Laboratory Investigations and Findings: Hematological Abnormalities, Biochemical Investigations, Free Light and Heavy Chains

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17.1 Introduction

Waldenström macroglobulinemia (WM) was first described by Jan Gosta Waldenström in 1944 when he reported the cases of two patients presenting with lymphadenopathy, infiltration of the bone marrow by lymphoid cells resulting in anemia and thrombocytopenia, and various symptoms (such as oronasal bleeding, elevated erythrocyte sedimentation rate, and high serum viscosity) which he attributed to the presence of an abnormally elevated high-molecular-weight serum protein; later to be demonstrated as a monoclonal pentameric IgM.

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Interestingly, decades later, the finding that IgM is a hallmark of WM remains in its diagnosis criteria [1]. The IgM monoclonal protein is also still used for prognostication [2], monitoring of response to therapy, and so on, despite many flaws in its measurement [3, 4].

Herein, we discuss the diagnostic workup of WM, along with the various attempts to either replace IgM or develop new tools to improve IgM serum measurement.

17.2 Hematological Abnormalities

As a result of bone marrow infiltration and the presence of large quantity of serum IgM, patients are often diagnosed with cytopenias, essentially anemia in WM. A complete blood count is, thus, mandatory at diagnosis.

Anemia Anemia is observed in more than half of WM patients. Anemia is usually normocytic, normochromic, nonregenerative. The mean corpuscular volume can be falsely macrocytic related to pseudoagglutination due to the excess of IgM coating the erythrocyte surfaces, also described as Rouleaux formation on blood smear.

The presence of anemia and importantly its relation to WM is important to recognize, as it is the most common indication for initiation of treatment. As treatment decision may rely on it, exclusion of other causes of anemia is critical; indeed, one is expecting to treat WM based on anemia when secondary to the presence of tumor cells in the bone marrow.

Anemia is often multifactorial; the most common causes being apoptosis of erythroid precursors mediated by Fas/FasL interactions, along with bone marrow involvement resulting in inadequate erythropoiesis, and anemia of chronic disease. The erythroid precursors indeed express the Fas receptor as a physiological mechanism of regulation of the erythroblast population in the bone marrow, but this death signal is exacerbated in WM due to the expression of FasL on WM tumor cells. An increased plasma viscosity has also been shown responsible for inappropriate erythropoietin formation by renal peritubular cells, resulting in lack of maturation and differentiation of erythroid colonies and erythroid progenitors in the bone marrow [5]. The level of hemoglobin is also partly decreased related to dilution induced by the presence of high concentrations of IgM monoclonal immunoglobulin in the serum—especially above 30 g/L. Interestingly, a falsely elevated hemoglobin can be found as well, due to abnormal reactions between reagents and monoclonal Ig present in excess [6].

Considering that the median age of WM is above 65 years old, other causes of anemia are frequently encountered in patients with WM, which may require specific therapy but rarely treatment of WM per se. For example, many patients display iron or vitamin deficiency. Interestingly, iron deficiency anemia if often refractory to oral treatment in WM, but not to intravenous iron repletion [7]. Furthermore, WM patients may present with occult hyperviscosity-related gastrointestinal bleeding, which should be considered and eventually explored in these patients who may

develop second malignancies. It is though worth noting that serum levels of ferritin can be falsely low due to interference related to the IgM monoclonal protein [8]. It has also been demonstrated that lymphoplasmatic cells produce hepcidin, which could contribute to anemia in WM [9]. Hepcidin indeed regulates iron metabolism by inhibiting ferroportin, which leads to sequestration of iron in enterocytes, monocytes, and macrophages. Therefore, since absorbed iron cannot be released (unless given intravenously), it is not available for erythroblasts; this situation can also be found in chronic inflammation.

Anemia can, as well, be a consequence of myelosuppressive chemotherapy. Late development of anemia may indicate development of myelodysplasia after treatment [10].

Hemolytic anemia can also occur in some patients with WM, either warm or cold agglutinin hemolytic anemia. If the reticulocytes count shows that anemia is regenerative, a test for cold agglutinins and an erythrocyte Coombs test should, thus, be performed. The Coombs test is positive in about 10 % of patients, but less than 5 % of patients develop significant hemolysis [11]. Warm autoimmune hemolytic anemia is mediated by IgG autoantibodies, resulting from an activation of autoreactive T cells stimulated by the tumor cells and is, thus, not directly related to tumor cells or the monoclonal IgM component. Warm autoimmune hemolytic anemia is likely to be primarily extravascular [7], mainly of splenic type. However, the monoclonal IgM may present with cold agglutinin activity, resulting in chronic immune hemolytic anemia with acute phases of intravascular hemolytic anemia [12]. This disorder occurs in less than 10 % of WM patients. In most cases, it is associated with cold agglutinin titers $> 1{:}1000$; the monoclonal component is usually an IgMk and it reacts most commonly with I/i antigens, with complement fixation and activation. Mild chronic hemolytic anemia can be exacerbated after cold exposure but is rarely responsible for a hemoglobin decrease below 70 g/L. Clinical manifestations can include Raynaud syndrome, acrocyanosis, and livedo reticularis. It is important to identify this hemolytic syndrome, as patients may benefit from a treatment of hemolytic syndrome and only get treated for WM later on if the primary proposed treatment failed.

