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Waldenström Macroglobulinaemia lymphoma patients have impaired platelet and coagulation function

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Abstract:
Clinical features in patients with the B-cell lymphoma, Waldenström Macroglobulinaemia (WM), include cytopenias, IgM-mediated hyperviscosity, fatigue, bleeding and bruising. Therapeutics such as Bruton's tyrosine kinase inhibitors (BTKis) exacerbate bleeding risk. Abnormal haemostasis arising from platelet dysfunction, altered coagulation or vascular impairment have not been investigated in WM patients. To evaluate haemostatic dysfunction in samples from WM patients. Whole blood (WB) samples were collected from 14 WM patients not receiving therapy, 5 patients receiving BTKis and 15 healthy donors (HDs). Platelet receptor levels and reticulation were measured by flow cytometry, plasma thrombin generation {plus minus} platelets by FRET assay, WB clotting potential by rotational thromboelastometry (ROTEM), and plasma soluble glycoprotein VI (sGPVI) and serum thrombopoietin (TPO) by ELISA. Donor platelet spreading, aggregation and ability to accelerate thrombin generation in the presence of WM-derived IgM were assessed. WM platelet receptor levels, responses to physiological agonists and plasma sGPVI were within normal ranges. WM platelets had reduced reticulation (p=0.0012) while serum TPO levels were increased (p=0.0040). WM plasma displayed slower thrombin generation (p=0.0080) and WM platelets contributed less to endogenous thrombin potential (ETP, p=0.0312). HD plasma or platelets incubated with IgM (50-60 mg/mL) displayed reduced spreading (p=0.0002), aggregation (p<0.0001) and ETP (p=0.0081). Alterations to thrombin potential and WB coagulation were detected in WM samples. WM IgM significantly impaired haemostasis in vitro. Platelet and coagulation properties are disturbed in well-managed WM patients.

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Abstract:

Clinical features in patients with the B-cell lymphoma, Waldenström Macroglobulinaemia (WM), include cytopenias, IgM-mediated hyperviscosity, fatigue, bleeding and bruising. Therapeutics such as Bruton's tyrosine kinase inhibitors (BTKis) exacerbate bleeding risk. Abnormal haemostasis arising from platelet dysfunction, altered coagulation or vascular impairment have not been investigated in WM patients. To evaluate haemostatic dysfunction in samples from WM patients. Whole blood (WB) samples were collected from 14 WM patients not receiving therapy, 5 patients receiving BTKis and 15 healthy donors (HDs). Platelet receptor levels and reticulation were measured by flow cytometry, plasma thrombin generation ± platelets by FRET assay, WB clotting potential by rotational thromboelastometry (ROTEM), and plasma soluble glycoprotein VI (sGPVI) and serum thrombopoietin (TPO) by ELISA. Donor platelet spreading, aggregation and ability to accelerate thrombin generation in the presence of WM-derived IgM were assessed. WM platelet receptor levels, responses to physiological agonists and plasma sGPVI were within normal ranges. WM platelets had reduced reticulation ($p=0.0012$) while serum TPO levels were increased ($p=0.0040$). WM plasma displayed slower thrombin generation ($p=0.0080$) and WM platelets contributed less to endogenous thrombin potential (ETP, $p=0.0312$). HD plasma or platelets incubated with IgM (50-60 mg/mL) displayed reduced spreading ($p=0.0002$), aggregation ($p<0.0001$) and ETP ($p=0.0081$). Alterations to thrombin potential and WB coagulation were detected in WM samples. WM IgM significantly impaired haemostasis *in vitro*. Platelet and coagulation properties are disturbed in well-managed WM patients.

Summary points:

- Slower thrombin generation and subdued enhancement by platelets were detected in WM samples.
- WM-derived IgM impaired HD platelet spreading and aggregation, and plasma thrombin generation.
- Measuring platelet properties and coagulation potential may aid in the stratification of bleeding propensity in WM patients.

Introduction

Waldenström Macroglobulinaemia (WM) is a rare incurable low-grade B-cell lymphoma, with lymphoplasmacytic infiltration in the bone marrow (BM) and immunoglobulin (Ig)M paraprotein. WM affects ~3 million people per year,[1] however this estimate inadequately captures asymptomatic WM.[2] Patients can present with symptoms in later stages of the disease including fatigue, recurrent infections, thrombocytopenia, hyperviscosity and bleeding.[3] Platelet counts below 100 x 10^9/L are independently associated with worse progression-free survival in WM patients,[4] and bleeding symptoms are often disproportionate to the observed platelet counts. Whether platelet or coagulation defects, or elevated IgM contribute to bleeding propensity in WM remain important and incompletely resolved questions.[5,6]

Platelets, as primary haemostatic agents, rapidly adhere, activate and spread upon detection of vascular injury or infection via coordinated ligand-mediated activation of glycoprotein (GP) receptor signalling pathways. Platelet degranulation, calcium flux, phosphatidylserine (PS) exposure and thrombin generation ensue, culminating in fibrinogen-αIIbβ3 binding and aggregate formation.[7] GPIbα of the GPIb-IX-V complex binds von Willebrand Factor (VWF) and other vascular ligands,[8,9] while GPVI binds collagen and fibrin.[10] Younger 'reticulated' platelets are more responsive to agonists compared to their older counterparts[11] and contain megakaryocyte (MK)-derived ribonucleic acid (RNA).[12] As platelets circulate, receptor ectodomains are metalloproteolysed and intraplatelet RNA decreases. Platelet surface assembly of coagulation factors and capacity to generate thrombin may diminish.[9,10] Loss of platelet receptors occurs in patients with bleeding diatheses[13-15] and lymphoproliferative diseases[16] and is speculated to be caused by a platelet production defect.[16] Monitoring of platelet and coagulation indices in patients with haematological malignancies may help detect disease progression.[17]

In WM, hyperviscosity is caused by high concentrations of IgM (>50-60 mg/mL) produced by the malignant B cells, which can electrostatically bind and agglutinate RBCs.[18] The subsequent increase in rheological drag can result in physical tearing of capillaries and dysregulated unfolding and proteolysis of high-molecular-weight VWF multimers, resulting in acquired von Willebrand syndrome.[19] Elevated levels of IgM in WM can cause cryoglobulinaemia,[20] amyloidosis,[21] specific functional deficiencies in VWF[22] and FVIII,[23] accelerated clearance of coagulation factors[24] and functional impairment of platelets.[25] In other paraproteinemias such as monoclonal gammopathy of undetermined significance or multiple myeloma, paraproteins have been shown to engage with platelet surface proteins[26,27] plasma proteins[28,29] and coagulation factors[30-32] to perturb platelet aggregation and

coagulation. All of these sequelae may contribute to bleeding phenotypes and are rapidly reversed with plasmapheresis.[33]

