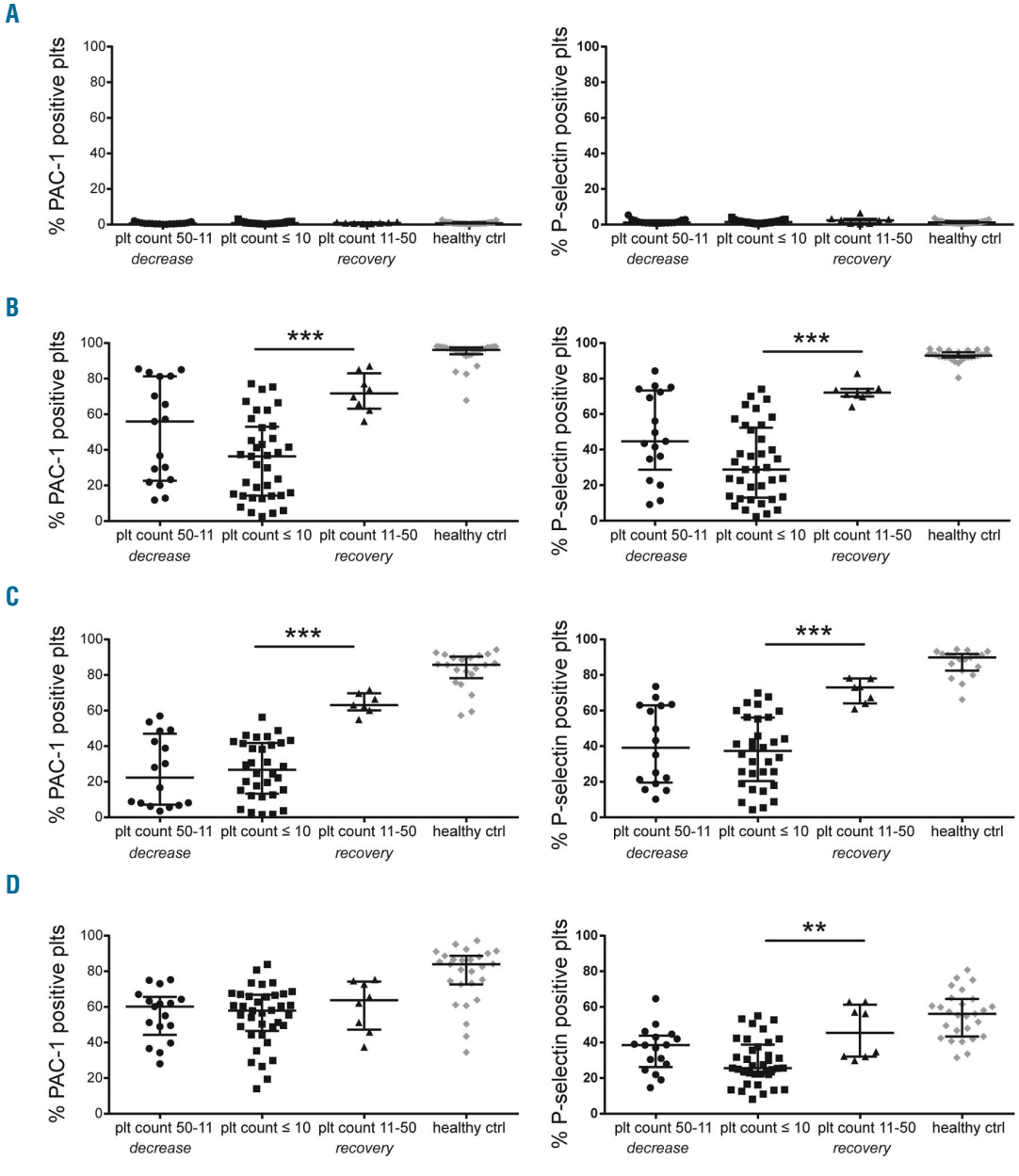

Figure 2. Impaired platelet responsiveness in relation to phase of treatment and/or recovery. Platelet integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression were measured (see Figure 1). Patients (n=52) were divided into two categories: (i) decreasing platelet count 50-11 x10^9/L (n=15) and (ii) decreasing platelet count ≤10 x10^9/L (n=37). Furthermore, from a subset of patients a sample could be collected when the platelet count increased independently of platelet transfusion (iii): 11-50 x10^9/L (n=8). Data are expressed as % of platelets positive for PAC-1 or anti-P-selectin staining in the absence of stimulation (A), or after stimulation with thrombin (B), CRP-XL (C) or 2MeS-ADP (D). Medians with IQR for patients and healthy controls (n=27); $^{**}P<0.01$ and $^{***}P<0.001$. plt: platelet.

ed a depolarization of the platelet mitochondria, which was independent of diagnosis or treatment class (Kruskal-Wallis H test, P=0.656 and P=0.126, respectively). The low TMRE fluorescence correlated well with the reduced platelet responsiveness (Spearman's rho=0.569, P=0.001). However, cyclosporin A-induced inhibition of mitochondrial permeability pore formation did not affect PS exposure (*data not shown*).

We subsequently assessed platelet mitochondrial activity by measuring mitochondrial respiration *via* high-resolution respirometry.[27] With saturating amounts of complex I-II substrates of the oxidative phosphorylation (OXPHOS) chain, i.e., pyruvate, malate, ADP, glutamate and succinate, the maximal ADP-supported respiration of mitochondria was significantly lower in platelets from patients than from controls (Figure 5B). To exclude that the mitochondrial content was altered, we measured the citrate synthase activity.[28] However, this was unchanged in the patients'

