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Original Article

High Prevalence of Acquired Platelet Secretion Defects in Multiple Myeloma

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Abstract

Thrombocytopenia at admission predicts mortality in multiple myeloma (MM) and might link to disease progression. Although thrombocytopenia is known to be associated with MM, a possible thrombopathy is clinically less known. We conducted a case–control study comparing platelet responses of MM patients to controls via flow cytometry, integrin αIIbβ3 activation and P-selectin exposure, and a bioluminescent assay, ATP release. No difference was found at baseline, but upon platelet stimulation, MM patients had decreased αIIbβ3 activation, partly impaired P-selectin exposure, and reduced δ-granule (ATP) secretion. Aspirin treatment in patients did not account for these diminished platelet responses. In total, 29% of patients had thrombocytopenia, while 60% had decreased αIIbβ3 activation and 67% had reduced platelet secretion capacity. Importantly, as secretion capacity was corrected for platelet count, granule release per platelet was reduced in patients versus controls. Of 6 patients with thrombocytopenia 4 displayed a thrombopathy, while for 15 patients with normal count, 64% had reduced αIIbβ3 activation and 73% had reduced platelet secretion capacity. Of all patients, 10% had thrombocytopenia combined with reduced αIIbβ3 activation plus low secretion capacity (one patient showed no qualitative or quantitative platelet defect). Our data suggest that beyond the known thrombocytopenia, MM patients also have reduced platelet function, which could reflect impaired platelet vitality. Combined measurement of platelet count and function, especially secretion capacity, gives a more comprehensive view of platelet phenotype than count alone. Large prospective follow-up studies are needed to confirm the importance of the acquired platelet secretion defect on the prognosis of MM patients.

Keywords
- multiple myeloma
- thrombocytopenia
- platelet secretion

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Introduction

Multiple myeloma (MM) is a malignant proliferation of plasma cells that produce a monoclonal immunoglobulin, M protein (also called paraprotein). Although platelets are not directly involved in the disease pathophysiology, they may give insight into the severity of the disease and the prognosis of the patient.[1–3] Remarkably, low platelet count (thrombocytopenia) at the start of MM is an independent predictor of mortality.[4] Thrombocytopenia is a common complication in MM that may cause serious bleeding, even after minor scrapes, cuts, or bruises.[5] In addition to the increased bleeding risk, a paradox in diseases with thrombocytopenia, in general, is a high incidence of thrombotic complications. In MM, this high thrombosis risk may be associated with disease-related complications or it may be a treatment side-effect.[6] For example, the risk of venous thromboembolism (VTE) increases by thalidomide plus dexamethasone combination therapy in MM patients.[7] Studies on platelet phenotype and function in addition to platelet count are required to further understand the paradoxical relation between thrombocytopenia and bleeding versus high thrombosis risk.

The pathophysiology of thrombocytopenia in MM is largely unknown but might be a result of reduced platelet formation resulting from the infiltration of neoplastic plasmocytes in the bone marrow, which inhibit megakaryocytopoiesis.[8,9] Chemotherapy is another important contributor to thrombocytopenia.[10] Alternatively, thrombocytopenia can be caused by a reduced platelet lifespan due to platelet exhaustion. This platelet exhaustion is a consequence of chronic degranulation of platelets, as observed in clinical conditions with prolonged platelet triggering, for example, in patients with malignancies, cardiopulmonary bypass surgery, disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, or hemolytic uremic syndrome.[11,12] Platelet hyperactivation is another common feature in newly diagnosed MM patients.[13]

While thrombocytopenia is a known side-effect of many malignant disorders including MM, the prevalence of impaired platelet activity in MM is less well described. The overall platelet conditions, such as the activation response to stimuli combined with the granule content per platelet, have not been studied in these patients. We introduce a novel approach to studying the granule release capacity of platelets with a rapid whole-blood platelet granule secretion test in patients with MM. The combination of platelet count and function, specifically the platelet secretion capacity, provides more insight into the platelet phenotype and function than measuring platelet count alone.[14] Low platelet number in combination with impaired platelet granule secretion may indicate a more severe disease state, but the importance of the acquired platelet defect on the prognosis of MM patients remains to be confirmed.

Materials and Methods

Materials

We used the following commercial chemicals: 2-methylthioadenosine-5′-diphosphate (2-MeSADP, Tocris, Abingdon, UK), TRAP-6 peptide SFLLRN (TRAP, H-2936; Bachem, Bubendorf, Germany), and cross-linked collagen-related peptide (CRP-XL, purchased from Professor Farndale, University of Cambridge, UK).[15] Recombinant luciferase was from Synapse B.V. (Maastricht, the Netherlands). Luciferin was purchased from Synchem UG & Co KG (Felsberg, Germany). The monoclonal antibodies used for flow cytometry, that is, APC-conjugated anti-CD42b (GPIb, clone HIP1) to select for platelets, FITC-conjugated PAC1, directed against the activated integrin αIIbβ3, and PE-conjugated anti-P-selectin (CD62P, clone AK4), were purchased from BD Pharmingen (New Jersey, United States).[16]