Bruton's tyrosine kinase (BTK) is widely expressed in haematopoietic cells and participates in signalling pathways mediating B cell proliferation and platelet function, through activation of nuclear factor-κB.[34] BTK inhibitors (BTKis) ibrutinib, zanubrutinib and acalabrutinib are effective and well-tolerated treatments for B-cell lymphomas,[35,36] helping achieve deep and durable responses.[37,38] BTKis disrupt signalling pathways initiated by the canonical B cell and Toll-like receptors, thus reducing B cell proliferation. Early studies using ibrutinib recorded an attendant risk of bleeding and importantly, noted defective platelet function in B-cell malignancy patients prior to BTKi treatment.[39] Newer BTKis with greater selectivity that target different regions of BTK have demonstrated reduced (but not absent) rates of haemorrhage in patients with leukaemias and lymphomas.[40,41]

The cause of the bleeding phenotype in WM has not yet been explored but is likely to involve several intersecting mechanisms. Here, we present data evaluating coagulation potential and platelet function in samples from WM patients with stable and well-managed disease both on and off treatment, and from healthy donors (HDs) treated with high concentrations of WM-derived IgM *in vitro*. We identify a reduction in thrombin generation capacity and platelet contribution to thrombus formation in WM patients, and significantly reduced platelet spreading and aggregation and plasma thrombin generation in IgM-treated HD samples. Laboratory assessment of platelet function and coagulation in patients prior to receipt of BTKis or other medical interventions may help identify those with strong bleeding potential and stratify patients for bleeding risk.

Materials and Methods

Details of extra materials and methods are included in the Supplementary File.

Patient samples: 18 WM patients were prospectively recruited into this study along with 15 contemporaneously-collected HDs, who were not age- or sex-matched (Table 1). Patients were diagnosed with WM according to the International Workshop on WM guidelines.[35,42] All blood samples were collected after obtaining informed consent, according to protocols approved by the Australian National University (2020/102 and 2022/372) and the Canberra Hospital (2023.LRE.00053) Human Research Ethics Committees. All work was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). 72% (13/18) of WM patients were not on active therapy while 28% (5/18) were

prescribed BTKis (ibrutinib or zanubrutinib, Table 1). Mean characteristics between patients not on therapy and BTKi-treated patients were: 73 vs 74 years of age, 62% (8/13) vs 40% (2/5) male, 23 vs 7 g/L IgM and 43% vs 22% BM infiltration (Table 1). Most patients were heavily pre-treated and possessed a wide range of comorbidities (Table 1), likely requiring simultaneous anticoagulant or anti-platelet therapy (Table 1). Generally, WM patients showed lower levels of RBCs, haemoglobin, WBCs and platelets (Table 1, Figure S4, Figure 1A). No patients had bleeding symptoms at the time of blood collection.

Platelet flow cytometry: Platelet surface proteins GPIbα, GPVI, CD9, αIIb and α2 integrin subunits, a disintegrin and metalloproteinase (ADAM)10, P-selectin and Trem-like transcript (TLT)-1, and estimates of platelet RNA content were measured by flow cytometry using citrated platelet-rich plasma (PRP) or whole blood (WB), as described in the supplementary methods.

Platelet function assays: Resting and agonist-induced αIIbβ3 activation and P-selectin exposure were measured by flow cytometry as described in supplementary methods.

Thrombin generation: A calibrated automated thrombogram protocol[43] was modified for measurement in platelet-poor plasma (PPP) ± washed platelets (WPs). Briefly, duplicate wells of 96-well plates contained 20 µL EDTA- or TSC-anticoagulated PPP, 10 µL TS, 10 µL WPs (10^7, pre-activated with 10 µM thrombin receptor activator peptide (TRAP)-6 at 37°C for 30 min), 10 µL trigger (containing tissue factor and phospholipids), and 10 µL thrombin substrate (Z-Gly-Gly-Arg-AMC, Diagnostica Stago S.A.S, Asnières-sur-Seine, France). Separate standard curves were prepared for each donor using a thrombin calibrator. Fluorescence was measured at 390/460 nm at 37°C for 30 min using an Infinite 200 PRO microplate reader (Tecan, Zürich, Switzerland), with 6 s shaking at 432 rpm between each cycle. Thrombin concentrations were interpolated from the standard curve and transformed to generate thrombograms.

Thromboelastometry: WB coagulation and platelet contributions to thrombus formation were evaluated by rotational thromboelastometry (ROTEM) according to the manufacturer's instructions.

Plasma and serum proteins: Soluble GPVI (sGPVI) ectodomain fragments were quantified by enzyme-linked immunosorbent assay (ELISA).[44] Serum TPO was quantified in a human TPO Quantikine ELISA (R&D Systems, Minneapolis, USA) following the manufacturer's instructions. Concentrations were interpolated from standard curves.

Platelet spreading: Monoclonal IgM was enriched from the plasma of patient WM13 (Supplementary File). WPs (10^7) were incubated with IgM (60 mg/mL) or an equimolar

amount of BSA (4.11 mg/mL) or vehicle (6 mM K_2HPO_4, pH 8.0, 0.006% w/v NaN_3) for 30 min at 37°C and allowed to spread on coverslips for 20-60 min at 37°C, as described.[45] 5 images were taken per coverslip using a SP5 Confocal Microscope (Leica, Wetzlar, Germany) and analysed using ImageJ. Circularity describes how closely the platelet shape approximates a circle, reflecting its degree of surface invaginations, while aspect ratio is calculated as the ratio of the width to the height of the platelet, reflecting its degree of elongation.

Statistical analyses: Data were analysed using GraphPad Prism version 10.1.0. Additional information is included in figure legends and the Supplementary File.

Results

WM platelet production is dysregulated

We assessed a range of platelet parameters in the WM cohort, including the platelet-specific activation marker sGPVI. WM patients displayed reduced mean platelet counts (Figure 1A, Table 1) while only BTKi-treated patients displayed significantly enlarged platelets (Figure 1B, Table 1). Median TPO levels were increased 3.8-fold and 1.7-fold respectively in WM patients not receiving therapy or receiving BTKis compared with HDs (Figure 1C). Flow cytometric analysis of platelet RNA content was evaluated to provide an estimate of circulating platelet age. Samples from WM patients not receiving therapy showed significantly reduced total platelet RNA (Figure 1D) and percentage of TO^{bright} reticulated platelets (Figure 1E) compared with HDs, suggesting that circulating WM platelets were significantly aged. Interestingly, proportions of reticulated platelets in WM patients receiving BTKis were similar to HDs (Figure 1E). In the WM patients not receiving therapy, we observed a mean 43% BM involvement and 23 g/L IgM (Table 1), which are 43% and 15-fold higher than the healthy means respectively, both infiltrate the BM and possibly disrupt the exquisite microenvironment in which the MKs reside to produce platelets.

By flow cytometry, levels of αIIb and α2 integrin subunits, GPIbα, GPVI, ADAM10, P-selectin and TLT-1 were all within ranges observed in HDs (Figure 1F). Plasma sGPVI was also within normal range (Figure 1G). Two WM patients displayed elevated sGPVI levels (>100 ng/mL, Figure 1G), however these levels were not associated with changes to any other markers measured (data not shown). Tetraspanin CD9 was significantly increased on the surface of platelets from WM patients receiving BTKis (Figure 1F).

