



Bleeding Propensity in Waldenström Macroglobulinemia: Potential Causes and Evaluation

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Thromb Haemost 2022;122:1843–1857.

Abstract

Waldenström macroglobulinemia (WM) is a rare, incurable, low-grade, B cell lymphoma. Symptomatic disease commonly results from marrow or organ infiltration and hyperviscosity secondary to immunoglobulin M paraprotein, manifesting as anemia, bleeding and neurological symptoms among others. The causes of the bleeding phenotype in WM are complex and involve several intersecting mechanisms. Evidence of defects in platelet function is lacking in the literature, but factors impacting platelet function and coagulation pathways such as acquired von Willebrand factor syndrome, hyperviscosity, abnormal hematopoiesis, cryoglobulinemia and amyloidosis may contribute to bleeding. Understanding the pathophysiological mechanisms behind bleeding is important, as common WM therapies, including chemoimmunotherapy and Bruton's tyrosine kinase inhibitors, carry attendant bleeding risks. Furthermore, due to the relatively indolent nature of this lymphoma, most patients diagnosed with WM are often older and have one or more comorbidities, requiring treatment with anticoagulant or antiplatelet drugs. It is thus important to understand the origin of the WM bleeding phenotype, to better stratify patients according to their bleeding risk, and enhance confidence in clinical decisions regarding treatment management. In this review, we detail the evidence for various contributing factors to the bleeding phenotype in WM and focus on current and emerging diagnostic tools that will aid evaluation and management of bleeding in these patients.

Keywords

- ▶ platelet
- ▶ Waldenström macroglobulinemia
- ▶ receptor
- ▶ bleeding

Introduction

Waldenström macroglobulinemia (WM) is the clinical manifestation of lymphoplasmacytic lymphoma, which is a rare, low-grade, B cell lymphoma. WM is characterized by bone marrow infiltration with malignant cells and hypersecretion of immunoglobulin (Ig) M paraprotein. It constitutes less than 5%

of all non-Hodgkin lymphomas, with an incidence of approximately 0.3/100,000 cases/year.¹ Many patients are asymptomatic at the time of their initial diagnosis and do not require treatment.^{2,3} However, approximately 30% of these patients are likely to need therapy within 2 years of diagnosis, and 80% within 10 years.⁴ Indications for treatment are varied, but most commonly include constitutional symptoms, bone marrow or organ dysfunction, hyperviscosity and neuropathy.

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received

March 8, 2022

accepted after revision

June 22, 2022

accepted manuscript online

July 11, 2022

article published online

October 17, 2022

DOI <https://doi.org/>

10.1055/a-1896-7092.

ISSN 0340-6245.

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Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

With an evolving treatment landscape in WM involving Bruton's tyrosine kinase (BTK) inhibitors proteasome inhibitors and newer agents targeting BCL2 and CXCR4, understanding potential mechanisms of bleeding is important, as some of these therapies increase bleeding risk and necessitate the use of alternative agents in a personalized medicine approach.

Molecular Basis of WM

WM patients carry one or more somatic genetic mutations within malignant lymphoplasmacytoid cells (– Fig. 1), which have been reported to be present in less mature lymphoid and hematopoietic progenitor cells in some cases.⁵ Whether common WM mutations can be detected in megakaryocytes and platelets remains an open research question. Understanding the WM genomic landscape is important, not only because specific mutations can influence disease presentation and treatment options,^{3,6} but also because they can potentially affect platelet function (see

below). The most common genetic defect in WM is a gain-of-function mutation within the myeloid differentiation factor (*MYD*) 88 gene resulting in a leucine-265 to proline (L265P) substitution within the cytoskeletal adaptor protein Myd88, detected in lymphoplasmacytoid cells in over 90% of WM patients.^{7–13} Of note, the *MYD88*^{L265P} transcript has also been detected in WM plasma cells, mature B lymphocytes, phenotypically normal B cell precursors and CD34⁺ hematopoietic precursor cells.⁵ There are several other less common mutations now identified^{14,15} and at least two distinct WM signature DNA methylation profiles specific for memory B cells or plasma cells.¹⁶

Through association with toll-like receptors (TLR) and the interleukin-1 receptor (IL-1R), Myd88 has an important role in coordinating innate immune cell responses. Cells expressing *Myd88*^{L265P} protein exhibit constitutive activation of TLR and IL-1R pathways, leading to nuclear translocation of transcription factor nuclear factor (NF)-κB and enhanced B cell proliferation and survival.^{17,18} *MYD88*^{L265P} has also been detected in approximately 61% of IgM monoclonal gammopathy of undetermined significance (IgM-MGUS) patients.^{9,10,12,19} This mutation is likely to be an early oncogenic event in WM development, with IgM-MGUS acting as a precursor condition. However, additional genetic mutations likely contribute to WM onset.^{14,20} Of note, Myd88 is expressed in platelets and megakaryocytes and is essential for appropriate TLR-driven platelet responses to viremia.²¹ A link between Myd88 activation and platelet function will be discussed below.

Gain-of-function mutations in the gene encoding the C-X-C chemokine receptor (CXCR) 4 also occur in approximately 30% of WM patients.^{22–24} CXCR4 engages with stromal-derived factor (SDF) 1 to mediate the homing of cells to the bone marrow. A common activating mutation involving serine-338 in the C-terminal region of CXCR4 prevents CXCR4 internalization following SDF-1 stimulation.²⁵ This leads to persistent CXCR4 activation and signaling via AKT, ERK and BTK pathways, and bone marrow myeloid cell migration, adhesion, proliferation, and survival.^{26,27}

The mutations in WM lymphoplasmacytoid cells, within genes involved in cell proliferation and survival, cause an over-proliferation of these cells, resulting in overproduction of IgM (– Fig. 1). Increased IgM contributes to the neurological and bleeding symptoms observed in WM, as correction of blood IgM levels often resolves symptoms.²⁴ WM malignant infiltration of the bone marrow leads to cytopenias, which increase bleeding risk and predispose to infections. While some of these mutations have been detected in lymphoid and hematopoietic precursors, it is not evident that the mutations arise in platelet-producing megakaryocytes or contribute to a bleeding phenotype. This review will explore the multifactorial nature of bleeding encountered in WM and explore considerations that may aid clinical decisions around therapy for these patients.

