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Conventional diagnostics in multiple myeloma

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ABSTRACT

This paper reviews the most relevant laboratory techniques currently used for the evaluation of patients with multiple myeloma (MM) and other monoclonal gammopathies. Although the bone marrow morphological examination and electrophoretic analysis of the monoclonal paraprotein still remain the 'gold standard' techniques for fast, accurate and cost-effective diagnosis, other assays such as immunophenotyping, DNA cell content and cell cycle analysis measured by flow cytometry may contribute to a better assessment of myeloma patients. Here, we will discuss not only the contribution of each technique to differential diagnosis of monoclonal gammopathies, but also the value of each parameter as prognostic factor and for monitoring treatment efficacy. In addition, possible technical pitfalls inherent to each technique will be analysed.

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1. Introduction

Multiple myeloma (MM) is a clonal B-cell disorder in which malignant plasma cells (PC) accumulate in the bone marrow (BM) and produce lytic bone lesions and excessive amounts of monoclonal protein. The diagnosis of MM requires the examination of BM, showing PC infiltration, detection and quantification of monoclonal protein in the serum or urine and evidence of end-organ damage (hypercalcemia, renal insufficiency, anaemia or bone lesions).[1] Tables 1 and 2 summarize the new reviewed criteria for myeloma diagnosis.[2] Conventional diagnosis in multiple myeloma (MM) is based on a series of laboratory investigations with three main objectives: i) to contribute to the diagnosis and differential diagnosis of monoclonal gammopathies; ii) to yield information about prognostic factors[3] in order to facilitate the therapeutic-decision making process; and iii) to afford appropriate tools to monitor treatment efficacy (disease activity). Nevertheless, it should be pointed out that many of the laboratory parameters contribute to more than one objective; thus, the

evaluation of the monoclonal component serves both to diagnose MM and to monitor response to treatment. In this review, we have grouped the different laboratory investigations into 6 areas: i) morphology; ii) analysis of M-component; iii) haematological features; iv) biochemical parameters; v) immunophenotyping; and vi) DNA ploidy and Labelling Index –proliferative activity of PC.

2. Morphology

The morphological assessment of the proportion of BM PC, based on the evaluation of May-Grünwald Giemsa stained smears, is one of the three major criteria for the diagnosis of MM. Moreover, the number of PC in BM is also an important criterion for differentiating MM from MGUS and solitary plasmacytoma. Thus, in MM there are usually more than 10% PCs, although, due to the heterogeneous distribution of PCs in BM, the percentage of PC may significantly vary depending on site of sample aspiration, and therefore it is not a consistent prognostic factor. In spite of this, cytomorphology remains as the

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Table 1 – Diagnostic criteria of multiple myeloma according to International Myeloma Working Group		
Monoclonal gammopathy of undetermined significance (MGUS)	Asymptomatic myeloma	Symptomatic myeloma
M-protein in serum <30 g/L Bone marrow clonal plasma cells <10% No evidence of end-organ damage	M-protein in serum ⩾30 g/L Bone marrow clonal plasma cells ⩾ 10% No evidence of end-organ damage or plasmacytoma	M-protein in serum and/or urine Bone marrow clonal plasma cells[a] Related organ or tissue impairment
a If flow cytometry is performed, most plasma cells (>90%) will show a "neoplastic" phenotype.		

Table 2 – Myeloma-related organ or tissue impairment (end organ damage) (ROTI) due to the plasma cell proliferative process
*Calcium levels increased: serum calcium >0.25 mmol/L above normal or >110 mg/dL (2.75 mmol/L). *Renal insufficiency: creatinine >2 mg/dL (173 mmol/L) *Anaemia: haemoglobin 2 g/dL below the normal level or <10 g/dL *Bone lesions: lytic lesions or osteoporosis with compression fractures Other: symptomatic hyperviscosity, amyloidosis, recurrent bacterial infections (>2 episodes in 12 months)
*CRAB (calcium, renal insufficiency, anaemia or bone lesions).