Patients and Control Subjects

In total, 21 patients who had been diagnosed with MM and were receiving regular therapy (with a stable condition) were recruited at the Meander Medical Center, Amersfoort, the Netherlands. The medication use of MM patients is described in detail in ►**Supplementary Table S1** (available in the online version only) and summarized in ►**Table 1**. Among these patients, 11 (52.4%) received antiplatelet drugs (aspirin); 14 (66.7%) received immunomodulatory imide drugs (IMiDs, including lenalidomide or thalidomide); 5 (23.8%) received proteasome inhibitors (PIs, bortezomib); 4 (19%) received anticoagulants (acenocoumarol (vitamin K antagonist), Xarelto (rivaroxaban: direct factor Xa inhibitor), or fraxiparine (nadroparin: low-molecular-weight heparin); 11 (52.4%) received corticosteroids (dexamethasone, prednisolone or triamcinolone acetonide); and 12 (57.1%) received antibiotics. As a representative control group ($n = 21$), we invited a closely related person (a family member or friend with a comparable age) of each patient to donate blood at the same time and location. Most controls (17 of 21) did not take any oral anticoagulant or antiplatelet drugs for at least 2 weeks. Two controls received aspirin and two controls received vitamin K antagonist at the time of enrollment. For platelet function analysis by flow cytometry, one donor and one patient sample were unavailable. Where indicated, in separate experiments, blood from healthy (nonrelated) donors was used. The study was approved by the Medical Ethical Committee of Maastricht University Medical Center, and patients and volunteers gave full informed consent according to the Helsinki Declaration (2013).

Blood Collection and Cell Count

Peripheral venous blood from patients and controls was collected aseptically by antecubital puncture via a 21-gauge needle into two 3.2% (109 mM) trisodium citrate vacuum tubes and one K2EDTA (7.2 mg) tube (BD Vacutainer System; Greiner Bio-One, Kremsmünster, Austria). Citrate blood was used for whole blood experiments and EDTA blood was used to measure cell count (Cedex HiRes Analyzer, Roche, Basel, Switzerland). All blood samples were kept at room temperature (RT) and used within 4 hours after collection. Age and medication use of patients and controls were registered. Blood of the related control was taken directly before or directly after the blood was taken from the patient in the same setting by the same nurse. Further preanalytical

Table 1 Baseline characteristics of MM patients and controls

	All ($n = 42$)	Patients ($n = 21$)	Controls ($n = 21$)	p-Value
Age, years	67 (53–74)	66 (57–75)	67 (51–73)	0.571
Female, %	43	38	48	0.533
M protein, g/L	N/A	0 (0–5.9)	N/A	
Cell count, median (IQR)				
White blood cell count,[a] 10^9/L	5.8 (4.2–7.2)	4.5 (4.0–6.9)	6.2 (5.3–7.5)	0.03*
Red blood cell count,[a] 10^{12}/L	4.5 (3.9–5)	4.0 (3.4–4.5)	4.8 (4.4–5.4)	0.001***
Hemoglobin, mmol/L	8.8 (7.9–9.4)	8.1 (6.7–9.1)	9 (8.4–9.5)	0.014*
Hematocrit, %	41.9 (38.7–44.9)	39.6 (32.6–44.3)	43 (41–46.3)	0.014*
Immature reticulocyte fraction,[a] %	12.3 (9.7–17.1)	15.9 (12.9–26.7)	10.1 (8.3–11.6)	<0.0001****
Platelet count,[a] 10^9/L	222 (156–285)	178 (76–222)	250 (222–292)	<0.01**
Mean platelet volume, fL	10.1 (9.6–11)	10.3 (9.8–11.4)	9.8 (9.5–10.5)	0.041*
Platelet distribution width, fL	11.2 (10.2–12.7)	11.6 (10–13.6)	10.7 (10.1–11.9)	0.365
Medication, *n* (%)				
Platelet inhibitor	13 (31%)	11 (52.4%)	2 (9.5%)	
Anticoagulants	6 (14.3%)	4 (19%)	2 (9.5%)	
Immunomodulatory imide drugs	14 (33.3%)	14 (66.7%)	0	
Corticosteroid	11 (26.2%)	11 (52.4%)	0	
Proteasome inhibitor	5 (11.9%)	5 (23.8%)	0	
Antibiotics	11 (26.2%)	11 (52.4%)	0	
Laxation	8 (19%)	8 (38.1%)	0	
Gastric acid inhibitor	9 (21.4%)	9 (42.9%)	0	
Calcium + vitamin D3	5 (11.9%)	4 (19%)	1 (4.8%)	
Antivirus	7 (16.7%)	7 (33.3%)	0	
Antihypertension	10 (23.8%)	7 (33.3%)	3 (14.3%)	
Asthma medication	4 (9.5%)	4 (19%)	0	
Uric acid inhibitor	5 (11.9%)	5 (23.8%)	0	
Cholesterol lowering drug	6 (14.3%)	4 (19%)	2 (9.5%)	

Abbreviation: N/A, not applicable.
Notes: Median and interquartile ranges (IQR, 25–75%) are indicated. The chi-square test was used to compare the percentage of males/females between the control and patient groups. Mann–Whitney test was used to compare the results from the patient and control group. The significance level is 0.05.
[a]Measured for 17 of 21 patients and corresponding controls.
*<0.05, **<0.01, ***<0.001, ****<0.0001

handling of the blood tubes, including all laboratory tests, was identical between the patient and control samples.

Platelet Function Analysis by Whole Blood Flow Cytometry

Platelet activation tests for flow cytometric analysis were prepared as published earlier.[16] Whole blood was treated with no agonist (vehicle control), 30 µM TRAP, 5 µg/mL CRP-XL, or 2 µM 2-MeSADP. A flow cytometer (Accuri C6; BD Biosciences, Franklin Lakes, New Jersey, United States) was used to analyze the samples. Platelets were identified based on forward and sideward scatter patterns and CD42b positivity. Median fluorescence intensity (MFI) of 5,000 events per sample was recorded in the FITC gate or PE gate to determine integrin αIIbβ3 activation or P-selectin exposure, respectively.