Thrombocytopenia Thrombocytopenia $< 100 \times 10^9$/L is not infrequent, seen in about 15 % of patients and mainly related to bone marrow involvement; while a thrombocytopenia $< 50 \times 10^9$/L is observed in less than 5 % of patients [11]. Of note, immune thrombocytopenia (ITP) is a frequent etiology in patients with WM, an important condition to diagnose since these patients may also benefit from a specific treatment prior to be treated for the underlying WM [8]. Other causes of thrombocytopenia include hypersplenism or myelosuppressive chemotherapy. Similarly to anemia, late development of thrombocytopenia may be related to development of myelodysplasia secondary to treatment [10]. Severe thrombocytopenia may increase the risk of bleeding associated with hyperviscosity, particularly when hyperviscosity presents with acquired von Willebrand syndrome [8].

White Blood Cells In most cases, the white blood cell count is normal. Neutropenia is rare, with less than 5 % of patients presenting with neutropenia $< 1 \times 10^9$/L. However, a lymphocytosis can be observed that rarely exceed 15×10^9/L in about 10 % of patients [11]. Even if not detected in standard white blood cell count, a circulating clonal population can be detected by flow cytometry immunophenotyping in many more patients. This is the source of several research projects to try and understand the significance and utility of such identification. Performing a flow cytometry immunophenotyping to seek for circulating WM tumor cells is not part of the recommended diagnosis check up so far.

Recommendation

To perform a CBC count with smear, plus reticulocyte count and hemolytic workup at diagnosis of WM.

If hemolysis is suspected (cold or warm hemolysis or ITP), a workup, including peripheral smear, reticulocyte count, LDH, bilirubin, haptoglobin, and Coombs test, should be performed. Hemolysis is, however, most often extravascular. Testing for cold agglutinins, and direct and indirect Coombs antibody testing if cold agglutinins are negative, is advised as part of the workup of hemolytic anemia.

17.3 Bone Marrow Investigations

Characterization of the bone marrow infiltration is essential in WM and should be performed in all symptomatic patients to confirm the diagnosis of WM prior to start treatment. In asymptomatic patients, its value is not clearly established but an arbitrary threshold of 10 g/L of serum IgM has been proposed to consider performing a bone marrow assessment [13].

A bone marrow biopsy is the recommended exam, as it allows better quantification of the infiltration and reveals the classically intratrabecular pattern of bone marrow infiltration, which may be diffuse, interstitial, or nodular. In contrast, a solely paratrabecular pattern is rare in WM and should raise suspicion of other low-grade B-cell lymphoma subtypes. A myelofibrosis with reticulin fibers can be detected in some cases. Intranuclear and cytoplasmic immunoglobulin inclusions, termed as Dutcher and Russell bodies, respectively, are frequently observed in the WM tumor lymphoplasmacytic cells. Interestingly, an increased number of mast cells close to lymphoid aggregates are also a common finding in WM, although the exact meaning of this histologic finding remains to be fully unraveled [12]. Besides the demonstration that mast cells and WM tumor cells cross talk, it is not yet known whether the tumor cells stimulate the production and aggregation of the mast cells or whether the mast cells are part of the modified bone marrow microenvironment which helps provide survival and proliferation advantage to the WM tumor cells.

If the marrow biopsy cannot be performed, a bone marrow aspiration along with bone marrow smear and flow cytometry immunophenotyping can be proposed as an adequate alternative.

Concerning the amount of infiltration, the Second International Workshop on Waldenstrom's Macroglobulinemia criteria does not require a minimum amount of bone marrow involvement to make the diagnosis of WM or recommend to start WM treatment [1]; however, the Mayo Clinic criteria require at least 10 % bone marrow involvement to differentiate IgM MGUS from WM [14].

The bone marrow infiltration is characterized by a lymphoplasmacytic clone constituted of a mixture (also called a continuum) of clonal B lymphocytes, plasma cells, and plasmacytoid/lymphoplasmacytic lymphocytes [15].

Immunophenotypic studies (flow cytometry and/or immunohistochemistry) should always be performed to confirm the diagnosis, showing the following profile: sIgM+, CD19+, CD20+, CD22+, and CD79+. The lymphoid component typically lacks CD10 (unlike follicular lymphoma) and can show some degree of CD5 expression, however, weak, clearly not the kind of strong CD5 expression observed in chronic lymphocytic leukemia/small lymphocytic lymphoma or mantle cell lymphoma, similar to CD23. The plasmacytic component expresses the same Ig light chain as the lymphocytic component, is positive for CD38 or CD138, and shows normal expression of B-cell-associated antigens (CD19, CD20, PAX5, etc.), which allows differential diagnosis from IgM secreting myeloma plasma cells. WM cells have also been shown to be $CD25^+$, $CD27^+$, $CD75^-$, $FMC7^+$, $Bcl2^+$, and $Bcl6^+$ [1, 16, 17], although the latter panel is not required in routine practice for the diagnosis of WM. Characteristics of the immunophenotype in WM is summarized in Table 17.1.

To note, tissue biopsy is not recommended in WM since the primary recommended biopsy for the diagnosis is the bone marrow biopsy; however, a

Table 17.1 Characteristic immunophenotype in WM

Markers	Proteins	%	Remarks
Clonality	Surface IgM	100	High level of expression
	Light chain	100	Kappa > Lambda
B cell phenotype	CD19/CD20	100	
	CD22	100	Low intensity
	CD79	100	
	FMC7	>85	Low intensity
	CD5/CD19	10–27	Low intensity
	CD23	14–33	Low intensity
	CD10	<10	
Plasma cell phenotype	CD38	0–60	Important variability
	CD138	<10	
Memory lymphocytes	CD27	42	
Other	CD25	90–100	Homogeneous expression
	CD103	0	Always negative
	CD43	<20	Low level of expression
	CD11c	0–31	Low level of expression
	CD52	100	Always positive

tissue biopsy is recommended in all patients with suspected histological transformation. Detailed pathological assessment should include assessment of Epstein–Barr virus (EBV) [16].