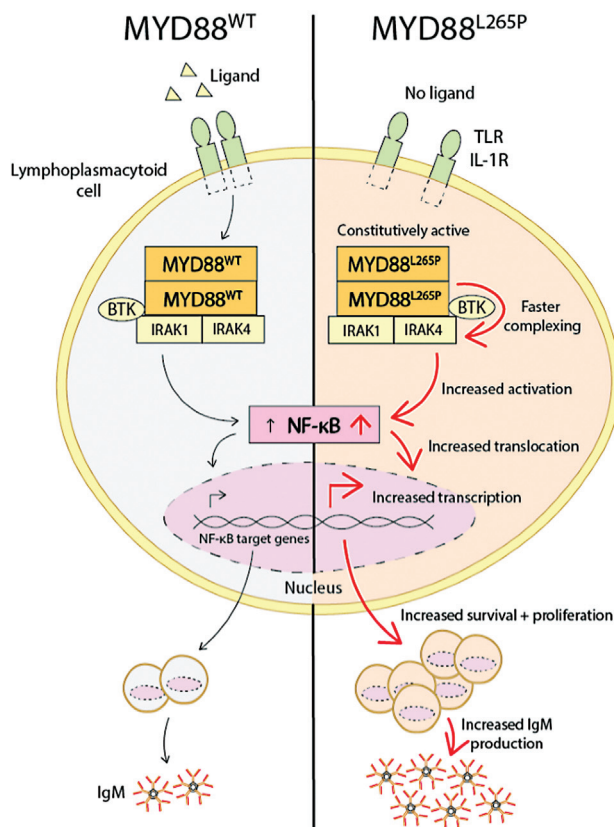


Fig. 1 Pathways of development of lymphoplasmacytoid B cells in healthy individuals and in WM patients. In WM patients, mutations and loss of DNA methylation occur to the lymphoplasmacytoid cells, with enrichment in B memory cells at an earlier stage of differentiation. The most common mutation, *MYD88*^{L265P}, results in increased BTK phosphorylation and faster MYD88 complexing with IRAK1/4 in response to low or absent TLR or IL-1R stimulus, which results in increased NF-κB translocation to the nucleus, increased target gene transcription, uncontrolled proliferation of WM lymphoplasmacytoid cells and overproduction of IgM. BTK, Bruton's tyrosine kinase; IgM, immunoglobulin M; IL-1R, interleukin-1 receptor; IRAK, IL-1 receptor-associated kinase; MYD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TLR, toll-like receptor; WM, Waldenström macroglobulinemia.

Waldenström Macroglobulinemia Patients Can Present with a Bleeding Phenotype

When Jan Waldenström first described WM in 1944, the symptoms in the two patients included oronasal bleeding.²⁸ While bleeding is often a feature of the initial presentation of

Table 1 Studies reporting the frequency of bleeding in treatment-naïve WM patients

Study	Sample size	% Bleeding	Nature of bleeding
Perkins et al 1970 ¹⁴⁵	62	36	Not specified
Merlini et al 2003 ¹⁴⁶	215	7	Hemorrhagic manifestations
García-Sanz et al 2001 ²	217	23	Not specified
Merchionne et al 2011 ³⁰	121	12.3	Epistaxis and gum bleeding

Abbreviation: WM, Waldenström macroglobulinemia.

WM, bleeding symptoms usually resolve after therapeutic intervention, implying that WM disease etiology does not directly affect platelet production and function. However, four studies have evaluated the frequency of bleeding in patients with WM before treatment intervention (► **Table 1**). When considered together, approximately 17% of patients displayed bleeding symptoms,^{2,30,145,146} with limited description of magnitude. Most other studies describe easy bruising and chronic oronasal bleeding as a common symptom of WM. It is worth noting that due to a paucity of large studies on this issue, evidence-based guidelines and standard of care management for treatment of bleeding in these patients are lacking. Thus, reported treatment approaches are variable, with inconsistent reporting of key diagnostic information. Standardized reporting of laboratory findings and outcomes is also lacking.

Laboratory Test Abnormalities Associated with Bleeding in WM

The cause of the WM bleeding phenotype is unknown, but laboratory studies have identified several vascular and platelet abnormalities that often co-occur (► **Table 2**). All of these sequelae are likely to contribute to bleeding symptoms in WM patients prior to initiation of treatment; however several of these findings could occur as a result of IgM protein binding to and/or inhibition of coagulation factor(s) function.

Thrombocytopenia

Thrombocytopenia is a common occurrence in WM, often coincident with anemia. One large WM study ($n=454$) classified 18% of WM patients with thrombocytopenia.³² Mechanisms by which thrombocytopenia arises are likely to be complex, potentially resulting from combinations that are autoimmune- and drug-mediated, as well as marrow infiltration which can cause overcrowding of hematopoietic stem cells and progenitors, resulting in disturbed megakaryopoiesis and thrombopoiesis.³³ Occasionally thrombocytopenia is secondary to peripheral platelet sequestration within the spleen (splenomegaly).³⁴ In isolation, thrombocytopenia rarely explains the observed bleeding, and the degree of bleeding is often out of step with the platelet count.

Acquired von Willebrand Syndrome

Von Willebrand factor (VWF) is a biorheological shear sensitive glycoprotein (GP) produced by the vascular endothelium and megakaryocytes. VWF plays a vital role in primary

hemostasis by triggering GPIb-IX-V-mediated platelet activation and formation of an adhesive bridge between platelets and the vasculature at sites of endothelial injury. VWF is also a carrier protein for factor VIII (FVIII) and contributes to fibrin clot formation.³⁵ Acquired von Willebrand syndrome (AVWS) is an uncommon disorder caused by a loss of high-molecular-weight VWF multimers, either by specific autoantibody-mediated destruction, absorption onto malignant cells, or increased fluid shear stress resulting in VWF multimer unfolding and proteolysis by ADAMTS-13.³⁶ In hematological cancer, AVWS is reported in lymphoproliferative neoplasms and several myeloproliferative disorders.³⁷ AVWS occurs in 6% of WM patients, where incidence is strongly correlated with elevated IgM levels (30–60 g/L).³⁸ Symptoms include mucosal and GI bleeding, which generally resolve following WM therapy.³⁸ To mitigate bleeding, specific therapeutic approaches aim to increase VWF antigen levels (treatment with desmopressin and/or transfusion of FVIII/VWF concentrate), remove an offending autoantibody (plasmapheresis), or disturb destructive autoantibody functions, via transfusion of intravenous immunoglobulin (IVIg).^{39,40} IVIg has been reported to successfully increase VWF/FVIII levels and reduce bleeding times in AVWS linked to IgMGUS.^{41,42} Although the mechanism of action of IVIg is unclear, isolated case reports clearance of VWF in the setting of IgM-MGUS may also be ablated by IVIg therapy, implying that IVIg could be a favourable therapeutic option for AVWS associated with WM.^{39,41}

Hyperviscosity

Hyperviscosity, caused by the accumulation of large (approximately 925 kDa), pentameric, positively charged IgM paraprotein in the blood, is a classic manifestation of WM. The IgM proteins electrostatically interact with sialic acid-rich red blood cells, resulting in an agglutinating effect and contributing to increased viscosity.⁴³ Healthy individuals have around 1.5 g/L of IgM, of which 80% is intravascular,⁴⁴ and hyperviscosity emerges when IgM levels exceed 50 to 60 g/L.⁴⁴ Hyperviscosity causes the physical tearing of small venules from increased rheological drag,⁴⁵ and the suspected inhibitory coating of platelets by IgM protein, resulting in reduced platelet adhesion and aggregation.⁴⁶ Symptoms include bleeding, vision problems, and neurological symptoms, which occur in 13% of WM patients.⁴⁷ Acute management of hyperviscosity involves plasmapheresis, while longer term management with chemo-immunotherapy or targeted agents works by depleting the IgM-producing cells in the marrow.