Platelet Secretion Kinetics

Citrated whole blood was kept at RT and processed between 30 minutes and 4 hours after collection. Whole blood (25 µL) was added to a reaction mixture (25 µL) that consisted of luciferin, luciferase, and $CaCl_2$ at final concentrations of 1.3 mg/mL, 25.4 µg/mL, and 16.7 mM, respectively, and an agonist (2.5 µM TRAP, 5 µg/mL CRP-XL, or 2.5 µM 2-MeSADP) in modified HEPES buffer pH 7.4 (10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$, 0.49 mM $MgCl_2$, 5 mM KCl, 1 mM dithiothreitol, and 5 mg/mL BSA).[17] The luminescent signal was measured every second and monitored for 5 minutes on a Glomax20/20 luminometer (Promega, Madison, Wisconsin, United States). The initiation and potential of platelet secretion were quantified by defining two parameters of the raw luminescence curve (i.e., lag time [LT]), and area under the curve (AUC; supplementary text and

►Supplementary Fig. S1A [available in the online version only]). Another parameter, time to peak (TtP), was quantified from the first derivative curve and reflects the rate of platelet secretion. The peak was the maximum value of the first derivative curve (►Supplementary Fig. S1B [available in the online version only]). To investigate the relationship between platelet count and granule secretion, reconstitution experiments were performed in platelet-rich plasma (PRP) obtained from three healthy donors. Platelet counts were adjusted with platelet-poor plasma to obtain PRP with the indicated platelet counts, and TRAP-stimulated ATP secretion was measured. The response obtained at a platelet count of 200×10^9/L was set at 100% (►Supplementary Fig. S1C [available in the online version only]). The performance of this test was evaluated in the supplementary text and in ►Supplementary Tables S2–S5 (available in the online version only).

Data Analysis

Statistical analyses were performed with SPSS version 25 and graphs were generated using GraphPad Prism software version 8. Continuous variables are expressed as median with interquartile ranges (IQR; 25–75%). All samples were tested for normality using the Shapiro–Wilk test before intergroup comparison. Most of the investigated parameters are not normally distributed, except for platelet count and mean platelet volume. Therefore, the Mann–Whitney *U*-test was used to compare the differences between groups. The chi-square test was used to compare the percentage of males/females between the control and patient groups. All intergroup comparisons were based on the mean rank of each group. Pearson or Spearman correlation was used after testing for normality.

Results

Baseline Characteristics of MM Patients and Controls

In total, 21 MM patients of the Meander Medical Center and 21 controls (partners or friends) were included in this study. Demographic data of this study population together with blood counts and medication use are summarized in ►Table 1. White and red blood cell counts, hemoglobin levels, hematocrit, and platelet count were decreased, and mean platelet volume and immature reticulocyte fraction were increased in patients compared with controls (►Table 1). In total, 29% (6 in 21) of MM patients were defined as thrombocytopenic (platelet count $<100 \times 10^9$/L). M protein level was lower than 10 g/L for most patients (19 of 21), and lower than 15 g/L for all MM patients.

Platelet Activation Phenotype in MM Patients Determined with Flow Cytometry

To study the impact of MM on platelet function, we measured platelet response (i.e., integrin activation and granule secretion to TRAP [30 µM], CRP-XL [5 mg/mL], and 2-MeSADP [2 µM] using flow cytometry (►Table 2). We first established that baseline values (no activation) were not different between patients and controls. TRAP-induced activation of integrin αIIbβ3, quantified by PAC1-binding, was severely reduced in MM patients compared with controls; the median (IQR) MFI was 151 (107–262) versus 467 (272–561) in MM patients and controls, respectively. This equals a 68% decrease in integrin αIIbβ3 activation in patients compared with controls. P-selectin exposure in response to TRAP was also reduced in MM patients, although this difference was 18% less pronounced. In line with these findings, CRP-XL- and 2-MeSADP-induced αIIbβ3 activation was also strongly reduced in MM patients (27 and 40%, respectively) versus controls. For 2-MeSADP- and CRP-XL-induced P-selectin expression, no significant effect was found in MM patients compared with controls (►Table 2).

Additional data analysis demonstrated that integrin αIIbβ3 activation strongly correlated with P-selectin exposure for all agonists used. Furthermore, for the integrin activation, we found strong correlations between the response to the different agonists (Spearman TRAP – CRP-XL, $r = 0.71$, $p = 0.001$; TRAP – 2-MeSADP, $r = 0.56$, $p > 0.001$ and CRP-XL – 2-MeSADP, $r = 0.37$, $p = 0.0197$).

Table 2 Agonist-induced platelet activation in MM patients and controls

	Patients ($n = 20$)	Controls ($n = 20$)	*p*-Value
	Median (IQR)	Median (IQR)	
αIIbβ3 activation (MFI)			
Baseline	105 (92–149)	115 (104–138)	0.192
TRAP	151 (107–262)	467 (272–561)	<0.001***
CRP-XL	3,253 (2510–4,069)	4,429 (3,329–4,840)	0.008**
2-MeSADP	1,363 (986–1,963)	2,254 (1,354–3,239)	0.018*
P-selectin exposure (MFI)			
Baseline	90 (84–112)	89 (86–113)	0.883
TRAP	5,842 (5,434–7,014)	7,124 (6,306–7,897)	0.03*
CRP-XL	6,321 (5,480–7,261)	6,554 (6,085–8,060)	0.242
2-MeSADP	2,314 (1,190–2,586)	2,383 (1,052–5,071)	0.512

Notes: Median and IQR (25–75%) are indicated. Mann–Whitney test was used for comparing the difference between the patient and control group. The significance level is 0.05.
*< 0.05, **<0.01, ***<0.001.