standard method for quantitation of PC infiltration. The use of BM biopsies is probably a more accurate method for evaluation of PC infiltration, but it would require substantial standardization.[4] Furthermore, although changes in BM infiltration is not used as a guideline-criterion for assessing response to treatment, the detection of <5% PC is one of the requisites for complete remission. Surprisingly, in routine laboratory techniques, usually only the number of BM PC is taken into account, and little attention is paid to the morphological characteristics of these PC, an attitude which is completely different from that observed in other haematological malignancies. In MM the morphology of PC is quite heterogeneous. Greipp and colleagues[5] have defined four morphological subtypes of PC (Fig. 1): mature (small nucleus eccentrically placed with a prominent hof and dense chromatin clumping, and a well developed cytoplasm); intermediate (do not fulfill criteria for the other subtypes of PC); immature (large nucleus eccentrically placed with a hof and diffuse chromatin pattern, with or without nucleolus >2 μm, and abundant cytoplasm); and plasmablasts (very high nucleus/cytoplasmic ratio with scanty cytoplasm and a centrally placed immature large nuclei with a reticular chromatin pattern, prominent nucleolus and little or no hof). According to these morphological subtypes, four MM groups were established: Mature MM (>10% mature PC, <2% plasmablasts and < 13% immature PC); Immature MM (>12% immature PC, <10% mature PC and <2% plasmablasts); Plasmablastic MM (>2% plasmablasts); and Intermediate MM (do not fulfill criteria for other subtypes). The prognostic value of PC morphology has been widely recognized by several groups.[4–8] Thus, a high number of PC in BM, as well as a diffuse pattern of infiltration and plasmablastic morphology are generally associated with a poor prognosis.[6,7]
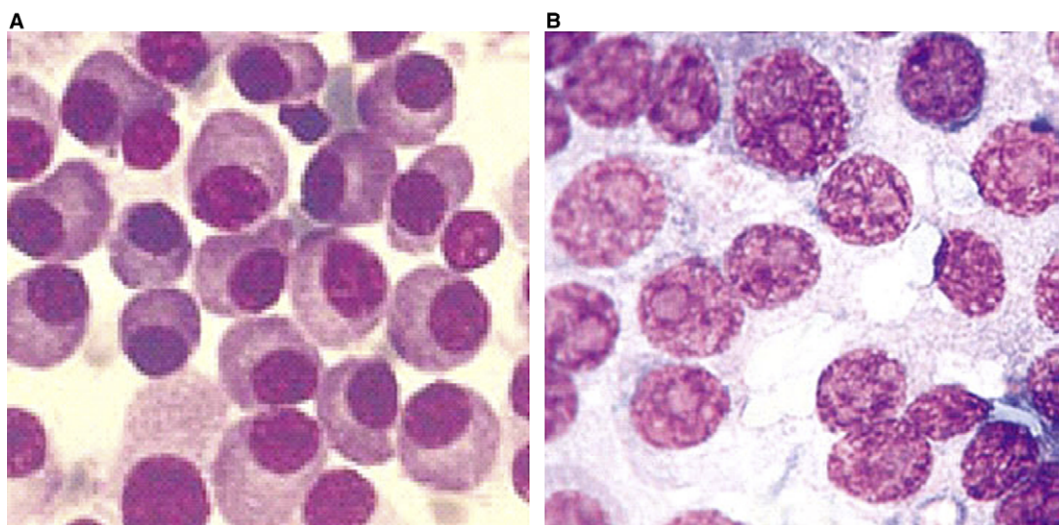

Fig. 1 – Morphological characteristics of myeloma plasma cells. A) Mature differentiated plasma cell. B) Immature-plasmablastic cells (kindly provided by "Fondo de Imagen en Hematología, AEHH").

3. Electrophoretic analysis of monoclonal proteins

The detection of a monoclonal (M-) component in serum or urine is the most characteristic biological maker of MM. Although the quantity of M-component has frequently been used for the differential diagnosis of MM and MGUS (the latter has <3 g/dl), it is not a parameter that can be used exclusively, since many MM patients show less than 3 g/dl. In addition, the M-component is the most widely used marker for monitoring the course of the disease. Serum protein studies provide information not only about the M-component but also on the level of polyclonal Igs, which are usually depressed in MM, and the level of albumin, where a decrease is associated with an adverse prognosis.