Table 2 Laboratory test abnormalities associated with bleeding in WM patients

Reference	Sample size	Finding	Comments and observations that may explain bleeding symptoms
Hivert et al 2012 ¹³³	43/72 (59%)	Reduced VWF levels	<ul style="list-style-type: none"> - Possibly due to IgM-mediated inhibition.⁴⁸ - Possibly due to specific autoantibody-mediated destruction, absorption onto malignant cells, altered blood rheology resulting in VWF multimer proteolysis by ADAMTS-13.³⁶ - Results in reduced VWF-GPIb-IX-V-mediated platelet activation and bleeding.
Gavriatopoulou et al 2019 ¹³⁴ Hivert et al 2012 ¹³³	6/42 (14%) 10/72 (14%)	Increased VWF levels	<ul style="list-style-type: none"> - Correlated with poor prognosis and low circulating ADAMTS-13 levels.¹³⁴ - Reflects greater engagement between WM lymphoplasmacytoid cells and endothelium.^{133,134} - Could trigger thrombosis due to increased VWF-GPIb-IX-V-mediated platelet activation. - Could result in increased bleeding due to low numbers of VWF multimers.¹³⁵
Castillo et al 2019 ³⁸	49/320 (15%)	Reduced FVIII levels	<ul style="list-style-type: none"> - FVIII is produced in the liver. Low FVIII levels possibly caused by liver dysfunction.¹³⁶ - FVIII is bound to VWF in plasma. Low FVIII levels possibly coincide with AVWS. - Possibly due to IgM-mediated inhibition of FVIII or VWF.⁴⁸⁻⁵⁰ - Results in decelerated FX activation and bleeding.¹³⁷
Saraya et al 1972 ¹³⁸ Kasturi et al 1978 ¹³⁹	3/3 (100%) 4/4 (100%)	Reduced platelet adhesion	<ul style="list-style-type: none"> - Reduced in vivo platelet adhesion to a wound.¹³⁸ - Results in bleeding
Kasturi et al 1978 ¹³⁹	4/4 (100%)	Reduced platelet activation	<ul style="list-style-type: none"> - Platelets take up microparticles containing tissue factor. Platelet activation measured via release of tissue factor in response to ADP.^{140,141} - Results in bleeding
Saraya et al 1972 ¹³⁸ Kasturi et al 1978 ¹³⁹	2/3 (67%) 3/4 (75%)	Reduced platelet aggregation	<ul style="list-style-type: none"> - Reduced aggregation in response to ADP and adrenaline. Within two standard deviations for noradrenaline, thrombin, and collagen.¹³⁸ - Results in bleeding

Abbreviations: ADAMTS-13; a disintegrin and metalloprotease; ADP, adenosine diphosphate; aPTT; activated partial thromboplastin time; AVWS; acquired von Willebrand syndrome; FVIII, factor VIII; PT; prothrombin time; TF; tissue factor; VWF; von Willebrand factor.

Note: Sample size indicates number of patients and percentage of cohort with clinically significant bleeding.

Hemostasis-Inhibiting Paraproteins

Circulating paraproteins have been reported to have VWF and FVIII-inhibitory activity in WM in vivo, caused by IgM⁴⁸⁻⁵⁰ or IgG^{51,52} antibodies. This has also been observed in other paraproteinemias, including multiple myeloma, MGUS, lymphoma, chronic lymphocytic leukemia, and amyloidosis.^{53,54} Reports showed that the monoclonal IgM isolated from a WM patient demonstrated antiplatelet activity and immune thrombocytopenia in vivo,^{55,56} implying a derangement causing autoimmunity. Additionally, WM IgM cryoglobulins can suppress erythroid and granulocyte progenitor cells grown in culture in vitro,⁵⁷ and possibly megakaryocyte progenitor cell maturation, which could alter platelet quality and function.

Cryoglobulinemia

Cryoglobulinemia, where temperature-sensitive Igs form concentration-dependent insoluble aggregates that precipitate below 37°C, can occur in WM.⁵⁸ These cryoglobulins may form immune complexes, where monoclonal IgM antibodies

bind to the Fc region of polyclonal IgG antibodies.⁴⁴ The symptoms of cryoglobulinemia occur at varied cryoglobulin concentrations depending on the individual. These include purpura and mucosal bleeding, caused by the tearing of small blood vessels by the aggregates, and are observed in approximately 5% of WM patients.⁴⁴

Amyloidosis

Amyloidosis can be associated with potentially life-threatening hemorrhage, by causing coagulation factor deficiency, hyperfibrinolysis, platelet dysfunction, angiopathy, and/or vascular fragility.⁵⁹ Amyloidosis is characterized by the production of misfolded proteins, often monoclonal Ig light and/or heavy chains, which form insoluble amyloid fibrils, that accumulate and form plaques, leading to tissue and organ dysfunction. IgM amyloidosis occurs in 7.5% of WM patients and is associated with a dramatic reduction in overall survival, from 12.1 to 2.5 years.⁶⁰ Bleeding occurs in 5 to 41% of amyloidosis patients, ranging from ecchymoses and purpura, to gastrointestinal (GI) and postprocedural bleeding.⁶¹⁻⁶⁵ Bleeding

results from increased vessel-wall fragility from perivascular amyloid deposition, and/or from acquired factor X (FX) deficiencies. Acquired FX deficiencies occur in 5 to 10% of amyloidosis patients and are caused by the absorption of FX and pentraxin-2 onto amyloid fibrils, particularly in the spleen, resulting in direct FX removal from circulation and indirect FX internalization by macrophages.⁶⁶ This can be corrected by splenectomy, chemotherapy treatment, or autologous hematopoietic cell transplant.⁶⁶

To summarize, WM patients display significantly elevated concentrations of serum IgM and one or more symptoms of blood hyperviscosity, cryoglobulinemia, coagulation irregularities, thrombocytopenia, amyloidosis and bleeding. The factor(s) responsible for bleeding in untreated WM remains to be fully defined, but bleeding is likely to be the result of a combination of all of the above observations.

