CAT
Critically Appraised Topic

Immunophenotypic Minimal Residual Disease Detection of Plasma Cells: Optimization and Clinical Usefulness

Author: Matthijs Oyaert
Supervisor: Prof. Dr. N. Boeckx
Date: 20 March 2014

CLINICAL BOTTOM LINE

Flow cytometric immunphenotyping is considered to be mandatory for the diagnosis, classification and monitoring of disease in monoclonal gammopathies. Moreover, it a useful diagnostic tool for clinical practice and has various applications, such as its ability to distinguish between normal, reactive and malignant plasma cells, to evaluate the risk of progression from monoclonal gammopathy of undetermined significance to plasma cell myeloma, to provide prognostic information, to evaluate the presence of minimal residual disease and to identify new therapeutic targets. The incorporation of novel therapies in the management of patients diagnosed with plasma cell neoplasms has increased extent and frequency of response, as well as prolonged progression free and overall survival. Along with these improvements in therapeutic strategies, the definition of responses to treatment has evolved over time. It was therefore necessary to develop reproducible and sensitive assays for detection and monitoring of minimal residual disease and to define its prognostic value in predicting progression free and overall survival, to allow for consolidation and maintenance therapeutic strategies, and to evaluate the efficacy of novel therapies. The aim of this critically appraised topic is to review the clinical value of flowcytometry in plasma cell neoplasms, and emphasize those areas were consensus exists to incorporate flowcytometry into routine evaluation of multiple myeloma and other clonal plasma cell related disorders.

CLINICAL/DIAGNOSTIC SCENARIO

Plasma cell neoplasms are monoclonal proliferations of plasma cells in the bone marrow (BM) and are characterized by the secretion of monoclonal immunoglobulins (heavy and/or light chain M-protein (or paraprotein)) [1,2] Presence of an M-protein in serum or urine can be due to the presence of malignant diseases, like multiple myeloma (MM) and lymphoplasmocytoid lymphoma, or it can be a result of a benign or (pre-) malignant disease of which monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM) are the most important. In most of the cases, there is a decreased concentration of polyclonal, normal immunoglobulins. According to the World Health Organization (WHO), plasma cell neoplasms are categorized into five entities that are listed in table 1 [1].

Monoclonal gammopathy of undetermined significance (MGUS)
Plasma cell myeloma (multiple myeloma)
Asymptomatic (smoldering) myeloma
Non-secretory myeloma
Plasma cell leukemia
Plasmacytoma
Solitary plasmacytoma of bone
Extra-osseous (extramedullary) plasmacytoma
Immunoglobulin deposition diseases
Primary amyloidosis
Systemic light and heavy chain deposition disease
Osteosclerotic myeloma (POEMS syndrome)

Table 1. Overview of the different plasma cell neoplasms according to the WHO [1].

MGUS and SMM are asymptomatic, premalignant disorders characterized by monoclonal plasma cell proliferation in the BM and absence of end-organ damage, such as osteolytic bone lesions, anemia, or renal failure [1,3]. Classification of both diseases is mainly based on serum monoclonal M-protein concentrations and BM plasmacytosis (see attachment 1) [1,4]. As in most cases, patients with MGUS are asymptomatic and M-protein is detected accidently. Risk of progression to malignancy is substantially different between both disorders, i.e. 1% per year for MGUS versus 10-20% per year for SMM. This difference in the risk of progression implicates that patients with MGUS and SMM are managed differently in terms of frequency, follow-up and development of chemo-preventive strategies [3]. MM arises from an asymptomatic premalignant proliferation of monoclonal plasma cells that accumulate in the BM and produce lytic bone lesions and excessive amounts of monoclonal protein. The diagnosis of MM requires the examination of BM, showing plasma cell infiltration, detection and quantification of monoclonal protein in the serum or urine and evidence of end-organ damage (hypercalcemia, renal insufficiency, anemia or bone lesions) (see attachment 1). Today, MM is still considered an incurable disease; although the introduction of novel therapies has changed the way the disease is approached and managed (see below) [5].

In the absence of definitive cure, the goal of treatment is to improve patients' long-term outcomes, including prolonging progression-free survival (PFS) and overall survival (OS). Numerous disease-related factors are of prognostic importance for OS, including the International Staging System (ISS), β_2-microglobulin, albumin, C-reactive protein and lactate dehydrogenase levels, cytogenetic abnormalities, plasma cell labeling index and renal impairment. Given this range of factors, and the highly heterogeneous nature of MM patients, determining the prognosis for long term OS is a rather complex issue.

One important factor widely associated with improved PFS and OS in MM, is a patient's quality of response to treatment, and in particular the achievement of a sustained complete response (CR). CR represents elimination of detectable disease by currently available laboratory methods (see table 2) [6]. From a historical point of view, the chance of achieving CR in newly diagnosed MM patients was low, as CR was merely obtained with conventional chemotherapy, such as melphalan [7,8,9,10] and prednisone or vincristine, doxorubicin, and dexamethasone [11,12,13,14,15,16]. Although these regimens demonstrated anti-myeloma activity in more than half of the patients, treatment was generally not sufficient intensive to eliminate residual disease burden. This changed with the introduction of high-dose melphalan therapy plus autologous stem-cell transplant (ASCT) therapy; substantially higher CR rates (up to 50%) were achieved in patients undergoing transplantation, and this was associated with improved outcomes, including PFS and OS [17,18,19,20]. More recently, regimens incorporating the novel agents bortezomib, thalidomide, and lenalidomide have demonstrated very high CR rates in both newly diagnosed and relapsed MM compared with previous conventional chemotherapeutic approaches [21,22]. Nonetheless, within those patients considered to be in CR, still a significant fraction of them relapse as a consequence of the persistence of minimal residual disease (MRD) that remained undetectable by conventional criteria for CR (i.e. BM morphology, protein electrophoresis with immunofixation and light chain quantification). An overview of the different diagnostic and analytical techniques for detection of myeloma disease burden in patients with plasma cell neoplasms is listed in table 2.

Technique	**Disease burden assessment**
Serum/urine protein electrophoresis	Detection and quantitation of monoclonal proteins/light chains in the serum/urine (sensitivity 1-2 g/L)
Serum/urine immunofixation	Detection of monoclonal proteins in the serum/urine (sensitivity 150-500 mg/L)
Serum free light chain assay	Quantitation of free kappa and lambda immunoglobulin light chains in the serum; ratio of kappa/lambda light chains (sensitivity < 1 mg/L)
Bone marrow aspirate/biopsy	Cytomorphological assessment of percentage of myeloma plasma cells in the BM marrow (sensitivity < 5%)
Immunohistochemistry/immunofluorescence	Quantitation of myeloma plasma cells in BM tissue/cell samples via antibody-antigen interaction (sensitivity 10^{-2} – 10^{-3})
Immunophenotyping	Automated cell-by-cell quantitation of myeloma plasma cells in BM samples via multiple antibody-antigen interactions (sensitivity 10^{-4})
Real-time quantitative polymerase chain reaction	Identification of residual tumor cells based on presence of patient-specific selected immunoglobulin heavy chain genomic rearrangements (sensitivity 10^{-6})
Magnetic resonance imaging	Identification of focal lesions in the BM (sensitivity 0.5 cm lesion size)

Table 2. Overview of the diagnostic and analytical techniques for the detection of myeloma disease burden.

Multiparameter flow cytometry (MFC) immunophenotyping is considered to be mandatory for the diagnostic characterization of neoplastic cells and monitoring of MRD in a vast number of hematological malignancies [23]. The input of MFC into the clinical management of patients with clonal plasma cell disorders has faced some reluctance. This was mainly attributed to the lack of plasma cell specific markers and the lower plasma cells frequencies usually detected in BM samples by MFC compared to morphological approaches. Moreover, variable or even discrepant results have been reported concerning the precise phenotype of clonal plasma cells and its clinical correlations, especially in MM and MGUS. On the contrary, immunophenotyping has shown to provide accurate assessment of the expression of multiple specific plasma cell markers, specific information on individual plasma cells, and to allow clear discrimination between aberrant and both normal and reactive plasma cells, even when they are present at very low frequencies [23].

QUESTION(S)

1) Which immunophenotypic markers can be used for the differentiation between normal, reactive and aberrant plasma cells?
2) What is the prognostic value of the different immunophenotypic markers used for characterization of plasma cells in plasma cell neoplasms?
3) What is the role of MRD monitoring of plasma cells in patients with plasma cell neoplasms? Is flow-based MRD a well suited technique for MRD assessment in plasma cell neoplasms?
4) Can flow cytometric detection of plasma cells tailor therapy in patients with plasma cell neoplasms?

SEARCH TERMS

1) MeSH Database (PubMed): MeSH term: "multiple myeloma"[MeSH], "flow cytometry"[MeSH], "monoclonal gammopathies" [MeSH], "immunophenotyping" [MeSH], "minimal residual disease" [MeSH], "plasma cells" [MeSH], "diagnosis" [MeSH], "follow-up" [MeSH], Amyloïdosis [MeSH]
2) Pubmed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic revieuws; Clinical Queries using Research Methodology Filters (diagnosis + specific, diagnosis + sensitive, prognosis + specific)
3) Pubmed (Medline; from 1966), SUMSearch (http://sumsearch.uthscsa.edu/), National Guideline Clearinghouse (http://www.ngc.org/), Institute for Clinical Systems Improvement (http://www.icsi.org), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane, Health Technology Assessment Database (http://www.york.ac.uk/inst/crd/htahp.htm)
4) IMWG guidelines (http://myeloma.org/IndexPage.action?tabId=0&indexPageId=155&categoryId=0&parentNuggetId=33); EMN guidelines (http://myeloma-europe.org.linux9.curanetserver.dk); Euroflow protocols (http://www.euroflow.org/usr/pub/pub.php);
5) UpToDate Online version 22.2 (2014)

RELEVANT EVIDENCE/REFERENCES

1. World Health Organization Classification of Tumors. WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues Edited by S.H. Swerdlow, E. Campo, N.L. Harris, E.S. Jaffe, S.A. Pileri, H. Stein, J. Thiele, J.W. Vardiman. (2008)
2. Bain BJ, Clard DM, Wilkins BS. Bone Marrow Pathology. Fourth Edition. Blackwell Science. 2010
3. Rajkumar V. MGUS and smoldering multiple myeloma: update on pathogenesis, natural history, and management. Hematology Am Soc Hematol Educ Program 2005; 340-345.
4. Kyle A and Rajkumar V. Criteria for diagnosis, staging, risk stratification and response assessment of multiple myeloma. Leukemia 2009; 23:3-9.
5. Palumbo A and Anderson K. Multiple myeloma. N Engl J Med 2011; 364:1046-1060.
6. Chanan-Khan A and Giralt S. Importance of achieving a complete response in multiple myeloma, and the impact of novel agents. J Clin Oncol 2010; 28:2612-2624.
7. Boccadoro M, Marmont F, Tribalto M et al. Multiple myeloma: VMCP/VBAP alternating combination chemotherapy is not superior to mephelan and prednisone even in high-risk patients. J Clin Oncol 1991; 444-448.
8. Cavo M, Benni M, Ronconi S et al. Mephelan-prednisone versus alternating combination VAD/MP or VND/MP as primary therapy for