3.1. Detection and identification of monoclonal proteins

The screening of monoclonal proteins in serum and urine requires a rapid and sensitive assay.[9–15] Electrophoresis on agarose or cellulose acetate membrane is the method of choice for detection of a serum monoclonal protein. Monoclonal light chains are rarely detected on the cellulose acetate electrophoresis, and IgD paraproteins may be difficult to be seen; in these latter cases a more sensitive technique, such as high resolution agarose gel electrophoresis can be used. [9–12] IgD or Bence-Jones myeloma should be suspected in the absence of serum M component if there is a severe hypogammaglobulinemia and BM plasmocytosis. Once a monoclonal protein has been detected, the heavy and light chain isotypes must be identified by immunoelectrophoresis or immunofixation. Immunofixation is more sensitive than immunoelectrophoresis and therefore is particularly useful for the assessment of small residual M-components following treatment.[9–12] Immunoisoelectric focusing is probably the most sensitive method for the detection of monoclonal proteins, but it requires technically skilled personnel. Nephelometry has frequently been used to detect the light-chain type of a monoclonal gammopathy, based on the existence of a marked imbalance in the κ/λ ratio. Nevertheless, cases with small monoclonal proteins may display a normal κ/λ ratio and be overlooked with the rate nephelometer.[12] Several groups have analyzed different protocols for a cost-effective detection of monoclonal gammopathies. Probably, one of the best alternatives is the combination of electrophoresis followed by immunofixation, which is a rapid approach and avoids the need for immunoelectrophoresis.[9,10] A new immunoassay, based on the measurement of serum free light chain (FLC) has been reported to be particularly useful for the diagnosis and monitoring of monoclonal light chain diseases, non-secretory myeloma and primary amyloidosis. Serum FLC measurements are more sensitive than urine presumably because of the kidney's high capacity for protein catabolism. Thus, serum assays could replace Bence-Jones protein urine test for patients with light chain MM. Another potential use is earlier identification of disease recurrence in myeloma and amyloid patients after chemotherapy or transplantation.[16,17] Because of their serum half-life, changes in serum FLC concentrations provide a rapid indication of response to treatment.

3.2. Quantitation of immunoglobulins and evaluation of response to treatment

Nephelometry is the method of choice for quantitation of Igs. However, for evaluation of changes in the M-component, either nephelometry or electrophoresis can be used, the latter probably being the more accurate. What is really important is to select one of the two techniques, and to always use the same method throughout the course of the disease. As previously mentioned, immunofixation is required to confirm disappearance of the M-component. It should be emphasized that not only the serum but also the urine M-component should be monitored since there are cases in which the former has disappeared following treatment and the urine M-protein may increase as the only indication of relapse.[18]

3.3. Analysis of urine

For the analysis of urinary monoclonal proteins a 24-hour collection of urine is mandatory for the determination of the total amount of protein excreted per day. An aliquot of the 24-hours sample must be concentrated (150–200-fold) (2–4 g/dl) for electrophoresis, which will show a dense band on the cellulose acetate or a peak on the densitometer tracing. To define the type of monoclonal light chain an immunoelectrophoresis of concentrated urine using monospecific antisera to κ and λ light chains (that recognize both free and combined light chains) should be performed. Immunofixation may be of value in cases of small amounts of proteinuria.[9–11]

4. Haematological parameters

Half of MM patients display a moderate normochromic, normocytic anaemia but, it can be severe in 20% (Hb < 8 g/dl). Thrombocytopenia is uncommon at diagnosis (only 10%–15% of cases show a platelet count of less than 100,000/mm^3) and usually reflects a severe degree of BM infiltration by PC. Leukocyte counts are usually normal, and in fact leukopenia only occurs in far advanced disease or after chemotherapy.[12–14]

The PB film may occasionally show circulating PC, but the incidence of these cases is low (<15%) and usually associated with advanced disease.[11] Nevertheless, if instead of morphological examination, more sensitive techniques (such as immunophenotyping or PCR) are used, the incidence of MM cases with circulating PC reaches up to 70%.[19–21]

Increased erythrocyte sedimentation rate (ESR) (>50 mm in 1 hour) as well as rouleaux formation, due to hypergammaglobulinaemia, are present in about two thirds of the patients.[13,14] In unselected patients, the detection of an ESR value >100 mm should point to a possible diagnosis of MM. Nevertheless, 10% of MM patients, mainly those with Bence-Jones myeloma, have ESR values <20 mm.

