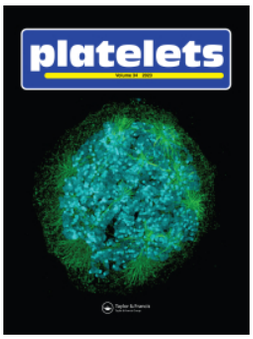

Platelets

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Multiple myeloma and its treatment contribute to increased platelet reactivity

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BRIEF REPORT

Multiple myeloma and its treatment contribute to increased platelet reactivity

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Abstract

Multiple myeloma (MM) and its precursor states, smoldering myeloma (SM) and monoclonal gammopathy of undetermined significance (MGUS) are associated with increased incidence of thrombosis, however the cause of this is unknown. Lenalidomide treatment of MM substantially improves patient survival, although significantly increases thrombotic risk by an unknown mechanism. This pilot study aimed to establish the impact of MM and its treatment with Lenalidomide on platelet function. We analyzed platelet function in MGUS, SM and MM compared to healthy controls. We report an increase in platelet reactivity in MGUS, SM, and MM where increases in fibrinogen binding, P-selectin exposure, altered receptor expression, elevated levels of aggregation and enhanced sensitivity to agonist stimulation were observed. We also demonstrate an increase in patient platelet reactivity post Lenalidomide treatment compared to pre-treatment. We show Lenalidomide treatment of platelets *ex vivo* increased reactivity that was associated with formation of larger thrombi at arterial shear rates but not venous shear rates. This study demonstrates a clear increase in platelet reactivity and prothrombotic potential in patients with MGUS, SM and MM which is elevated further upon treatment with Lenalidomide. Our observations suggest that more detailed studies are warranted to determine mechanisms of thrombotic complications to enable the development of new preventative strategies that specifically target platelets.

Plain Language Summary

What is the context?
Multiple myeloma is associated with increased risk of thrombosis, although the potential role of platelets in this has not been evaluated.
What is new?
We show in this pilot study that multiple myeloma and its precursor states of smoldering myeloma and monoclonal gammopathy of undetermined significance are associated with increased levels of platelet responses. This is further exacerbated by treatment with the immunomodulatory drug lenalidomide.
What is the impact?
This study suggests that more detailed studies are warranted to explore the mechanisms that cause these effects in a larger population of patients, since this may reveal new approaches to prevent myeloma-associated thrombotic complications

Keywords

Haemostasis, IMiD, Lenalidomide, multiple myeloma, platelets, thrombosis

History

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Introduction

Multiple myeloma (MM) is an incurable cancer characterized by malignant proliferation of clonal plasma cells in the bone marrow.[1] MM is preceded by two precursor states, premalignant Monoclonal Gammopathy of Undetermined Significance (MGUS),[2] and smoldering myeloma (SM). MM patients have an increased prothrombotic state[1,3] and coagulation profiles are altered in MGUS, SM and MM patients.[4] Platelets play a key role in driving thrombosis in cardiovascular disease and while aspirin can prevent thrombosis in low-risk MM patients, greater understanding of the impact of MM and its treatment on the

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regulation of platelet function is necessary to develop more effective and safer treatment combinations and reduce rates of thrombosis. The etiology of the increased thrombotic complications is currently unknown.

The introduction of IMiD therapy in MM, including Thalidomide, Lenalidomide and Pomalidomide, has substantially improved patient outcomes.[5] These IMiD treatments, however, further increase the risk of thrombotic complications.[6] Large datasets have been used to develop clinical predictors of venous thromboembolism in IMiD-treated patients, however the cause for this remains unclear.[7]

In this pilot study, we therefore sought to determine whether these conditions, and the treatment of MM are associated with alterations in platelet function.

Methods

Patients with MGUS, SM and MM were recruited prospectively and serially from outpatient clinics at the Oxford University Hospitals and the Royal Berkshire Hospital NHS Trust. Healthy donors were recruited from the Oxford University Hospitals Trust, the Royal Berkshire Hospital NHS Trust and the University of Reading. Ethical approval was granted by the UK National Health Service Research Ethics Committee (Project ID: 163700). Data analysis in this study was performed in GraphPad Prism 8.3.0. Image analysis was performed in FIJI by ImageJ. Statistical analysis was performed on either MFI values from flow cytometry or EC50 values calculated from dose response curves using either one-way or two-way Analysis of Variance (ANOVA) for multiple comparisons or Student's t-test for comparing two variables in thrombus formation studies. All data were confirmed to be normally distributed before statistical analyses. $P < .05$ was considered significant.