Platelet Dysfunction in WM

Platelets circulate throughout the vasculature and are the primary mediators of hemostasis (►Fig. 2). These are produced in the bone marrow by megakaryocytes, via controlled endomitosis. A healthy individual generally has a very stable platelet count; however, the numbers of circulating platelets can range from 150 to 400 × 10⁹ platelets/L. Thrombocytopenic individuals (<100 × 10⁹ platelets/L) can have a heightened risk of bleeding, which significantly increases if the platelet count falls below 20 × 10⁹ platelets/L. However, as mentioned earlier, bleeding can also occur in the absence of thrombocytopenia. Thus, the prediction of an imminent bleeding event should not rely solely on a low platelet count.^{67–69} Besides the platelet count, platelet quality and functionality are critical components of an effective hemostatic response. This involves detection of injury-exposed collagen and other matrix proteins, and sensing alterations to local blood rheology. Platelets respond by adhering to a site of vascular injury and undergoing platelet activation. Platelet surface receptors coordinate this response⁷⁰ as well as the subsequent formation of a thrombus (platelet aggregate or blood clot), which acts to seal the blood vessel, reduce blood loss and begin the process of wound repair (►Fig. 2).^{71,72}

Platelet Function Is Controlled by Surface Receptors and Signaling Pathway Proteins

Levels of platelet receptors and their attendant surface densities mediate platelet responsiveness to molecular cues in the vasculature. Low receptor numbers and densities have been associated with bleeding in patients receiving mechanocirculatory support⁷³ and in trauma patients.⁷⁴ Further, a loss of platelet receptors, including GPIIb/IIIa and GPVI,⁷⁵ and a diminution of platelet function prior to therapy⁷⁶ have been demonstrated in leukemia patients. The molecular explanation for these losses remains undefined, but, may be linked to disturbances in bone marrow cellularity and megakaryocyte maturation.⁷⁷ As GPIIb/IIIa and the collagen/fibrin receptor GPVI also contribute to efficient thrombus generation by binding thrombin and other coagulation proteins,^{78–81} any alteration to normal levels of plate-

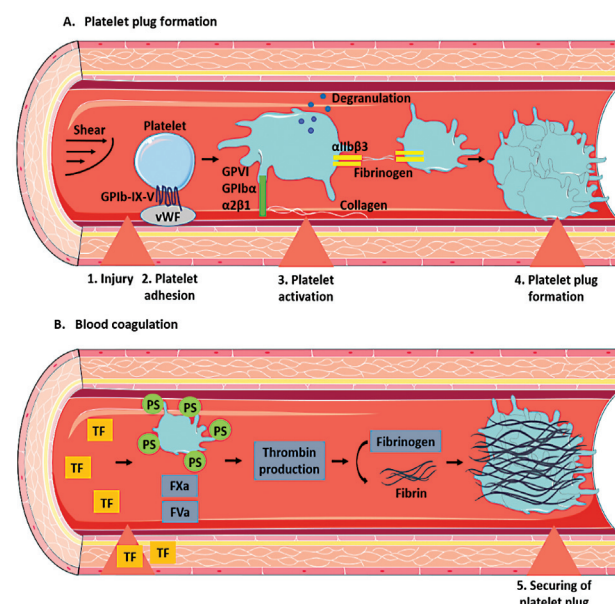


Fig. 2 Simplified hemostasis. (A) Platelet plug formation. Following an injury, GPIIb/IIIa and GPVI bind exposed extracellular matrix ligands such as von Willebrand factor (vWF) and collagen respectively to enable platelet adhesion to the endothelium. Engagement of these receptors triggers platelet signaling, and platelets undergo changes to the cytoskeleton and release granules. The platelet-specific integrin, α IIb β 3, becomes active and binds fibrinogen, bridging adjacent platelets. Platelets aggregate forming a platelet plug at the site of the injury. (B) Blood coagulation and the securing of the platelet plug. Tissue factor (TF) is exposed at the site of the injury triggering the extrinsic coagulation cascade, resulting in thrombin production. Thrombin converts fibrinogen into insoluble fibrin which secures the platelet plug in place. Blood hyperviscosity and high levels of IgM are likely to interfere with these hemostatic pathways, and potentially underpin bleeding events. GPVI, glycoprotein VI; IgM, immunoglobulin M.

let adhesion receptors due to changes in megakaryocyte maturity could disrupt efficient thrombin generation at the platelet surface.

Bleeding has been observed in WM patients who do not have hyperviscosity, thrombocytopenia, cryoglobulinemia or AVWS,^{30,82} implying that there are other potential causes of bleeding in WM. It is possible that many WM patients do not display chronic bleeding symptoms but possess an underlying platelet lesion. When these patients experience vascular and hemostatic challenges such as surgery or trauma, or when the platelet lesion is present in combination with one or more common bleeding causes as outlined above, the platelet lesion can become more evident and unexplained bleeding complications ensue.^{83,84} Studies specifically evaluating platelet function in WM would help identify patients with reduced hemostatic capacity and enhanced bleeding risk, and this information could aid in clinical decisions regarding therapeutic approaches.

Standard WM Therapies May Accentuate the Bleeding Phenotype

WM is an incurable disease where the treatments aim to alleviate the symptoms and achieve prolonged remissions.^{1–3}

Treatment decisions in this regard are generally based on the symptoms, diagnostic laboratory profile and the availability of drugs and clinical trials.⁸⁵ For asymptomatic patients, a “wait and watch” approach is routinely implemented.³ Options for treatment of symptomatic WM patients include alkylating chemotherapy agents (bendamustine, cyclophosphamide), proteasome inhibitors (bortezomib, carfilzomib, ixazomib), or the first-generation BTK inhibitor (ibrutinib), alone or in combination with rituximab. Newer therapeutic options include new, irreversible and, more selective BTK inhibitors (acalabrutinib, zanubrutinib) administered as a monotherapy, and emerging options include BCL2 antagonists (venetoclax).⁸⁵ Many of these treatments carry an attendant bleeding risk, which can be enhanced in WM patients and will be discussed in this context below.

Rituximab

Rituximab is an anti-CD20 monoclonal antibody that specifically causes B cell depletion and thus acts to reduce IgM production.⁸⁶ Although rituximab is commonly used in combination with chemotherapy agents, if used as monotherapy, it can be associated with >25% rises in IgM (an IgM flare), which can exacerbate hyperviscosity-related bleeding symptoms.⁸⁷ This risk is reduced when used in combination with other drugs. Alternatively, rituximab can be associated with acute thrombocytopenia, linked with cytokine release syndrome and complement activation.⁸⁸

Alkylating Chemotherapies

Alkylating chemotherapies, such as bendamustine and cyclophosphamide, have been used effectively as a frontline therapy in WM to kill rapidly dividing cells.⁸⁹ However, hematopoietic stem cells and their progenitor lineages are also sensitive to these therapies, resulting in cytopenias, particularly thrombocytopenia, and increasing the risk of bleeding.