5. Biochemical markers and cytokines

5.1. Conventional markers

Renal function has a major impact on disease outcome.[22] Around 25%–30% of MM patients have creatinine values >2

mg/dl at diagnosis and the proportion increases to double during disease evolution.[13,14,22] Hypercalcaemia is detected in a similar proportion of patients, while severe hyperuricaemia is less frequent (15% in our series), although in other series its incidence reached almost one-third of the patients.[14] Lactic dehydrogenase (LDH) activity, reflecting cell turnover, is increased in very aggressive forms of MM (approximately 20% of cases).[22]

Beta 2-microglobulin (B2M) is one of the most relevant prognostic factors in MM.[23–30] B2M levels may increase as a result of both tumour burden growth and renal impairment.[23–28] Initially, some groups suggested that B2M should be corrected according to the level of serum creatinine. However, further studies showed that it was better to use the uncorrected value since it then provides dual prognostic information: tumour burden and renal function.[23–28] The most important application of serum B2M measurements is as a prognostic factor.[3] Recently, a new International staging system (ISS), derived from >11,000 patients, has shown that B2-M and albumin are the best combination of easily available markers to discriminate prognostic subgroups: Stage I (B2M <3.5 & Albumin >3.5 mg/dl), Stage III (B2M > 5.5) and Stage II (the rest).[31] In contrast, B2M is not helpful for monitoring the course of the disease since there are patients that relapse without a previous increase in B2M level (false negative), while others show increased levels without any evidence of disease progression.[18]

5.2. Cytokines and acute phase reactants

IL-6 is a pleiotropic cytokine that induces differentiation of B-lymphocytes into Ig-secreting cells, and proliferation of PC.[32,33] Soluble IL-6R is generated through the shedding of the receptor from the cell membrane or by alternative RNA splicing,[34] but s-IL-6R levels do not show a linear correlation with IL-6.[35] Serum IL-6 and sIL-6R may be measured by ELISA or radioimmunoassays.[29,36] Serum IL-6 (s-IL-6) and sIL-6R levels are increased in 30%–50% of MM patients.[36–41] Although the levels in MM are higher than in patients with MGUS, none of these two markers are reliable parameters for differential diagnosis between MM and MGUS.[35] In MM patients, high s-IL-6 levels have been shown to reflect active disease and a poor prognosis.[35,36]

C-reactive protein (CRP) is an acute phase reactant protein whose synthesis by human hepatocytes is induced by IL-6. Serum CRP levels reflect IL-6 activity and represent a surrogate for IL-6 concentration.[27,37] In MM patients, 40% of cases display increased CPR values (>6 mg/l in serum) while this only occurs in 8% of MGUS patients. Moreover, MM patients who remain in remission show significantly lower CRP values than cases at relapse. However, CRP is not a specific marker of disease activity in MM since it may increase because of many different factors. Evaluation of CRP is important in MM at diagnosis, since it is an independent prognostic factor that can substitute IL-6 (its determination is more rapid, simpler and cheaper), and together with B2M constitutes an excellent combination for the prognostic stratification of MM patients.[27]

6. Immunophenotypic studies in MM

6.1. Immunophenotype of myelomatous plasma cells

Immunophenotypic studies of myelomatous PC have now been performed for more than 15 years.[42–52] However, a careful review of the literature shows discrepancies in results regarding the exact phenotype of PC and its clinical significance. These controversies could be due to methodological problems, such as the use of different MoAb clones,[59] different fluorochromes[51] and the use of single vs multiparametric antigen staining procedures.[51] Nowadays, flow cytometry represents an attractive approach, not only for research purposes but also in clinical practice. In this sense, flow cytometry has many advantages over fluorescence microscopy and APAAP (immuno-alkaline phosphatase) techniques: i) a higher number of cells are analyzed, ii) it permits the assessment of fluorescence intensity, iii) it has a higher sensitivity and iv) it facilitates the performance of multiple marker analysis.