multiple myeloma: final analysis of a randomized clinical study. Haematologica 2002; 87:934-942.
9. Facon T, Mary Y, Pégourie B et al. Dexamethasone-based regimens versus mephelan-prednisone for elderly multiple myeloma patients ineligible for high-dose therapy. Blood 2006; 1292-1298.
10. Hernandez M, Garcia-Sanz R, Golvano E et al. Randomized comparison of dexamethasone combined with mephelan versus mephelan with prednisone in the treatment of elderly patients with multiple myeloma. Br J Haematology 2004; 127:159-164.
11. Alexanian R, Barlogie B and Tucker S. VAD based regimens as primary treatment for multiple myeloma. Am J Hematology 1990; 33:86-89.
12. Dimopoulos A, Pouli A, Zervas K et al. Prospective randomized comparison of vincristine, doxorubicin and dexamethasone (VAD) administered as intravenous bolus injection and VAD with liposomal doxorubicin as first-line treatment in multiple myeloma. Ann Oncol 2003; 1039-1044.
13. Gertz A, Kalish A, Hyle A, Hahn G, Tormey C and Oken M. Phase III study comparing vincristine, doxorubicin (Adriamycin), and dexamethasone (VAD) chemotherapy with VAD plus recombinant interferon alfa-2 in refractory or relapsed multiple myeloma. An Eastern Cooperative Oncology Group study. Am J Clin Oncol 1995; 18:475-480.
14. Harousseau L. Bortezumib/dexamethasone versus VAD as induction prior to autologous stem cell transplantation (ASCT) in previously untreated multiple myeloma: updated data from IFM 2005/01 trial. J Clin Oncol 2008; Abstr 8505.
15. Segeren M, Sonneveld P, van der Holt B et al. Vincristine, doxorubicin and dexamethasone (VAD) administered as rapid intravenous infusion for first-line treatment in untreated multiple myeloma. Br J Hematology 1999; 105:127-130.
16. Sonneveld P. First analysis of HOVON-65/GMMG-HD4 radomized phase II trial comparing bortezomib, adriamycin, dexamethasone (PAD) vs VAD as induction treatment prior to high dose mephelan (HDM) in patients with newly diagnosed multiple myeloma. Haematologica 2009.
17. Attal M, Harousseau L, Stoppa M et al. A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. Intergroupe Français du Myélome. N Engl J Med 1996; 335:91-97.
18. Barlogie B, Jagannath S, Vesole H et al. Superiority of tandem autologous transplantation over standard therapy for previously untreated multiple myeloma. Blood 1997; 89:789-793.
19. Child A, Morgan J, Davies E et al. High dose chemotherapy with hematopoietic stem cell rescue for multiple myeloma. N Engl J Med 2003; 348:1875-1883.
20. Lenhoff S, Hjorth M, Holmberg E et al. Impact on survival of high dose therapy with autologous stem cell support in patients younger than 60 years with newly diagnosed multiple myeloma: a population based study. Nordic Myeloma Study Group. Blood 2000; 95:7-11.
21. Richardson G, Mitsiades C, SChlossman R, Munshi N and NAderson K. New drugs for myeloma. Oncologist 2007; 12:664-689.
22. Richardson G, Mitsiades C, Schlossman R et al. Bortezomib in the front line treatment of multiple myeloma. Expert Rev Anticancer Ther 2008; 8:1053-1072.
23. Paiva B, Almeida J, Pérez-Andrés M et al. Utility of flow cytometry immunophenotyping in multiple myeloma and other clonal plasma cell-related disorders. Cytometry B Clin Cytom 2010; 78: 239-252.
24. Raja R, Kovarova L and Hajek R. Review of phenotypic markers used in flow cytometric analysis of MGUS and MM, and applicability of flow cytometry in other plasma cell disorders. Br J Haematol 2010; 149:334-351.
25. Rawstron AC, Orfae A, Beksac M et al. Report of the European Myeloma Network on multiparametric flow cytometry in multiple myeloma and related disorders. Haematologica 2008; 93:431-438.
26. Paiva B, Vidriales MB, Pérez JJ et al. Multiparameter flow cytometry quantification of bone marrow plasma cells provides more prognostic information than morphological assessment in myeloma patients. Haematologica 2009; 94:1599-1602.
27. Smock KJ, Perkins SL and Bahier DW. Quantitation of plasma cells in bone marrow aspirates by flow cytometric analysis compared with morphologic assessment. Arch Pathol Lab Med 2007; 131:951-955.
28. Bataille R, Jégo G, Robillard N et al. The phenotype of normal, reactive and malignant plasma cells. Identification of “many and multiple myelomas” and new targets for myeloma therapy. Haematologica 2006; 91:1234-1240.
29. Rawstron AC. Immunophenotyping of plasma cells. Curr Protoc Cytom 2006; May, Chapter 6.
30. Jégo G, Robillard N, Puthier D et al. Reactive plasmacytoses are expansions of plasmablasts retaining the capacity to differentiate into plasma cells. Blood 1999; 94:701-712.
31. Jégo G, Bataille R, Pellat-Deceunynck C. Interleukin-6 is a growth factor for nonmalignant human plasmablasts. Blood 2001; 97:1817-1822.
32. Lin P, Owens R, Tricot G, Wilson CS. Flow cytometric immunophenotypic analysis of 306 cases of multiple myeloma. Am J Clin Pathol 2004; 121:482-488.
33. Caraux A, Klein B, Paiva B et al. Circulating human B and plasma cells. Age-associated changes in counts and detailed characterization of circulating normal CD138- and CD138+ plasma cells. Haematologica 2010; 1016-1020.
34. Harada H, Kawano MM, Huang N et al. Phenotypic difference of normal plasma cells from mature myeloma cells. Blood 1993; 81:2658-2663.
35. Pellat-Deceuninynck C, Bataille R, Robillard N et al. Expression of CD28 and CD40 in human myeloma cells: a comparative study with normal plasma cells. Blood 1994; 84:2597-2603.
36. Terstappen LW, Johnsen S, Segers-Nolten IM, Loken MR. Identification and characterization of plasma cells in normal human bone marrow by high-resolution flow cytometry. Blood 1990; 76:1739-1747.
37. Thomas ML. The leukocyte common antigen family. Annu Rev Immunol 1989; 7:339-369.
38. Towbridge IS and Thomas ML. CD45, an emerging role as a protein tyrosine kinase phosphatase required for lymphocyte activation and development. Annu Rev Immunol 1994;. 12:85-116.

39. Pellat-Deceuninynck C and Bataille R. Normal and malignant human plasma cells: proliferation, differentiation and expansions in relation to CD45 expression. Blood Cells Mol Dis 2004; 32:293-301.
40. Asosingh K, De Raeve H, Van Riet I, Van Camp B and Vanderkerken K. Multiple myeloma tumor progression in the 5T2MM murine model is a multistage and dynamic process of differentiation, proliferation, invasion and apoptosis. Blood 2003; 101:3136-3141.
41. Bataille R, Robillard N, Pellat-Deceunynck C and Amiot M. A cellular model for myeloma cell growth and maturation based on an intraclonal CD45 hierarchy. Immunol Rev 2003; 194:105-111.
42. Jensen GS, Belch AR, Mant MJ, Ruether BA and Pilarski LM. Expression of multiple adhesion molecules on circulating monoclonal B cells in myeloma. Curr Top Microbiol Immunol 1992; 182:187-193.
43. Moreau P, Robillard N, Avet-Loiseau H et al. Patients with CD45 negative multiple myeloma receiving high dose therapy have a shorter survival than those with CD45 positive multiple myeloma. Haematologica 2004; 89:547-551.
44. Pérez-Andrés M, Santiago M, Almeida J et al. Immunophenotypic approach to the identification and characterization of clonal plasma cells from patients with monoclonal gammopathies. J Biol Regul Homeost Agents 2004; 18:392-398.
45. Pellat-Deceunynck C, Barillé S, Puthier D et al. Adhesion molecules on human myeloma cells: significant changes in expression related to malignancy, tumor spreading, and immortalization. Cancer research 1995; 3647-3653.
46. Pellat-Deceunynck C, Barillé S, Jégo G et al. The absence of CD56 (NCAM) on malignant plasma cells is a hallmark of plasma cell leukemia and of a special subset of multiple myeloma. Leukemia 1998; 12:1977-1982.
47. Blaheta RA, Hundemer M, Mayer G et al. Expression level of neural cell adhesion molecule (NCAM) inversely correlates with the ability of neuroblastoma cells to adhere to endothelium in vitro. Cell Commun Adhes 2002; 9:121-147.
48. Blaheta RA, Beecken WD, Engl T et al. Human cytomegalovirus infection of tumor cells downregulates NCAM (CD56): a novel mechanism for virus-induced tumor invasiveness. Neoplasia 2004; 6:323-331.
49. Rawstron AC, Barrans S, Blythe D et al. Distribution of myeloma plasma cells in peripheral blood and bone marrow correlates with CD56 expression. Br J Haematol 1999; 104:138-143.
50. Ely SA and Knwoles DM. Expression of CD56/neural cell adhesion molecule correlates with the presence of lytic bone lesions in multiple myeloma and distinguishes myeloma from monoclonal gammopathy of undetermined significance and lymphomas with plasmacytoid differentiation. Am J Pathol 2002; 160:1293-1299.
51. Martin P, Santon A and Bellas C. Neural cell adhesion molecule expression in plasma cells in bone marrow biopsies and aspirates allows discrimination between multiple myeloma, monoclonal gammopathy of uncertain significance and polyclonal plasmacytosis. Histopathology 2004; 44:375-380.
52. Kremer M, Ott G, Nathrath M et al. Primary extramedullary plasmacytoma and multiple myeloma: phenotypic differences revealed by immunohistochemical analysis. J Pathol 2005; 205:92-101.
53. Leo R, Boeker M, Peest D et al. Multiparameter analysis of normal and malignant human plasma cells: CD38++, CD56+, CD54+, cIg+ is the common phenotype of myeloma cells. Ann Hematol 1992; 64:132-139.
54. Zandecki M, Facon T, Bernardi F et al. CD19 and immunophenotype of bone marrow plasma cells in monoclonal gammopathy of undetermined significance. J Clin Pathol 1995;. 48:548-552.
55. Mahmoud MS, Fujii R, Ishikawa H and Kawano MM. Enforced CD19 expression leads to growth inhibition and reduced tumorigenicity. Blood 1999; 94:3551-3558.
56. Ocqueteau M, Orfae A, Almeida J et al. Immunophenotypic characterization of plasma cells from monoclonal gammopathy of undetermined significance patients. Implications for the differential diagnosis between MGUS and multiple myeloma. Am J Pathol 1998; 152:1655-1665.
57. Sezer O, Heider U, Zavrski I and Possinger K. Differentiation of monoclonal gammopathy of undetermined significance and multiple myeloma using flow cytometric characteristics of plasma cells. Haematologica 2001; 86:837-843.
58. Kovarova L, Buresova I, Buchier T et al. Phenotype of plasma cells in multiple myeloma and monoclonal gammopathy of undetermined significance. Neoplasma 2009; 56:526-532.
59. Shapiro VS, Mollenauer MN and Weiss A. Endogenous CD28 expressed on myeloma cells up-regulates interleukin-8 production: implications for multiple myeloma progression. Blood 2001; 98:187-193.
60. Robillard N, Jego G, Pellat-Deceunynck C et al. CD28, a marker associated with tumoral expansion in multiple myeloma. Clin Cancer Res 1998;. 4:1521-1526.
61. Zhang XG, Gaillard JP, Robillard N et al. Reproducible obtaining of human myeloma cell lines as a model for tumor stem cell study in human multiple myeloma. Blood 1994; 83:3654-3663.
62. Camarini D, Walz G, Loenen WA, Borst J and Seed B. The T cell activation antigen CD27 is a member of the nerve growth factor/tumor necrosis factor receptor gene family. J Immunol 1991; 147:3165-3169.
63. Hintzen RQ, de Jong R, Lens SM and van Lier RA. CD27: marker and mediator of T-cell activation? Immunol Today 1994; 15:307-311.
64. Agematsu K, Nagumo H, Oguchi Y et al. Generation of plasma cells from peripheral blood memory B cells: synergistic effect of interleukin-10 and CD27/CD70 interaction. Blood 1998; 91:173-180.
65. Zhan F, Hardin J, Kordsmeier B et al. Global gene expression profiling of multiple myeloma, monoclonal gammopathy of undetermined significance, and normal bone marrow plasma cells. Blood 2002; 99:1745-1757.
66. Moreau P, Robillard N, Jego G et al. Lack of CD27 in myeloma delineates different presentation and outcome. Br J Haematol 2006; 132:168-170.
67. Guikema JE, Hovenga S, Vellenga E et al. CD27 is heterogeneously expressed in multiple myeloma: low CD27 expression in patients with high-risk disease. Br J Haematol 2003; 121:36-43.
68. Katayama Y, Sakai A, Oue N et al. A possible role of the loss of CD27-CD70 interaction in myelomagenesis. Br J Haematol 2003; 120:223-234.