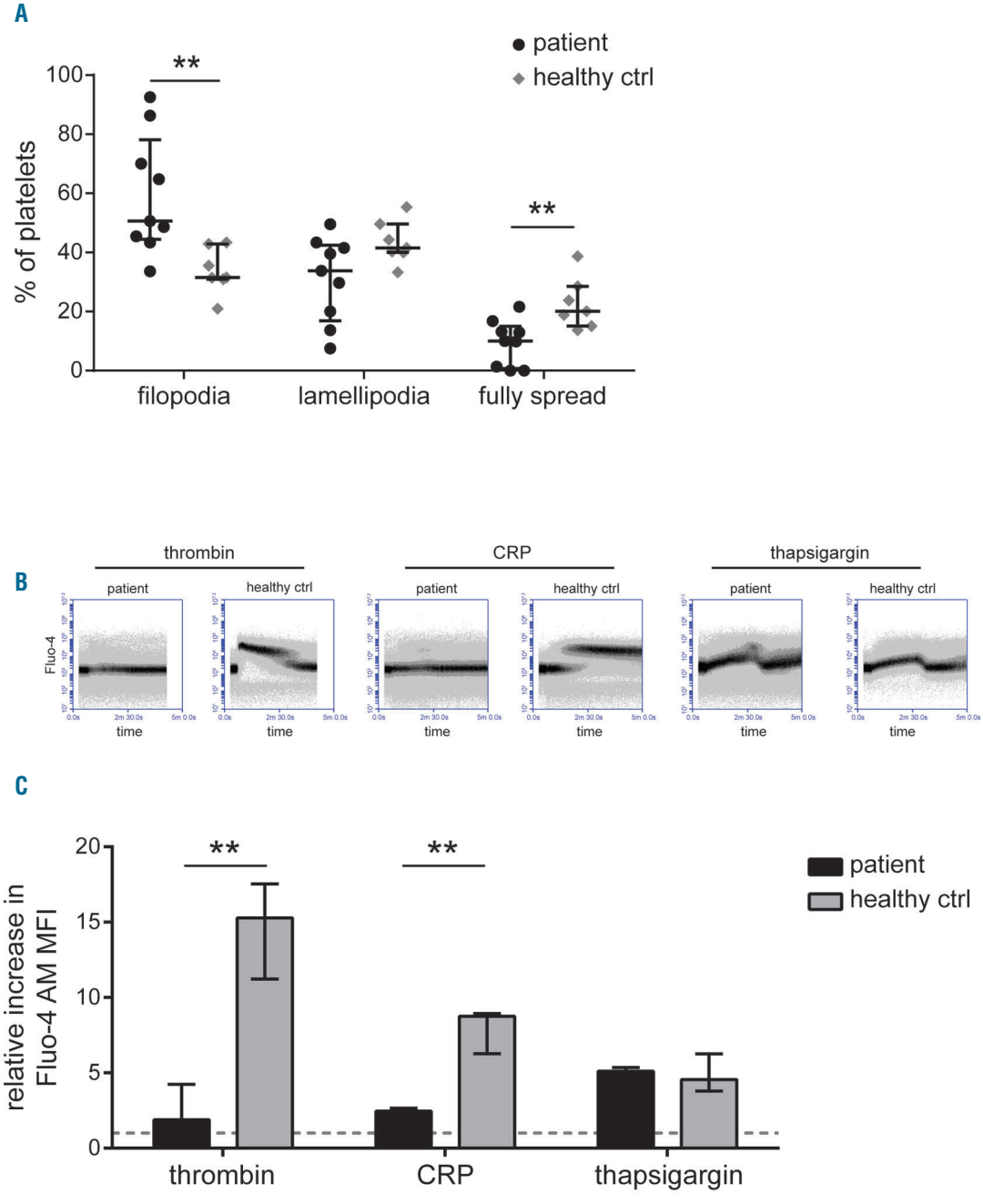

Figure 3. Impaired platelet spreading and Ca^{2+} signaling of platelets from patients. (A) Platelets from patients or healthy controls were allowed to spread on a fibrinogen surface for 10 min, after which microscopic images were captured. Spreading state per platelet was classified in three stages based on morphology: (i) filopodia, (ii) lamellipodia, or (iii) fully spread. Percentages of platelets per category are shown. Medians (with IQR) for nine patients, seven control subjects. (B, C) Fluo-4-loaded platelets from patients (n=7) and controls (n=5) were stimulated with thrombin (4 nM), CRP-XL (10 μg/mL) or thapsigargin (0.5 μM) in the presence of 2 mM $CaCl_2$. Changes in Fluo-4 fluorescence were measured in time by flow cytometry. (B) Representative Fluo-4 traces in time. (C) Relative increases in cytosolic Ca^{2+}. Medians with IQR, $^{**}P$<0.01. Overall platelet responsiveness of the patients was 31.5 – 57.9% (IQR). CRP: collagen-related peptide.

platelets (Figure 5C). Transmission electron microscopic images were also recorded, and these did not reveal structural abnormalities of the mitochondria (*data not shown*).

Chemotherapeutics like anthracycline analogues can cause (cardio)myopathy and neuropathy by inducing mitochondrial damage, a process mediated by oxidative stress.