Although CD38 antigen is widely distributed in the haematopoietic system, flow cytometry has shown that the intensity of CD38 expressed on PC is clearly higher than that observed on any other cell type (Fig. 2A), and this strong reactivity (CD38+++) has converted it into a "specific PC marker", ideal for multiple-staining studies in which PC must be identified.[49,51,56,60,61] The BB-4 MoAb, which recognizes CD138,[43] is highly specific for the detection of both benign and malignant PC, since the CD138 antigen is not expressed in other cells
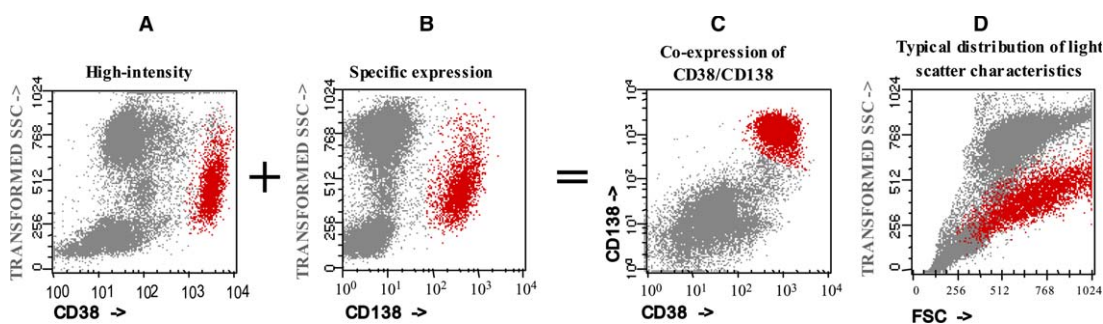

Fig. 2 – Plasma cell selection strategy. A) Flow immunophenotypic diagrams showing a PC population (painted in red) with strong positivity for CD38; B) and exclusive expression for CD138 antigen. C) The coexpression of CD38 and CD138 antigens allows a more specific discrimination of PC from other cells present in the sample and, at the same time; D) PC can be further identified by a well-defined area of a light scatter characteristics (SSC and FSC).

within the haematopoietic compartment (Fig. 2B). The combination of CD38 and CD138 is widely used[57,58] for a more efficient identification of PC which is crucial for the correct assessment of other immunophenotypic characteristics of PC using multiple-staining procedures (Fig. 2C). In addition, PC displays singular light scatter characteristics, which also contribute to their unequivocal identification at flow cytometry[51] (Fig. 2D).

Although early studies on MM patients showed that PC usually lose most B-lineage-associated markers,[49,50,53,59] latter studies in larger series of patients, in which more sensitive techniques were applied, showed that a minority of MM patients (less than 15%) retained molecules such as CD19,[48,56,58] CD20[58,64,65] or CD22.[56,64] Likewise, the presence of sIg has been reported in up to one third of all MM patients.[53,65] These heterogeneous findings suggest that the neoplastic clone may be able to undergo a certain degree of differentiation/maturation (early PC and plasmablast should be positive for these markers).[60,66,68,69]

Myeloma PC interacts with BM microenvironment through a wide type of adhesion cell surface molecules:[54,55,63,70–72] i) β1 integrins, including the VLA-1 (CD29-CD49a), VLA-4 (CD29-CD49d) and VLA-5 (CD29-CD49e) antigens; ii) β2 integrins, including LFA-1 (CD11a-CD18); iii) proteoglycans: syndecan-1 (CD138) and CD44; and iv) molecules of the immunoglobulin superfamily: CD56 (N-CAM), CD54 (ICAM-1), CD102 (ICAM-2) and CD50 (ICAM-3). It is well-know that the myeloma cell-cell and cell-extracellular matrix unions promote not only the localization of the tumour cells in the BM microenvironment, but also provide important functional and clinical sequelae. In fact, several studies have shown that the CD56 molecule is strongly expressed in up to 60% of MM patients,[47,58,73] mediates homing of MM cells and it is usually absent in advanced phases of the disease.[70,74]