BTK Inhibitors

The B cell receptor (BCR) signaling pathway is a central determinant of B cell fate and function. This pathway is activated in WM, particularly in patients bearing the Myd88 mutation.¹⁴ When an antigen binds to the BCR, this triggers BCR clustering and initiation of signal transduction via phosphorylation of BCR cytoplasmic tyrosine-based activation motifs (ITAMs). ITAM clustering enables recruitment of Src-family kinases, which serve to phosphorylate Syk and activate phosphoinositide 3-kinase (PI3K) δ . PI3K δ mediates the conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-triphosphate, which engages BTK. BTK phosphorylates phospholipase C (PLC) γ 2, activating NF- κ B, NF of activated T cells, and mitogen-activated protein (MAP) kinase pathways (**► Fig. 3**). These are all key elements of survival, development and cell proliferation pathways.

The clinical use of BTK inhibitors for the treatment of B cell malignancies has grown remarkably, resulting in improved outcomes. At present, three different covalent irreversible BTK inhibitors are approved for clinical use. These inhibitors (ibrutinib, zanubrutinib and acalabrutinib) all bind cysteine 481

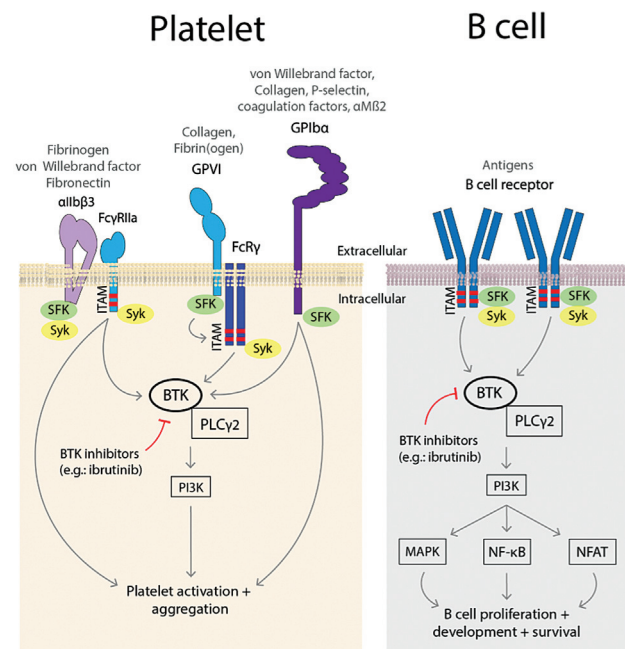


Fig. 3 Platelets and B lymphocytes utilize common signaling pathways. BTK is differentially involved in the downstream signaling pathways triggered by ligand engagement of the major platelet adhesion/signaling receptors (α IIb β 3, GPVI, and GPIb α of the GPIb-IX-V complex) and the BCR. In receptors with ITAMs, following ligand binding and receptor clustering, phosphorylation of the cytoplasmic ITAMs and recruitment of SFKs ensue, resulting in phosphorylation of Syk and activation of PI3K. PI3K mediates the conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-triphosphate, which engages BTK, resulting in phosphorylation of PLC γ . In platelets, this leads to activation and aggregation. In B cells, activation of MAPKs, NF- κ B, and NFAT leads to B cell proliferation, development, and survival. BCR and GPVI ITAM signaling are more reliant on the BTK pathway compared with GPIb α and α IIb β 3, thus signaling downstream of these receptors is more sensitive to BTK inhibition. BCR, B cell receptor; BTK, Bruton's tyrosine kinase; GP, glycoprotein; ITAM, immunoreceptor tyrosine-based activation motif; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor κ B; NFAT, nuclear factor of activated T cells; PI3K, phosphoinositide 3-kinase; PLC γ , phospholipase C γ ; SFK, Src-family kinases.

within the ATP-binding pocket of BTK with different avidities and selectivities, and serve to inhibit the phosphorylation of downstream kinases in the BCR signaling pathway, blocking B cell activation. As many as 20 new BTK inhibitors are under development.⁹⁰ Nonetheless, bleeding remains a significant side effect that is associated with the use of these therapies.⁹¹

Platelet activation requires intra-platelet signaling, triggered by platelet receptor–ligand interactions, leading to enhanced platelet aggregation. Several of these pathways involve activation of BTK^{92,93} (**► Fig. 3**), which is important in receptor signaling. Importantly there is redundancy in this pathway, as studies of genetically engineered mice or patients with X-linked agammaglobulinemia with loss-of-function mutations to the *BTK* gene did not reveal any bleeding propensity, most likely due to compensatory signaling by Tec and other kinases.⁹⁴ BTK inhibitors interfere with BTK activity by irreversibly and covalently binding to the kinase domain,⁹⁵ preventing autophosphorylation of BTK

Table 3 Inhibited kinases and adverse events associated with various Bruton's tyrosine kinase inhibitors

BTK inhibitor	Number of studies evaluated ¹⁴²	Total number of patients ¹⁴²	Type of hematological malignancies included ¹⁴²	Inhibited kinases: IC ₅₀ ± SD (nM) ¹⁴³	% Adverse events: grade 1–2 (≥ grade 3) ¹⁴²
Ibrutinib	12	1,263	TN + R/R CLL/SLL with P53 aberrations or del(17)p TN + pretreated WM R/R MCL Pretreated GvHD	BTK: 1.5 ± 0.2 TEC: 10 ± 12 ITK: 4.9 ± 1.2 TXK: 2.0 ± 0.3 EGFR: 5.3 ± 1.3	Bleeding: 35 (4.4) ¹⁴⁴ AF: 12.4 (5.9) Hypertension: 19.6 (15.6) Rash: 15.8 (1.1) Diarrhea: 47.3 (3.8)
Acalabrutinib	9	937	TN + R/R + ibrutinib-intolerant CLL/SLL TN + R/R WM R/R MCL	BTK: 5.1 ± 1.0 TEC: 126 ± 11 ITK: >1,000 TXK: 368 ± 141 EGFR: >1,000	Bleeding: 40.7 (2.5) AF: 4.3 (1.1) Hypertension: 8.6 (3.9) Rash: 16.1 (0.3) Diarrhea: 37.7 (1.9)
Zanubrutinib	6	756	TN + R/R CLL/SLL with del(17)p TN + R/R + symptomatic WM R/R MCL	BTK: 0.5 ± 0.0 TEC: 44 ± 19 ITK: 50 ± 5 TXK: 2.2 ± 0.6 EGFR: 21 ± 1	Bleeding: 39.2 (3.9) AF: 2.0 (0.7) Hypertension: 10.9 (2.8) Rash: 17.7 (0) Diarrhea: 20.8 (1.4)
Tirabrutinib	5	178	R/R CLL TN + R/R WM R/R MCL R/R B cell malignancies or NHLs	BTK: 5.6 ± 1.0 TEC: 77 ± 7 ITK: >1,000 TXK: 116 ± 35 EGFR: >1,000	Bleeding: 17.8 (1.8) AF: 0 (0) Hypertension: 0 (0) Rash: 24.7 (3.7) Diarrhea: 19.8 (1.1)