Table 3 Agonist-induced platelet secretion parameters in MM patients and controls

	Patients ($n = 21$)	Controls ($n = 21$)	p-Value
	Median (IQR)	Median (IQR)	
LT(s)			
TRAP	16 (13–18)	14 (11–16)	0.172
CRP-XL	32 (24–41)	31 (28–35)	0.840
2-MeSADP	9 (0–13)	12 (9–14)	0.045
AUC (kRLU*s)			
TRAP	930 (568–2,333)	3,952 (3,172–6,000)	<0.001***
CRP-XL	1,354 (628–1,772)	2,411 (1,645–2,983)	0.001**
2-MeSADP	487 (210–1,217)	1,610 (762–2,198)	0.001**
AUC/PLT (RLU*s)			
TRAP	0.25 (0.16–0.47)	0.66 (0.55–0.93)	<0.001***
CRP-XL	0.28 (0.23–0.35)	0.39 (0.30–0.47)	0.028*
2-MeSADP	0.13 (0.08–0.23)	0.26 (0.13–0.35)	0.05
TtP (s)			
TRAP	36 (25–41)	24 (18–26)	0.001**
CRP-XL	54 (46–63)	63 (44–80)	0.217
2-MeSADP	20 (10–26)	21 (14–29)	0.319

Abbreviations: AUC, area under the curve; kRLU*s, kilo relative light units per second; LT, lag time; PLT, platelet; RLU*s, relative light units per second; TtP, time to peak.
Notes: Median and IQR (25–75%) are indicated. Mann–Whitney test was used for comparing the difference between the patient and control group. The significance level is 0.05.
*< 0.05, **<0.01, ***<0.001

As antiplatelet medication could influence platelet reactivity measured in vitro, we tested whether the decrease in platelet function seen in MM patients could be attributed to the intake of aspirin. In total, 10 patients assessed for integrin activation and P-selectin exposure were treated with aspirin. No difference was found in platelet activation for any of the agonists between the patients with and without aspirin treatment.

The Platelet Secretion Capacity in MM Patients

The platelet secretion capacity was determined by the kinetic ATP release parameters in response to TRAP, CRP-XL, and 2-MeSADP in whole blood of MM patients and controls. AUC in response to all agonists was decreased in patients compared with controls (►**Table 3**). There was a strong correlation between platelet count and total platelet granule release for all the agonists (Spearman $r = 0.78$, $p < 0.001$ for TRAP, $r = 0.74$, $p < 0.001$ for CRP-XL, and $r = 0.73$, $p < 0.001$ for 2-MeSADP), indicating that the platelet number contributed to the total granule release capacity. This is confirmed in ►**Supplementary Fig. 1C** (available in the online version only), which shows that, within the same subject, there is a linear relation between platelet count and granule release (AUC) in a range from 25 to 500×10^9/L platelets. Middle to strong correlation was found between platelet count and total granule release for patients and controls separately (data not shown). As expected, after adjusting for platelet count, mean granule release per platelet (AUC/platelet) was no longer correlated with platelet count (Spearman $r = 0.275$, $p = 0.078$ for TRAP, $r = 0.216$, $p = 0.17$ for CRP-XL, and $r = 0.297$, $p = 0.057$ for 2-MeSADP).

Interestingly, after the AUC correction for platelet count (AUC/platelet), this parameter was still lower in patients compared with controls for TRAP and CRP-XL stimulation (►**Table 3**). This indicates that MM patients have lower granule content per platelet compared with controls in addition to the lower platelet count. When results from this novel assay were compared with the flow cytometry data, TRAP-stimulated AUC/platelet correlated with TRAP-stimulated integrin activation (Spearman $r = 0.5157$, $p > 0.001$), and P-selectin exposure (Spearman $r = 0.3377$, $p = 0.0331$).

To establish that medication use did not affect platelet granule release in our patient cohort, we further categorized patients according to their medication type for the subset of TRAP-induced platelet granule secretion (►**Fig. 1**). No significant effects were found in the AUC (either or not corrected for platelet count) by the use of platelet inhibitors, anticoagulants, IMiDs or corticosteroids in MM patients. As mentioned earlier, especially the combined therapy of thalidomide (IMiD) plus dexamethasone (corticosteroid) for MM patients increases the risk of VTE. Thus, we checked whether the combined therapy of IMiD (thalidomide or lenalidomide) and corticosteroid (dexamethasone or prednisone) affected platelet granule release (AUC/platelet upon TRAP stimulation). No significant difference was found for the platelet granule release in these patients ($n = 5$) compared with patients without this treatment combination