[29,30] To determine whether a similar process is operative in the platelet lineage, activation markers, mitochondrial function (TMRE) and reactive oxygen species (ROS) levels were measured in platelets from seven patients prior to chemotherapy, and two days after said chemotherapy. Additional blood samples were analyzed when severe thrombocytopenia occurred (median ten days after last treatment; median count 11x10^9/L). Before the start of chemotherapy, platelet reactivity in these patients was comparable to that of healthy controls (Figure 6A,B). After two days of therapy, the platelet count was slighter lowered (median decrease: 15x10^9/L, IQR: 12.5-24.5), but platelet reactivity was not significantly changed. In contrast, reactivity in response to all agonists decreased markedly when the patients became thrombocytopenic. Similarly, TMRE fluorescence only decreased in the latter case (Figure 6C), which only then was accompanied by a higher ROS production (Figure 6D). The reduction in TMRE fluorescence correlated with the production of ROS (Spearman's rho=-0.459, *P*=0.012). Treatment of control platelets *in vitro* with chemotherapeutics affected neither the mitochondrial membrane potential nor the production of ROS (*Online Supplementary Figure S3C,D*). Together, these results strongly suggest that mitochondrial dysfunction is not caused by a direct effect of chemotherapeutics on platelets, but by affecting the platelet precursor cells, the megakaryocytes.