Cytogenetics and Molecular Biology The normal equivalent of the WM malignant cell is believed to be a post-germinal center B cell that has undergone somatic hypermutation but transforms before isotype class switching and terminal differentiation to plasma cells.

The molecular study of IgVH sequence, thus, reveals somatic hypermutation without intraclonal diversity (no switch). There is a biased use of the heavy chain gene repertoire with an overrepresentation of VH3 and JH4 families.

Cytogenetic analysis is required for the routine diagnostic assessment of WM patients [16]. Conventional karyotyping without specific stimulation has limited applicability in WM, as it is difficult to obtain tumor metaphases because of the low rate of cell proliferation. The absence of IgH involving translocation can be used in difficult cases for differential diagnosis; indeed, it was shown that although WM never harbors IgH translocations, multiple myeloma producing IgM frequently has this abnormality, particularly the t(11;14)(q13;q32) [18].

Several frequent cytogenetic abnormalities, such as del(6q) and trisomy 4, can be explored but are better detected by FISH analysis than conventional karyotyping.

Fluorescence in situ hybridization (FISH) improved cytogenetic studies, even though it only looks at prespecified probes. Although not completely specific for WM, deletion of the long arm of chromosome 6 encompassing del(6)(q21) is the most common genetic abnormality detected by FISH, being detected in 40–50 % of patients [19, 20]. This genetic abnormality is rarely seen in other lymphoproliferative or plasmaproliferative malignancies, but can be found in IgM gammopathy of unknown significance (MGUS) [21]. Several candidate tumor suppressor genes in this region are under study, in order to provide an explanation for the frequency of this abnormality. Among these genes is BLIMP-1, which regulates the transition of mature B cells to terminally differentiated plasma cells and is, thus, a major candidate [22]. Deletion of TP53 occurs in a minority of patients and appears to confer a poor outcome [23]. The deleted region of TP53 varies a lot between patients; a minimal deleted region has been described by Poulain et al. [24].

Other cytogenetic abnormalities can be found in WM: trisomy 4 is reported in up to 20 % of cases, and trisomy 5, monosomy 8, and deletion of the long arm of chromosome 20 have also been reported, along with del17p and del13q [20].

MYD88 L265P Mutation, A Molecular Signature The recent finding of MYD88 L265P mutation in about 90 % of cases of WM made it a major marker of the disease [25].

Our understanding of the pathogenesis of WM has improved considerably since this discovery. MYD88 (myeloid differentiation primary response 88) indeed plays a significant role in Toll-Like Receptor (TLR) and interleukin-1 receptor signaling.

The acquired gain-of-function mutation of MYD88 gene on the short arm of chromosome 3, resulting in the change in the amino acid leucine to proline at position 265 (L265P), activates downstream signaling of the transcription protein complex NF-kB (nuclear factor kappa-light chain-enhancer of activated B cells), thereby promoting WM cell growth and survival [26]. MYD88L265P also activates Bruton's tyrosine kinase (BTK), another crucial enzyme involved in NF-kB regulation [27].

This single point somatic mutation, detected by PCR, is also present in 87 % of IgM MGUS, suggesting that this may be an early oncogenic event [28]. It remains, however, unclear whether MYD88 is a driver mutation for the transformation of MGUS to WM or solely a marker of MGUS with predisposition to progress to WM [29, 30].

The MYD88 mutation can also be useful in distinguishing WM from splenic marginal-zone lymphoma and IgM MM, as it is present in more than 90 % of WM patients but is uncommon in IgM MM and found in only 5–7 % of patients with marginal-zone lymphoma [25, 31, 32].

Its presence has been associated with early onset of WM presentation and a greater bone marrow involvement compared to MYD88 WT [28]. It has been described that WM patients with the absence of MYD88 mutation were characterized with a female predominance, a splenomegaly, a gain of chromosome 3, and CD27 expression [32].

Inhibition of MYD88 signaling has been shown to induce cytotoxicity and inhibit cell growth of cell lines issued from patients with WM [32].

All of these recent data confirmed the value of screening for the MYD8 L265P mutation in the routine diagnostic setting, and promising novel treatments resulting from its discovery are under study [28, 33].

Mutations of CXCR4 at the carboxyl (C) terminal domain have also been observed in approximately 30 % of patients with WM and 20 % of IgM MGUS patients [28].

WM cells are known to express high levels of chemokine and adhesion receptors. The C–X–C chemokine receptor type 4 (CXCR4), via its interaction with its ligand, stromal derived factor-1 (SDF-1/CXCL-12), is believed to play a critical role, particularly in modulating clonal lymphoplasmacytic cell homing to the bone marrow niches [26].

CXCR4 mutations in WM are the first reported acquired somatic mutations of CXCR4 in any human cancer. They have been described in patients with WHIM syndrome—a rare, congenital immunodeficiency disease characterized by Warts, Hypogammaglobulinemia, recurrent Infections and bone marrow Myelokathexis resulting in chronic neutropenia. In WM, two types of CXCR4 mutations have been observed in equal proportions: the frame shift mutations ($CXCR4^{WHIM/FS}$) and nonsense mutations ($CXCR4^{WHIM/NS}$) [26].