TPO levels and platelet counts were significantly inversely correlated in HDs (p=0.0368) and in WM patients (p=0.0072, data not shown). There were no other significant correlations between TPO, TO-bound RNA, GPIbα, IgM levels, BM involvement or platelet counts in HDs or WM patients (data not shown). Taken together, the slightly lower platelet counts, elevated TPO and higher proportions of aged platelets in WM patients not receiving therapy suggest dysregulated platelet production in WM.

WM platelets respond normally to platelet agonists

To evaluate WM platelet responsiveness to agonists, αIIbβ3 activation (Figure 2A) and P-selectin exposure (Figure 2C) were measured by flow cytometry. Patient sample volumes collected were insufficient to permit light transmission aggregometry. Levels of active αIIbβ3 (Figure 2B) and P-selectin (Figure 2D) on untreated WM platelets were similar to levels in HD samples. Treatment of platelets from either untreated WM patients or HDs with various doses of cross-linked collagen-related peptide (CRP-XL), TRAP-6 or adenosine diphosphate (ADP) resulted in increased Oregon-green 488 fibrinogen (OgFg) binding and P-selectin exposure, suggesting that WM platelets responded normally in this assay and had normal amounts of protein packaged in their α granules by MKs (Figure 2B, D). As expected, WM patients receiving BTKis showed minimal responses to 0.5-30 μg/mL CRP-XL, and normal responses to all other agonists (Figure 2B, D).

WM patient samples display reduced thrombin generation potential

Platelet aggregation, PT and aPTT generally remain within healthy ranges for well-managed WM patients and our cohort showed no abnormalities in these assays. Plasma thrombin generation studies and platelet contributions to thrombin generation have only been reported in two hyperviscous WM patients[25] and not in well-managed patients. We developed an assay to quantify plasma thrombin generation in WM patients and HDs in the absence or presence of HD platelets using a fluorogenic thrombin substrate, in a modified calibrated automated thrombogram protocol, triggered by phospholipids and tissue factor.[43] Compared with plasma from HDs, plasma from WM patients not receiving therapy displayed significantly prolonged lag times (Figure 3A-B) and times to peak thrombin generation (Figure 3C). The peak thrombin (Figure 3D), thrombin generation rate (Figure 3E) and endogenous thrombin potential (ETP) (Figure 3F) were all markedly reduced, which became significant if WM patients were assessed as a single group (data not shown). Plasmas from 3 patients receiving BTKis showed normal rates and extents of thrombin generation (Figure 3B-F). The observed

differences were not explained by plasma IgM (Figure 3G). Differences between HD and WM plasma were corrected if PAR-1 agonist-activated non-autologous HD platelets were included (Figure 3H). Addition of activated platelets enhanced thrombin generation significantly more in WM plasma compared to HD plasma (data not shown). When comparing the capacity of WM and HD platelets to accelerate thrombin generation, the ETP in WM plasma was significantly reduced in the presence of WM platelets compared to HD platelets (Figure 3M). No significant differences were observed for the ETP in HD plasma (Figure 3M), nor for the lag time (Figure 3I), time to peak thrombin (Figure 3J), peak thrombin (Figure 3K) or velocity (Figure 3L) in HD or WM plasma.

ROTEM reveals a diminution of clotting rates in WM blood

To specifically assess the platelet contribution to WM blood clotting capacity, ROTEM was performed. WM blood displayed normal EXTEM and INTEM values, including clotting time (CT, Figure 4A, D), rate (α angle, Figure 4B, E) and amplitude (A10, Figures 4C, F). However, in FIBTEM where clotting is triggered as for EXTEM, but the platelet contribution is minimised by inclusion of the actin polymerisation inhibitor cytochalasin D, WM samples returned similar CTs to HDs (Figure 4G), but increased α angles (Figure 4H) and A10 (Figure 4I). These data suggest that WM blood exhibited enhanced clotting activity that was not evident in EXTEM. The platelet contribution (PLTEM) can be extrapolated by subtracting FIBTEM amplitude values from EXTEM.[14] However, as the A10 and α angle do not change in a linear fashion and the A10 parameter and platelet count do not have a linear relationship, not all ROTEM parameters can be used to infer platelet contributions.[46] Therefore, we performed a PLTEM maximum clot elasticity (MCE), which is a function of the maximum clot amplitude and has been shown to more accurately express the platelet component to clot strength compared to other PLTEM amplitude measurements.[47] We found no significant difference in PLTEM CT (Figure 4J) or MCE (Figure 4K) between HDs and WM patients. This shows that WM coagulation factors contributed more to clotting rate and amplitude compared to HDs, while WM platelets functioned normally.

High concentrations of patient-derived IgM impair platelet function *in vitro*

To evaluate the effects of hyperviscosity on platelet function, IgM was isolated from a plasmapheresis bag generated as part of treatment of a WM patient and used to assess platelet spreading under hyperviscous experimental conditions, by mixing WM-derived IgM or an

equimolar concentration of BSA with HD plasma or platelets. WM-derived IgM (60 mg/mL) bound to HD platelets (Figure S5). Unfortunately, this could not be compared to similar concentrations of healthy human IgM due to challenges in obtaining sufficient quantities. Therefore, we used equimolar amounts of BSA as the control for future experiments.

HD platelets, pre-incubated with IgM and allowed to adhere to fibrinogen-coated surfaces, displayed reduced numbers of adherent platelets (Figure 5A, 5C), reduced platelet surface area (Figure 5A, 5D) and reduced aspect ratios (Figure 5A, 5F) compared to vehicle and BSA-treated platelets. On collagen-coated surfaces, HD platelets incubated with IgM or BSA displayed reduced adhesion after 60 minutes (Figure 5B, 5G), increased circularity after 20 minutes (Figure 5B, 5I) and increased aspect ratios after 60 minutes compared to vehicle-treated platelets (Figure 5B, J). This indicates that under static conditions, these concentrations of BSA or IgM interfere with platelet adhesion and initial filopodia extension events on collagen. However, only IgM delayed spreading on collagen, resulting in more invaginated and elongated platelets and impaired lamellipodia formation at 60 minutes. We conclude that under static conditions, IgM interferes with rates and extents of platelet adhesion and spreading on substrates, resulting in fewer and smaller platelets with altered membrane projections.

We also assessed agonist-induced platelet aggregation in the presence of IgM. HD platelets preincubated with IgM displayed significantly reduced aggregation responses to collagen and TRAP-6 compared to vehicle- or BSA-treated platelets (Figures 5K-L). In thrombin generation assays, HD plasma mixed with WM IgM in the absence or presence of donor platelets, displayed reduced thrombin generation potential compared to vehicle and/or BSA-treated plasma (Figures 5M, 5O, 5S), but normal lag times (Figures 5N, 5R), peak thrombin (Figures 5P, 5T) and thrombin generation velocity (Figures 5Q, 5U). The observed muted aggregatory and thrombin generation responses when IgM was present may disrupt ligand-receptor interactions because of steric hindrance due to the large molecular weight of IgM relative to BSA (~970 vs 66 kDa). Additionally, IgM may interfere with exposure of PS on the membrane surface which would also reduce thrombin generation.