69. Miettinen M and Lasota J. KIT (CD117): a review on expression in normal and neoplastic tissues, and mutations and their clinicopathologic correlation. Appl Immunohistochem Mol Morphol 2005; 13:205-220.
70. Ocqueteau M, Orfae A, Garcia-Sanz R, Almeida J, Gonzalez M and San Miguel JF. Expression of the CD117 antigen (c-Kit) on normal and myelomatous plasma cells. Br J Haematol 1996; 95:489-493.
71. Kraj M, Poglod R, Kopec-Szlezak J, Sokolowska U, Wozniak J and Kruk B. C-kit receptor (CD117) expression on plasma cells in monoclonal gammopathies. Leuk Lymphoma 2004; 45:2281-2289.
72. Pruneri G, Ponzoni M, Ferreri AJ et al. The prevalence and clinical implications of c-kit expression in plasma cell myeloma. Histopathology 2006; 48-529-535.
73. Mateo G, Castellanos M, Rasillo A et al. Genetic abnormalities and patterns of antigenic expression in multiple myeloma. Clin Cancer Res 2005; 11:3661-3667.
74. Bataille R, Pellat-Deceunynck C, Robillard N, Avet-Loiseau H, Harousseau JL and Moreau P. CD117 (c-kit) is aberrantly expressed in a subset of MGUS and multiple myeloma with unexpectedly good prognosis. Leuk Res 2008; 32:379:382.
75. Barrena S, Almeida J, Yunta M et al. Aberrant expression of tetraspanin molecules in B-cell chronic lymfoproliferative disorders and its correlation with normal B-cell maturation. Leukemia 2005; 19:1376-1383.
76. Freeman SD, Kelm S, Barber EK and Crocker PR. Characterization of CD33 as a new member of the sialoadhesin family of cellular interaction molecules. Blood 1995; 85:2005-2012.
77. Wagner JE, Collins D, Fuller S et al. Isolation of small primitive human hematopoietic stem cells: distribution of cell surface cytokine receptors and growth in SCID-Hu mice. Blood 1995; 86:512-523.
78. Almeida J, Orfao A, Acqueteau M et al. High-sensitive immunophenotyping and DNA ploidy studies for the investigation of minimal residual disease in multiple myeloma. Br J Haematol 1999; 107:121-131.
79. Sahara N, Ohnishi K, Ono T et al. Clincopathological and prognostic characteristics of CD33-positive multiple myeloma. Eur j Haematol 2006; 77:14-18.
80. Morice WG, Hanson CA, Kumar S, Frederick LA, Lesnick CE and Greipp PR. Novel multi-parameter flow cytometry sensitively detects phenotypically distinct plasma cell subsets in plasma cell proliferative disorders. Leukemia 2007; 21:2043-2046.
81. de Tute RM, Jack AS, Child JA, Morgan GJ, Owen RG and Rwastron AC. A single tube six-colour flow cytometry assay for the detection of minimal residual disease in myeloma. Leukemia 2007; 21:2046-2049.
82. Saraquete ME; Garcia-Sanz R, Gonzalez D et al. Minimal residual disease monitoring in multiple myeloma: a comparison between allelic-specific oligonucleotide real-time quantitative polymerase chain reaction and flow cytometry. Haematologica 2005; 90:1365-1372.
83. Robillard N, Béne MC, Moreau P and Wuillème S. A single tube multiparameter seven colour flow cytometry strategy for the detection of malignant plasma cells in multiple myeloma. Blood Canc J 2013; doi:10.1038/bcj.2013.33.
84. van Dongen JJ, Hermitte L, Böttcher S et al. Euroflow antibody panels for standardized n-dimensional flow cytomatric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia 2012; 26:1908-1975.
85. Pérez-Persona E, Vidriales MB, Mateo G et al. New criteria to identify risk of progression in monoclonal gammopathy of uncertain significance and smoldering multiple myeloma based on multiparameter flow cytometry analysis of bone marrow plasma cells. Blood 2007; 110:2586-2592.
86. Uckun, FM. Regulation of human B-cell ontogeny. Blood 1990; 76:1908-1923.
87. Paiva B, Vidriales MB, Mateo G et al. The persistence of immunophenotypicaly normal residual bone marrow plasma cells at diagnosis identifies a good prognostic subgroup of symptomatic multiple myeloma patients. Blood 2009; 114:4369-4372.
88. Robillard N, Avet-Loiseau H, Garand R et al. CD20 is associated with a small mature plasma cell morphology and t(11;14) in multiple myloma. Blood 2003; 102:1070-1071.
89. San Miguel JF, Gonzalez M, Gascon A et al. Immunopohenotypic heterogeneity of multiple myeloma: influence on the biology and clinical course of the disease. Castellano-Leones (Spain) Cooperative Group for Study of Monoclonal Gammopathies. Br J Haematol 1991; 1991:185-190.
90. Linsley PS and Ledbetter JA. The role of the CD28 receptor during T cell responses to antigen. Annu Rev Immunol 1993; 11:191-212.
91. Mateo G, Montalban MA, Vidriales MB et al. Prognostic value of immunophenotyping in multiple myeloma: a study by the PETHEMA/GEM cooperative study groups on patients uniformly treated with high-dose therapy. J Clin Oncol 2008; 26:2737-2744.
92. Kumar S, Rajkumar SC, Kimlinger T, Greipp PR and Witzig TE. CD45 expression by bone marrow plasma cells in multiple myeloma: clinical and biological correlations. Leukemia 2005; 19:1466-1470.
93. Van Camp B, Durie BG, SPier C et al. Plasma cells in multiple myeloma express a natural killer cell associated antigen: CD56 (NKH-1; Leu-19). Blood 1990; 76:377-382.
94. Sahara N and Takeshita A. Prognostic significance of surface markers expressed in multiple myeloma: CD56 and other antigens. Leuk lymphoma 2004; 45:61-65.
95. Schmidt-Hieber M, Pérez-Andrés M, Paiva B et al. CD117 expression in gammopathies is associated with an altered maturation of the myeloid and lymphoid hematopoietic cell compartments and favorable disease features. Haematologica 2011; 96:328-332.
96. Moreaux J, Hose D, Reme T et al. CD200 is a new prognostic factor in multiple myeloma. Blood 2006; 108:4194-4197.
97. Alapat D, Coviello-Malle J, Owens R et al. Diagnostic usefulness and prognostic impact of CD200 expression in lymphoid malignancies and plasma cell myeloma. Am J Clin Pathol 2012; 127:93-100.
98. Paiva B, Gutiérrez NC, Chen X et al. Clinical significance of CD81 expression by clonal plasma cells in high-risk smoldering and symptomatic multiple myeloma patients. Leukemia 2012; 26:1862-1869.
99. Bataille R, Robillard N, Avet-loiseau H, Harousseau JL and Moreau P. CD221 (IGF-1R) is aberrantly expressed in multiple myeloma, in relation to disease severity. Haematologica 2005; 90:706-707.

100. Atakanovic D, Panse J, Hildebrandt Y et al. Surface molecule CD229 as a novel target for the diagnosis and treatment of multiple myeloma. Haematologica 2011; 96:1512-1520.
101. Olteanu H, Wang HY, Weina C, McKenna RW and Karandikar NJ. Immunophenotypic studies of monoclonal gammopathy of undetermined significance. BMC Clin Pathol 2008; 8:13.
102. Bahlis NJ, King AM, Kolonias D et al. CD28-mediated regulation of multiple myeloma cell proliferation and survival. Blood 2007; 109:5002-5010.
103. Shapiro VS, Mollenauer MN and Weiss A. Endogenous CD28 expressed on myeloma cells up-regulates interleukin-8 production: implications for multiple myeloma progression. Blood 2001; 98:187-193.
104. Sahara N, Takeshita A, Shigeno K et al. Clinicopathological and prognostic characteristics of CD56-negative multiple myeloma. Br J Haematol 2002; 117:882-885.
105. Hekimgil M, Cagirgan S, Pehlivan M, Doganavsargil B, Tombuloglu M and Soydan S. Immunohistochemical detection of CD 95 (Fas) & Fas ligand (Fas-L) in plasma cells of multiple myeloma and its correlation with survival. Leuk lymphoma 2006; 47:271-280.
106. Robillard N, Pellat-Deceunynck C and Bataille R. Phenotypic characterization of the human myeloma cell growth fraction. Blood 2005; 105:4845-4848.
107. Menke DM, Horny HP, Griesser H, Atkinson EJ, Kaiserling E and Kyle RA. Immunophenotypic and genotypic characterization of multiple myelomas with adverse prognosis characterised by immunological expression of the T cell related antigen CD45RO. J Clin Pathol 1998;. 51:432-437.
108. Robillard N, Wuillème S, Lodé L, Magrangeas F, Minvielle S and Avet-loiseau H. CD33 is expressed on plasma cells of significant number of myeloma patients, and may represent a therapeutic target. Leukemia 2005; 19:2021-2022.
109. Fernandez de Larrea C, Kyle RA, Durie BG et al. Plasma cell leukemia: consensus statement on diagnostic requirements, response criteria and treatment recommendations by the International Myeloma Working Group. Leukemia 2013; 27:780-791.
110. Garcia-Sanz R, Orfao A, Gonzalez M et al. Primary plasma cell leukemia: clinical, immunophenotypic, DNA ploidy and cytogenetic characteristics. Blood 1999; 93:1032-1037.
111. Cha CH, Park CJ, Huh JR, Chi HS, Suh CW and Kang YK. Significantly better prognosis for patients with primary plasma cell leukemia than for patients with secondary plasma cell leukemia. Acta Haematol 2007; 118:178-182.
112. Gertz MA, Kyle RA and Greipp PR. The plasma cell labeling index: a valuable tool in primary systemic amyloidosis. Blood 1989; 74:1108-1111.
113. Yoshida T, Matsuda M, Katoh N et al. Long term follow-up of plasma cells in bone marrow and serum free light chains in primary systemic AL amyloidosis. Intern Med 2008; 47:1783-1790.
114. Perfetti V, Casarini S, Palladini G et al. Analysis of V(lambda)- J(lambda) expression in plasma cells from primary AL amyloidosis and normal bone marrow identifies 3r (lambda III) as a new amyloid associated germline gene segment. Blood 2002; 100:948-953.
115. Kyle RA and Gertz MA. Primary systemic amyloidosis: clinical and laboratory features in 474 cases. Semin Hematol 1995; 32:45-59.
116. Matsuda M, Gono T, Shimojima Y, Sakashita K, Koike K and Ikeda S. Evaluation of intensive chemotherapy in AL amyloidosis: usefulness of flow cytometric analysis of plasma cells in bone marrow. Amyloid 2003; 10-250-260.
117. Haghighi B, Yanagihara R and Combleet PJ. IgM myeloma case report with immunophenotypic profile. Am J Hematol 1998; 59:302-308.
118. Zarrabi MH, Stark RS, Kane P, Dannaher CL and Chandor S. IgM myeloma, a distinct entity in the spectrum of B-cell neoplasms. Am J Clin Pathol 1981; 75:1-10.
119. Annibali O, Petrucci MT, Del Bianco P et al. IgM multiple myeloma: report of 4 cases and review of the literature. Leuk lymphoma 2006; 47:1565-1569.
120. Kyle RA, Therneau TM, Rajkumar SV et al. Long-term follow-up of IgM monoclonal gammopathy of undetermined significance. Blood 2003; 102:3759-3764.
121. San Miguel JF, Caballero MD, Gonzalez M, Zola H and Lopez Borrasca A. Immunological phenotype of neoplasms involving the B cell in the last step of differentiation. Br J Haematol 1986; 62:75-83.
122. Feyler S, O'Connor SJ, Rawstron AC et al. IgM myeloma a rare entity characterized by CD20-CD56-CD117- immunophenotype and the t(11;14). Br J Haematol 2008; 140:547-551.
123. Munshi NC and Kenneth CA. Minimal residual disease in multiple myeloma. J Clin Oncol 2013; 31:2523-2525.
124. Martinez-Lopez J, Blade J, Mateos MV et al. Long-term prognostic significance of response in multiple myeloma after stem cell transplantation. Blood 2011; 118:529-534.
125. van de Velde HJ, Liu X, Chen G, Cakana A, Deraedt W and Bayssas M. Complete response correlates with long-term survival and progression free survival in high-dose multiple myeloma. Haematologica 2007; 92:1399-1406.
126. Attal M, Harousseau HL, Stoppa AM et al. A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma: Intergroupe Français du Myélome. N Engl J Med 1996; 335:91-97.
127. Child JA, Morgan GJ, Davies FE et al. High dose chemotherapy with hematopoietic stem cell rescue for multiple myeloma. N Engl J Med 2003; 348:1875-1883.
128. Lahuerta JJ, Martinez-Lopez J, Serna JD et al. Remission status defined by immunofixation vs. electrophoresis after autologous stem cell transplantation has a major impact on outcome of multiple myeloma patients. Br J Haematol 2000; 109:438-446.
129. Rawstron AC, Child JA, de Tute RM et al. Minimal residual disease assessed by multiparameter flow cytometry in multiple myeloma: impact on outcome in the medical research council myeloma IX study. J Clin Oncol 2013; 10:2540-2547.
130. Lahuerta JJ, Mateos MV, Martinez-lopez J et al. Influence of pre- and post-transplantation responses on outcome of patients with multiple myeloma: sequential improvement of response and achievement of complete response are associated with longer survival. J Clin Oncol 2008; 26:5775-5782.