Ninety-eight percent of CXCR4 mutations coexist with MYD88 L265P mutations. While the MYD88 L265P mutation has a role in WM cell proliferation and survival, CXCR4 mutation has been implicated in tumor progression and drug

resistance. CXCR4 mutation indeed mediates drug resistance to mTOR, PI3K, and BTK inhibitors by blocking CXCR4 receptor internalization and leading to a persistently activated CXCR4, which prolongs and enhances activation of AKT1 and MAPK1 [34]. $CXCR4^{WHIM/NS}$ mutations have been reported to be associated with more aggressive disease features such as greater marrow involvement and higher serum IgM levels with increased risk of hyperviscosity syndrome, but without impact on overall survival [26].

Anti-CXCR4 drug, plerixafor, has been shown to successfully inhibit this pathway.

As multiple CXCR4 mutations can be found, the development of a PCR-based assay is difficult, and routine testing for these mutations is not recommended yet.

Recommendation
To perform a bone marrow biopsy accompanied by immunophenotyping. Cytogenetic is recommended, particularly to identify whether del6q is present; however, to date the identification of MYD88 L265P mutation has clearly became a hallmark of the diagnosis of WM along with histology. It seems CXCR4 is not yet to be recommended in routine check up for WM identification, as its role may be more into understanding the risk of chemoresistance.

17.4 Hemostasis

Viscosity Hyperviscosity syndrome is a classical feature of WM but occurs in less than 15 % of patients. It is related to an increased vascular resistance and blood viscosity secondary to the presence of elevated serum IgM levels. Common symptoms include headache, blurred vision, vertigo, oronasal bleeding, or hearing defects. Other manifestations can be observed, such as neurological symptoms or aggravation of congestive heart failure due to the increased blood viscosity and expanded plasma volume arising from increased osmotic pressure. Thromboembolic events can also be observed. Symptoms typically occur when the serum viscosity reaches 4–5 cP, which usually correlates with a serum IgM level > 30 g/L [12]. Although it can prove a very useful adjunct to the clinical assessment of patients, measurement of plasma viscosity is not routinely available. A high sedimentation rate is another marker for hyperviscosity.

In symptomatic patients or if IgM levels are > 30 g/L, a fundoscopic examination is recommended in order to screen for indicators of hyperviscosity, such as retinal venous engorgement with dilated and tortuous veins and focal constrictions ("venous sausaging") or flame-shaped retinal hemorrhages in the macular area [7].

If hyperviscosity syndrome is suspected, plasmapheresis and treatment of WM should be started quickly to rapidly remove IgM.

Thromboembolism Patients with WM present an increased risk of venous thromboembolism, especially during the first year following diagnosis but also up to 10 years after [35]. Unlike patients with multiple myeloma, no excess risk of

arterial thrombosis was described. There is no consensual approach concerning venous thromboembolism prophylaxis for WM patients yet; however, this prophylaxis should be considered in periods of additional risk, such as surgery [16].

Bleeding Syndrome Monoclonal IgM component may interact with circulating proteins or with proteins expressed on cell membranes. Interaction with platelets, fibrinogen, and coagulation factors (especially factors V, VII, and VIII) may cause thrombopathy and coagulation disorders, such as acquired hemophilia, acquired von Willebrand disease, or defects in fibrin formation.

Clinically, these alterations are responsible for abnormalities in bleeding and clotting times [12].

Acquired von Willebrand Syndrome (AVWS) [36] Among manifestations caused by IgM in WM, the occurrence of acquired von Willebrand syndrome (AVWS) is a rare event, characterized by bleeding related to the decrease of von Willebrand factor (VWF) activity [37]. By contrast with congenital von Willebrand disease, patients do not have any personal or family history of bleeding.

VWF is an adhesive glycoprotein produced by the vascular endothelium and megakaryocytes, which plays a key role in primary hemostasis. Multiple pathogenic mechanisms are described in AVWS, including selective VWF adsorption on tumoral cells, increased VWF proteolysis, and the presence of both neutralizing and non-neutralizing anti-VWF antibodies [38].

In a recent study including 72 patients with WM, 13 % fulfilled criteria of AVWS [37]. Patients with AVWS had higher serum IgM concentration, higher serum viscosity, and more frequent cryoglobulinemia than unaffected patients; it appeared to be unrelated to the bone marrow infiltration.

AVWS could offer protection against venous thromboembolism and can lead to sometimes severe hemorrhagic events, even though patients often present biological features without clinical manifestation [37].

Systematic screening of AVWS should be performed in patients with WM as it may have important clinical implications. If an AVWS is detected, treatment includes plasma exchanges which by reducing hyperviscosity allow a quick resolution of the VWF defect, desmopressin, or replacement therapy to cover invasive procedures [37].

Recommendation

It is recommended to identify patients at risk for hyperviscosity and, in parallel, patients that will present with bleeding disorders, particularly prior to performing biopsies.

17.5 Biochemical Investigations

Diagnostic biological workup for WM is summarized in Table 17.2.