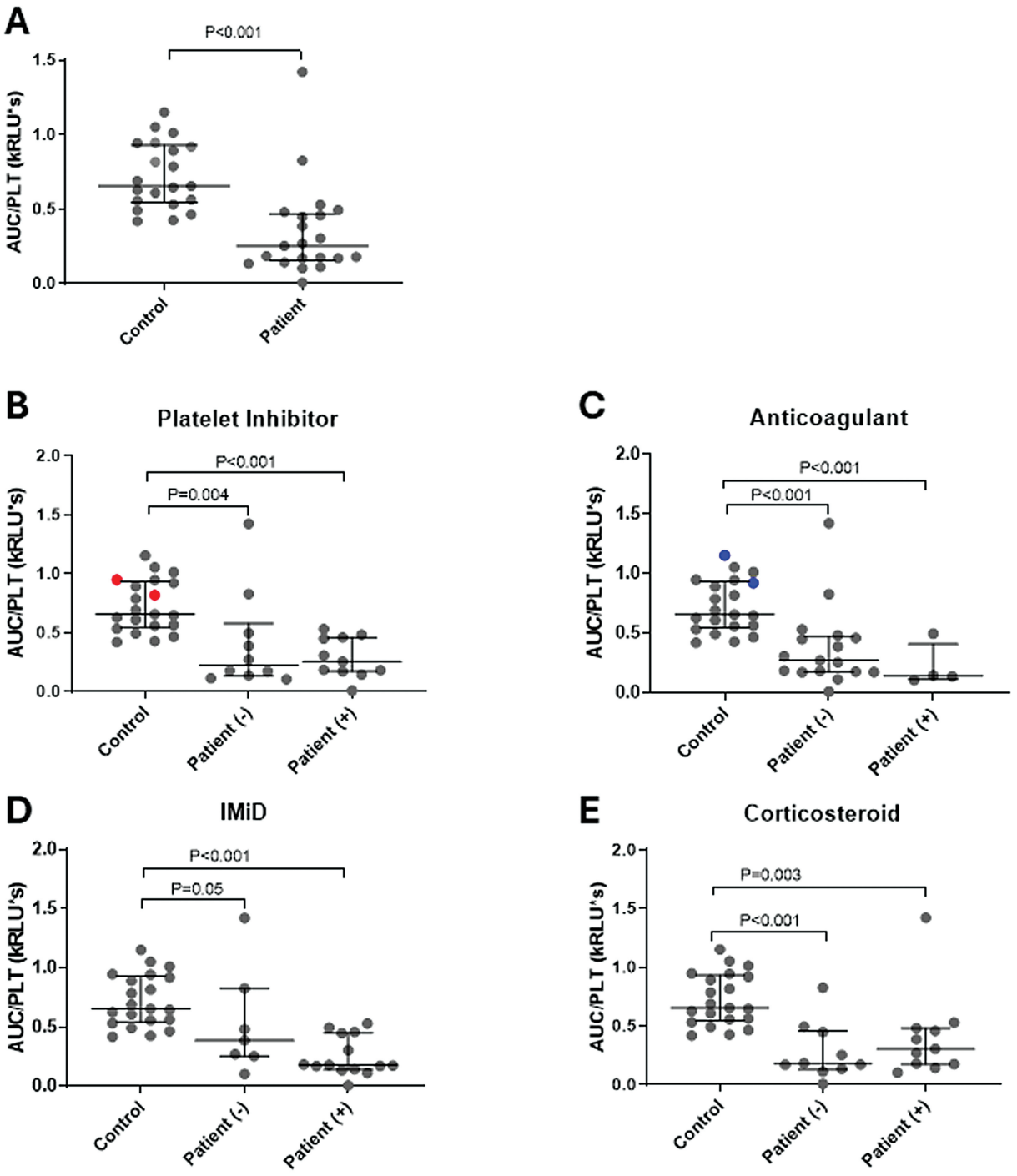

Fig. 1 Medication effect on platelet secretion capacity in response to TRAP. Platelet secretion in response to 2.5 µM TRAP was determined (**A**) for all controls ($n = 21$) and patients ($n = 21$); split for platelet inhibitor (**B**); anticoagulant (**C**); IMiD (**D**); corticosteroid (**E**). The median with IQR range is shown. Red dots: controls ($n = 2$) who received platelet inhibitors; blue dots: controls ($n = 2$) who received anticoagulant therapy. Patient (−): patients who did not take the related drug; patient (+): patients who received the related drug. AUC, area under the curve; IMiDs, immunomodulatory imide drugs; PLT, platelet.

(median [IQR] is 0.31 [0.18–0.50] vs. 0.47 [0.14–0.47], $p = 0.445$). Additionally, we checked for all therapies that were given to at least four patients (as counted from data in ►**Supplementary Table S1** [available in the online version only]) whether there was an effect on the platelet granule release outcome. Only with allopurinol (to reduce uric acid levels in the blood by lowering its production) the AUC/platelet in response to CRP was significantly lower ($p = 0.003$) compared with the patient group without allopurinol treatment (data not shown).