131. Niesvizky R, Rochardson PG, Rajkumar SV et al. The relationship between quality of response and clinical benefit for patients treated on the bortezomib arm of the international, randomized phase 3 APEX trial in relapsed multiple myloma. Br J Haematol 2011; 143:46-53.
132. Gay F, Larocca A, Wijermans P et al. Complete response correlates with long-term progression free and overall survival in elderly myeloma treated with novel agents: analysis of 1175 patients. Blood 2011; 117:3025-3031.
133. Chanan-Khan AA and Giralt S. Importance of achieving a complete response in multiple myeloma, and the impact of novel agents. J Clin Oncol 2010; 28:2612-2624.
134. Harousseau JL, Attal M and Avet-loiseau H. The role of complete response in multiple myeloma. Blood 2009; 114:3139-3146.
135. Chee CE, Kumar S, Larson DR et al. The importance of bone marrow examination in determining complete response to therapy in patients with multiple myeloma. Blood 2009; 114:2617-618.
136. Durie BG, Harousseau J, Miguel JS et al. International uniform response criteria for multiple myeloma. Leukemia 2006; 20:1467-1473.
137. Rajkumar SV, Harousseau JL, Durie B et al. Consensus recommendations for the uniform reporting of clinical trials: report of the International Myeloma Workshop Consensus Panel 1. Blood 2011; 117:4691-4695.
138. San Miguel JF, Almeida J, Mateo G et al. Immunophenotypic evaluation of the plasma cell compartment in multiple myeloma: a tool for comparing the efficacy of different treatment strategies and predicting outcome. Blood 2002; 99:1853-1856.
139. Rawstron AC, Davies FE, DasGupta R et al. Flow cytometric disease monitoring in multiple myeloma: the relationship between normal and neoplastic plasma cells predicts outcome after transplantation. Blood 2002; 100:3095-3100.
140. Paiva B, Vidriales MB, Cervero J et al. Multiparameter flow cytometric remission is the most relevant prognostic factor for multiple myeloma patients who undergo autologous stem cell transplantation. Blood 2008; 112:4017-4023.
141. Liu H Yuan C, Heinerich J et al. Flow cytometric minimal residual disease monitoring in patients with multiple myeloma undergoing autologous stem cell transplantation: a retrospective study. Leuk Lymphoma 2008; 49:306-314.
142. Paiva B, Martinez-Lopez J, Vidriales MB et al. Comparison of immunofixation, serum free light chain, and immunophenotyping for response evaluation and prognostication in multiple myeloma. J Clin Oncol 2011; 29:1627-1633.
143. Harousseau JL, Palumbo A, Richardson PG et al. Superior outcomes associated with complete response in newly diagnosed multiple myeloma patients treated with nonintensie therapy: analysis of the phase 3 VISTA study of bortezomib plus mephelan-prednisone versus mephelan-prednisone. Blood 2010; 116:3743:3750.
144. Harousseau JL, Dimopoulos MA, Wang et al. Better quality of response to lenalidomide plus dexamethasone is associated with improved clinical outcomes in patients with relapsed or refractory multiple myeloma. Haematologica 2010; 95:1738-174.
145. Kyle RA, Leong T, Li S et al. Complete response in multiple myeloma: Clinical trials E9486, an Eastern Cooperative Oncology study not involving stem cell transplantation. Cancer 2006; 106:1958-1966.
146. Paiva B, Gutiérrez NC, Rosinol L et al. High-risk cytogenetics and persistent minimal residual disease by multiparameter flow cytometry predict unsustained complete response after autologous stem cell transplantation in multiple myeloma. Blood 2012; 119:687-691.
147. Paiva B, Vidriales MB, Montalban MA et al. Analysis of immunophenotypic response (IR) by multiparameter flow cytometry in 516 myeloma patients included in three consecutive Spanish trials. Blood 2010. 116 (Abstr 1910).
148. Kantarjian H, Schiffer C, Jones D and Cortes J. Monitoring the response and course of chronic myeloid leukemia in the modern era of BCR-ABL tyrosine kinase inhibitors: practical advice on the use and interpretation of monitoring methods. Blood 2008; 111:1774-1780.
149. Brüggeman M. Standerdized MRD quantification in European ALL trials: proceedings of the Second International Symposium of MRD assessment in Kiel, Germany, 18-20 september 2008. Leukemia 2010.
150. Grimwade D, Jovanovic JV, Hills RK et al. Prospective minimal residual disease monitoring to predict relapse of acute promyelocytic leukemia and to direct pre-emptive arsenic trioxide therapy. J Clin Oncol 2009; 27:3650-3658.
151. Hadzidimitriou A, Stamatopoulos K, Belessi C et al. Immunoglobulin genes in multiple myeloma: expressed and non-expressed repertoires, heavy and light chain pairing and somatic mutation patterns in a series of 101 cases. Haematologica 2006; 91:781-787.
152. Corradini P, Voena C, Astolfi M et al. High-dose sequential chemoradiotherapy in multiple myeloma: residual tumor cells are detectable in bone marrow and peripheral blood cell harvests and after autografting. Blood 1995; 85:1596-1602.
153. Björkstrand B, Ljungman P, Bird JM, Samson D and Gahrton G. Double high dose chemoradiotherapy with autologous stem cell transplantation can induce molecular remissions in multiple myeloma. Bone marrow transplant 1995; 15:367-371.
154. Swedin A, Lenhoff S, Olofsson T, Thuresson B and Westin J. Clinical utility of immunoglobulin heavy chain rearrangement identification for tumour cell detection in multiple myeloma. Br J Haematol 1998; 103:1145-1151.
155. Corradini P, Voena C, Tarella C et al. Molecular and clinical remissions in multiple myeloma: role of autologous and allogeneic transplantation of hematopoietic cells. J Clin Oncol 1999; 17:208-215.
156. Martinelli G, Terragna C, Zamagni E et al. Polymerase chain reaction-based detection of minimal residual disease in multiple myeloma patients receiving allogeneic stem cell transplantation. Haematologica 2000; 85:930-934.
157. Martinelli G, Terragna C, Zamagni E et al. Molecular remission after allogeneic or autologous transplantation of hematopoietic stem cells for multiple myeloma. J Clin Oncol 2000; 18:2273-2281.
158. Cavo M, Terragna C, Matinelli G et al. Molecular monitoring of minimal residual disease in patients in long-term complete remission after allogeneic stem cell transplantation for multiple myeloma. Blood 2000; 96:355-357.
159. Bird JM, Russell NH and Samson D. Minimal residual disease after bone marrow transplantation for multiple myeloma: evidence for cure in long-term survivors. Bone Marrow Transplant 1993; 12:651-654.
160. Galimberti S, Benedetti E, Morabito F et al. Prognostic role of minimal residual disease in multiple myeloma patients after non-myeloablative allogeneic transplantation. Leuk Res 2005; 29:961-966.

161. Corradini P, Cavo M, Lokhorst H et al. Molecular remission after myeloablative allogeneic stem cell transplantation predicts a better relapse-free survival in patients with multiple myeloma. Blood 2003; 102:1927-1929.
162. Raab MS, Cremer FW, Breitkreutz IN et al. Molecular monitoring of tumour load kinetics predicts disease progression after non-myeloablative allogeneic stem cell transplantation in multiple myeloma. Ann Oncol 2005; 611-617.
163. Ladetto M, Donovan JW, Harig S et al. Real time polymerase chain reaction of immunoglobulin rearrangements for quantitative evaluation of minimal residual disease in multiple myeloma . Biol Blood Marrow Transplant 2000; 6:241-253.
164. Rasmussen T, Poulsen TS, Honoré L and Johnsen HE. Quantitation of minimal residual disease in multiple myeloma using an allele-specific real-time PCR assay. Exp Hematol 2000; 28:1039-1045.
165. Bakkus MH, Bouko Y, Samson D et al. Post-transplantation tumour load in bone marrow, as assessed by quantitative ASO-PCR, is a prognostic parameter in multiple myeloma. Br J Haematol 2004; 126:665-674.
166. Fenk R, Ak M, Kobbe G et al. Levels of minimal residual disease detected by quantitative molecular monitoring herald relapse in patients with multiple myeloma. Haematologica 2004; 89:557-566.
167. Novella E, Giaretta I, Elice F et al. Fluorescent polymerase chain reaction and capillary electrophoresis for IgH rearrangement and minimal residual disease evaluation in multiple myeloma. Haematologica 2002; 87:1157-1164.
168. Korthals M, Sehnke N, Kronenwett R et al. The level of minimal residual disease in the bone marrow of patients with multiple myeloma before high-dose therapy and autologous blood stem cell transplantation is an independent predictive parameter. Biol Blood Marrow Transplant 2012; 18:423-431.
169. Ladetto M, Pagliano G, Gerrero S et al. Major tumor shrinking and persistent molecular remissions after consolidation with bortezomib, thalidomide, and dexamethasone in patients with autografted myeloma. J Clin Oncol 2010; 28:2077-2084.
170. Ladetto et al, 2011; Long term results of the GIMEMA VTD consolidation trial in autografted multiple myeloma patients (VEL-03-096) impact of MRD detection by real time quantitative PCR on late recurrences and overall survival ASH 2011.
171. Lioznov M, Badbaran A, Fahse B, Bacher U, Zander AR and Kröger NM. Monitoring of minimal residual disease in multiple myeloma after allo-SCT: flow cytometry versus PCR-based techniques. Bone marrow transplant 2008; 41:913-916.
172. Zhao X, Huang Q, Slovak M and Weiss L. Comparison of ancillary studies in the detection of residual disease in plasma cell myeloma in bone marrow. Am J Pathol 2006; 125:895-904.
173. Owen R and Rawstron AC. Minimal residual disease monitoring in multiple myeloma: flow cytometry is the method of choice. Br J Haematol 2005; 128:732-733.
174. Davies F, Forsyth PD, Rawstron AC et al. The impact of attaining a minimal residual disease state after high-dose mephelan and autologous transplantation for multiple myeloma. Br J Haematol 2001; 112:814-819.
175. Baer MR, Stewart CC? Dodge RK et al. High frequency of immunophenotypic changes in acute myeloid leukemia at relapse: implications for residual disease detection (Cancer and Leukemia Group B Study 8361). Blood 2001; 97:3574-3580.
176. Chen W, Karandikar NJ, McKenna RW and Kroft SH. Stability of leukemia-associated immmunophenotypes in precursor B-lymphoblastic leukemia/lymphoma: a single institution experience. Am J Clin Pathol 2007; 127:39-46.
177. Cao W, Goolsby CL, Nelson BP, Singhal S, Mehta J and Peterson LC. Instability of immunophenotype in plasma cell myeloma. Am J Clin Pathol 2008; 129:926-933.
178. Gupta R, Bhaskar A, Kumar L, Sharma A and Jain P. Flow cytometric immunophenotyping and minimal residual disease analysis in multiple myeloma. Am J Clin Pathol 2009; 132:728-732.
179. Foran JM, Norton AJ, Micallef IN et al. Loss of CD20 expression following treatment with rituximab (chimaeric monoclonal anti-CD20): a retrospective cohort analysis. Br J Haematol 2001; 114:881-883.
180. Spears MD, Olteanu H, Kroft SH and Harrington AM. The immunophenotypic stability of plasma cell myeloma by flow cytometry. Int J Lab Hematol 2011; 33:483-491.
181. Liu D, Lin P, Hu Y et al. Immunophenotypic heterogeneity of normal plasma cells: comparison with minimal residual plasma cell myeloma. J Clin Pathol 2012; 65:823-829.
182. Martinez-Sanchez P, Montejan L, Saraquete ME, et al. Evaluation of minimal residual disease in MM patients by fluorescent-polymerase chain reaction: the prognostic impact of achieving molecular response. Br J Haematol 2008; 142:766-774.
183. Puig N, Sarasquete ME, Balanzategui A et al. Critical evaluation of ASO RQ-PCR for minimal residual disease evaluation in multiple myeloma. A comparative analysis with flow cytometry. Leukemia 2014; 28, 391-397.
184. Novel three-and four-drug combination regimens of bortezomib, dexamethasone, cyclophosphamide, and lenalidomide, for previously untreated multiple myeloma: results from the Multi-center, Randomized, Phase 2 EVOLUTION Study. ASH Meeting 2010 Abstract 621.
185. van de Donk MWCJ, Kamps S, Mutis T and Lokhorst HM. Monoclonal antibody based therapy as a new treatment strategy in multiple myeloma. Keukemia 2012; 26:199-213.
186. Munshi N and Anderson KC. New strategies in the treatment of multiple myeloma. Clin Cancer Res 2013; 19:3337-3344.
187. Pilarski LM, Biagorri E, Mant MJ et al. Multiple myeloma includes phenotyipcaly defined subsets of clonotypic CD20+ B cells that persist during treatment with rituximab. Clin Med Oncol 2008; 2:275-287.
188. Allegra A, Penna G, Alonci A et al. Monoclonal antibodies: potential new therapeutic treatment against multiple myeloma. Eur J Haematol 2013; 90:441-468.
189. Chanan-Khan A, Wolf JL, Farci J et al. Efficacy analysis from phase I study of lorvotuzumab mertansine (IMGN901), used as monotherapy, in patients with heavily pre-treated CD56-positive multiple myeloma. A preliminary efficacy analysis. ASH 2010. 116:1162.
190. Chanan-Khan A, Wolf J, Gharibo M et al. Phase I study of IMGN901, used as monotherapy, in patients with heavily pre-treated CD56-positive multiple myeloma.- A preliminary safety and efficacy analysis. ASH 2009; 114: 2883.