Patient exclusion criteria included history of bleeding diatheses, use of anticoagulants prior to study recruitment, use of drugs that interact with anti-coagulants, use of NSAIDs (e.g. aspirin, other than to prevent venous thromboembolism in MM patients), pregnancy or breast feeding, moderate and severe liver disease with coagulopathy, history of active or recently treated malignancy (within the last 3 years) other than myeloma, evidence of alcohol or drug abuse.

Platelet-rich plasma (PRP) was isolated from normal healthy controls ($n = 10$) and patients with MGUS ($n = 3$), SM ($n = 5$), and MM ($n = 13$). In studies comparing disease groups, no patients in any groups were on direct oral anticoagulant or antiplatelet treatment. The MM group was further divided into 2 treatment cohorts; (1) treatment with proteasome inhibitor (PI) and dexamethasone (Dex), ($n = 6$) and (2) treatment with PI, Dex, immunomodulatory drug (IMiD) and direct oral anticoagulant ($n = 7$). Patients with myeloma undergoing treatment had blood samples drawn at baseline, ie. prior to treatment (visit A), 2 weeks after (visit B) and 4 weeks after (visit C) therapy commencement. No patients in this study experienced any thrombotic events on treatment.

Age, platelet number and mean platelet volume (MPV) were not altered between MGUS, SM and MM groups (Table I). Patients treated with proteasome inhibitors and dexamethasone received the following regimens: VCD (bortezomib, cyclophosphamide and Dex), CCD (carfilzomib, cyclophosphamide and Dex), LD (Lenalidomide, Dex) or ILD (Ixazomib, Lenalidomide and Dex). Patients treated with IMiD drugs PIs and Dex received the following regimens: VTE (Bortezomib, Thalidomide, Dex) and LVD (Lenalidomide, bortezomib, Dex).

Results

Flow cytometry was used to measure surface levels of CD61, CD41b, CD9, CD36, CD31, CD49b, GPVI, CD148, CD41a, CD42a, CD29, and CD151 on a minimum of 10 000 events per sample as described previously.[8] The results showed that platelet receptor expression levels were altered in MGUS and MM patients. Changes were observed in levels of select platelet receptors compared to healthy controls (Figure 1A). CD31 (PECAM-1), a negative regulator of function increased by 59% and 52% ($P < .05$) in MGUS and SM patients, respectively, while the activatory oxidized low-density lipoprotein scavenger receptor CD36 was increased 145% ($P < .001$) in MGUS patients. Subtle decreases in CD42a levels in SM and CD29 in SM/MM patients were observed. These changes in receptor levels may influence both activatory and inhibitory aspects of platelet function.

Agonist-stimulated platelet fibrinogen binding (FITC-anti-fibrinogen, Agilent, UK) and P-selectin exposure (PE-Cy5-anti-

Table I. Patient platelet and coagulation characteristics. The table depicts the average age of patients in each disease group as well as platelet and blood coagulation parameters including platelet count, mean platelet volume (MPV), prothrombin time (PT), partial thromboplastin time (APTT), D-dimer levels and thrombin generation lag and peak times. One-way ANOVA was performed on all groups with Dunnett's post-hoc test for multiple comparisons and results were all non-significant compared to normal healthy controls.