Combined Prevalence of Individuals with Thrombocytopenia and Thrombopathy

To explore whether platelet functional responses gave additional insights into platelet count measurements, we first determined cut-off values to categorize which patients had

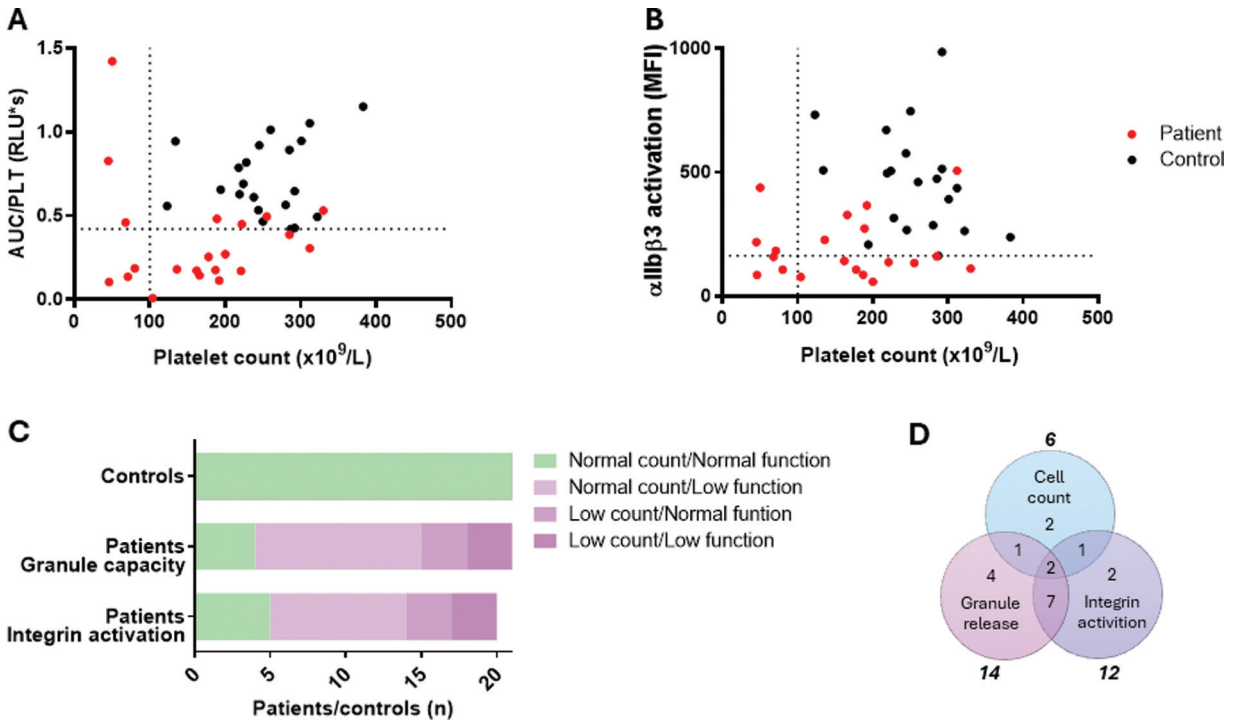

Fig. 2 Subgroups of patients and controls based on mean platelet secretion capacity (AUC/PLT) and platelet count (PLT). Whole blood platelet granule release (**A**, $n = 42$) and integrin activation by PAC1 binding in washed platelets (**B**, $n = 40$) in response to TRAP for all participants were classified into four subgroups according to their platelet count (PLT) and functional response (AUC/PLT or αIIbβ3 activation): upper left—"low PLT/normal function," lower left—"low PLT/low function," lower right—"normal PLT/low function," and upper right—"normal PLT/normal function." Grid line is depicted as the cut-off value for platelet count (100×10^9/L); and for AUC/PLT (0.42 relative light units per second [RLU*s]) or αIIbβ3 activation (163 MFI). Red dots represent patients, black dots represent controls. Absolute counts for the quadrants are presented in (**C**), and overlaps of the platelet traits are shown in the Venn diagram (**D**, the bold number represents the total number of patients with affected trait). AUC, area under the curve; PLT, platelet.

decreased functional responses. Thereto the variables were categorized in quadrants for the subset of TRAP-induced platelet activation. The quadrants formed were for patients with low platelet count and low secretion capacity (low AUC/platelet), low platelet count and normal secretion capacity, normal platelet count and low secretion capacity, or normal platelet count and normal secretion capacity (►Fig. 2A) and similarly for platelet count and αIIbβ3 activation (►Fig. 2B). Cut-off value for low platelet count was the clinical cut-off point of 100×10^9 platelets/L[18–20] and for platelet secretion capacity and αIIbβ3 activation, the 2.5th percentile of the controls (AUC/platelet < 0.42 relative light units per second and MFI < 163, respectively). None of the controls displayed a platelet quantitative or qualitative defect, while 29% (6/21) of MM patients had low platelet count; 67% (14/21) had a low secretion capacity and 60% (12/20) had a low αIIbβ3 activation response to TRAP (►Fig. 2A, B). As mentioned earlier, antiplatelet medication may influence platelet responses in vitro and thus we checked this for the 11 patients on aspirin. We observed a secretion capacity defect in 7/11 patients on aspirin (vs. 7/10 without aspirin) and an integrin activation defect in 6/10 (vs. 6/10 without aspirin).

Further categorization showed that there was only one patient without any disturbance of either platelet function or platelet numbers. We observed that two patients (10%) had thrombocytopenia with reduced αIIbβ3 activation plus low platelet secretion capacity. In addition, of the six patients with thrombocytopenia, four patients also displayed a platelet granule release and/or integrin activation defect (►Fig. 2C, D). Importantly, for patients with a normal platelet count, 9 (64%) had reduced αIIbβ3 activation and 11 (73%) had a reduced platelet granule secretion capacity. Furthermore, we found that for patients without thrombocytopenia and normal integrin αIIbβ3 activation, an additional four patients were found to have a platelet granule release defect, highlighting the added value of the latter assay.