Other antigens frequently found on myelomatous PC are CD117 (also named c-kit) that is present in approximately one third of MM patients,[47,57,58] the granulomonocytic antigens CD13 and CD33 (in a quarter of MM cases)[56] and co-stimulatory molecules involved in the activation of B- and T- lymphocytes such as CD40 and CD28 (positive in 70% and 40% of MM patients, respectively).[62,75] Several groups have explored the prognostic implications of the antigenic profile of PC.[59,66,70,76–78] However, these studies are usually based on small series of patients and nor uniformly treated, which may account, at least in part, to the existence of discrepant results. Some studies suggest that markers associated to an early PC phenotype (CD20, CD45, sIg) correlate with a poor outcome.[59,66,67,79] Down-regulation of CD56 and a greater expression of CD44 have been associated with the extramedullary spreading of malignant PC.[70] The expression of CD28 has been related to disease activity, probably confined to highly proliferative accelerated phases of the disease.[76,77] We have recently analysed in a series of 587 transplanted patients uniformly treated, the prognostic influence of the antigenic expression.[78] Although the results should still be considered as preliminary due to short follow-up, it was observed that the lack of CD56 or the overexpression of CD19 and CD28 antigens conferred an adverse prognosis. By contrast, acquisition of CD117 was associated with a favourable outcome. Moreover, patients with a CD56$^-$CD117$^-$ or CD56$^-$CD28$^+$ phenotypic profile had adverse prognosis.

6.2. Immunophenotypic studies for the differential diagnosis of different monoclonal gammopathies

It is well-established that the phenotypic characteristics of myelomatous PC differ from those of normal PC,[80–84] specially on the basis of their different pattern of expression for CD19, CD45, CD56 and CD38:[48,81] malignant PC usually display lower levels of CD38, and strong reactivity for CD56, generally associated with the lack of positivity for CD19 and CD45; this aberrant phenotype is never present in BMPC from healthy individuals (CD38^{+++}CD56$^-$CD19^{++}CD45$^-$) (Fig. 3A, B). Interestingly, the coexistence of residual polyclonal/normal PC and clonal/malignant PC is a constant finding in MGUS patients whereas it is a rare event in MM (Fig. 3B).[81] Moreover, on multivariate analysis the proportion of normal residual PC (> or <3% from the total amount of PC) was the most powerful sin-
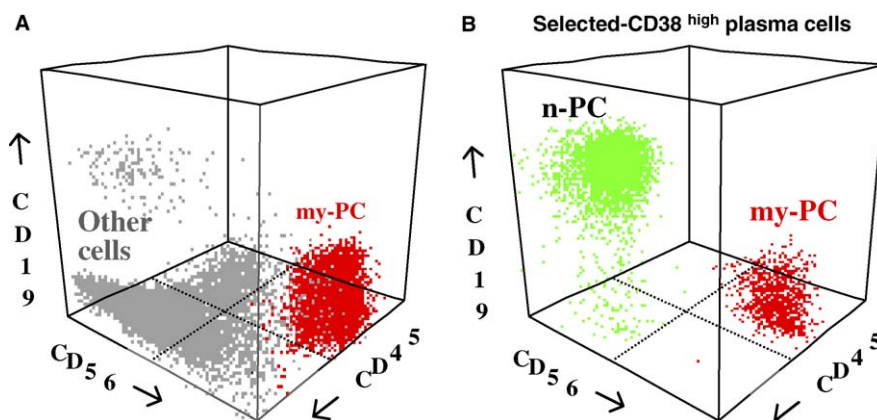

Fig. 3 – Antigenic profile of both myelomatous (my-PC) and normal PC (n-PC). A) Representative trivariate dot-plot of a bone marrow sample for a MM patient analysed at diagnosis showing my-PC (in red) characterized by the CD38$^+$CD56$^+$CD19$^-$CD45$^-$ phenotype. B) This figure corresponds to the same patient following autologous stem cell transplantation. A small proportion of residual my-PC coexists with a predominant population of n-PC (CD38$^+$CD56$^-$CD19$^{-/+}$CD45$^+$) (in green). This latter situation resembles to that observed in MGUS patients.

gle parameter for the discrimination between MGUS and MM patients at diagnosis.[81] Interestingly, immunophenotypic profile of PC from MM differs from that observed in PC from Waldenström Macroglobulinaemia (WM). In this latter condition, the antigenic profile of PC is CD5+, CD19+, CD20+, CD22−/+, CD38++, CD45+, CD56−, FMC7+ and sIg+, typically with κ light chain restriction. This phenotype has intermediate features between the clonal B-lymphocyte and mature PC, and it may reflect a possible derailment in the differentiation pathway of the MW clone.[84]