Abbreviations: AF, atrial fibrillation; BTK, Bruton's tyrosine kinase; CLL, chronic lymphocytic leukemia; EGFR, epidermal growth factor receptor; GvHD, graft-versus-host disease; ITK, interleukin-2-inducible T cell kinase; MCL, mantle cell lymphoma; NHL, non-Hodgkin lymphoma; R/R, relapsed/refractory; SD, standard deviation; SLL, small lymphocytic lymphoma; TEC, tyrosine kinase expressed in hepatocellular carcinoma; TN, treatment-naïve; TXK, T and X cell expressed kinase; WM, Waldenström macroglobulinemia.

and phosphorylation of PLC γ 2 and MAP kinases. Ibrutinib is less specific for BTK and can inhibit other tyrosine kinases such as TEC (tyrosine kinase expressed in hepatocellular carcinoma), ITK (interleukin-2-inducible T cell kinase), TXK (T and X cell expressed kinase), and EGFR (epidermal growth factor receptor), while the second-generation therapeutics zanubrutinib and acalabrutinib are more specific to BTK (►Table 3). As BTK is critical for BCR signaling, there is a level of specificity achieved by targeting BTK. However, because of the broad kinase target spectrum of most tyrosine kinase inhibitors, they inevitably have off-target adverse events (►Table 3). Platelets rely on tyrosine kinase activity for their activation. BTK is involved in platelet signaling pathways, mediated by GPVI, CLEC-2, GPIb, and α IIb β 3, that enable platelet adhesion in flowing blood.⁹⁶ Platelet function has been shown to be inhibited in patients being treated with ibrutinib,^{97,98} as well as several other tyrosine kinase inhibitors.⁹⁹ Platelets from ibrutinib- but not zanubrutinib-treated patients showed reduced levels of GPIb-IX-V and α IIb β 3 and an ablation of platelet aggregate formation.¹⁰⁰

Consistent with an off-target effect of BTK inhibitors on platelets, a meta-analysis by Brown and colleagues found that approximately 40% of patients with B cell malignancies receiving ibrutinib experienced bleeding, with 4.4% experiencing major hemorrhage (> grade III) (►Table 3).¹⁰¹ Reports of bleeding in WM patients as a result of ibrutinib treatment were slightly lower than calculated for all B cell malignancy patients,⁹⁶ where approximately 23% of WM patients on ibrutinib experienced bleeding (►Table 4). While differences in study design and definition of what constitutes a major or

minor bleed may account for some of the disparity, it is likely that differences in disease pathogenesis may also contribute to incidence of bleeding across these different malignancies. Ibrutinib discontinuation reverses major toxicities observed in WM patients (bleeding, GI toxicity), despite causing IgM rebound in 73% and withdrawal symptoms (fever, body aches, night sweats, arthralgia, headaches) in 19%.^{102,103}

Antiplatelet and Anticoagulant Therapies

WM affects an older demographic, many of whom have existing comorbidities. Treatments of these comorbidities can include antiplatelet or anticoagulant drugs, such as aspirin, clopidogrel, warfarin or direct oral anticoagulants, which carry an attendant bleeding risk. Treatment of WM patients with antiplatelet or anticoagulant therapies in combination with ibrutinib is common because of a significantly increased risk (approximately 10%) of atrial fibrillation with ibrutinib.^{24,104–110} The use of these therapeutics concomitantly elevates the bleeding risk. In one study of B cell malignancy patients, major bleeding occurred in 3.7% of patients receiving ibrutinib monotherapy compared with 5.1% receiving ibrutinib in combination with antiplatelet reagents or anticoagulants.¹⁰¹

Taken together, WM treatments increase bleeding risk, and treatments for WM comorbidities can enhance this risk. Therefore, it is important to strengthen our understanding of the molecular basis underlying the WM bleeding phenotype to accurately estimate bleeding risk, to adjust clinical management plans accordingly, minimize bleeding potential and improve patient quality of life.

Table 4 Reports of bleeding frequency in Waldenström macroglobulinemia patients treated with ibrutinib

Reference	Sample size	Number of WM patients	Study type	n, minor bleeding (% grade 1–2)	n, major bleeding (% grade 3–5)	Thrombocytopenia	Bleeding description
Abeykoon et al 2020	80	80 (100%)	Nonclinical trial (84% previously treated, 16% treatment naïve)	3 (4%) hemorrhage, 1 (1%) petechiae, 2 (3%) hematuria	1 (1%)	8 (10%)	Unspecified hemorrhage, petechiae, hematuria; intracranial hemorrhage due to CNS involvement
Ali et al 2017	45	8 (18%)	Retrospective observational cohort analysis Previously treated	14 (30.5%)	0 (0%)	NA	Bruising, epistaxis; gastrointestinal (rectal) bleeding
Dimopoulos et al 2017	31	31 (100%)	Previously treated	12 (39%) bleeding, 7 (23%) bruising	0 (0%)	5 (16%)	Grade 1–2 bleed, bruising
Dimopoulos et al 2018	75	75 (100%)	Phase 3 trial of ibrutinib plus rituximab	51% in ibrutinib + rituximab cf. 31% placebo + rituximab	4% incidence in each arm	0 (0%)	Unspecified low-grade bleeding; unspecified major hemorrhage
Treon et al 2018	30	30 (100%)	Treatment-naïve	2 (7%) bruising, 1 (3%) postprocedural hemorrhage	1 (3%)	1 (3%)	Bruising, postprocedural hemorrhage; rectal bleeding
Treon et al 2015	63	63 (100%)	Previously treated	2 (3%)	0 (0%)	9 (14%)	Epistaxis, postprocedural hemorrhage

Abbreviations: CNS, central nervous system.

Note: n: number of individuals; minor bleeding consisted of grade 1–2 bleeds; major bleeding comprised grade 3–5 bleeds.

Evaluating the Bleeding Phenotype in the Diagnostic Laboratory

Current approaches to WM patients with bleeding include coagulation testing, measurement of plasma viscosity and specialized blood and platelet testing (►Fig. 4). Further platelet quality and function tests utilizing research tools are emerging (►Fig. 4). However, these tests present several challenges which will be discussed below.

Laboratory Tests Assessing Blood Coagulation

Coagulation assays measuring prothrombin time (PT)/international normalized ratio and activated partial thromboplastin time (aPTT) are routinely performed.¹⁵ It should be noted that these tests are influenced by several preanalytical variables which can result in considerable intra- and inter-laboratory variation.¹¹¹ Variables include phlebotomy technique, anticoagulant volume based on patient hematocrit, sample mixing and centrifugation, sample transport conditions, delays in transport (over 4 hours), patient age, gender (females have increased levels of certain coagulation factors and antithrombin, and reduced Protein S), physiological states (postsurgery, pregnancy), and drugs (anticoagulants, antiplatelets, anti-inflammatories).