Discussion

Published data on haemostatic function in WM are lacking, however anecdotally, WM patients often present to clinics with mild but chronic bleeding symptoms, disproportionate to the platelet count or medications.[5,25] WM patients also have an increased risk of venous thrombosis,[48] infection[49] and chronic inflammation,[49,50] which together are consistent with

primary haemostatic failure. BTKis are effective in the long-term treatment of B-cell malignancies,[36] so it is important to understand haemostatic function as BTKis can impact platelet function, particularly in susceptible individuals. Here, we characterised platelet and coagulation function in samples from eighteen WM patients and explored the effects of high concentrations of IgM on haemostasis. In our cohort, of which 72% were not receiving active treatment, we identified substantial differences in WB clotting and thrombin generation assays, as well as impairment of donor platelet function *in vitro* in the presence of high WM-derived IgM concentrations.

Flow cytometric analysis of platelets from WM patients revealed no significant differences in levels of αIIb integrin subunit, GPIbα and GPVI. Plasma sGPVI was also within normal ranges, consistent with levels measured in patients with other BM malignancies.[16] Reduced levels of GPIbα and αIIbβ3 on platelets from chronic lymphocytic leukaemia (CLL) patients have been reported,[51] and reduced levels of GPIbα and GPVI in treatment-naïve patients with CLL and mantle cell lymphoma have been noted.[16,39] Reasons for observed platelet receptor differences between WM and CLL patients remain obscure, and whether the degree of BM involvement impacts MK maturation and thrombopoiesis has not yet been studied. WM platelets responded normally to physiological agonists by upregulating active αIIbβ3 and increasing P-selectin exposure. As expected, GPVI-mediated platelet function was abolished in WM samples from patients receiving BTKis due to on-target effects of the BTKi.[52]

We observed significant increases in TPO levels in our WM cohort, consistent with other studies where TPO levels were elevated 2- to 12-fold in haematological malignancies[53] and commensurate with the observed irregular platelet count. Increased interleukin-6 which can stimulate TPO production[54] have been reported in WM patients.[55,56] Levels of TPO generally vary inversely with the platelet count[57] and TPO levels are elevated in CLL and correlate with disease progression and prognosis.[58] We found that WM patients not receiving active treatment had reduced proportions of reticulated 'new' platelets displaying elevated CD9 levels, which are normally tightly regulated on MKs during megakaryopoiesis.[59] CD9 coordinates MK interactions with BM stromal cells,[60] and elevated levels may perturb stromal signals that provide molecular cues for normal megakaryopoiesis and thrombopoiesis. Our patients had clinically elevated BM involvement and IgM levels. Both properties may disrupt MK function and platelet production however this remains to be addressed experimentally. Purified IgG antibodies from thrombocytopenic patients can bind to MKs *in vitro*[61] and inhibit MK maturation and proplatelet formation.[62,63] Whether WM

paraprotein engages MKs and impairs platelet production remains to be experimentally validated. Nevertheless, the TPO, reticulated platelet and CD9 irregularities are consistent with disturbed thrombopoietic pathways, possibly due to BM disease-associated changes. It would be of interest to evaluate haematopoietic progenitors and MK numbers as well as CD9 levels in WM BM and correlate with BM involvement and platelet function.

None of our patient cohort displayed platelet counts below 35 x 10^9/L or significant bleeding symptoms. However, to explore subtle coagulation aberrations, we evaluated thrombin generation potential in WM plasma alone or mixed with donor platelets.[64] WM plasma displayed prolonged lag times and times to reach peak thrombin, with trends towards reduced rate and extent of thrombin formation and ETP compared to HD plasma. These findings align with an isolated case study reporting reduced ETP associated with elevated IgM levels in two WM patients.[25] Our work shows that WM patients receiving BTKis have equivalent capacity for thrombin generation compared to those not receiving treatment. In our study, observed deficits in thrombin generation in WM plasma were largely corrected by the inclusion of sensitised donor but not WM platelets, indicating a thrombocytopathy was present in well-managed WM patients. The mean serum IgM level in our WM cohort was 22 ± 24 g/L (no treatment) and 7 ± 4 g/L (BTKi therapy) and we found no relationship between IgM levels and ETP in WM patients. This question should be further explored in WM cohorts with significant hyperviscosity (>60 g/L). Agonist-treated WM platelets exposed P-selectin to an extent similar to HD platelets, suggesting platelet degranulation pathways were intact. Observed differences between WM and HD platelets in accelerating thrombin generation may reflect dysregulated PS exposure or release of mediators such as VWF, FV FXIII and polyphosphates from WM platelets. Future work will evaluate this possibility by assessing PS exposure and releasate components from activated WM platelets. Together, our findings suggest that thrombin generation assays using plasma and PRP may better reflect the haemostatic capacity of WM patients compared to routine coagulation assays, with utility in clinical decisions around changes to therapy and management of patients through medical procedures.

Using ROTEM to evaluate WB clotting, we found that WM samples performed similarly to HD samples in intrinsic and extrinsic coagulation assays. In FIBTEM where clotting activity is triggered extrinsically but the platelet contribution is minimised by inclusion of cytochalasin D, we observed no change to clot time, but significant enhancements to the rate and extent of clot formation over HD values, which fell within normal ranges. Whilst WM plasma had reduced thrombin generation potential, in the

presence of a platelet inhibitor, WM blood showed a faster onset and extent of clotting in ROTEM, suggesting increased contribution from fibrinogen in this cohort. WB assays allow contributions from numerous other cells, such as PS-exposing erythrocytes and TF-bearing neutrophils, which are absent from thrombin generation assays. When focusing on the platelet contribution to ROTEM (the relative differences between FIBTEM and EXTEM values), WM platelets showed a similar per-platelet contribution to clot amplitude compared with HDs.

Elevated IgM is likely to contribute to the exacerbated bleeding phenotype often reported in hyperviscous WM patients.[35,65] We observed that purified WM IgM bound to platelets and significantly impaired spreading and aggregation responses when compared with equimolar concentrations of a control protein. WM IgM is reported to coat blood cells and platelets and interfere with platelet filopodia formation[25] and thrombin generation.[66] Expression of the known IgM receptor FcμR is restricted to lymphocytes,[67] so it is likely that IgM engages non-specifically with platelets, possibly via electrostatic interaction to interfere with platelet function. Under experimental conditions used here, IgM had no major effect on coagulation, with only the ETP being slightly reduced. Future experiments aim to assess the effects of hyperviscosity on endothelial cell function and vascular integrity, and to assess an electrostatic interaction of IgM with platelets by use of different polyanionic compounds. In summary, it is likely that WM-derived IgM interacts with platelets electrostatically and interferes with platelet function and thrombin generation, which may contribute to bleeding.