Discussion

In this paper, we provide novel evidence that the platelets from thrombocytopenic patients suffering from hematological malignancies and treated with myeloablative chemotherapy are dysfunctional in multiple aspects. We found that key agonist-induced responses of the patients' platelets, such as integrin activation, secretion and Ca^{2+} fluxes are impaired, at a remarkably variable extent. Furthermore, the platelets from almost all patients showed agonist-independent exposure of PS upon storage, which was not linked to apoptotic caspase activity, in contrast to the platelets from healthy subjects which did not display PS exposure. In the patients' platelets, the defective activation could be linked to an impaired mitochondrial membrane potential and a decreased mitochondrial respiratory activity.

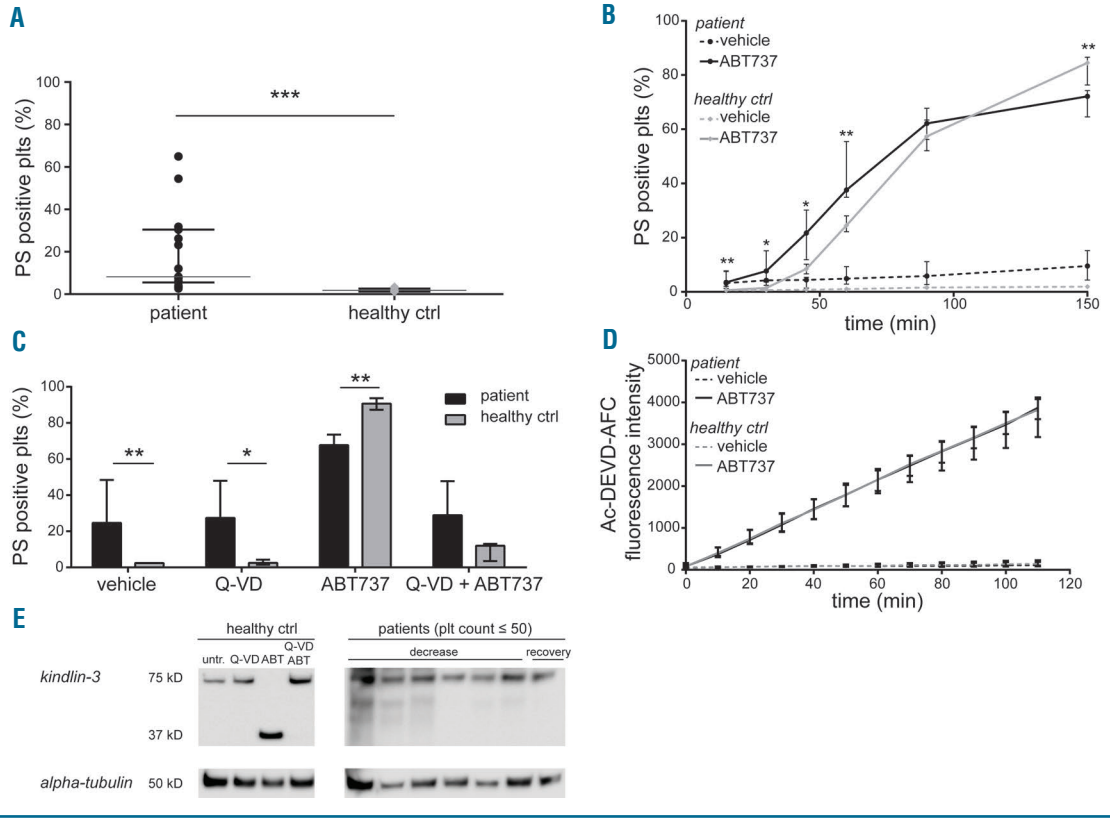

Figure 4. Increased PS exposure in platelets from patients receiving chemotherapy in the absence of apoptosis. Isolated platelets from patients and controls were incubated at 37 °C for 90 min, and stained for PS exposure with FITC-annexin A5. (A) Percentages of PS-exposing platelets, (patients n=15, controls n=12). (B) PS exposure measured after indicated times with vehicle or 5 μM ABT-737 to induce apoptosis, (n=6-9). Platelets (10x10^9/L) from patients or controls were pretreated with caspase inhibitor Q-VD-OPh (10 μM), as indicated, and then stimulated with ABT-737 (5 μM) or vehicle. (C) Fractions of platelets with PS exposure, measured with FITC-annexin A5, (n=8). (D) Caspase-3 activity determined with a fluorometric assay, (n=4). (E) Absence of caspase-dependent kindlin-3 cleavage in western blots from patient platelets. Control platelets were stimulated with ABT-737 with(out) Q-VD-OPh pretreatment; patient platelets were analyzed during the decreasing and recovery phases of platelet count, (n=7). Overall platelet responsiveness of the patients was 30.5 – 48.4% (IQR). Medians with IQR, **P*<0.05, ***P*<0.01 and ****P*<0.001. plt: platelets.

The impaired platelet responsiveness after myeloablative chemotherapy (median of eight days) was only weakly correlated to the whole blood platelet count, thus indicating that the extent of thrombocytopenia was not a main factor in the dysfunction. In agreement with this conclusion, in patients with a recovering platelet count after transplantation, the functionality of the platelets was enhanced. Detailed analysis indicated that neither disease type nor chemotherapy regimen could explain the inter-patient variation in platelet responsiveness. This points to other factors determining the severity of dysfunction, such as a different sensitivity of megakaryocytes in the bone marrow to the previous chemotherapy treatment. As the sensitivity of megakaryocytic precursor cells to chemotherapeutics is known to vary,[31] the extent of platelet dysfunction might be a combined result of the sensitivity of the precise drugs administered and their dosage.

The dysfunction of platelets identified in this patient group differs markedly from the so-called 'exhausted' platelets, which have been described for patients with solid tumors.[32] Exhausted platelets were characterized by a high integrin activation and P-selectin expression in the absence of stimulating agents, and a reduced increase in the parameters after agonist stimulation. These changes might point to platelet activation *in vivo*, resulting in a sec-