Table 17.2 Diagnostic biological workup recommended for WM, adapted from [12]

Hematological workup	
Cell blood count Peripheral smear	Normocytic or macrocytic anemia, normochromic, reticulocyte count, thrombocytopenia Rouleaux on smear
Hemolytic workup	Elevated reticulocyte count, bilirubin, haptoglobin, LDH, Coombs, cold agglutinin
Hemostasis workup	Acquired VWD
Bone marrow	
Histological analysis	Lymphoplasmacytic cells (small lymphocytes with plasmacytoid differentiation and plasma cells)
Immunophenotype	sIg^+, $CD19^+$, $CD20^+$, $CD22^+$, $CD79^+$, $CD5^{+/-}$, $CD23^{+/-}$, $CD138^+$, $CD10^-$, $CD103^-$
Cytogenetic	6q deletion by FISH in 30–50 % of cases
Molecular biology	MYD88 L265P mutation in 90 % of cases Discussion on CXCR4 mutation to identify chemoresistance and decide for specific treatment
IgM immunoglobulin measurement	
SPEP, IFS	Monoclonal Ig difficult to detect if cryoglobulin, sampling to be done, maintained, and performed at 37°. Lower sensitivity in low level, migration defect. Monitor hypogammaglobulinemia
Proteinuria	It is possibly more important to check for albuminuria (glomerulopathy of cryoglobulinemia type, AL amyloidosis type, renal infiltration by tumor cells) and that can simply be done with urine bandelette UPEP and IFU: Bence-Jones proteinuria frequently positive but exceptionally associated to occurrence of CAST nephropathy
sFLC	Serum free light chain is increased similar to IgM to perform possibly in patients informative to sFLC and pauci informative on IgM measurement
Plasma viscosity	Elevated viscosity > 3cp: indication of treatment and plasmapheresis
Beta-2 microglobulin	Prognostic marker

LDH lactate dehydrogenase, *SPEP* serum protein electrophoresis, *IF* immunofixation, *sFLC* serum free light chain, *UPEP* urine protein electrophoresis

Serum IgM Monoclonal Protein Biochemically, IgM monoclonal protein remains the hallmark of WM; indeed, the definition of WM [1] requires the presence of a monoclonal IgM protein, irrespective of its serum level—although most of the time, this level is > 5 g/L.

The first clue to diagnosis of WM is often the identification of the presence of an excess of serum protein (elevated serum sedimentation rate and/or elevated serum protein level) suggesting an IgM monoclonal component. The presence of a monoclonal component is often characterized using serum protein electrophoresis (SPEP). An immunofixation is then recommended to demonstrate clonality and further characterize the isotype of the monoclonal component; this immunofixation

should be performed in all cases, since SPEP could miss small quantities of IgM. The IgM M-spike can sometimes migrate into the beta region rather than the gamma region. In rare cases, two M-spikes can be identified, which represent either the association of monomeric and pentameric forms of IgM or true biclonality. Class switching with corresponding IgG or IgA M-spikes is unusual.

An elevated IgM is usually associated with a reciprocal hypogammaglobulinemia in WM, made of a profound decrease in polyclonal IgM along with IgG and IgA. There is no correlation between the serum level of IgM and the percentage of bone marrow involvement or to the depth of hypogammaglobulinemia.

However, many limitations are associated with the use of this IgM marker, and development of more accurate markers is needed as discussed below.

Limitations to IgM Measurement in WM Even though IgM monoclonal protein remains the key marker in WM, many limitations can be argued against its use:

- The definition of WM [1] requires the presence of a monoclonal IgM protein, irrespective of its serum level, which means that serum IgM level is not sensitive enough to differentiate WM from Monoclonal Gammopathy of Undetermined Significance (MGUS).
- Although the presence of serum monoclonal IgM protein is the hallmark of WM, it is not a disease-specific finding. A study found that nearly 60 % of patients with IgM paraprotein-related lymphoid neoplasms had WM; other causes included CLL (20 %), marginal-zone lymphoma (7 %), follicular lymphoma (5 %), DLBCL (5 %), and MCL (3 %) [39]. Furthermore, serum IgM paraprotein level was not a reliable discriminator in the differential diagnosis.
- The measurement of IgM can be obtained by SPEP (serum protein electrophoresis) or by nephelometry, but none of these techniques allow measurement of the exact concentration of monoclonal IgM. Furthermore, a number of studies have identified discordance between the two methods, preventing direct comparison of results [40]. IgM measurement using nephelometry is not a valuable technique, not only does it not allow differentiation between clonal and non-clonal Ig but it is affected by technical problems.
- It is also difficult and unreliable to compare IgM-spike concentrations from different laboratories.
- Moreover, monoclonal Ig can be difficult to detect in some WM patients, due to the presence of cryoglobulins, because of the migration defect of the IgM protein or because low IgM concentrations can be obscured by the polyclonal background.
- In the IPSS WM score [2], IgM has an adverse prognostic impact only when >70 g/L, a rare occurrence at such a very high serum level: IgM serum level is, thus, not a sensitive prognostic marker.
- In addition, even a high IgM serum level is not a sufficient indication by itself to initiate therapy [41] because IgM does not correlate to tumor mass.

- However, although serum IgM level is not correlated to tumor mass, it is included in the response criteria for WM [42] but with considering the above limitations in its measurement.
- As IgM has a prolonged half-life (IgM clearance from the serum takes about 3 weeks) [43], it is not a sensitive marker for early response.
- Moreover, serum IgM can vary differently depending on the treatment. Serum IgM level may indeed increase following rituximab therapy, and distinction between progression and flare effect might be difficult to affirm [44], making response or progression assessment impossible in this situation. Following nucleoside analogues, response can be delayed by months, sometime a 1-year period of time, and, therefore, patients with delayed response can be miscategorized as non- or poor responders [42]. Conversely, bortezomib-containing regimens may demonstrate excellent IgM responses, while suboptimal bone marrow responses [45].