This study has some limitations. First, the patients were from a single centre and require standardisation and validation before being adopted more widely. Second, our patient cohort were clinically stable, with only a few currently requiring therapeutic interventions and none with symptomatic hyperviscosity. Future work evaluating haemostatic properties of blood from WM patients on therapeutic regimens including expansion of data from patients in receipt of BTKis and/or with hyperviscosity will be important. Further, a direct comparison with patients with other types of B-cell lymphomas without hyperviscosity symptoms as additional controls to delineate how specific the observed haemostatic dysfunctions are to WM would be valuable. Third, our findings could not be linked to clinical outcomes as no bleeding events were observed in our cohort. Fourth, age and other demographic differences between our HDs and WM cohort should be noted when considering the findings. Finally, we had opportunity to isolate IgM from only one WM patient in our cohort with hyperviscosity, thus it remains unclear whether the IgM-mediated haemostatic impairment was specific to that patient.

Our study describes significant differences in platelet parameters and haemostatic functions in a clinically stable WM cohort. Whether these findings can be extended to other lymphomas and leukaemias with similar burdens of BM disease[6] awaits further investigation. The extent to which haemostatic changes contribute to clinically significant thrombotic and bleeding risks in WM patients also remains to be evaluated, however monitoring of platelet function and thrombin generation data could be incorporated in a patient's management plan and re-evaluated in the event of clinically significant bleeding or prior to changes in therapy or to surgical intervention.

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Authorship contributions: SAB, EEG and DT designed the study and interpreted the data. SAB drafted the manuscript. EEG and DT planned the study. MM, MGM, PJC and DT acquired informed consent from patients, provided patient-specific data and samples and clinical advice on interpretation of data. SAB, SMH, JIH, SAA, VB, AK and YLT contributed to experimental data and intellectual discussions. JCW provided key reagents. EEG acquired funding for the project. All authors contributed to the review of the manuscript.

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	HD	WM + no BTKi	WM + BTKi
Number	15	13	5
Age (years)	NR (18-60)	73 (50-87)	74 (52-91)
Sex			
Male	NR	8 (62%)	2 (40%)
Female	NR	5 (38%)	3 (60%)
BM involvement (% cellularity)	NT	43 (5-80)	22 (2-70)
IgM (g/L)	NT	23 (3-78)	7 (2-11)
Active treatments			
None	15 (100%)	13 (100%)	0 (0%)
Ibrutinib		0 (0%)	3 (60%)
Zanubrutinib		0 (0%)	2 (40%)
Past treatments*			
None	NR	4 (33%)	2 (40%)
R-CVP		6 (50%)	1 (20%)
BR		3 (25%)	1 (20%)
DRC		2 (17%)	0 (0%)
Ibrutinib		1 (8%)	1 (20%)
Rituximab		2 (17%)	0 (0%)
R-FC		1 (17%)	0 (0%)
CR		1 (8%)	0 (0%)
Chlorambucil		1 (8%)	0 (0%)
CP		1 (8%)	0 (0%)
Comorbidities			
None	NT	0 (0%)	1 (20%)
Cardiovascular			
Hypertension		4 (33%)	2 (40%)
Atrial fibrillation		2 (17%)	2 (40%)
Ischemic heart disease + stent		2 (17%)	0 (0%)
Dyslipidaemia		1 (8%)	1 (20%)
Cerebral vascular accident		1 (8%)	0 (0%)
Aortic stenosis		1 (8%)	0 (0%)
Dilated cardiomyopathy		1 (8%)	0 (0%)
Patent ductus arteriosus		1 (8%)	0 (0%)
Infection/ inflammation			
Arthritis		3 (25%)	0 (0%)
Recurrent infections		3 (25%)	0 (0%)
Sepsis		2 (17%)	0 (0%)
Asbestosis		1 (8%)	0 (0%)
Glomerulonephritis		1 (8%)	0 (0%)
Meningitis		1 (8%)	0 (0%)
Sarcoidosis		1 (8%)	0 (0%)
Severe chronic obstructive airway disease		1 (8%)	0 (0%)
Vasculitis		1 (8%)	0 (0%)
Shingles		0 (0%)	1 (20%)
Additional malignancy			
Squamous cell carcinoma		0 (0%)	2 (40%)
Myelodysplastic syndrome		0 (0%)	1 (20%)

Prostate cancer		1 (8%)	0 (0%)
Cryoglobulinaemia		2 (17%)	0 (0%)
Thoracic mass		1 (8%)	0 (0%)
Schwannoma of lumbar spine		1 (8%)	0 (0%)
Lower abdomen mass		1 (8%)	0 (0%)
RBC (x 10^{12}/L)	4.1 (3.5 – 5.8)	3.0 (1.8 – 4.4)	3.4 (2.8 – 3.9)
Haemoglobin (g/L)	132 (113 – 199)	105 (749 – 142)	110 (74 – 128)
WBC count (x 10^9/L)	5.6 (3.8 – 8.0)	4.3 (2.0 – 6.3)	5.6 (2.3 – 8.5)
Lymphocyte count (x 10^9/L)	1.9 (1.0 – 2.5)	1.3 (0.3 – 2.1)	0.5 (0.7 – 2.6)
Platelet count (x 10^9/L)	207 (73 – 264)	151 (35 – 285)	143 (72 –202)
MPV (fL)	7.4 (6.7 – 8.7)	7.6 (6.7 – 9.4)	8.5 (7.3 –9.7)

Table 1: Waldenström Macroglobulinaemia patient and healthy donor information. Values displayed as number (%) or mean (range). P-values were determined by Mann-Whitney t-test. Data for HD age, sex and any past treatments were not collected due to ethics protocol requirements. All current HD medications were screened prior to blood collection. NR: not recorded, NT: not tested, RBC: red blood cell, IgM: immunoglobulin M, BM: bone marrow, WBC: white blood cell, MPV: mean platelet volume, R-CVP: rituximab, cyclophosphamide, vincristine, prednisolone; BR: bendamustine, rituximab; DRC: dexamethasone, rituximab, cyclophosphamide; R-FC: rituximab, fludarabine, cyclophosphamide; CR chlorambucil, rituximab, CP: chlorambucil, prednisolone. Patients may have had more than one prior treatment. * Not all medications were recorded, including but not limited to historical use of anticoagulant and anti-platelet therapies and non-steroidal anti-inflammatory drugs to treat comorbidities.