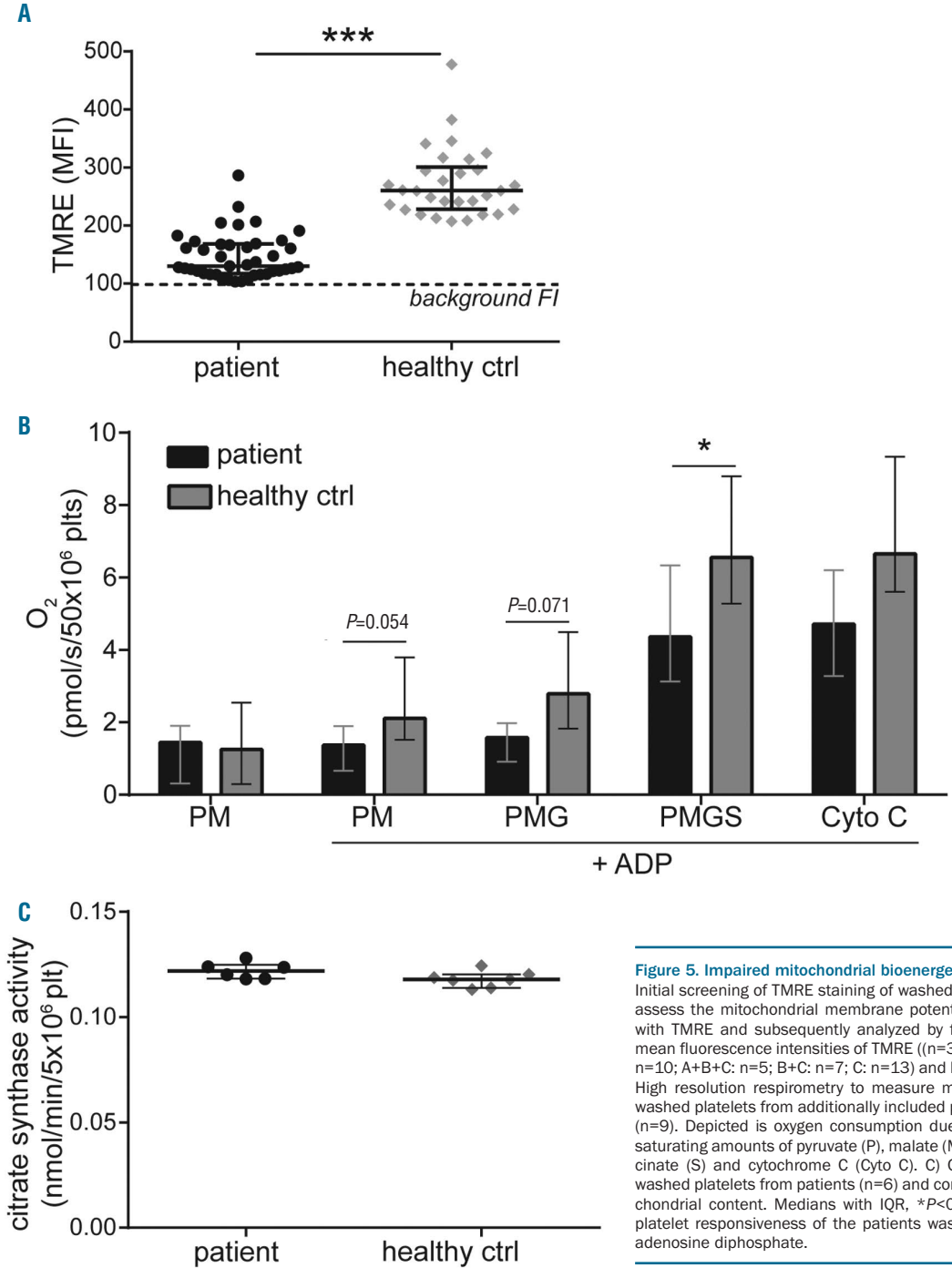

Figure 5. Impaired mitochondrial bioenergetics in patient platelets. A) Initial screening of TMRE staining of washed platelets from patients. To assess the mitochondrial membrane potential, platelets were stained with TMRE and subsequently analyzed by flow cytometry. Shown are mean fluorescence intensities of TMRE ((n=39: treatment classes: A+B: n=10; A+B+C: n=5; B+C: n=7; C: n=13) and healthy controls (n=27)). B) High resolution respirometry to measure mitochondrial respiration in washed platelets from additionally included patients (n=7) and controls (n=9). Depicted is oxygen consumption due to sequential addition of saturating amounts of pyruvate (P), malate (M), ADP, glutamate (G), succinate (S) and cytochrome C (Cyto C). C) Citrate synthase activity in washed platelets from patients (n=6) and controls (n=7) to assess mitochondrial content. Medians with IQR, *P<0.05, ***P<0.001. Overall platelet responsiveness of the patients was 28.7– 46.9% (IQR). ADP: adenosine diphosphate.

ondary loss of function.[33] Given that in the present patient group P-selectin expression and integrin activation were low without stimulation, there is no evidence for *in vivo* platelet activation linked to chemotherapy treatment. On the other hand, the patients' platelets showed a tendency to expose PS, which is compatible with an apoptotic process, as apoptotic platelets are known to be defective in aggregation and secretion.[22] However, ongoing apoptotic signaling could be excluded, since: (i) treatment with the pancaspase inhibitor Q-VD-OPh did not prevent PS exposure, (ii) measurable caspase-3 activity was absent, and (iii) caspase-dependent cleavage of kindlin-3 could not be detected.