1. IMMUNOPHENOTYPIC CHARACTERISTICS OF PLASMA CELLS

1.1. APPLICATION OF IMMUNOPHENOTYPING IN PLASMA CELL NEOPLASIA

Many studies have shown high clinical sensitivity of flow cytometry in the analysis of malignant plasma cells when compared to conventional morphology [24]. The identification of markers that allow the identification of plasma cells among other hematopoietic cells, and the identification of aberrant plasma cell phenotypes that enable us to discriminate between normal and neoplastic plasma cells, means we can identify, characterize and enumerate plasma cells even when few cells are present [25]. Although significant differences in the BM plasma cell percentages have been found between morphological and flow cytometric analysis, in many cases flow cytometry results showed less plasma cells than morphological analysis. Proposed reasons for this have been described as **(i)** contamination by peripheral blood, **(ii)** existence of small plasma cell clusters, **(iii)** fragility of plasma cells during sample preparation and **(iv)** unavailability of first pull BM aspirate for flow cytometry [25,26,27].
The advantages of flow cytometry in the diagnosis and monitoring of monoclonal gammopathies can be categorized into three main topics: (a) primary diagnosis of myeloma and other plasma cell disorders, based on the presence of plasma cells in the BM and demonstration that a proportion are immuno-phenotypically abnormal, monoclonal or not reactive; (b) the identification of independent prognostic markers, in particular those predicting the risk of progression for patients with MGUS and smoldering myeloma based on the relative proportions of abnormal and normal plasma cells, (c) quantitative evaluation of MRD levels for assessing efficacy of treatment and prediction of outcome, as well as the determination of stringent complete remission as defined by the International Myeloma Working Group (IMWG) (see below). Additionally, it may also be useful for (iv) the definition of prognosis associated antigenic profiles and (v) the identification of new therapeutic targets [23,24].

1.2. IMMUNOPHENOTYPIC IDENTIFICATION AND CHARACTERIZATION OF PLASMA CELLS

Multiparameter immunophenotyping based on multiple monoclonal antibody staining's allows unequivocal identification, quantification and further characterization of plasma cells, even when they are present in small percentages. It displays unique features for the study of biological samples containing plasma cells: it allows (i) simultaneously analysis of multiple parameters on a single cell basis, (ii) the study of high numbers of cells within a relatively short period of time, (iii) storage of information about individual cells for latter analyses, (iv) quantitative evaluation of antigen expression, and (v) combined detection of surface and intracellular antigens.

1.2.1. Universal markers to detect plasma cells

In a first step, a primary gating strategy aimed at the specific identification of plasma cells should be used. CD138 and CD38 are the most efficient antibodies for specific and universal identification of plasma cells. These markers, along with CD45 and light scatter characteristics; represent the best combination for the specific identification of plasma cells in hematological samples and its discrimination from other populations of leucocytes and hematopoietic cells [25,28,29].
CD138 or syndecan-A is a molecule belonging to the heparin sulfate family that mediates cell-to-cell adhesion by heparin binding molecules expressed by adjacent cells, like epithelial, mesenchymal and carcinoma cells. In human hematopoietic cells, CD138 expression is restricted to both plasma and myeloma cells. Studies of plasma cell differentiation show that CD138 must be considered as a differentiation antigen: CD138 expression appears after the plasma blastic stage. CD138- plasma blasts are plasma cell progenitors that differentiate into CD138-bright positive plasma cell precursors retaining some proliferative ability before final maturation into non-dividing CD138+ plasma cells [30,31]. CD138 expression is specific for plasma cells, although plasma cells expressing low levels of CD138 have been frequently reported and CD138- plasma cells are present in peripheral blood [23,32,33].

In contrast, CD38 is an antigen widely expressed on both hematopoietic and non-hematopoietic cells (mainly T and B-cells) that shows uniquely high amounts on normal plasma cells but can be expressed at lower levels in myelomateous plasma cells. Although widely expressed on hematopoietic cells, the uniquely bright intensity of CD38 typically observed on plasma cells, clearly higher than that found for other hematopoietic cell populations, is considered as a "specific" plasma cell profile [34]. In 1994, Pellat-Deceuninynck identified the applicability of CD138 and CD38 in recognition of normal and malignant plasma cells by flow cytometry [35]. Further, Terstappen has shown strong association between CD38 and CD138 in plasma cells [36].

Along with CD138 and CD38, CD45, which is a leucocyte common antigen found in all haematopoietic cells, with the exception of platelets and red blood cells [37], can be used in the primary gating strategy to identify plasma cells. CD45 is well known for its function as a key regulator of antigen-mediated signaling and activation in B and T lymphocytes [38]. In MM patients, two distinct plasma cell populations can be identified based on CD45 expression. These populations are characterized by diminished to negative expression of CD45 and intermediate to bright expression of CD45 [39,40,41]. Most reports agree that CD45- phenotype represents the malignant plasma cell population in MM. CD56 expression strongly correlates with CD45- plasma cells. Similarly, CD138 is also highly expressed on CD45- cells rather than CD45+ cells. Conversely, CD45+ plasma cells were reported to express more often CD44 and CD11b [42]. The presence of CD45- plasma cells is associated with poorer outcome and worse overall survival rate compared to CD45+ plasma cells. The poor outcome of CD45- phenotype might be due to other intrinsic factors influencing malignancy, such as change of ploidy or cell kinetics, especially at the time of relapse [43].

The process of gating plasma cells based on CD138, CD38, CD45 and light scatter characteristics, together with the usage of additional multiple staining provides the basis for the accurate immunophenotypic characterization of plasma cells and subsequent discrimination between phenotypically aberrant (clonal) and normal/reactive (polyclonal) plasma cells [25]. The different possible gating strategies are presented in figure 1.

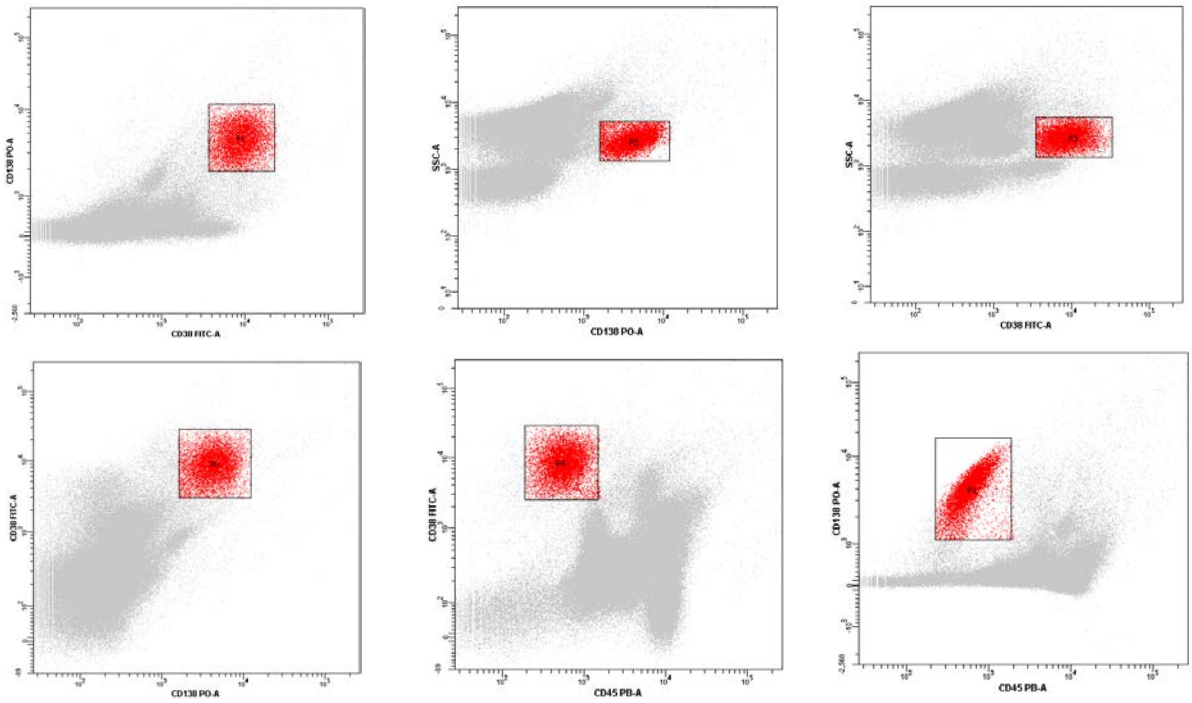

Figure 1. Gating strategies for the identification and enumeration of plasma cells in a patient with MM. The boxed events represent gated plasma cells. FITC, fluorescein isothiocyanate; PO-A, Pacific Orange, PB-A, Pacific Blue.

1.2.2. Further characterization of plasma cells

Multicentric studies have clearly shown that the phenotypic characteristics of clonal plasma cells differ from their normal counterpart in terms of antigenic expression [25,28,34,44]. Antigens associated with aberrant plasma cell expression include CD28, CD56 and/or CD117, and frequent loss of CD19 and CD45 [28]. No single marker can differentiate neoplastic plasma cells from normal plasma cells.

1.2.2.1. Expression of CD19 and CD56 in myeloma cells

As compared to normal plasma cells, myeloma cells overexpress CD56 (NCAM – Neural Cell Adhesion Molecule), a marker of NK cells [28]. However, myeloma cells circulating into the peripheral blood usually lack CD56, whereas myeloma cells located in pleural or ascites fluids express CD56 [35,45,46]. CD56 mediates cell-cell and cell-matrix interactions and loss of CD56 expression could accelerate the process of metastatic spread [47,48]. Additionally, a lack of CD56 expression is associated with less osteolysis, which is confirmed by the fact that patients with CD56 MM have more osteolytic lesions [35,49,50]. CD56 is very frequently overexpressed in BM, but less in extra medullary blood sites [46]. Measurement of CD56 expression and its intensity may therefore be helpful for distinguishing MGUS, MM, extra medullary plasmacytoma and plasma cell leukemia [46,51,52].
Lack of CD19, a pan B-cell marker, is observed frequently in patients with MM and constitutes a marker of plasma cell malignancy. Normal plasma cells retain CD19 expression, but a subpopulation may lack CD19 expression. An early study reported negative CD19 expression in MGUS [53]; however contrary to this study, Harada and Zandecki defined CD19 expression in MGUS [34,54]. CD19 intensity and expression on MGUS plasma cells was diminished when compared to normal plasma cells, but higher than on myeloma cells. Loss of CD19 was found to be associated with tumor progression in MGUS and MM patients. Mahmoud showed that increased expression of CD19 on myeloma cell lines lead to growth inhibition and reduced tumorigenicity [55]. CD19 expression in MGUS plasma cells thus defines the non-malignant population; loss of CD19 by MGUS plasma cells in parallel with alterations of other phenotypic markers might represent disease progression.
Several groups described two different populations of normal and malignant plasma cells in the BM of patients diagnosed with MGUS and MM. Normal plasma cells are characterized by low forward/side scatter (FSC/SSC) along with high CD38 expression, and CD19+ and CD56- immunophenotype. Malignant plasma cells are CD19- / CD56+ or CD19-/ CD56- with high FSC/SSC and low CD38 expression [56,57,58].