Figure Legends:

Figure 1: Waldenström Macroglobulinaemia platelets have altered thrombopoietic and age-related phenotypic markers. A) Platelet count, (B) MPV, (C) serum TPO, (D) normalised TO staining of platelets in WB (divided by HD mean), and (E) relative proportion of TO^{bright} WM platelets falling within the gate encompassing the top 10% of HD platelets (log_{10} WM/HD %), (F) platelet surface receptor levels in PRP and (G) sGPVI levels in PPP, from HDs (n=9-15, blue), WM patients not on therapy (n=9-13, red) or receiving BTKis (n=0-5, green). A-C, E, G) Mean +/± SD. D, F) Normalised geomeans (divided by mean HD geomean) ± SEM. P-values were determined by (A-C) Kruskal-Wallis one-way ANOVA with Dunn's multiple comparisons test, performed on the raw data, (E) Mann-Whitney t-test, or (E) one-sample t-test. HD: healthy donor, WM: Waldenström Macroglobulinaemia, BTKi: Bruton's tyrosine kinase inhibitor, MPV: mean platelet volume, TPO: thrombopoietin, TO: thiazole orange, GP: glycoprotein, CD: cluster of differentiation, ADAM: a disintegrin and metalloproteinase, TLT: Trem-like transcript, sGPVI: soluble GPVI, PRP: platelet-rich plasma, PPP: platelet-poor plasma.

Figure 2: Waldenström Macroglobulinaemia platelets respond appropriately to standard agonists. A-B) OgFg binding and (C-D) P-selectin exposure in WB and PRP samples respectively, from HDs (n=7-14, blue), WM patients not on therapy (n=8-13, red) or receiving BTKis (n=0-5, green). Representative histograms from a HD showing (A) OgFg- or (C) P-selectin-positive events. Data (mean ± SD) for (B) OgFg binding or (D) P-selectin exposure (%) in platelets treated with 0.5, 5 or 30 μg/mL CRP-XL, 10 μM TRAP-6 or 5 μM ADP. P-values were determined by Mann-Whitney t-test with multiple comparisons. OgFg: Oregon-green 488 fibrinogen, CRP-XL: cross-linked collagen-related peptide, TRAP-6: thrombin receptor-activating peptide 6, ADP: adenosine diphosphate, WB: whole blood.

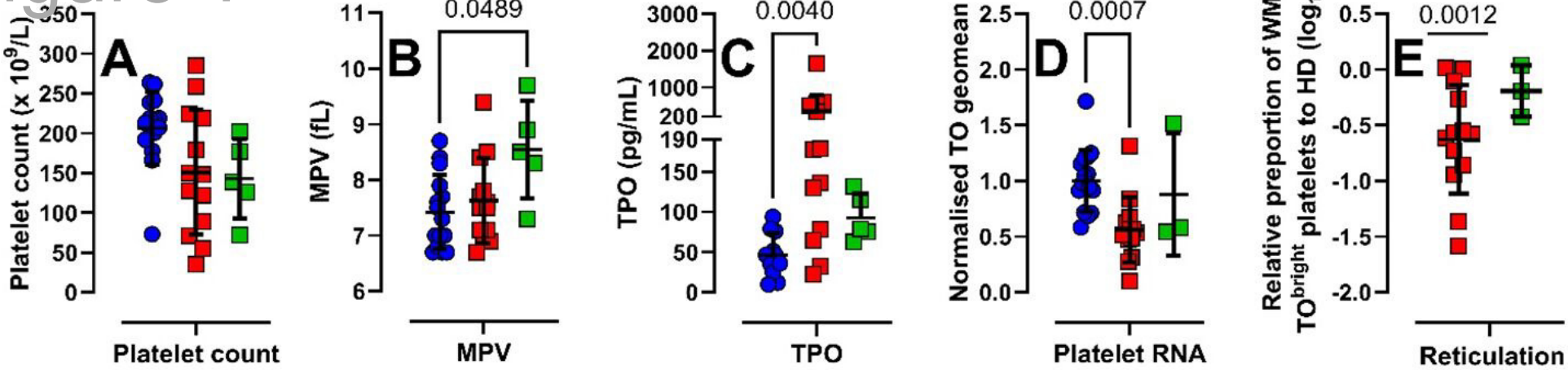
Figure 3: Waldenström Macroglobulinaemia patients have delayed and reduced thrombin generation. Thrombin generation in (A-G) EDTA-anticoagulated PPP, (H) EDTA-anticoagulated PPP + PAR1-activated HD WPs, or (I-M) TSC-anticoagulated PPP + PAR1-activated HD or WM WPs, in HDs (n=6-25, blue), WM patients not on therapy (n=5-13, red) and receiving BTKis (n=1-3, green). A, H) Representative thrombograms, mean of duplicates. B-F, I-M) Mean ± SD. G) Correlation between WM patient ETP and IgM levels. P-values were determined by Kruskal-Wallis one-way ANOVA with Dunn's multiple comparisons test (B-F) or Wilcoxon matched-pairs signed rank test (I-M). R^2 values were

determined by simple linear regression. ETP: endogenous thrombin potential, WP: washed platelet, IgM: immunoglobulin M.

Figure 4: Waldenström Macroglobulinaemia coagulation factors contribute robustly in a whole blood clotting assay. ROTEM measurements in WB samples from HDs (n=31, blue), WM patients not on therapy (n=13, red) or receiving BTKis (n=5, green). A-C) INTEM is triggered by calcium, phospholipids and ellagic acid. D-F) EXTEM is triggered by calcium and tissue factor, (G-I) while FIBTEM additionally inhibits platelet cytoskeletal changes with cytochalasin D and reflects the coagulation factor contribution to thrombus formation. J-K) EXTEM-FIBTEM (PLTEM) reflects the platelet contribution to thrombus formation. Mean ± SD. P-values were determined by Kruskal-Wallis one-way ANOVA with Dunn's multiple comparisons test. ROTEM: rotational thromboelastometry, CT: clot time, α angle: clotting rate, A10: clot amplitude after 10 min, MCF: maximal clot firmness, MCE: maximum clot elasticity (MCF x 100) / (100 - MCF).

Figure 5: High concentrations of WM-derived IgM impair platelet spreading, aggregation and thrombin generation. HD WP spreading (n=4-5) for 20-60 min on (A, C-F) fibrinogen or (B, G-J) collagen, then visualised for actin (red) and α-tubulin (green), in the presence of (C-F, G-J) vehicle (blue), BSA (equimolar to IgM, 4.11 mg/mL, pink) or IgM (60 mg/mL, red). C, G) Number of adhered platelets; (D, H) platelet surface area; (E, I) circularity; or (F, J) aspect ratio, average of 5 field of views. K) Representative aggregation trace, mean of duplicates. L) WP aggregation responses to collagen (5 µg/mL), TRAP-6 (10 µM) and ADP (5 µM). M) Representative thrombogram, mean of duplicates. Thrombin generation in HD samples (n=8), with (N, R) lag times, (O, S) ETP, (P, T) peak thrombin generated, and (Q, U) thrombin generation velocity in (N-Q) PPP or (R-U) PPP + autologous WPs. T) Mean ± SD. P-values were determined by two-way ANOVA with multiple comparisons (C-F, G-J, L) or Friedman test (N-U). BSA: bovine serum albumin, AUC: area under the curve.