	Disease group			
	Control	MGUS	SM	MM
Average age	59 (SD ± 5.6)	73 (SD ± 9.6) ns	66 (SD ± 8.1) ns	68 (SD ± 9.6) ns
Average mean platelet volume (MPV)	9.47 (SD ± 1.05)	9.33 (SD ± 0.55) ns	9.12 (SD ± 1.01) ns	8.67 (SD ± 1.11) ns
Average platelet count	196.1 (SD ± 47.2)	163 (SD ± 30.1) ns	135.8 (SD ± 74.9) ns	185 (SD ± 47.1) ns
Prothrombin time (PT) (s)	10.73 (SD ± 0.67)	10.68 (SD ± 0.55) ns	10.79 (SD ± 0.54) ns	10.78 (SD ± 0.88) ns
partial thromboplastin time (APTT)(s)	25.8 (SD ± 1.94)	26.57 (SD ± 1.88) ns	25.69 (SD ± 2.79) ns	26.57 (SD ± 3.04) ns
D-dimer (ng/ml)	421.4 (SD ± 374.5)	935.1 (SD ± 976.1) ns	503.7 (SD ± 416.8) ns	1366 (SD ± 2070) ns
Thrombin generation (lag time) (min)	3.79 (SD ± 0.6)	3.84 (SD ± 0.72) ns	3.885 (SD ± 1.00) ns	4.354 (SD ± 1.12) ns
Thrombin generation (peak) (nM)	240.1 (SD ± 47.94)	227.7 (SD ± 42.63)	274.4 (SD ± 53.79)	230.6 (SD ± 69.51)

Ns – non-significant vs. normal healthy controls.