For all these reasons, there is a need to identify new surrogate markers for measurement of tumor burden in WM, especially for patients with low or high IgM levels, and extramedullary disease.

Study of Urine Proteinuria in WM There are numerous reasons for renal alteration in WM, and one must be aware of the various types of syndromes that can be encountered to properly guide the tests needed to optimally treat the renal alterations in WM, to avoid worsen the prognosis often associated to renal insufficiency.

Although Bence-Jones proteinuria [identified using urine electrophoresis (UPEP) with subsequent urine immunofixation (IFU)] is frequently present in WM patients (40–80 %), it rarely exceeds 1 g/24 h (about 3 % of cases) and is, therefore, not always routinely performed [12]. Furthermore, CAST nephropathy as a consequence of excess of Bence-Jones proteinuria is rarely seen in WM [46].

Glomerular nephropathy is more often seen in WM as a consequence of several kidney alterations, among which the most frequent are glomerulopathy of cryoglobulinemia type, AL amyloidosis type, and renal infiltration by tumor cells [47]. The diagnosis of these various types of renal alteration is performed on a kidney biopsy using histopathology tests, along with red congo staining, polarized birefringence, and immunofluorescence identification of light chain deposits for AL amyloidosis. Consequently, one would then recommend to seek for the presence of albuminuria and to quantify the importance of albuminuria, using, for example, a very simple test in routine practice, i.e., the urine bandelette.

Cryoglobulins Cryoglobulins (type I anti-IgM monoclonal component related or type II with immune complex of monoclonal IgM anti-IgG type) can occur in WM and can lead to underestimation of the serum IgM levels and increased blood viscosity. Diagnosis is confirmed by measurement of cryoglobulins using a warm

bath collection; this test should be repeated if necessary. The temperature range of cryoglobulins can be wide.

Type I cryoglobulinemia may be detected in 7–20 % of WM patients but is symptomatic in less than 5 % [12]. Due to impaired blood flow in small vessels, manifestations such as Raynaud phenomenon, acrocyanosis, arthralgias, purpura, and skin ulcers can occur [48, 49]. It is usually associated with lymphoproliferative disorders.

Type II cryoglobulinemia results from the autoantibody activity of the monoclonal IgM against the Fc portion of IgG, acting as a monoclonal rheumatoid factor [12, 48, 49]. Rheumatoid factor blood test is, thus, usually positive. The cryoprecipitating property results from the size and limited solubility of the IgM–IgG immune complex. Deposition of these immune complexes on the walls of small vessels and subsequent activation of the complement cascade can result in a systemic vasculitis—clinical features of which are purpura, arthralgias, weakness, liver involvement, renal involvement (cryoglobulinemic glomerulonephritis), peripheral neuropathy, and widespread vasculitis [12]. Type II cryoglobulinemia has been associated with the hepatitis C virus.

Dosage of antinuclear antibodies and syphilis serology can be falsely positive in case of the presence of cryoglobulins. Patients with cryoglobulinemia should be screened for hepatitis C infection [16].

If a symptomatic cryoglobulinemia is identified, plasmapheresis is indicated to rapidly remove cryoglobulins from the blood circulation.

Complement Exploration Among abnormalities in complement exploration, serum C4 levels can be found very low. Exceptionally, an acquired C1 esterase inhibitor deficiency can be observed, leading to angioedema [50].

Serum Free Light Chain Test The serum free light chain (sFLC) assay is a nephelometric measurement of serum kappa and lambda light chains that circulate as light chain monomers or dimers and that are not bound to immunoglobulin heavy chain. It can be analyzed whether using iFLC (involved FLC level), ratio kappa/lambda, or dFLC (difference between clonal and non-clonal sFLC); the most informative being iFLC, although the difference or the ratio are recommended for patients with some degree of renal insufficiency. This test has been widely used in the assessment of response in patients with multiple myeloma and other plasma cell dyscrasias and is now relatively widely available among laboratories worldwide.

It has been previously shown that the use of iFLC values accurately diagnosed patients with WM and differentiated them from patients with IgM MGUS (monoclonal gammopathy of undetermined significance) who presented with significantly lower values [51, 52]. sFLC assay was further demonstrated to be a useful and sensitive marker of tumor measurement in WM that correlates well with IgM and M-spike measurement [52]. Response according to iFLC was indeed comparable with the response obtained using SPEP or IgM measurement by nephelometry, indicating that it can be used in the future as a reliable measurement of disease response [52]. However, there are two particular interests in iFLC measurement:

first, it allowed a more rapid detection of response and progression than standard tests studying intact immunoglobulin, due to its significantly shorter half-life [53], and next, it has been proven a sensible marker to assess lower tumor burden compared with SPEP.

sFLC could also be useful in situations where SPEP is defective. For instance, a case of Waldenstrom's macroglobulinemia with type I cryoglobulinemia was reported where quantification of the paraprotein was not possible using conventional serum protein electrophoresis due to the high serum viscosity, but monitoring using serial sFLC measurements was proved successful [54].