Figure 1



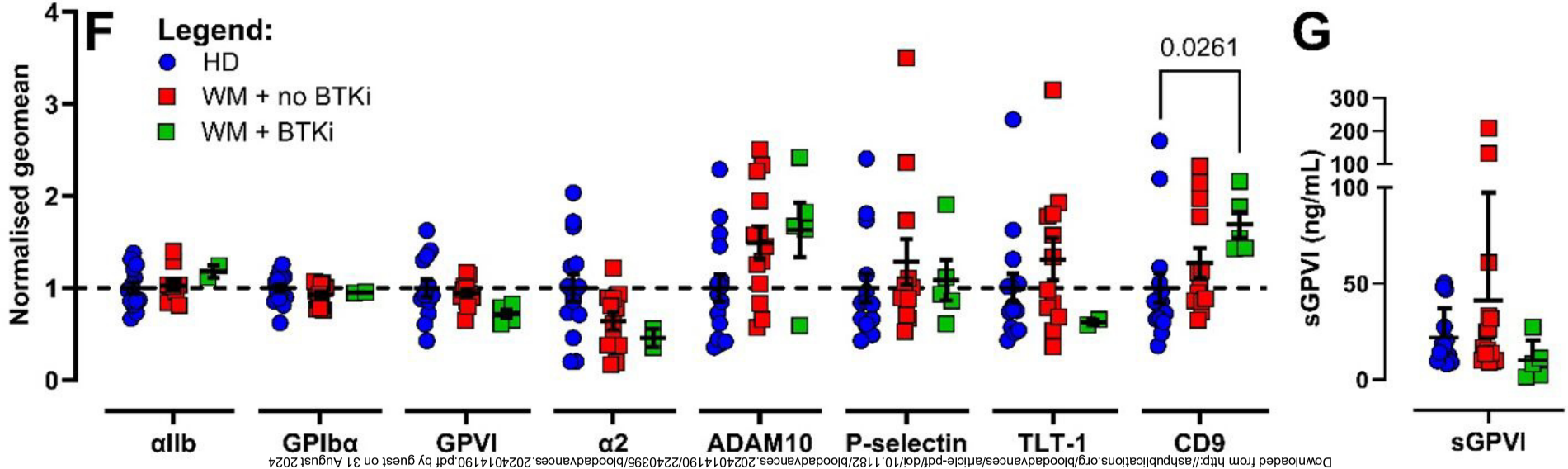


Figure 2

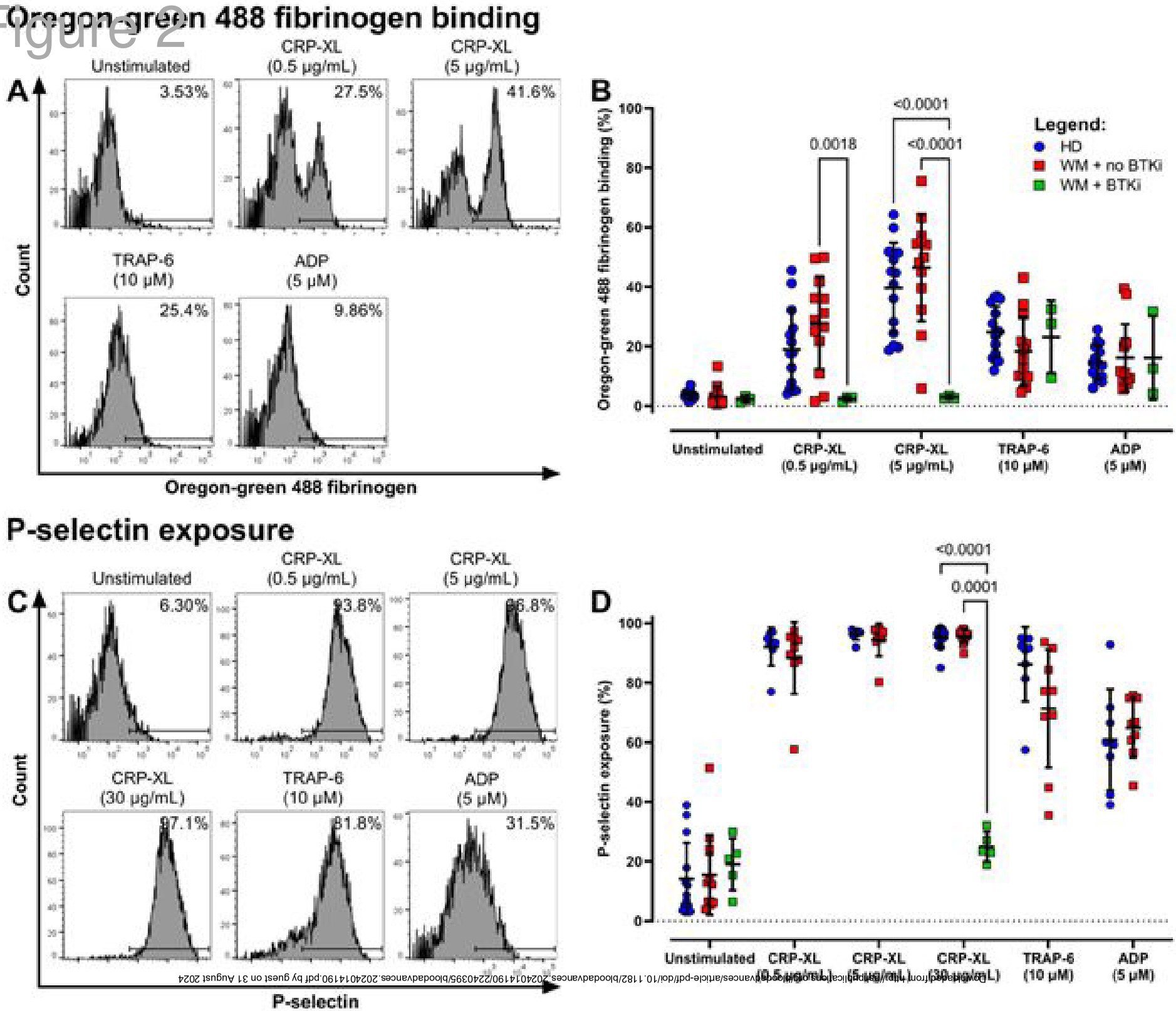