1.2.2.2. Expression of CD28 in myeloma cells

CD28 antigen, a T-cell specific marker, is not expressed on normal plasma cells but is found consistently and brightly on malignant plasma cells [24]. Myeloma cells express one co-receptor of CD28, CD86, but not the other one, CD80. CD28 is not involved in myeloma proliferation and survival, but CD28 triggering induced chemokine secretion [59]. Expression of CD28 increases with disease progression since its expression frequency increases with relapse [60]. CD28 expression studies on myeloma patients have found higher reactivity in advanced disease stage [35]. This finding is further supported by the fact that most myeloma cell lines were obtained at the terminal stage of disease or from patients with aggressive forms of myeloma cells expressing CD28 [61]. 41% of MM patients were found to express CD28 on BM plasma cells, but its expression was higher in the cases of relapsed myeloma. High reactivity for CD28 expression was found in 59% of medullary relapsed patients and 93% of extramedullary relapsed patients. In contrast to relapsed and active myeloma patients, MGUS patients (19%) expressed very low levels of CD28 on plasma cells [60]. Therefore, the intensity of CD28 expression on plasma cells correlates with stage and could be useful for diagnostic assessment on MGUS and MM patients [24].

1.2.2.3. Expression of CD27 in myeloma cells

CD27 is a 110-kDa homodimeric transmembrane glycoprotein of the tumor necrosis factor receptor family [62]. The ligand molecule for CD27 is CD70 and the interaction plays a role in the differentiation of memory B-cells into mature plasma cells [63,64]. Gene expression studies of normal plasma cells and myeloma cells have identified CD27 as being one of the most significant genes lost by myeloma cells and CD27 loss in MGUS has been linked to progression to MM [65,66]. The lack of CD27 expression is usually coupled to the loss of CD19. Conversely, CD19 loss is not always associated with CD27 loss in monoclonal gammopathies [67]. Negative CD27 expression on plasma cells has been found in stage II and stage III MM patients. In MGUS and stage I MM, half of plasma cells are CD27 positive, and probably all might be negative for CD19 expression [68]. In accordance with immunophenotyping data, gene expression profile studies also revealed that low CD27 expression in myeloma cells contrast to normal plasma cells with high CD27 expression [65].

1.2.2.4. Expression of CD117 in myeloma cells

The C-kit receptor (CD117) is an essential hematopoietic growth factor receptor with tyrosine-kinase activity. Kit-mediated signal transduction was found to be critical for normal development and hematopoietic progenitor cell survival [69]. Normal plasma cells and tumors, including sarcomas, carcinomas and lymphomas, do not express CD117.
For the time, expression of CD117 was reported in malignant plasma cells of some myeloma patients in 1996 [70]. Although these data, as well as data of other studies [71,72] suggest that c-kit positive MM might represent a poor-risk category (i.e. poor predictive marker), data from Mateo, as well as from Bataille, indicate that patients with CD117+ MM could have a better outcome [73,74]. The sample size and treatment heterogenicity may account for such discrepancies (see below).
Bataille identified CD117 expression in 36 of 122 MGUS (30%) and 169 of 617 MM (27%) cases [74]. CD117 expression in MGUS was significantly higher compared to myeloma patients. Therefore, it is possible that CD117 could be one of the markers for transition from MGUS to MM. Moreover, myeloma patients often acquire antigen CD221 in parallel with the loss of CD117 in a process associated with disease progression and poor prognosis. Mateo identified that the CD117+ patient group had a better outcome compared to the CD117- patient group [73]. No significant differences were observed between the CD117+ and CD117- groups when comparing clinical and biological parameters, such as M-protein, albumin, β2-microglobulin, LDH, disease stage, response to chemotherapy and survival time in a study of Kraj [71].

1.2.2.5. Expression of CD81 in myeloma cells

CD81 is a tetraspin cell surface protein that regulates CD19 expression in mature B-lymphocytes and is involved in the regulation of cell growth, motility, signaling and BM homing (or cell adhesion). Immunophenotypic studies of CD81 expression in patients with MM are scanty. Barrena analyzed the distribution and the pattern of expression of several tetraspanin (i.e. CD9, CD37, CD53 and CD81) antigens in normal and neoplastic human B-cells. They found that the more mature BM B-lymphocytes become, loss of tetraspanins increases, and BM lymphocytes become ready to migrate and leave the BM [75]. Less than 50% of MM cases express CD81 on plasma cells and expression is heterogeous in most of the cases (ranging from 5% to 92%).

1.2.2.6. Expression of myeloid markers in myeloma cells

CD33 is a 67-kDa glycoprotein found on the myeloid cell surface and belongs to the sialo-adhesion molecule family [76]. CD33 is expressed in 90% of leukemia patients (especially acute myeloid leukemia), but is not detected on normal haematopoietic stem cells [77]. Few studies have reported CD33 expression on plasma cells but the reactivity of the marker has been found in 6.5-12% of myeloma patients [73,78]. CD33 expression in myeloma patients correlates with clinical parameters, suggesting its clinicopathological significance. Patients were divided into two groups based on CD33 expression on plasma cells (cut-off 20%). No significant

differences were observed between CD33+ and CD33- group in terms of age, sex, bone lesion extension or extra medullary disease [79].

A summary of these and other immunophenotypic markers of clinical relevance in the detection of plasma cells neoplasms are listed in attachment 3. Clinically important and necessary antigens allowing discrimination of abnormal from normal plasma cells are presented in table 3 [25]. An example of a MFC analysis of plasma cells in a patient with MGUS and MM is presented in figure 2.

Antigen	Normal expression	Abnormal expression	Patients with abnormal expression	Requirement for diagnostic monitoring
CD19	Positive (>70%)	Negative	95%	Necessary
CD56	Negative (<15%)	Strongly positive	75%	Necessary
CD117	Negative (0%)	Positive	30%	Recommended
CD20	Negative (0%)	Positive	30%	Recommended
CD28	Weak positivity (<15%)	Strongly positive	15-45%	Recommended
CD27	Strong positivity (100%)	Weak/negative	40-50%	Recommended

Table 3. List of surface antigens useful for detection of normal and abnormal CD38+/CD138+ plasma cells in monoclonal gammopathies [25].

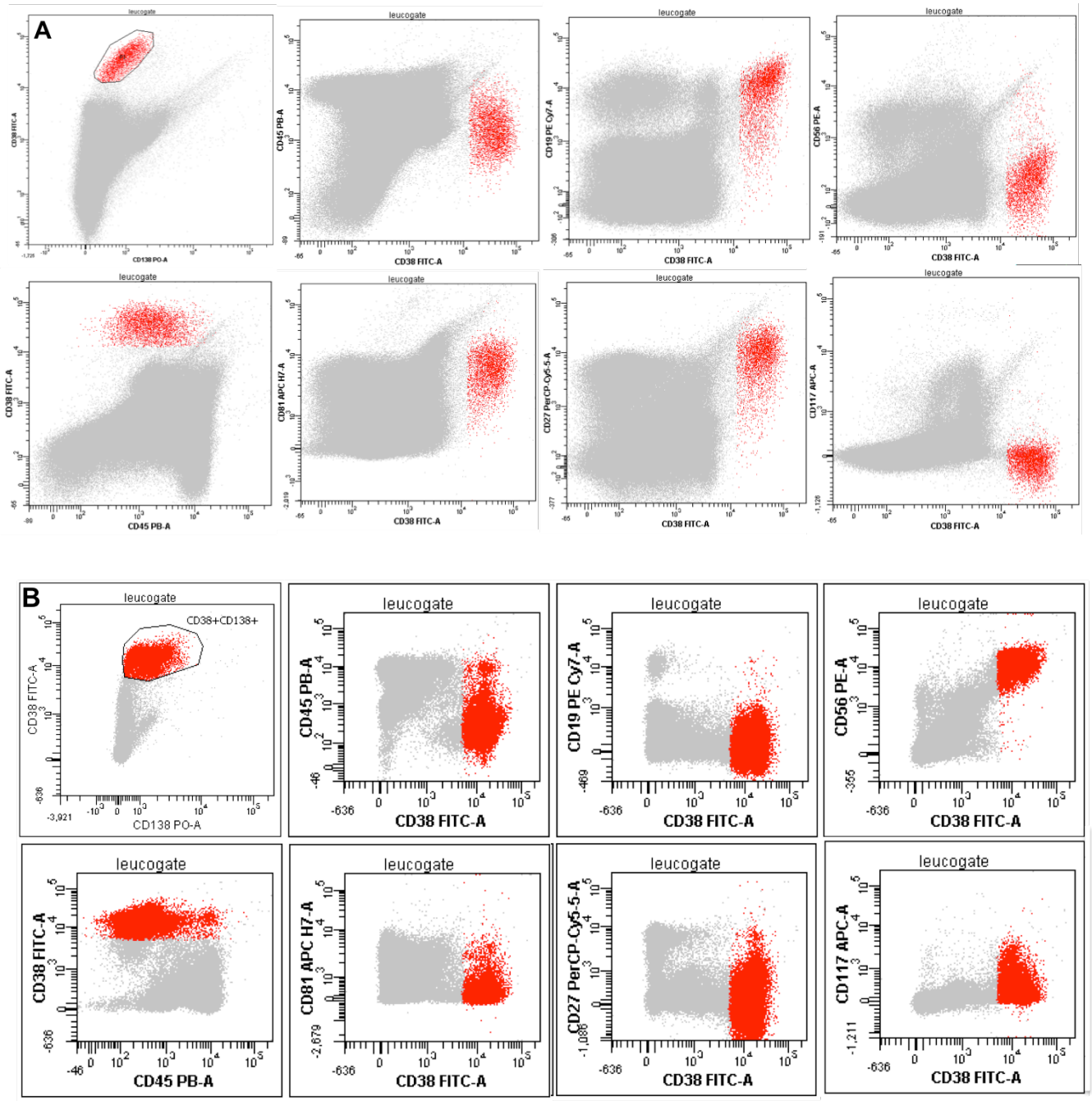

Figure 2. Multiparameter flow cytometric immunophenotypic analysis of normal antigen expression in a patient diagnosed with **(A)** MGUS and unusual antigen expression in a patient diagnosed with **(B)** multiple myeloma.

1.2.3. Plasma cell clonality assessment

After chemotherapy or stem cell transplantation, monoclonal neoplastic plasma cells are mixed with normal polyclonal plasma cells in the BM compartment. Neoplastic plasma cells can be identified either by restricted patterns of cytoplasmatic immunoglobulin (Cy-Ig) light chain expression or by aberrant patterns of surface antigen expression (see above). Although neoplastic plasma cells can be identified using either of these approaches, both have important shortcomings. It can be difficult to confidently identify abnormal plasma cells by Cy-Ig light chain restriction alone, particularly when the number of cells is low, when they are obscured by a background population of normal polytypic plasma cells, or when the disorders are bi-clonal with subsets for kappa and lambda light chains, respectively [80]. Moreover, it was demonstrated that in 6-color immunophenotyping assays, all plasma cells with aberrant phenotypes exhibit monoclonality [80,81]. Thus, in an MFC-based MRD assay for MM, assessment based on detection of plasma cells with aberrant phenotypes rather than light chain restriction is preferable for enumerating low numbers of neoplastic plasma cells in a background of polyclonal plasma cells. The introduction of 8-color flow cytometry, in which a larger subset of monoclonal antibodies can be combined, possibly brings a solution to this problem. On the contrary, the surface immunophenotype is not always sufficiently distinctive to allow for the malignant plasma cells to be identified in up to 10% of all plasma cells neoplasms due to the fact that aberrant phenotypes may be found in healthy donors as well [82,83]. Furthermore, in all immunophenotypic analyses, it is difficult to formulate a gating strategy, which allows the plasma cells to be captured. This problem can be solved when evaluating Cy-Ig light chain expression as described in a recent study of Robillard and presented in **figure 3** [83].