Moreover, sFLC should be measured and followed if amyloidosis is suspected.

sFLC can also be used as a prognostic marker in WM, as elevated iFLC has been shown to correlate to shorter time to first treatment [55]. Interestingly, patients with early iFLC response seem more likely to have intermediate/high ISS-WM stage, elevated β-2 microglobulin or low hemoglobin levels [52, 56]. These results may be indicating that high iFLC and rapid reductions after therapy could reflect a more aggressive disease.

There are caveats to sFLC test in WM, such as the flare effect previously reported with IgM measurement and which has also been observed with sFLC test. An iFLC flare was observed following rituximab therapy, with a lack of correlation with IgM flare, which suggests that iFLC cannot help to differentiate progression from flare in this situation [52], similar to serum IgM measuring tests. No correlation between iFLC flare and response rate was observed.

Further studies should help determine whether sFLC assay will help in making decision of treatment modification in WM.

Hevylite Assay (HLC for Heavy/Light Chain) The serum IgM Hevylite immunoassay measures specifically serum IgM kappa and IgM lambda separately [57, 58]. It is based on specific polyclonal antibodies, which recognize unique epitopes spanning the junction of the heavy and light chains of the individual immunoglobulin isotypes. This test allows true quantitative measurement of the intact immunoglobulin involved in the clone serum secretion, e.g., the IgMk M-spike for IgM kappa WM. The test measures separately the involved and uninvolved immunoglobulin for all existing immunoglobulins. Involved IgM HLC measurements indeed reflect the clonal IgM production, and quantification of uninvolved IgM HLC assesses the tumor-induced polyclonal suppression, e.g., the hypogammaglobulinemia, both more precisely than available tests.

It has been shown that the IgM Hevylite ratio (involved monoclonal immunoglobulin/uninvolved polyclonal immunoglobulin) correlated well with the M-spike measured by SPEP [55, 59].

Hevylite levels were indeed higher in WM than in IgM MGUS and higher in symptomatic than asymptomatic WM. Interestingly, high Hevylite serum levels predicted a shorter time to treatment for patients with asymptomatic WM, traducing a shorter survival free of evolution towards symptomatic WM requiring treatment. Koulieris et al. also showed that Hevylite was a potential prognostic marker of high tumor burden and poor prognosis. They have proposed a prognosis score including

Hevylite level above median, beta-2 microglobulin > 5.5 mg/L, and elevated LDH at diagnosis, which distinguished four groups of patients with significantly different overall survival (the four groups were constituted according to the number of adverse factors: 0, 1, 2, or 3) [60].

Hevylite, thus, represents a promising technique to monitor the monoclonal IgM in WM.

These novel M-component-based biomarkers appear to be reliable markers for the diagnosis of WM, for the monitoring of WM, and potentially the prognosis of WM. They are easy to perform in routine practice, which make these tools of potential choice to replace IgM measurement. However, they do not seem to be really superior to IgM measurement, at least for sFLC. More studies are needed to demonstrate the impact of Hevylite in WM and whether the couple sFLC and Hevylite could replace the current tests for all patients with WM or solely a subset yet to identify.

Spurious Results Monoclonal proteins can interfere in certain measurements because they may form precipitates during the procedure. This can result in artifactually increased total bilirubin concentration, low high-density lipoprotein and cholesterol, and low ferreting and transferring [61].

Recommendation
It is recommended to use SPEP and IFS to monitor IgM M-component. In certain cases, sFLC could be of some help. Cryoglobulinemia should be looked at particularly in symptomatic WM patients to prevent complications and to optimize measurement of IgM M-component in these patients. Urine albuminuria is far more important to investigate compared to Bence-Jones proteinuria, given the greater risk of glomerulopathy over CAST tubular nephropathy.

17.6 Flare Effect

IgM flare is defined as any increase (relative to baseline) in IgM levels that occurred before the end of therapy and that would not be related to disease escape, e.g., progression of WM. As such, this phenomenon should be transient and decrease thereafter to the level of the depth of response.

This phenomenon was first suggested when it was reported that some patients treated with rituximab or cladribine for WM developed transient increases in monoclonal protein levels [62, 63], and its exact mechanism remains unraveled. It was then further characterized by Ghobrial et al. who found that an IgM flare could be observed in up to 54 % of patients following rituximab monotherapy, but that 73 % of these patients will have a decrease of their IgM levels within 4 months of therapy [44]. This phenomenon, thus, should not discourage physicians from continuing to administer this treatment. Response rates were, however, poorer for patients who experienced an initial IgM flare compared with those who did not (28 % versus 80 %). In patients with high IgM levels (>40 g/L), the use of

rituximab as single-agent therapy should be avoided, as considerably lower response rates were reported in those patients [64, 65]. Similarly, the IgM flare can occur in the maintenance phase of rituximab therapy and can be mistaken for progression [7]. A bone marrow biopsy should be performed if differentiating these two situations appears difficult.

Patients with increased IgM levels after treatment might develop hyperviscosity and require plasmapheresis, and IgM flare may also lead to worsening of IgM-related neuropathy, cryoglobulinemia, and other IgM-related complications [7]. Serum IgM levels should, therefore, be closely monitored (at least weekly) during the time of risk to develop flare effect, for example, early on during the first cycle on rituximab-based therapy, particularly if not combined to regular chemotherapy. The IgM flare may last for several weeks and even months [44, 66]. No factor has been identified for predicting the occurrence of this IgM flare.