Figure 3

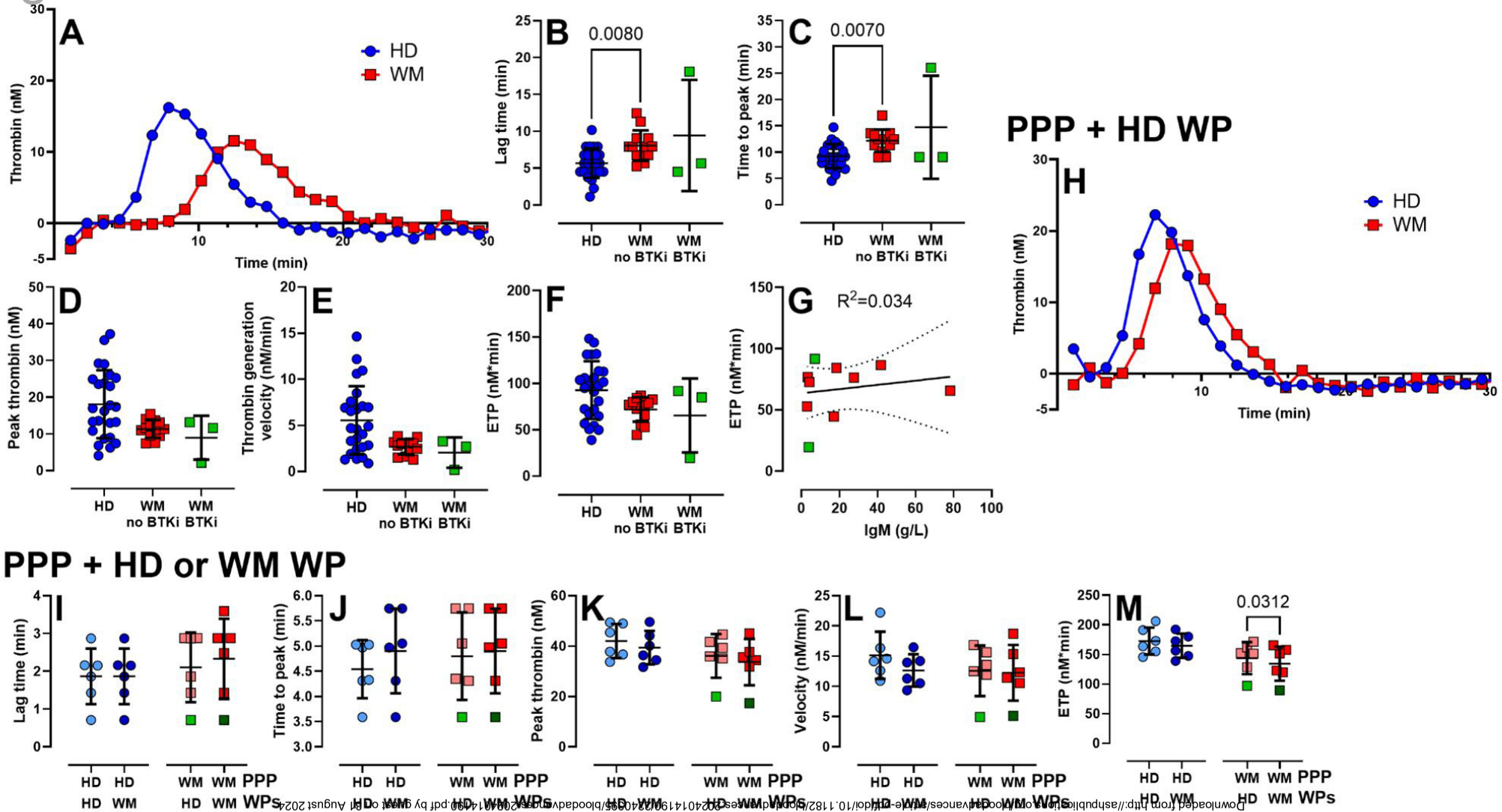


Figure 4

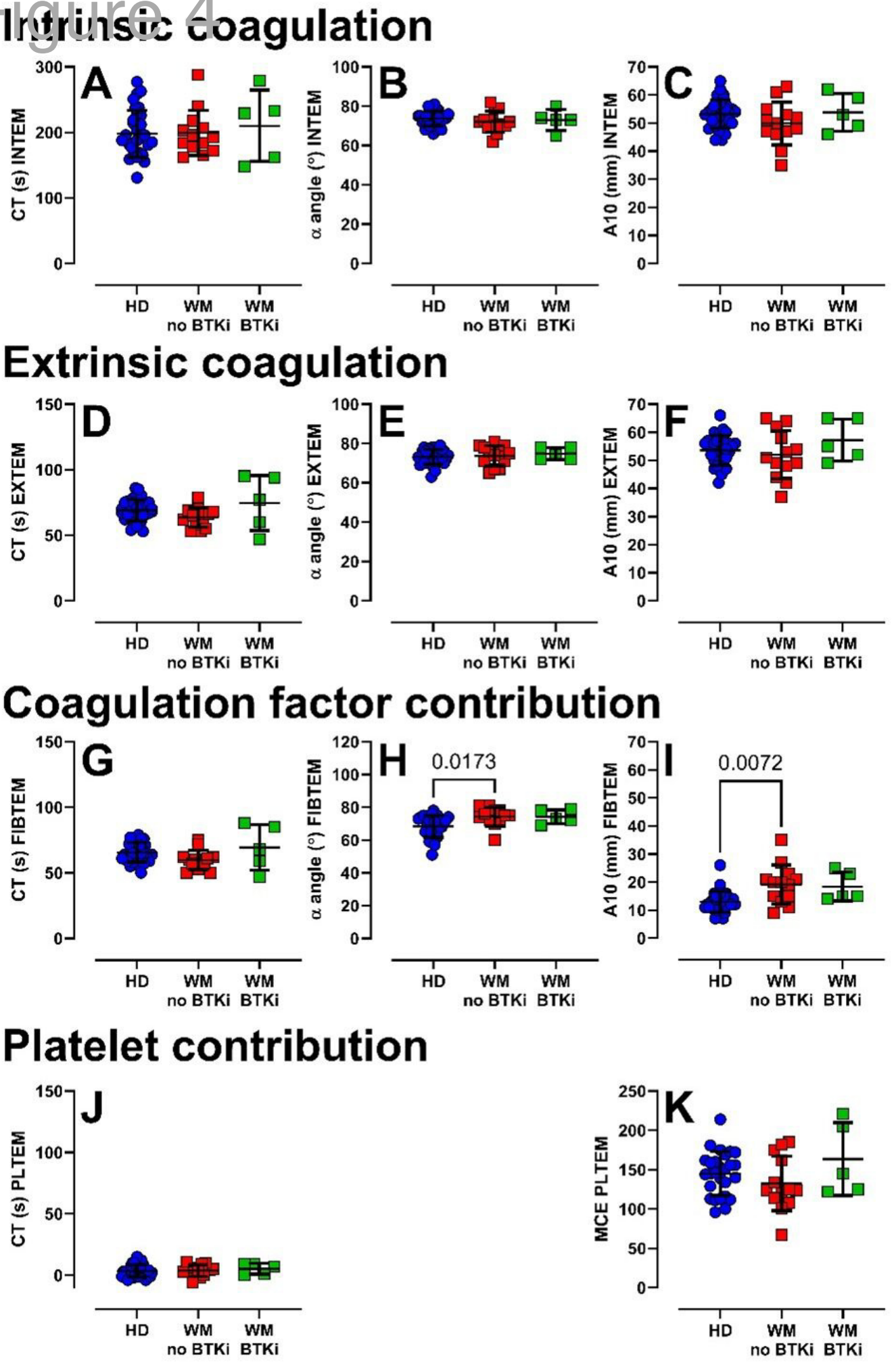


Figure 5