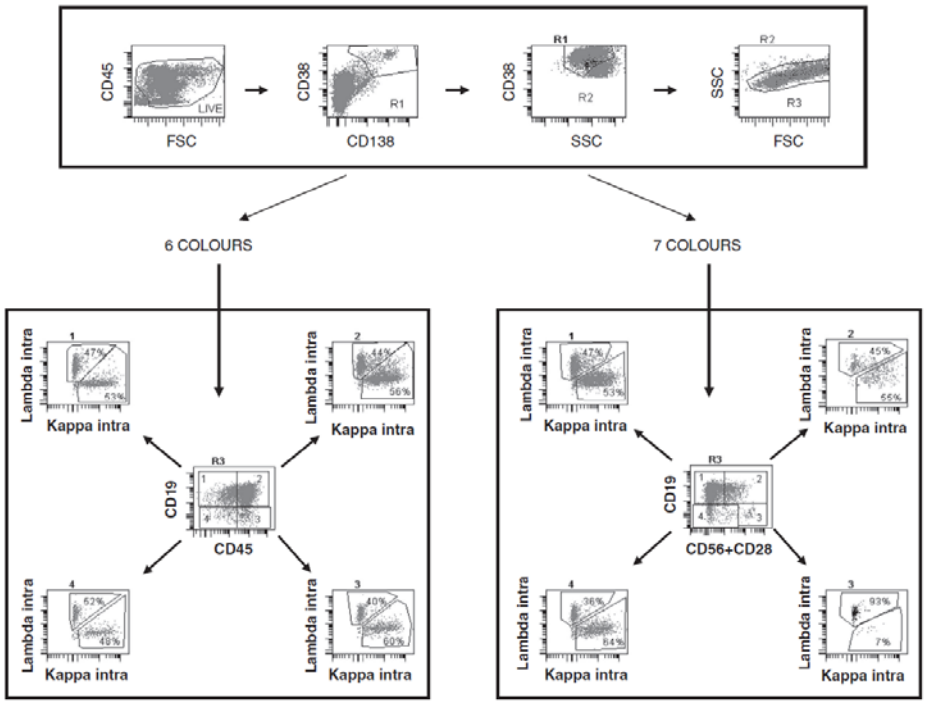

Figure 3. Analysis of data from a single-tube seven-color staining with a six-color strategy (left) or a seven-color strategy (right) as presented by Robillard [83]. In the first part, plasma cells are included in a broad R1 gate encompassing CD138+/CD38++ or CD38- cells. This population is further refined on an SSC/CD38 scattergram conditioned on R1, thereby defining gate R2. Cells satisfying both R1 and R2 are then displayed on a CD19/CD45 scattergram, allowing to define four populations as shown in de center of the panel. For each of them, a κ/λ scattergram is established to discriminate normal polyclonal plasma cells and MM-restricted plasma cells. The right bottom panel shows that, in the seven-color strategy, intracytoplasmatic light chain restriction is examined among four different populations, delineated on the basis of the expression or not of CD19 combined with the mixture of CD28 and CD56 (either or both antigens expressed when positive). CD45, which can be abnormally expressed on the clonal population as shown in the six-color strategy, is examined on a different plot. In this sample, the six-color strategy fails to identify light chain restriction, whereas the seven-color strategy reveals that 93% of the cells in subset 3 (CD19-/CD56/CD28+) use lambda chains, thereby characterizing abnormal plasma cells.

1.3. CONCLUSION

In addition to the plasma cell gating markers discussed above (CD38, CD138 and CD45), the minimal antigens for classifying abnormal plasma cells are CD19 and CD56. A preferred panel would additionally incorporate CD117, CD28 and CD27. The most commonly assessed antigens for discrimination between malignant and normal plasma cells include CD19, CD56, CD20, CD117, CD28, CD33, CD27, CD81, CD31, CD39, CD40, CD44, CyclinD1 and CD34 [25]. MFC (minimum of 6 markers, ideally 8 markers) is required for sufficient plasma cell analysis and combination of surface and intracellular antigens is necessary for identification and clonality assessment of plasma cells.

As stated by the European Myeloma Network, assessment of cytoplasmatic κ/λ restriction is generally apparent only when plasma cell myeloma cells represent 30% or more of the total plasma cells [25]. The demonstration of phenotypically abnormal plasma cells is more sensitive and specific for the detection of residual disease than clonality assessment by immunohistochemistry and/or flow cytometry. Combined assessment of clonality with basic immunophenotype may be useful for screening at diagnosis and follow-up [25]. Similar antigens are suggested in Euroflow panels (see table 4).

Tube/fluorochrom	Pacific Blue	Pacific orange	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7
1	CD45	CD138	CD38	CD28	CD27	CD19	CD117	CD81
2	CD45	CD138	CD38	CD28	CD56	β2m	cIgκ	cIgλ

Table 4. Euroflow plasma cell disease classification panel. Tube no. 1 is useful for phenotype characterization of plasma cells and evaluation markers with potential prognostic significance. Tube no. 2 is used for detection and discrimination of normal plasma cells from aberrant and clonal plasma cells [84].

2. PROGNOSTIC VALUE OF IMMUNOPHENOTYPIC DETECTION OF PLASMA CELLS

With increasing therapeutic choices, and improved outcomes in patients with MM, risk stratification is becoming important as treatment could be tailored for different groups of patients. Use of flow cytometry to investigate the prognostic value of immunophenotypic characteristics of plasma cells in patients with MM have frequently led to conflicting results, probably due to technical pitfalls (single *versus* multiparameter labeling, use of different clones of monoclonal antibody conjugates and distinct criteria for definition of positivity) or study design (small series of heterogeneously treated patients). We here present an overview of the different studies that examined the prognostic value of different monoclonal antibodies using MFC.

2.1. IMMUNOPHENOTYPIC DISCRIMINATION BETWEEN NORMAL AND ABERRANT PLASMA CELLS AT DIAGNOSIS

Different studies derived independent prognostic features based on flow cytometric evaluation of BM plasma cells in MGUS, SMM and MM. In patients with MGUS, the coexistence of normal and phenotypically aberrant plasma cells is a constant finding, with most cases displaying >5% normal plasma cells within the overall BM plasma cell compartment [85]. By contrast, only a small proportion (<15%) of all patients with symptomatic MM display >5% of normal plasma cells in the BM plasma cell compartment at diagnosis [26,87]. Therefore, the presence of >5% residual polyclonal plasma cells (from the whole BM compartment) at diagnosis have been found to be an accurate parameter for the discrimination between MGUS and MM [56,101]. Furthermore, this parameter (>5% normal plasma cells in BM plasma cells) is also of help to predict risk of transformation of MGUS and SMM into symptomatic disease, with time to progression rates at 5 years of 25% versus 5% and 64% versus 8%, respectively [85]. Accordingly, both MGUS and SMM patients who show >5% normal plasma cells in the BM at diagnosis, display a significantly lower risk of progression to symptomatic MM versus cases with ≤5% normal plasma cells in the BM. Similarly, those patients with symptomatic MM who have >5% normal plasma cells in the BM at diagnosis display a unique clinical and biological signature characterized by higher hemoglobin levels (12.1 g/dL *versus* 10.6 g/dL), lower levels of BM plasma cells (2% *versus* 13%) and lower levels of M-component (2 g/dL *versus* 4 g/dL). In addition, this unique subgroup of patients with MM also display a greater response rate to HDT/ASCT (rate of complete remission (CR) after HDT/ASCT of 64% vs. 33%), together with significant longer PFS and OS (5 year rates of 44% *versus* 33% and 71% *versus* 62%, respectively) [26,87].

2.2. PROGNOSTIC VALUE OF MULTIPARAMETER FLOW CYTOMETRIC IDENTIFICATION OF PLASMA CELLS

In 2009, Paiva showed, in a large series of uniformly treated MM cases, that quantification of BM plasma cells at diagnosis based on MFC provides more prognostic value than that obtained by morphological counts at diagnosis. More importantly, MFC was of independent prognostic value for predicting patient survival [87]. An update of these findings have been published on the prognostic impact of the immunophenotypic characteristics of BM plasma cells in MM, based on a series of 685 newly diagnosed, uniformly treated patients (HDT followed by ASCT) with MM [91]. The results of this extensive study showed that three individual markers may afford prognostic information: positive staining for CD19 and CD28, as well as absence of CD117 detected on clonal plasma cells were associated with significantly shorter PFS and OS in patients with MM. Moreover, the combination of CD28 and CD117 allow patient stratification into three risk categories: poor risk: CD28+ CD117- patients (23%), intermediate CD28-/CD117- and CD28+/CD117+ patients (56%), and good risk CD28-/CD117+ myeloma patients (21%). Therefore, simultaneous assessment of both CD28 and CD117 expression identifies one fourth of cases (CD28+/CD117-) that would not benefit from current therapeutic strategies based on HDT/ASCT, and in which alternative therapeutic strategies based on novel agents should be encouraged. In contrast, a similar proportion of patients who are CD28- and CD117+ enjoy a prolonged survival with ASCT [91] (figure 4). These results support the previous observations on the clinical relevance of CD28+ [102,103] and CD117 [28] phenotypic profiles.

Only a small proportion of patients express the CD19 antigen [28,73,91]. Mateo showed that, despite the small number of CD19+ patients (n=30; 4%), these patients have a poor outcome as compared with the CD19-

patients, with approximately 1 and 2 years shorter PFS and OS, respectively. Moreover, the majority of CD19+ patients did not express CD117, an antigen that also confers a worse prognosis to patients with MM. In contrast, CD20 antigen did not show any influence on disease outcome [91].

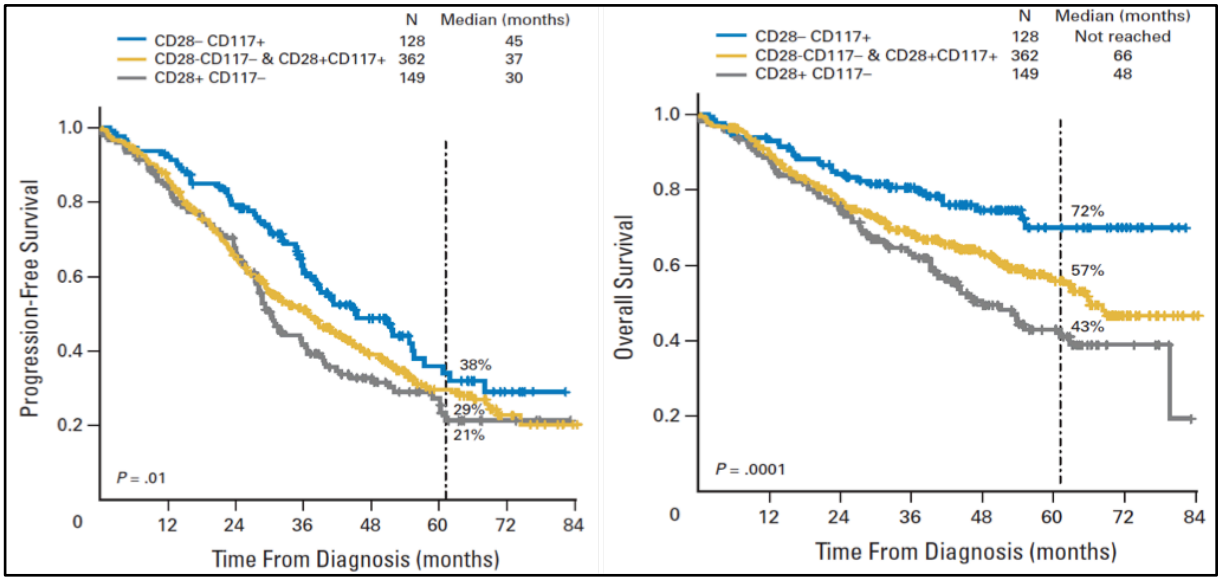

Figure 4. Prognostic impact of the phenotypic patterns of expression of CD28 and CD117 on survival of patients with multiple myeloma (n=685). PFS: progression free survival; OS: overall survival [91]

As described above, absence of CD56 expression is associated with extramedullary spreading, aggressive disease, and adverse outcome [39,94,104], although this was not confirmed in another (small) series of patients [105]. CD56 expression has been associated with fewer osteolytic lesions. In the series of Mateo, downregulation of CD56 in myelomatous plasma cells correlates with a tendency to short PFS, but no different OS [91].

Different groups have shown that the absence of expression of CD45 on MM plasma cells is associated with disease progression and adverse prognostic factors [43,92]. These data, which attribute poor prognosis for CD45- patients, would contradict the observation by Robillard, showing that the proliferating plasma cell compartment, which is theoretically involved in disease progression, is included within the CD45+ bright plasma cell fraction [106]. Moreover, Menke found that expression, and not the absence, of the 180kDa isoform of CD45, recognized by CD45RO antibodies, correlated with poor survival in patients with MM [107]. However, Mateo did not found any prognostic influence for CD45 expression in their large series of patients with MM [91].

Another study of the Spanish Salamanca group [98] prospectively analyzed the frequency and prognostic impact of CD81, assessed by MFC immunophenotyping, in a series of 230 newly diagnosed elderly symptomatic MM patients. Regarding the impact of CD81 on patient's survival, cases showing CD81+ expression showed a significant shorter PFS as compared with the CD81- patients (21 *versus* 37 months). The same finding was observed in an additional series of 325 newly diagnosed, symptomatic transplant candidates MM patients [98].