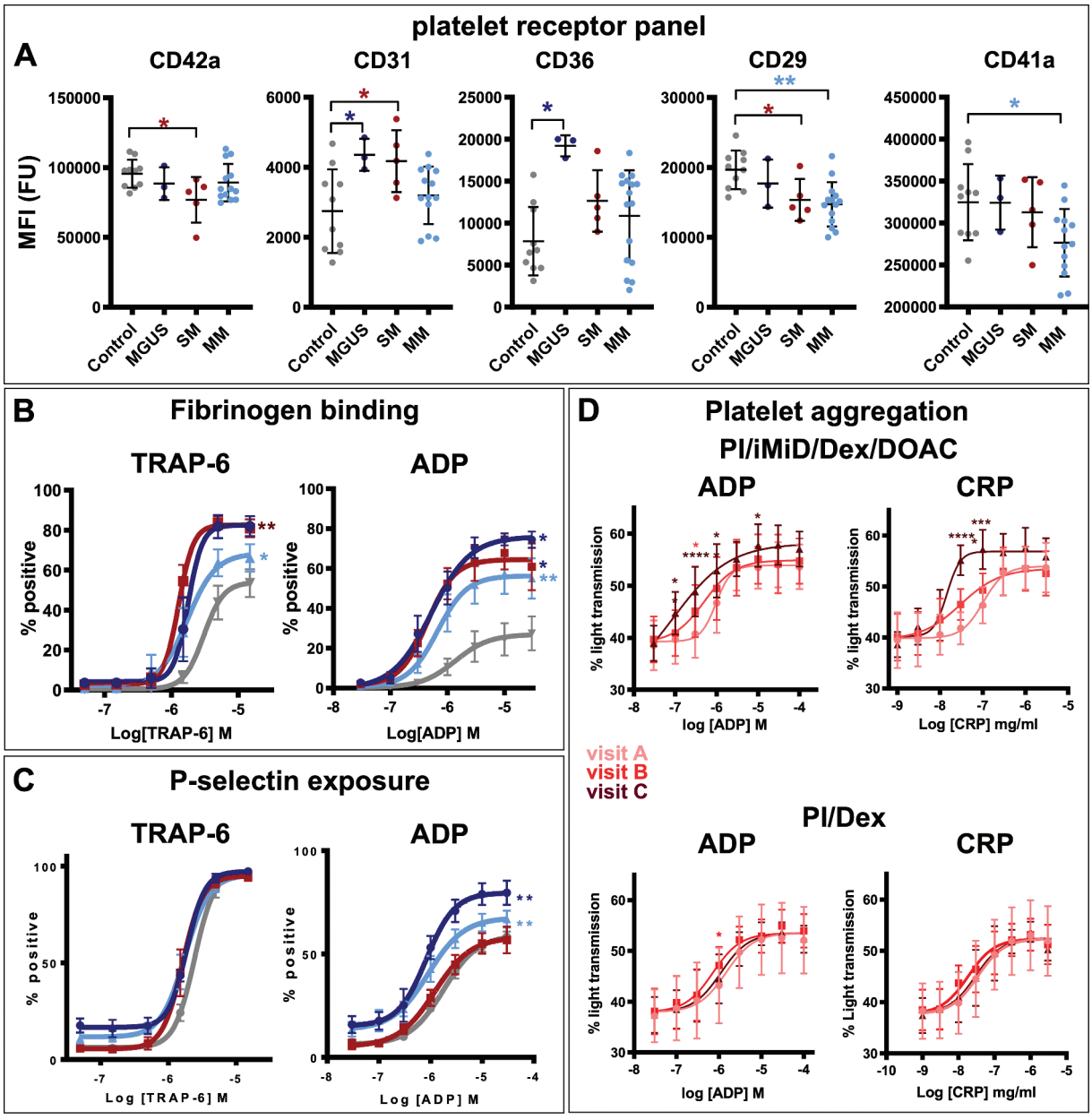

Figure 1. Platelet receptor expression levels are altered in MGUS, SM and MM patients and platelet activation is increased in MM patients before and after IMiD treatment. (A) levels of key platelet receptors (activatory and inhibitory) were monitored between MGUS, SM, MM (not on treatment) and control cohorts. Receptor levels of CD42a (left), CD31 (middle left), CD36 (middle), CD29 (middle-right), and CD41a (right) were altered in various MM cohorts. Data were analysed using one-way ANOVA. *$P < .05$, **$P < .01$, ***$P < .001$ vs. normal controls. (B) dose response curves showing activation of platelets in response to agonists TRAP-6 and ADP by flow cytometry. Data shows % positive events for platelet-bound fibrinogen, used as a measure of up-regulation in affinity of its receptor, integrin αIIbβ3, and (C) P-selectin exposure, a measure of platelet alpha-granule secretion. Data represent mean ± SEM of responses to TRAP-6 and ADP in healthy controls (controls; $n = 10$, grey multiple myeloma (MM (not on treatment); $n = 7$, light blue), smouldering multiple myeloma (SM; $n = 5$, dark red) and MGUS ($n = 3$, dark blue). One-way ANOVA performed on EC50 for each group, stars represent P < .05*, P < .01** for EC50 values. (D) platelet aggregation in response to ADP and CRP-XL was increased in response to lower concentrations of agonists after IMiD treatment but not after PI/Dex treatment alone. Visit A (pink) data from samples taken before treatment, visit B (red) is on treatment and visit C (dark red) is after treatment. Data was compared using two-way ANOVA. *$P < .05$, **$P < .01$, ****$P < .0001$.