Platelets rely on mitochondrial ATP production, in particular upon activation when their energy demand increases.[24] While the mitochondrial content and ultrastructure appeared normal in the patients' platelets, we noticed a marked reduction of the platelet mitochondrial membrane potential and the mitochondrial oxidative phosphorylation. Other authors have shown that anti-tumor antibiotics (anthracyclines), an important class of chemotherapeutic agents used to treat hematological malignancies, induce cardiotoxicity and muscle weakness due to the impairment of mitochondrial function *via* an increased production of ROS.[29,34,35] In cardiac cells, the accumulation of iron inside the mitochondria may contribute to the production of ROS.[36] Furthermore, the mitochondrial activity in myocardial and hepatic cells is known to be impaired by the chemotherapeutics cyclophosphamide and carmustine (BCNU).[37-39] Our results suggest that a similar mechanism of ROS-linked mitochondrial dysfunction is operative in the platelet precursor cells, as deduced from the strong correlation (at >2 days after treatment) between mitochondrial dysfunction and elevated ROS levels. The fact that platelet activation induced by strong agonists (CRP-XL, thrombin) was more affected than platelet activation by ADP suggests a relatively larger role of mitochondrial ATP production upon stimulation with stronger agonists.[40] The slight decrease in GPVI (and GPIbα) receptor levels might contribute to the lower responsiveness of platelets, although this can also be the consequence of receptor shedding induced by ROS and mitochondrial stress.[41]

Taken together, our findings suggest that ROS-induced dysfunction in the mitochondria (before the production of platelets) impairs platelet activity and induces PS exposure, thus leading to a shortened platelet lifetime. This conclusion is supported by a recent study in mice, developing thrombocytopenia after 5-fluororacil treatment. In these animals, low-level laser therapy was found to increase the mitochondrial activity of megakaryocytes, resulting in a normalization of hemostasis.[42] Another pos-