Discussion

Many patients with MM develop acquired thrombocytopenia. We have now demonstrated that MM patients also present with thrombopathy. This was assessed as a decrease compared with control in integrin activation and a selective reduction in P-selectin exposure upon stimulus but was more pronounced as a defect in platelet granule release capacity. We found that MM patients have lower granule content per platelet compared with controls in addition to the lower platelet count. This defect could not be attributed to intake of antiplatelet medication (i.e., aspirin).

We observed a prevalence of thrombocytopenia in 29% (6 in 21) of the stable MM patients, which is higher than a previously described prevalence of 10% in newly diagnosed MM patients.[21] This can partly be explained by chemotherapy or disease progression.[22–24] Furthermore, we showed that MM

patients had a reduced platelet secretion capacity (low ATP release/platelet), which was mainly independent of thrombocytopenia. Platelet ATP secretion capacity reflects the overall content of δ-granule components of platelets, but it could also indicate a signal transduction effect. In addition to the reduced granule content, we show that platelets of MM patients had a reduced αIIbβ3 activation in response to all agonists used and a decreased P-selectin exposure response to PAR1-mediated platelet activation by TRAP, indicating a reduced secondary platelet activation response via ADP release from δ-granules.[25] The observation of impaired integrin αIIbβ3 activation and reduced δ-granule content (ATP) secretion in MM patients implies a reduced vitality of circulating platelets. Some MM patients acquired both a thrombocytopenia and a thrombopathy.

Thrombocytopenia in these patients may be in part explained by disrupted platelet production. Both platelets and red blood cells are formed in the bone marrow from megakaryocytes and reticulocytes, respectively, and this process might be affected by MM and treatment. Although immature platelet fraction (IPF) was not determined for this study, some information relating to this can be obtained from the performed cell count analyses. First, MM patients had decreased red blood cell counts and the immature reticulocyte fraction was shown to be significantly higher in patients compared with controls. We found that MPV, as a proxy marker for immature platelets, was significantly increased in patients compared with controls. This could be indicative of a larger IPF in MM patients compared with control. An increased IPF is furthermore associated with elevated GPVI expression, which could thus be a functional link between platelet maturity and function.[26] Although we have not looked at GPVI expression levels, we did selectively study the effect of GPVI stimulation on platelet activation markers and granule release with GPVI agonist CRP-XL. We found a significant decrease in platelet responses to CRP-XL for αIIbβ3 activation and granule release parameters. Interestingly, although our findings point to an increased IPF, associated with increased platelet responses, in MM patients there is a strong decrease in platelet function.

The primary objective of the current study was to evaluate platelet secretion defects in MM patients. Our data show that low ATP secretion strongly correlates with reduced αIIbβ3 activation after TRAP stimulation, while correlations were much less strong for P-selectin expression. Previous work from our group has shown that the platelet activation response to TRAP is enhanced by secondary platelet activation by autocrine secretion of ADP from dense granules during the activation process. Patients taking P2Y12 inhibitors lack the secondary ADP activation route, which has a major impact on αIIbβ3 activation after TRAP stimulation, while it has minimal impact on P-selectin expression.[27] In other words, our findings may indicate that the secondary platelet activation response of MM patients via autocrine pathway activation is impaired, due to the low-dense granule content of the platelets.

Although there was a correlation between platelet count and total secretion capacity, we also observed MM patients with low platelet count and normal secretion capacity and patients with normal platelet count and impaired secretion capacity. This may be important because the combined phenotype of thrombocytopenia and impaired platelet secretion capacity is suspected to have the highest bleeding risk. Our case–control study does not allow to confirm this hypothesis, but it will be our aim in future studies to follow up on this. The finding that the presence of reduced platelet count and reduced platelet secretion capacity in MM are often independent of each other illustrates the importance of a dual measurement of both platelet number and platelet secretion capacity.[28,29] It is not expected that low platelet count in MM patients is the only explanation for bleeding complications. In general, severe spontaneous bleeding mainly occurs if platelet counts drop below 10×10^9/L,[30–32] which is extremely rare in MM patients.[19] Still, many MM patients show bleeding symptoms with platelet counts of $>100 \times 10^9$/L.[33] Therefore, we recommend testing the combination of platelet number and platelet function to get a better insight into the platelet phenotype. Defects in platelet function can lead to hemorrhagic complications, especially in combination with thrombocytopenia. It has been shown that platelet aggregation measured by light transmission aggregometry (LTA) was reduced in some MM patients with severe life-threatening bleeding.[34,35] This acquired platelet dysfunction has been linked to the elevated M protein in patients, which binds to platelet surface receptors specifically or nonspecifically.[9,28,36–41] We could not study the relation between platelet phenotype and M-protein levels, because all MM patients in our study were treated for high M protein levels. Our finding of a reduced platelet secretion capacity in most MM patients is in line with a previous study, evaluating whole blood platelet aggregation and secretion simultaneously.[33] It has been shown that MM disease progression is characterized by platelet hyporeactivity, as patients with active MM show reduced aggregation and P-selectin exposure compared with both monoclonal gammopathy of undetermined significance patients and MM patients posttreatment.[42] Furthermore, a study by Baaten et al[43] suggested that platelet mitochondrial dysfunction might be an important reason for the impaired platelet activity during chemotherapy. On the other hand, increased platelet activation is a common characteristic of newly diagnosed MM patients.[13,44] As mentioned, in addition to the increased bleeding risk, a paradox in diseases with thrombocytopenia in general is a high incidence of thrombotic complications, which may be associated with disease-related complications or treatment.