In combination therapy, the occurrence of the rituximab-mediated IgM flare can vary considerably and seems to depend both on the associated drugs and the administration schedule of rituximab [67]. For instance, bortezomib appears to suppress IgM production independently of tumor cell killing [7].

In order to try and avoid the rituximab-related IgM flare and its possible effects on IgM-related morbidity, the introduction of rituximab can be deferred in patients considered at high risk, arbitrarily defined by an IgM level > 40 g/L and/or a plasma viscosity > 4 cP [16].

17.7 Other

Workup for Peripheral Neuropathy Peripheral neuropathy is frequent in IgM dyscrasia, including WM, as it has been reported in about 25 % of patients, with various etiology to consider [7]. One of the most frequent mechanisms involved in IgM-related peripheral neuropathy, including WM, is related to demyelinization and is the consequence of the anti-myelin autoactivity of the IgM monoclonal component.

Common etiologies related to WM are lymphoplasmacytic infiltration of the nerve fibers, IgM deposition, autoantibody deposition, cryoglobulinemia, and amyloidosis; differential diagnosis includes radiculopathy, diabetic neuropathy, cobalamin deficiency, thyroid dysfunction, HIV infection, Lyme disease, autoimmune processes such as systemic lupus erythematosus and other vasculitides, and chronic inflammatory demyelinating polyneuropathy.

If a peripheral neuropathy is suspected, the etiologic workup should include: measurement of serum anti-MAG (anti-myelin-associated glycoprotein) antibodies, anti-ganglioside M1 antibodies, anti-sulfatide IgM antibodies, along with electromyography signature. The nerve biopsy should be avoided when it comes to diagnosis of anti-MAG neuropathy as it affects sensitive nerves that are not comprised in the biopsy. If performed, one could visualize IgM deposits at the site of MAG localization or sulfatide moieties on nerve sheaths. However, this biopsy might be helpful for the differential diagnosis such as peripheral neuropathy

related to cryoglobulin deposits and AL amyloidosis [7, 12]. Amyloidosis can also be ruled out using other histology exams such as salivary glands and/or fat pad biopsy and congo red staining.

Hepatic and Renal Function Renal and hepatic functions are to be assessed before any therapy.

Renal failure is rarely a manifestation of WM. Proteinuria can be observed due to deposition of the monoclonal protein in the kidneys. CAST nephropathy as a consequence of excess of Bence-Jones proteinuria is, however, a rare event in WM [46]. Proteinuria still needs to be assessed, but mainly to detect albuminuria. If albuminuria is detected, suggesting a glomerulopathy, prompt investigation should indeed be performed to detect AL amyloidosis, cryoglobulinemia, or infiltration of the kidney by tumor cells [7].

Hypercalcemia is unusual in WM.

HIV, Hepatitis B and C Status Viral status should be assessed before starting therapy. Screening for hepatitis B and C viruses is specifically required prior to the introduction of rituximab-containing regimens, and hepatitis C should be screened in case of cryoglobulinemia [16]. The role of chronic antigenic stimulation was suspected in WM; however, the implication of hepatitis C or HHV8 viruses remains controversial [68].

17.8 Prognostic Markers

As for most cancer types, the prognosis of WM can be summarized based on three main entities: (i) the disease itself, e.g., the intrinsic agressivity of tumor cells, (ii) the patient characteristics, e.g., a mix of age and comorbidities, particularly given that WM is a disease of the elderly, and finally (iii) the sensitivity of tumor cells to treatment, e.g., a prolonged progression-free survival that translates into a prolonged overall survival, in patients that would not die from WM but from other age-related causes.

Interestingly, one may consider that the patient- and disease-based entities of the prognosis of WM are summarized into the International Staging System (ISS WM) [2]. This prognosis score of WM depends on five major factors: age > 65 years, anemia < 11.5 g/dL, thrombocytopenia $< 100 \times 10^9$/L, β-2 microglobulin > 3 mg/L, and IgM level > 70 g/L. Low risk disease is defined by 0–1 adverse features excluding age, intermediate risk by two adverse factors or age, while high-risk is defined by >2 adverse features. These risk categories each comprise approximately 1/3 of patients and are associated with 5-year survival rates of 87 %, 68 %, and 36 %, respectively.

This staging system appears robust and was validated in the relapse setting as well, with the main caveats that it was created in the era of "old" WM-based

treatments. Another limitation would be that there is no evidence yet that it should influence treatment decisions for individual patients.

Elevated LDH, low albumin, B symptoms, poor performance status, and increased C-reactive protein, but not transformation to high-grade B cell non-Hodgkin lymphoma (DLBCL), have also been associated to poor prognosis.

17.9 New Biomarkers to Measure the Activity of Tumor Cells in WM Independently of the M-Component

Soluble CD27 Soluble CD27 (sCD27) is a tumor necrosis factor family member secreted by WM cells and was proposed to be a faithful marker of disease burden in WM [69]. It was demonstrated to strongly correlate to serum IgM levels and clinical responses in patients with WM and is unaffected by the rituximab-induced flare or by plasmapheresis. However, it cannot be used in routine lab practice and, therefore, did not develop beyond research purpose.

17.10 Conclusion

Major progress have been made in understanding the pathophysiology of WM, and biological tests are now of great interest in the characterization of this disease. Biological markers may lead to personalized treatments in patients with WM. New biomarkers are under investigation, offering a quantitative alternative to traditional electrophoretic techniques, and could, therefore, be of great use in the management of patients with WM.

Disclosures of Potential Conflicts of Interest None.

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