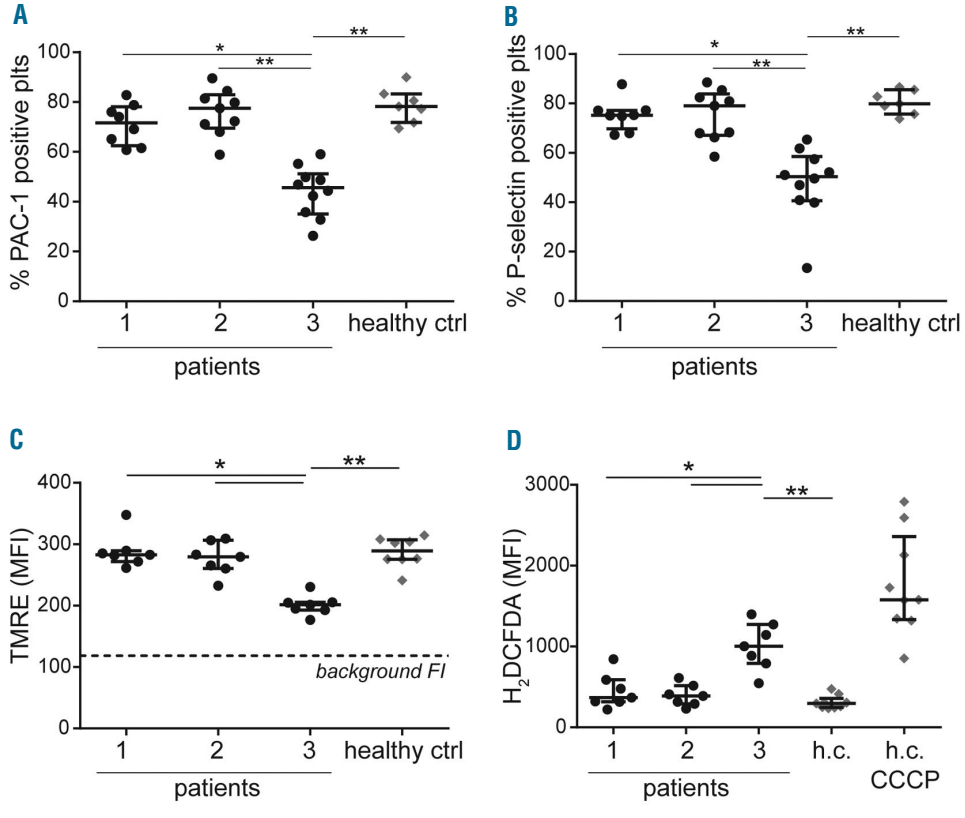

Figure 6. Decreased responsiveness of patient platelets is accompanied by mitochondrial membrane depolarization and ROS production. Platelets ($10x10^9$/L) were isolated from healthy controls (healthy ctrl) and from patients at three time points; namely 1: directly before the start of chemotherapy, 2: at two days of chemotherapy and, 3: upon severe thrombocytopenia (count ≤$50x10^9$/L). Washed platelets were activated with thrombin (4 nM), CRP-XL (10 μg/mL) or 2MeS-ADP (1 μM) in the presence of 2 mM $CaCl_2$. After 15 min activation, integrin $\alpha_{IIb}\beta_3$ activation (A) and P-selectin expression (B) were measured by flow cytometry using labeled PAC-1 and anti-P-selectin antibody, respectively. Depicted is mean platelet responsiveness to thrombin, CRP-XL and ADP. Platelet samples were loaded with TMRE (C) to assess mitochondrial membrane potential, indicative of mitochondrial function, or with H_2DCFDA (D) to measure ROS levels. Platelets from healthy controls were treated with CCCP as a positive control (h.c. CCCP). Medians with interquartile ranges (IQR); n=7-10 (patients) and n=7 (healthy controls), *P<0.05,**P<0.01.

sible strategy to improve mitochondrial function after chemotherapy are the administration of antioxidants to reduce ROS, considering that patients with hematological malignancies have low levels of vitamin C.[30,43] Alternatively, treatment with metformin to improve the mitochondrial energy metabolism could be beneficial.[30]

Due to ethical limitations, we could not assess whether the platelet dysfunction after chemotherapeutic treatment was linked to abnormal (pro)platelet formation from megakaryocytes in the bone marrow. The available literature suggests that the progenitor cells are more vulnerable towards chemotherapy than matured megakaryocytes.[31] In patients who received chemotherapy and had not yet developed thrombocytopenia, we observed a normal platelet activity comparable to that before treatment had started. Furthermore, *in vitro* treatment of whole blood from healthy controls with cytarabine and/or melphalan affected neither platelet reactivity nor mitochondrial function. This agrees with an indirect drug effect *via* the megakaryocytes or precursor cells, rather than a direct effect on the circulating platelets.