6.3. Immunophenotypic studies for the investigation of minimal residual disease (MRD)

Response to treatment in MM is generally assessed by changes in serum and urine paraprotein concentration and in the percentage of BM PC. Although patients receiving high-dose chemotherapy and peripheral blood stem cell transplant show higher complete remission rates, event-free survival and overall survival, relapse remains a major problem. Accordingly, more sensitive and specific methods, and stringent criteria, are needed to evaluate residual disease. Multiparametric immunophenotyping techniques are becoming increasingly useful for treatment monitoring in MM patients. As mentioned above, myelomatous PC can be unequivocal distinguished from their normal counterparts on the basis of aberrant expression of several markers (CD19, CD38, CD56 and CD45) (Fig. 3B). This specific cell-surface antigen combination offers a quick and efficient method not only for quantifying low levels of residual malignant PC but also for assessing the normal immune reconstitution. Flow monitoring is applicable to up 80% of patients[85] and it is possible to reach a detection limit of 0.01% of PC.[85,86] Although quantitative PCR strategies are considered to be the most sensitive and specific method for the assessment of MRD, they are more expensive and time-consuming than flow cytometry, and are only applicable to approximately only 50% of cases. Upon analysing MRD following high-dose therapy we and others have shown that residual malignant plasma cells are detectable in up to 40% of patients,[86,87] in otherwise theoretical complete response (CR) by immunofixation.[86] Normal plasma cells reappear in the majority of the patients and correlate with the recovery of normal immunoglobulin levels.[87] Moreover, the recovery of the normal plasma cell population has showed to be a high predictable marker to identify patients with low risk of early relapse.[86,87]

7. DNA ploidy studies and proliferative activity of PC

Measurement of cell DNA contents by flow cytometry provides rapid and objective information about the quantitative abnormalities in the total DNA content per cell. Different fluorochromes have been used for the quantitative measurement of cell DNA contents -ethidium bromide, propidium iodide, acridine orange, 4,6 diamidino-2-phenylindole (DAPI), chromomycin A3, mithramycine and the Hoechst dyes-although propidium iodide is the most widely used. This is mainly due to several factors such as: i) it is excited at 488

nm (a wavelength usually available in all flow cytometers); ii) it emits in the orange-red region of the visible light spectrum which makes it suitable for combination with green dyes, such as fluorescein isothiocyanate (FITC), to simultaneously stain cells with specific FITC-conjugated MoAbs; and iii) it provides relatively narrow peaks with acceptable coefficient of variation (cv). In order to reduce the inter-laboratory variation, a nomenclature consensus has been proposed.[88] Accordingly, the DNA ploidy assessed by flow cytometry is usually expressed as the DNA index, which is calculated as the ratio obtained between the modal fluorescence channel of the G_0/G_1 peak of the PC and the modal channel of the residual G_0/G_1 normal cells present in the sample. If this DNA index is equal to 1, the tumour cells are considered to be DNA diploid, while if it is different to 1, cells are considered to be DNA aneuploid (hyperdiploid, DNA index >1 or hypodiploid, DNA index <1).

Based on the different series published,[89–91] the overall incidence of DNA aneuploidy in MM ranges between 28% and 80%. It was stated in the DNA Cytometry Consensus Conference[92] that these discrepancies can be largely attributed to poorly designed studies with lack of significant numbers of patients as well as to technical artefacts (samples either containing few tumour cells or stored under inappropriate conditions). Using a high-sensitive method, in which a simultaneous staining for PC (with a combination of MoAb against the CD38 and CD138 antigens) and DNA (with propidium iodide) was used,[93] we have recently analyzed more than 800 untreated MM patients and DNA aneuploidy was present in 54% of cases, with the incidence of cases with a DNA index greater or lower than 1 being 45% and 1%, respectively. The remaining 8% of the cases correspond to a bi-clonal group, in which two populations of PC with different DNA ploidy coexist.[58]