VWF antigen and cofactor binding assays have value in the appropriate clinical context (bleeding phenotype).¹⁵ VWF antigen assays measure VWF levels using a monoclonal antibody in a sandwich-based enzyme-linked immunosorbent assay (ELISA). The original VWF ristocetin cofactor binding assay (VWF:RCo) remains the gold standard method to measure VWF activity in plasma, by evaluating donor platelet agglutination following ristocetin-mediated VWF unfolding and binding to platelet-GPIIb α , using light transmission aggregometry (LTA). Unfortunately, this method is insensitive at VWF levels below 20 U/dL, and subject to variation based on the source of ristocetin, variation in donor platelets, and the presence of VWF A1 domain mutations resulting in poor ristocetin-VWF binding.¹¹² Newer versions of the VWF:RCo assay address sensitivity limitations, for example through a chemiluminescence-based method which directly evaluates VWF binding to magnetic particles coated with recombinant GPIIb α in an active configuration (removing the requirement for ristocetin). Additionally, the VWF collagen-binding assay analyses VWF multimers by measuring the preferential binding of high-molecular-weight VWF multimers to collagen, using an ELISA.¹¹³ This assay has been shown to be more sensitive, reproducible, and less variable than the VWF:RCo assay, improving discrimination between functional and nonfunction VWF.¹¹⁴ VWF multimer analysis by gel electrophoresis is clinically informative but challenging to perform and not widely available as a diagnostic assay.

Thrombin generation assays measure the rate and “hemostatic potential” of plasma for thrombin generation, via the cleavage of a quenched synthetic fluorogenic or chromogenic substrate. Assays require calibration, and although several commercial kits exist, limitations including a lack of standardization and reference values across laboratories

have been highlighted and are being addressed.^{115,116} Thrombin generation assays are becoming more prevalent, but will require additional clinical trials with well-defined endpoints to fully determine utility. To date, these assays have been used to monitor anticoagulant or antiplatelet therapy,¹¹⁷ and abnormal measurements have been associated with bleeding in patients with rare inherited coagulation disorders such as hemophilia¹¹⁸ and the risk of venous thromboembolism recurrence.¹¹⁹ Capturing the contribution of blood cells to thrombin potential, however, remains a clear gap in clinical applications of this assay.¹²⁰ Platelets accelerate the initiation of thrombin production via provision of membranes bearing phosphatidylserine and receptors that interact with coagulation proteins (GPIIb-IX-V complex, GPVI and α IIb β 3 amongst others),⁷² as well as the release of granule contents. Platelets, erythrocytes, leukocytes and the endothelium are all likely to modulate thrombin potential in vivo. Further, in the setting of pathologies with high paraprotein levels such as WM, the degree to which high levels of plasma proteins interfere with normal production of thrombin remains to be determined.

Laboratory Tests Assessing Platelet Function

Both LTA and the platelet function analyzer (PFA-100 and PFA-200)¹²¹ are established diagnostic platelet function tests. Whole blood impedance aggregometry (Multiplate) is an emerging tool,¹²² and together with the PFA-100/200, these techniques enable rapid screening of platelet responses to physiological agonists (adenosine diphosphate, thrombin, collagen). These tests are influenced by the same preanalytical variables as the PT and aPTT tests and require platelet counts to be above $100 \times 10^9/L$, so are not suitable for thrombocytopenic patients. The LTA also requires a high sample volume, and elevated levels of bilirubin and lipids increase plasma turbidity and affect LTA data. The PFA-100/200 has the advantage of incorporating fluid shear stress into the assay, and so provides a readout that is more physiological, but remains dependent on an aperture closure time and does not evaluate platelet secretion defects.¹²³ All of these tests lack sensitivity to changes in receptor levels and can discriminate only certain platelet function disorders.¹²⁴

Laboratory Tests Assessing both Blood Coagulation and Platelet Function

Thromboelastography (TEG) and rotational thromboelastometry (ROTEM) are simple automated, highly sensitive, emerging viscoelastic tests that provide a global assessment of hemostasis and are widely utilized in massive transfusion and acute bleeding scenarios.¹²⁵ Parameters including time to clot initiation, rate of clot formation, clot firmness and strength and clot lysis time are quantified, and the contribution of platelets to clot parameters can also be gleaned.¹²⁶ Oncologic diseases can cause coagulopathic states that may be identifiable by TEG or ROTEM; however, more work is required to evaluate the utility of these tests for assessing hemostasis beyond surgical bleeding.¹²⁵ Further, ROTEM and TEG are not yet standardized for evaluation of