With regard to the coagulant state, the reduced level of factor VII found in combination with high circulating D-dimers in the patients' plasmas is suggestive for a mild ongoing state of tissue factor-triggered coagulation.[44] However, the data do not provide evidence for appreciable consumption of other coagulation factors. Given that factor VII has a short half-life in blood,[45] it will be the first coagulation factor to decline upon ongoing coagulation. Chemotherapy can induce endothelial cell activation and upregulate tissue factor levels,[46] which also can explain the elevated VWF levels in patients. The increased bleeding tendency is most likely the result of the impaired platelet function, without compensation by a higher coagulant activity. Moreover, although the relative number of PS positive platelets is high, given their fast clearance from circulation it is unlikely that this platelet population would significantly compensate for primary hemostasis.

The study herein has several limitations. Given that the number of isolated platelets was limited due to severe thrombocytopenia, only a restricted subset of measurements could be performed per patient blood sample, with the consequence that different patient samples needed to be used for some of the measurements. Furthermore, platelet samples were analyzed from patients with different disease types (AML/ALL, multiple myeloma and malignant lymphoma) after receiving chemotherapy in distinct treatment regimens. Herein, we wish to stress the fact that a reduced platelet function was detected in all patient groups and all therapeutic regimens.

Current guidelines for prophylactic transfusion during myelosuppression are based on platelet count only. Our novel findings indicate that, along with the platelet count, the activity of circulating platelets also needs to be considered for an optimal control of hemostasis. Hence, this work encourages an inclusion of platelet function assays for the prediction of bleeding in this patient group.

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