As far as the prognostic implications of the DNA ploidy status is concerned, it has been suggested that hypodiploid is associated with a poor response to treatment and short survival.[94] However, the incidence of DNA hypodiploid cases is very low. Interestingly, a high incidence of hypodiploid cases has been reported in plasma cell leukaemia,[90] which supports the hypothesis of a worse prognosis for DNA hypodiploid MM. As for hyperdiploidy, although some studies have suggested that it could be associated with a worse prognosis, especially when the DNA index is higher than 1.15,[95] in our experience, DNA hyperdiploid MM patients show a significantly better outcome as compared to the diploid cases.[88,96]

Heterogeneous results have been reported as regards the incidence of DNA aneuploidy in MGUS patients, ranging between 0% to 61% of cases.[80,89,97] We have analyzed the PC DNA ploidy status in a series of 76 MGUS cases using a simultaneous staining for PC and DNA. Based on this methodological approach, two clearly different PC subsets could be discriminated in 73% of the cases:[81] one showed a normal DNA content (corresponding to polyclonal PC) and the other displayed DNA aneuploidy (corresponding to the clonal PC population). It should be noted that the aneuploid subpopulation found in MGUS BM samples always showed a DNA index higher than 1.[81] These results are in line with the fact that the presence of DNA hyperdiploidy in MM patients is associated with favourable prognosis.

DNA aneuploidy constitutes a specific tumour marker, present in >50% of MM patients at diagnosis and has a detection limit of 10^{-4} (one aneuploid cell among 10 000 diploid cells). Therefore, it could be used as MRD technique for treatment monitoring in patients with DNA aneuploidy.

The proliferative activity of PC, also named PC labelling index (LI) correlated with the percentage of plasma cells in S-phase of the cell cycle. Initially, LI was assessed by the incorporation of [H^3]thymidine and it proved to be an excellent independent prognostic factor in MM. Accordingly, it has been shown that patients with a high tumour cell mass and more than 3% of myeloma cells incorporating [H^3]thymidine had a median survival of only 5 months.[98] Interestingly, Joshua and colleagues[99] have shown that the proliferative activity is almost entirely attributable to an increase of the LI of the immature PC. However, this technique uses *in vitro* radioactive labelling and therefore it is difficult to be applied in routine laboratories. Due to these limitations, non-radioactive approaches, such as the use of bromodeoxyuridine, another thymidine analogue, have been developed showing a similar prognostic impact.[100] The use of propidium iodide (PI) stainings for PC, and its subsequent analysis at flow cytometry, permits the discrimination of PC distribution along the different cell cycle phases, upon appropriate mathematical models.[51] However, due to the fact that in the BM of MM patients, many other cells apart from myelomatous PC exist, the first requisite for an accurate analysis of the S-phase PC in MM, based on the measurement of cell DNA content, is the simultaneous identification of the neoplastic cells present in the sample so that their cell cycle distribution can be analysed separately from that of the normal haematopoietic residual cells. In this sense, we have reported that the combined assessment of PC-related antigens (CD38^{+++} and CD138$^+$) and propidium iodide can be used to assess the cell cycle distribution of MM PC separately from that of normal residual haemopoietic cells, by flow cytometry.[94] With this method, we have found a clear correlation between a high percentage of S-phase plasma cells (>3%) and poor outcome.[96]

Although patients with active MM usually display higher LI values than those with MGUS or smoldering MM,[28,97,101,102] this is not a reliable parameter for differential diagnosis since at least one third of symptomatic MM patients have normal PC LI.[103]

8. Concluding remarks

Laboratory investigations are essential for myeloma diagnosis. Morphologic examination of bone marrow and evaluation of monoclonal protein are two laboratory procedures required for definitive diagnosis of MM. Moreover M-component is the most widely used marker for monitoring the course of the disease. Regarding biochemical parameters, beta 2-microglobulin is still the most relevant and straightforward prognostic factor. Nowadays, flow cytometry is fully integrated in myeloma clinical practice as a reliable technique for differentiating benign and malignant plasma cells as well as for the investigation of minimal residual disease. In addition, measurement of cell DNA contents by flow cytometry provides rapid and objective information about the quantitative abnormalities in the total DNA content per cell, as well as

the cell cycle distribution such as number of S-phase plasma cells.

Conflict of interest statement

None declared.

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