As many treatments can affect platelet count and function,[24,45] we analyzed the effect of medication hereon. However, because of the limitation of a small sample size per treatment group, we could mainly assess single medications and did not find evidence that medication use could explain the difference between MM patients and controls. Additionally, as especially the combined therapy of thalidomide (IMiD) plus dexamethasone (corticosteroid) for MM patients increases the risk of VTE, we also checked whether the combined therapy of IMiD and corticosteroid affected

platelet granule release, but found no significant effect. We specifically tested whether aspirin, as an antiplatelet agent, attributed to the in vitro decreased platelet functional responses. For the thrombopathy assessed by integrin αIIbβ3 activation and δ-granule release capacity, aspirin intake was not associated. It should be noted that our assay focuses on primary platelet activation and is not intrinsically dependent on the secondary response of thromboxane A2.

Our study inevitably also has some limitations. The total number of 21 patients and 21 controls were adequate to distinguish the variation in platelet release capacity between cases and controls. However, these numbers do not allow an in-depth subgroup analysis to link reduced platelet vitality to multiple medication therapies within the patient cohort. Furthermore, the case–control design makes a clear distinction between cases and controls, but it cannot be used for the prediction of the disease outcome in MM patients, because we do not have follow-up data. Based on the different platelet phenotypes of patients, it would be interesting to determine the association of platelet secretion defects with thrombotic or bleeding events or prognosis in future research. Our observation that integrin αIIbβ3 activation is lower in MM patients than in controls may be influenced by integrin closure. This closure of integrin αIIbβ3 is mainly observed in highly activated platelets, with high intracellular calcium levels, and is accompanied by phosphatidylserine exposure on the cell membrane.[46] However, we do not expect a large impact of αIIbβ3 closure on our results, because single agonists to trigger αIIbβ3 activation do not induce αIIbβ3 closure.[46] Furthermore, integrin αIIbβ3 activation can also be a reversible process. Our group recently demonstrated that this reversibility depends on the receptor and signaling path involved. Most versatile receptors in terms of transiency appeared to be PAR1 (triggered by TRAP6), ADP (by P2Y12), and GPVI (by CRP), compared with PAR4.[47] This will intrinsically affect the integrin activation measurements. For the granule release capacity assay with the high sensitivity for the primary platelet response, we perceive it to be optimal for clinical use to test for a possible thrombopathy.

A major strength of our study is the patient and control recruitment. We invited the partner or a close friend of each patient as a control, to ensure an equal age and gender distribution between patients and controls. Furthermore, patients and their related controls were invited together, to donate blood in the same room by the same technician, with a maximum of 5-minute time difference. This strategy guarantees an identical (pre-)conditioning of the blood during the whole process from blood collection to final data analysis. Another benefit is that testing of platelet secretion capacity, as used in the present study, requires maximally 100 µL whole blood, which is more efficient and requires less preanalytical steps than LTA (which requires larger blood volumes for the preparation of PRP). Consistent with a recently published whole blood ATP release test,[48] our test also showed robust sensitivity to the thrombocytopenic samples from some MM patients.

For a better understanding of the progression of the disease state (and treatment) with respect to platelet function, it would be beneficial to measure platelet responses in these patients before the start of treatment and during treatment. This would allow us to further determine the platelet function defect in MM disease and their reciprocal influence. A much larger patient group should be assessed to have large enough subgroups that allow in-depth analysis of the different stages of the disease, including accompanying treatments. Furthermore, we consider the publication of our pilot data on thrombopathy in MM patients a crucial step to a large-scale prospective cohort study. We now describe an exploratory study where we show a thrombopathy in stable MM patients alongside thrombopenia, which we could sensitively detect with a whole blood granule release assay. We consider this assay highly suited for this purpose since platelet secretion is of critical importance for (secondarily) regulating not only platelet functions but also other cells. To determine the association between these results on platelet function and clinical outcomes, a larger patient cohort would be required, which is beyond the scope of this article.

In summary, our data show that most MM patients have a thrombopathy (i.e., reduced platelet secretion capacity), which reflects impaired platelet function. Since platelet secretion is of critical importance for (secondarily) regulating not only platelet functions but also other cells, we have developed and included a novel assay to sensitively measure platelet granule release. We compared the outcomes of our test to the integrin activation response as a key step in the platelet functional response. Reduced platelet granule release was associated with reduced secondary activation of platelets, as shown by the reduced αIIbβ3 activation. The thrombopathy was neither related to the thrombocytopenia nor the antiplatelet therapy. The combined measurement of platelet number and platelet secretion capacity may give a more complete view of platelet phenotype than platelet number alone. Our study does not allow drawing firm conclusions about the importance of a reduced platelet storage pool on the prognosis of MM patients, but it opens the door for large prospective follow-up studies to follow MM patients from diagnosis to disease outcome on platelet number and platelet condition.

Authors' Contributions

J.K. and M.R. designed the research; R.F. recruited the patients; Y.S., L.L., and M.R. performed experiments and collected the data; S.J.A.K. provided the reaction kits for flow cytometric analysis; Y.S., D.H., F.S., and M.R. performed data analysis; Y.S. and M.R. wrote the original manuscript; Y.S., M.R., J.A.R., R.F., K.S., S.J.A.K., R.T.U., D.H., F.S., J.K., L.L., and B.d-L. revised the manuscript; J.K., M.R., and J.A.R. supervised the study.

Conflicts of Interest

Y.S. and L.L. reported grants from the China Scholarship Council (file no. 201606790009 and file no. 201706230245) during the conduct of the study.

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