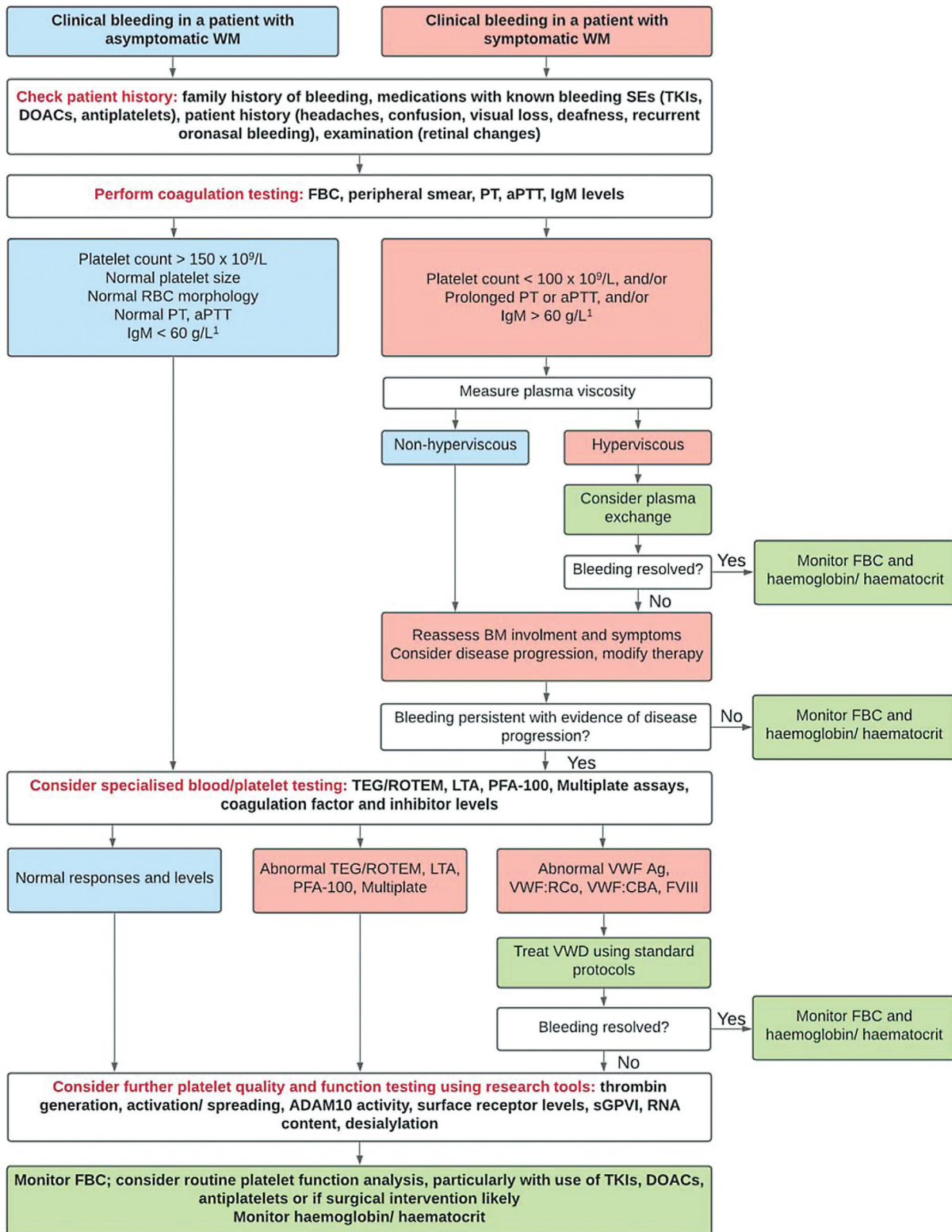


Fig. 4 Recommended pathway to evaluate bleeding phenotypes in Waldenström macroglobulinemia, with treatment options. Blue: asymptomatic WM; red: symptomatic WM; green: therapy recommendations¹ (Castillo et al 2019). ADAM10, a disintegrin and metalloproteinase 10; Ag, antigen; aPTT, activated partial thromboplastin time; BM, bone marrow; CBA, collagen binding assay; DOAC, direct oral anticoagulant; FBC, full blood count; FVIII, factor VIII; IgM, immunoglobulin M; LTA, light transmission aggregometry; PFA, platelet function analyzer; PT, prothrombin time; RBC, red blood cell; R:Co: ristocetin cofactor assay; ROTEM, rotational thromboelastometry; SE, side effect; sGPVI, soluble glycoprotein VI; TEG, thromboelastography; TKI, tyrosine kinase inhibitor; VWD, von Willebrand disease; VWF, von Willebrand factor.

thrombocytopenic samples, which may be relevant to patients with hematological diseases, particularly those receiving treatment. There are no data to date specifically evaluating whole blood clotting using TEG or ROTEM in WM.

In summary, evaluating platelet function and coagulation in WM patients presents several challenges. First, none of the standardized platelet functional assays can evaluate platelets in thrombocytopenic WM patients. Second, evaluation of platelet receptor levels, which govern platelet function and are often diminished in hematological malignancies,⁸⁴ is not routinely evaluated by flow cytometry. This is due to lack of standardized routine protocols for platelet flow cytometry and lack of clear understanding of its clinical implications. Finally, none of these assays evaluate platelet function under conditions that replicate vascular shear rates found in flowing blood.

Future Directions

In the research laboratory setting, several additional techniques can be used to evaluate platelet function. The bone marrow microenvironment is disrupted in WM, contributing to the initiation and propagation of WM lymphoplasmacytoid cells and likely disturbing megakaryocyte maturation and platelet production. Therefore, megakaryocyte and platelet flow cytometry can be used to evaluate the levels of receptors (α IIb β 3, GPIIb α , GPVI), as well as extent of platelet activation (P-selectin, active α IIb β 3) on circulating platelets. Platelet flow cytometry has the advantage of remaining viable even when the platelet count is extremely low, and useful data can be gathered on chemically fixed samples, meaning samples can be stored for short periods. Levels of shed receptor ectodomains can be quantified by ELISA¹²⁷ and microfluidic systems can be used to quantify platelet adhesion to immobilized substrates (collagen, fibrinogen, fibrin) under shear, providing direct readouts of platelet function under conditions that mimic a range of vascular rheological conditions.^{128–130}

The generation of thrombin and fibrin as part of the coagulation pathway plays a crucial role in the securing of platelet aggregates across sites of blood vessel injury. Insufficient levels or defective coagulation factors can lead to formation of an unstable thrombus. Whole blood coagulation, evaluated by ROTEM or TEG, assesses coagulation throughout all phases of clot formation, triggered via extrinsic or intrinsic coagulation pathways. While these parameters have not been previously evaluated in WM patients, it would be of interest to compare samples from newly diagnosed individuals with those on different therapies and ascertain whether a platelet defect can be determined. Additionally, it might be of value to evaluate the effect of WM plasma, particularly from patients with high levels of IgM and hyperviscosity, in mixing experiments using healthy donor plasma-depleted blood to assess the effect of elevated IgM on whole blood coagulation.

Besides enhancing the availability of these tests, it will also be important to define the situations in which these tests will be most helpful. As information on the platelet lesion and more broadly the hemostatic defects in WM emerge, existing diagnostic tools such as ROTEM or TEG may become

more widely applied. While platelet flow cytometry and testing for platelet activation markers using ELISAs remain distant from the diagnostic laboratory, these additional new approaches can potentially be developed and incorporated into diagnostic algorithms and may help guide therapy decisions in patients with compromised hemostatic pathways.

Finally, evaluation of WM-related genes within the megakaryocytic progenitor populations has not yet been explored. As platelets and megakaryocytes express both Myd88 and CXCR4,¹³¹ the prevalence of WM-related gene mutations in the megakaryocytes and megakaryocyte progenitor populations should be evaluated. Platelet α -granules possess functional membrane-bound CXCR4 as well as SDF-1. Activating CXCR4 mutations in megakaryocytes, like the CXCR4^{S338X} mutation, prevents CXCR4 internalization by platelets, following SDF-1 stimulation.²⁵ This may impair platelet aggregation, thromboxane A₂ production and dense granule secretion,¹³² and could contribute to a bleeding phenotype.

Concluding Remarks

With the advent of BTK inhibitors as efficacious and routine therapies for WM, it is important that patients are evaluated and monitored continually for bleeding propensity. By applying new approaches complemented by sensitive research-based techniques (flow cytometry, thrombin generation assays, platelet spreading assays), in combination with megakaryocyte-specific genetic approaches to evaluate common WM mutations, it may be possible to stratify WM patients for bleeding risk based on platelet functionality. One or more of these techniques could be integrated into routine testing for WM patients at diagnosis and then during treatment, particularly in patients who present with an elevated risk for bleeding.

Author Contributions

S.A.B., D.T., and E.E.G. planned and drafted the manuscript. All authors contributed to the review of the manuscript. Images were created using Smart Servier (<https://smart.servier.com/>).

Conflict of Interest

None declared.

Acknowledgments

The authors thank Dr. Philip J Crispin for helpful comments. This work was supported by the National Health and Medical Research Council of Australia, the Australian Research Council, the National Blood Authority of Australia, and the Australian Capital Territory Department of Health.

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