CD62P, Becton Dickinson (BD), UK) were also measured in samples containing at least 10 000 events using flow cytometry. The results demonstrate that both parameters were increased in patients in response to ADP and TRAP-6. The EC50 of fibrinogen binding was decreased in MM and SM patients in response to TRAP-6, and ADP in MGUS and MM (Figure 1B) indicating increased sensitivity to agonists compared to healthy controls. The EC50 of exposure of P-selectin on the platelet surface decreased significantly in response to ADP but not TRAP-6 in MGUS and MM compared to healthy donors (Figure 1C).

Plate-based platelet aggregation studies were performed as described previously.[8–10] Platelet aggregation was enhanced in MM patients after IMiD treatment. Platelet aggregation to ADP and collagen-related peptide (CRP-XL) was increased at mid-agonist concentrations after PI/IMiD/Dex/DOAC treatment at visit C compared to visit A, an effect not observed after PI/Dex treatment alone (Figure 1D). These data suggest that IMiD treatment enhances platelet activation levels. Baseline aggregation responses were comparable between PI/IMiD/Dex/DOAC and PI/Dex treatment groups at visit A before treatment.

Given the observed impact of Lenalidomide treatment on platelet activation *ex vivo*, potential direct effects of IMiDs on platelets were explored using healthy donor platelets treated *in vitro* with Lenalidomide or vehicle. Lenalidomide enhanced platelet aggregation (Figure 2A) and P-selectin exposure in response to ADP, TRAP, and CRP-XL (Figure 2B). Thrombus formation under flow was also measured in citrated whole blood using Cellix Vena8 Fluoro+ biochips pre-coated with collagen at shear rates of 200 s^{-1}, 500 s^{-1}, 1000 s^{-1} and 1500 s^{-1} as described previously.[11] The thrombus formation studies demonstrated that exposure of citrated blood to Lenalidomide increased thrombus size ($P < .01$; Figure 2C,D) at arterial (1000 s^{-1}, 1500 s^{-1}) but not venous (200 s^{-1}, 500 s^{-1}) shear rates.

Concentrations of coagulation proteins were also measured in MGUS, SM and MM patients and compared with healthy controls to determine whether the prothrombotic phenotype in these patients is due to altered coagulation. Plasma concentrations of FVIII (Figure 3, top panel), VWF (Figure 3, middle panel) and fibrinogen (Figure 3, bottom panel) were moderately increased in the MM group, compared to healthy controls. Prothrombin time (PT), partial thromboplastin time (APTT), D-dimer and thrombin generation were also measured in these patients; however, no

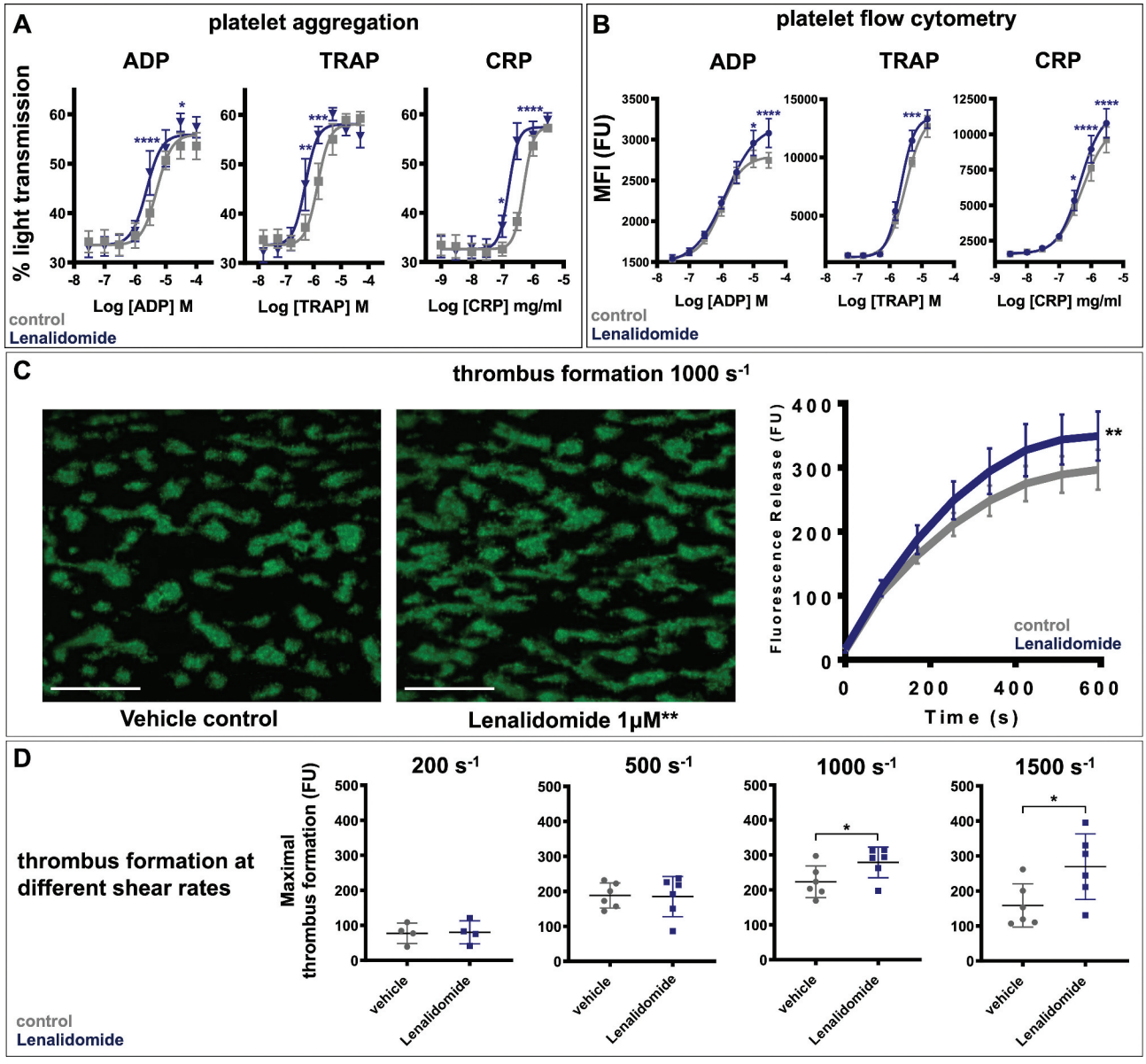

Figure 2. Lenalidomide increases platelet aggregation, degranulation and thrombus formation *in vitro*. (A) platelet aggregation in response to ADP, TRAP and CRP-XL, was increased in response to lower concentrations of agonists after Lenalidomide (1 μM; dark blue) treatment compared to 0.01% DMSO vehicle control (grey). Data compared using two-way ANOVA. *$P < .05$, **$P < .01$, ***$P < .0001$. (B) median fluorescence intensity (MFI) for P-selectin exposure, a measure of platelet alpha-granule secretion. Data represent mean ± SEM of responses to ADP, TRAP and CRP-XL in healthy controls in the presence of Lenalidomide (10 μM; dark blue) or vehicle control (0.01% DMSO; grey). Two-way ANOVA performed on each agonist concentration in vehicle vs. Lenalidomide treated. P < .05*, P < .01**, *$P < .001$, **$P < .0001$. (C) images on left representative of endpoint thrombus formation after 10 minutes in Lenalidomide (1 μM; right image) and vehicle (0.01% DMSO; left image) treated controls. Scale bar represents 100 μm. Graph on right shows Lenalidomide (1 μM; dark blue) treatment enhanced thrombus formation on collagen (100 μg/ml) at arterial shear (1000s^{-1}) compared to vehicle control (0.01% DMSO; grey) ($P < .01$, t-test of maximal thrombus formation FU vs. vehicle control). Data represent mean ± SEM, n = 6. (D) effects of Lenalidomide (1 μM; dark blue) treatment on maximal thrombus formation on collagen (100 μg/ml) after 5 mins at venous shear rates (200s^{-1}, left; 500s^{-1}, middle-left) and arterial shear rates (1000s^{-1}, middle-right; 1500s^{-1}, right) compared to vehicle control (0.01% DMSO; grey). ($P < .05$, t-test of maximal thrombus formation FU vs. vehicle control). Data represent mean ± SEM, n = 4–6.

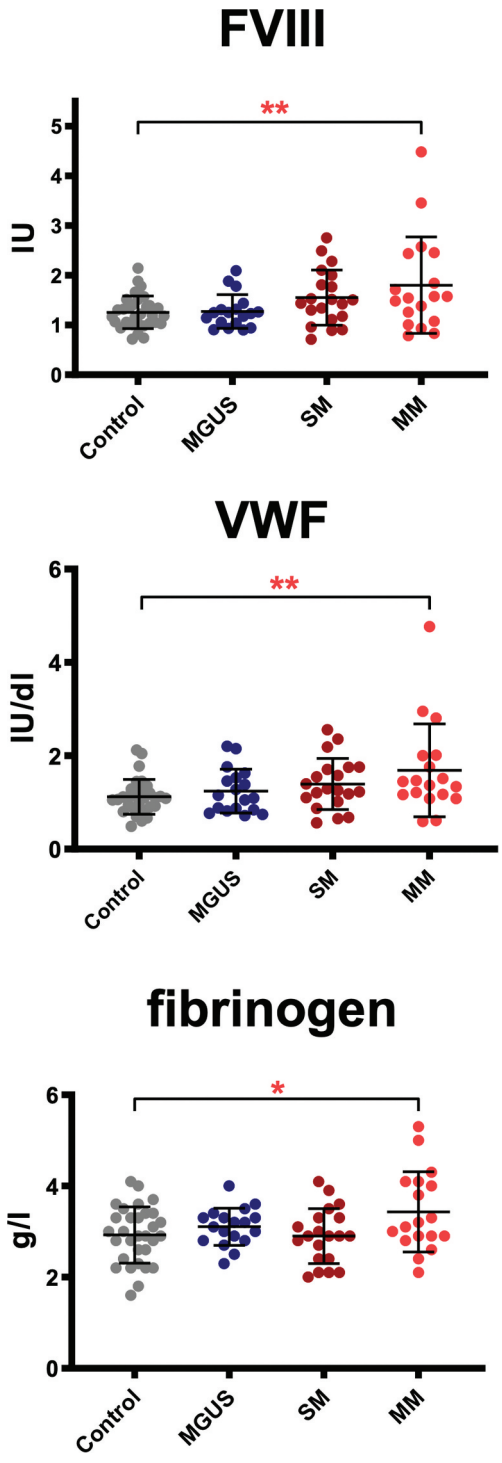

Figure 3. FVIII, VWF and fibrinogen levels are all increased in MM patients. Coagulation parameters were measured in MGUS, SM and MM patients and compared to normal healthy controls (one-way ANOVA in all groups vs. normal controls, $P < .01$**, $P < .05$*).

significant differences between normal healthy controls and disease groups were identified (Table I).

Discussion

Lenalidomide remains a backbone of antimyeloma therapy, however ongoing administration of antiplatelet and anticoagulant agents to target aberrant clotting requires justification and clarity on what escalates thrombotic risk. Patients with multiple myeloma are a difficult group to study due to the ever-changing medications, which stimulated the present study to take a broader look across the variety of medication combinations currently used. The impact of MGUS, SM and MM, and Lenalidomide administration on platelet function and the direct enhancing effects of the drug on platelet function suggest the involvement of more than one mechanism of action in this complex disease. Our data indicate that MM and its treatment with IMiD therapies may represent important and separable factors that contribute to the increased incidence of adverse thrombotic events through platelet activation. While understanding of underlying mechanisms is complicated by the range of medication combinations administered to patients, this pilot study provides compelling evidence that a larger mechanistic study is warranted to determine the basis of these effects. Notably, altered platelet receptor levels throughout the plasma cell dyscrasia spectrum suggests megakaryocyte or platelet biogenesis defects, while direct effects of lenalidomide on platelets enhanced their functional responses.

The primary target of Lenalidomide[12] is Cereblon whose presence has been detected in platelets.[13,14] Lenalidomide binding to Cereblon targets (neo)substrates and promotes their ubiquitination and subsequent degradation. There has been limited research into the specific role of Cereblon in platelets and the effects of IMiD-induced targeting of (neo)substrates on platelet function, however a recent study has successfully used IMiD-based drugs to bind to Cereblon and utilize its ubiquitination machinery to successfully degrade Bruton's tyrosine kinase and reduce platelet reactivity.[15] This demonstrates the ability of Cereblon to degrade platelet signaling molecules suggesting the plausibility that IMiDs recruit Cereblon to degrade intracellular substrates that impact upon platelet reactivity. Indeed, in megakaryocytes, IMiD therapy targets aromatase which reduces proplatelet formation, resulting in thrombocytopenia.[16]

This study reveals the complexity of changes observed in platelets and their function in myeloma and its treatment, even though the sizes of patient groups were small. We show that platelet function, and therefore prothrombotic potential, is elevated in MGUS, SM and MM, and further increased following treatment of MM with the IMiD Lenalidomide. Lenalidomide was also found to exert a direct effect on platelet function. In addition to the enhanced platelet reactivity observed herein, we also show an increase in plasma levels of pro-coagulant factors vWF, FVIII and fibrinogen in MM patients. Together, this increase in pro-coagulant proteins and platelet reactivity may explain enhanced thrombotic risk in these patients. These observations will inform the development of studies to determine underlying mechanisms of action and to explore interventions to limit thrombotic events experienced by MM patients.

Disclosure statement

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Author contributions

J.L.M performed experiments, analyzed data and wrote the manuscript, D.K. performed experiments, analyzed data and wrote the manuscript. R. H.R. designed governance and technical protocols, performed experiments and analyzed data. N.K, A.J.U, T.S and A.P.B analyzed data. M. L., S.S., H.G., A.T., K.R. designed the research and analyzed data, and J. M.G designed the research, analyzed data and wrote the manuscript.

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