Expression of myeloid markers, such as CD13 and CD33, in myelomateous plasma cells is exceptional. Some studies have previously reported that patients with MM with this phenotype tend to have a poor prognosis, but once again, the series of patients were small [79,108]. Mateo observed that, although the outcome of CD33 positive patients was slightly worse than that of CD33- patients, differences did not reach statistical significance [91].

A summary of the prognostic value of these and other immunophenotypic markers is listed in attachment 3.

2.3. USE OF FLOW CYTOMETRY IN PLASMA CELL LEUKEMIA, AL AMYLOIDOSIS AND IGM MYELOMA

2.3.1. Plasma cell leukemia

Plasma cell leukemia (PCL) is a rare form of malignant plasma cell disease accounting for 1-2% of all plasma cell neoplasms. The disease may be classified as primary to designate a '*de novo*' leukemia in patients with no evidence of previous MM or as secondary when it is observed as a leukemic transformation of relapsed or refractory disease in patients with previously recognized MM. Of them, 60-70% of PCL are primary, and the remaining 30-40% are secondary. Primary PCL is a distinct clinic-pathological entity with different cytogenetic and molecular findings [109]. Compared to MM patients, PCL patients have adverse prognosis, lower survival rate and response to chemotherapy [110]. MM and PCL clinical features are similar, although the involvement of visceral organs, fewer bone lesions and high frequency of anemia, LDH, β2-microglobulin and trombocytopenia make PCL a distinct clinical entity [110,111]. In contrast to studies performed on MM, the immunophenotypic information at diagnosis as well as on the MRD follow-up in PCL is limited. Flow cytometry immunophenotypic findings have shown that the expression of antigens such as CD38, CD138, CD2, CD3, CD10, CD13, CD16 and CD15 were similar for MM and PCL. On the other hand, CD20 expression was found in PCL whereas four other antigens (i.e. CD56, CD9, CD117 and HLA-DR) were more frequently negative in PCL compared to MM. Differentiation between primary and secondary PCL could be made by using CD28 antigen expression. Pellat-Deceunynck found that secondary PCL cases express more frequently CD28 compared to primary PCL (92% in PB, 55% in BM *versus* 33% either PB/BM). This is consistent with the fact that the acquisition of CD28 antigen on plasma cells correlates with increased plasma cell proliferation and disease progression [46]. Contrastingly, CD56 expression and negativity for CD19 were homogenous in primary and secondary PCL. In conclusion, independent from the immunophenotype of the plasma cells, unique prognostic features and worse outcome are common in PCL in contrast to MM. This indicates that PCL patients need different clinical management and treatment.

2.3.2. AL amyloidosis

Primary amyloidosis results from a plasma cell neoplasm in which there is production of an amyloidogenic light chain. The features of the disease are caused by amyloidosis rather than other features due to plasma cell proliferation. Transformation into MM occurs infrequently [112]. The disease is mostly associated with λ light chain, which is unlike other plasma cell disorders [113,114]. AL amyloidosis clinical features are represented as progressive dysfunction of multiple visceral organs (mainly heart and kidneys) and, pathologically, as the extracellular deposition of insoluble fibrils derived from Ig light chains [115]. A recent study demonstrated the prominent phenotype nature of malignant plasma cells in AL amyloidosis and derived specific markers for follow-up studies [113]. They reported that the phenotype of normal and malignant plasma cells of AL amyloidosis patients were similar to myeloma patients. Further characterization of plasma cell subsets has been based on lineage markers, such as MPC-1, CD45 and CD49e. Lineage specific plasma cell subsets were described as: immature cells: MPC-1$^-$CD45$^+$ or MPC-1$^-$CD45$^-$, intermediate cells: MPC-1$^+$CD45$^-$CD49e$^-$ and mature cells: MPC-1$^+$C45$^+$CD49e$^-$ or MPC-1$^+$CD45$^+$CD49e$^+$. Immature, intermediate and abnormal plasma cell and serum free light chain (FLC)/creatinine ratio were significantly higher in AL amyloidosis patients than in the control group (MGUS and other diseases). Also, a significant lower frequency of mature plasma cells was observed in AL amyloidosis patients compared to controls [113].
AL amyloidosis patients in remission and who received high-dose chemotherapy followed by ASCT were reported to have a decrease in the frequency of malignant plasma cells, intermediate plasma cells, serum FLC/creatinine ratio and disappearance of M-protein in serum/urine [113,116]. Highlighting intermediate plasma cells (MPC-1$^+$CD45$^-$CD49e$^-$) represents a prognostic marker for diagnosis and follow-up of AL amyloidosis patients.

2.3.3. IgM multiple myeloma

MM cases are frequently associated with IgG or IgA M-protein. However, cases of IgM myeloma have been described with clinic pathological features intermediate to those of MM and Waldenström Macroglobulinemia (WM). Classical MM (IgG and IgA) and WM are two different entities that could be diagnosed early based on involvement of M-protein. IgM M-protein is commonly found in WM and adds important diagnostic value. The additional feature in the WM patient is monoclonal proliferation or lymphoplasmocytoid cells both in PB and BM [117]. The clinical presentation of IgM myeloma have been characterized as a high incidence of lytic bone lesions, decreased IgG and IgA, renal failure, hypercalcemia, and Bence-Jones proteinuria, which are common findings in patients with MM. Yet, findings typical of WM also occur, including hyperviscosity symptoms, lymphadenopathy, and hepatosplenomegaly [117,118,119]. It seems that IgM myeloma might arise from IgM-specific MGUS, although a long-term follow-up study [120] found that any case of 213 patients diagnosed with IgM MGUS progressed to IgM myeloma. Only progression to disorders such as lymphoma, WM, primary amyloidosis and chronic lymphatic leukemia have been noticed [120].

IgM myeloma is an intermediate entity between MM and WM; immunophenotypic studies revealed a hybrid phenotype of plasma cells in one case. The phenotype of this case was represented as CD45-CD38++ cytoplasmic Ig++CD20+CD22+FMC7+ with strong positivity for surface and cytoplasmic Ig expression and variable expression for CD38 and CD5 [121]. A more recent study of 10 IgM myeloma cases clearly identified an IgM MM phenotype as CD20-C56-CD117-, which is in contrast to a case reported by Haghighi where CD20 expression was found on plasma cells [117, 122].

Although difficult to establish, it was found that the survival in patients diagnosed with IgM MM was <36 months, suggesting that IgM myeloma may be associated with an inferior clinical outcome compared with IgG/IgA MM.

3. MINIMAL RESIDUAL DISEASE DIAGNOSTICS FOR EVALUATION OF TREATMENT

As in most other hematological malignancies, continuous efforts are made in MM to improve efficacy of therapy. As a consequence, this is associated with both an overall increased rate of remission and a progressive decrease in the number of residual clonal plasma cells after therapy. To trace these low frequencies of malignant cells, so called minimal residual disease (MRD), sensitive techniques are required (figure 5). MRD-techniques should reach sensitivities of at least 10^{-4} (one malignant plasma cell in 10.000 normal cells), but sensitivities of 10^{-5} to 10^{-6} are preferred.

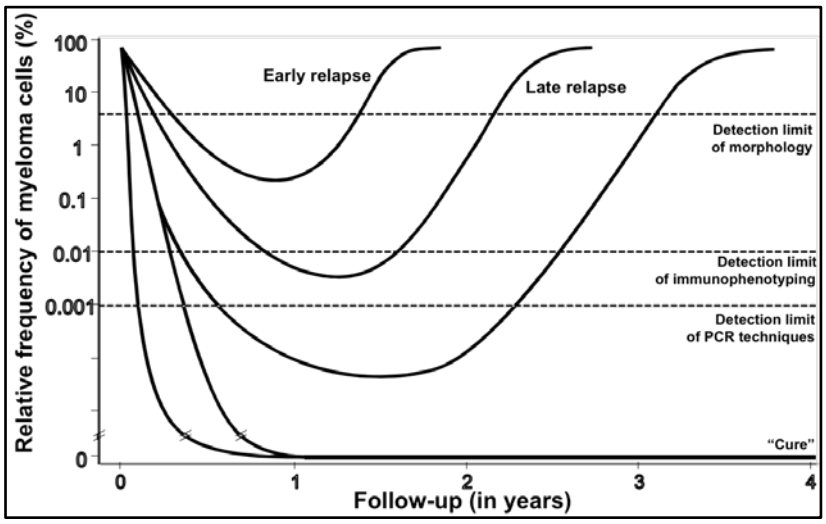

Figure 5. Diagram of relative frequencies of myelomatous plasma cells in BM of patients with MM, during and after therapy, and during development of relapse. The detection limit of cytomorphology, flow cytometric immunophenotyping and PCR techniques are indicated.

3.1. TECHNIQUES FOR MRD DETECTION

During the past years, several methods for MRD detection have been developed and evaluated, including conventional cytogenetics, fluorescent *in situ* hybridization (FISH), immunophenotyping, and polymerase chain reaction (PCR). Most of these techniques are not suitable for clinical MRD detection, due to limited sensitivity, limited specificity or limited applicability.

At this moment, two quantitative techniques reaching sensitivities of 10^{-3} to 10^{-6} are widely used for detection of MRD in MM patients. These methods include allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) capable of detecting up to one clonal cell in 10^5 normal cells and immunophenotypic assays detecting one clonal cell in 10^4 normal cells by use of ≥ seven-color MFC. Compared to the molecular methods, flow-MRD shows a clearly higher applicability (e.g. it can currently be applied to > 95% versus 70-75% of all myeloma patients) and a similar specificity. In addition, flow-MRD is a fast and easy-to-perform test, which is widely available in most clinical laboratories where MM patients are treated. At the same time, it provides information, not only about the myeloma plasma cell compartment, but also about the other cellular compartments in the sample. Although the ASO-PCR method may provide greater sensitivity than currently used flow MRD approaches, it has a lower applicability, it remains a difficult assay to be performed, and is time consuming as it requires prior information about patient-specific immunoglobulin sequences for the design of patient-specific primers. In turn, both approaches deserve standardization efforts at both an intra- and inter-laboratory level. A summary of the main characteristics of both techniques is shown in table 5 [113].

Technique	Sensitivity	Applicability in MM	Advantages	Disadvantages
Flow cytometric immunophenotyping	10^{-4}	≥95%	Quantitative Fast Disease specific Growing Standardization	Background of BM regeneration Immunophenotypic shifts Less color (≤ 4): lower sensitivity/applicability More colors (≥ 6): increased costs, knowledge and expertise being needed
ASO-PCR	10^{-5}	≤75%	Quantitative High Sensitivity Highly standardized	Time consuming Relatively expensive Patient specific

Table 5. Comparison of the most relevant features of flow-MRD and ASO-PCR MRD techniques for MRD assessment in MM. MFC: multiparameter flow cytometry; ASO-RQ-PCR: Allele-specific oligonucleotide real time quantitative PCR; MM: multiple myeloma; MRD: minimal residual disease.

3.2. CLINICAL RELEVANCE OF MRD DETECTION IN PATIENTS WITH MULTIPLE MYELOMA

Modern treatment protocols for MM (i.e. high dose melphalan and supporting ASCT) include a complete remission (IMWG guidelines), defined as a negative immunofixation of serum and urine along with the presence of less than 5% plasma cells in the BM. Achievement of complete response (CR) predicted outcome in a significant proportion of MM patients [4,123,124,125,126,127,128,129,130]. With the introduction of novel induction regimens, it has become apparent that the achievement of CR also predicts outcome in patients ineligible for ASCT [131,132]. Data from clinical trials on MM patients with and without ASCT show that reaching CR is associated with prolonged PFS and OS [133,134]. However, the measurement of the M-component has some limitations. For instance, response assessment at specific time points is influenced by M-protein type because clearance half-lives vary considerably, typically 2 to 4 hours for free light chains and approximately 25 days for immunoglobulin G [129]. Moreover, it does not directly reflect the remaining tumor burden. In addition, Chee [135] recently illustrated the clinical relevance of morphological BM evaluation, even in patients with MM showing negative immunofixation after therapy. They showed that approximately 14% of all the MM cases display > 5% BM plasma cells and therefore did not fulfill CR criteria. However, compared to acute leukemia, BM evaluation in MM based on conventional methods is generally not capable to distinguish normal from aberrant plasma cells and therefore, has limited sensitivity [78]. Because of this, the International Myeloma Working Group (IMWG) has proposed additional criteria to define stringent CR (sCR): complete response plus the absence of clonal plasma cells evaluated by serum free light chain levels and BM immunohistology or immunofluorescence [136,137].

3.2.1. Multiparameter flow cytometry for MRD detection

Early MRD-studies in MM based on MFC investigation showed that treatment of patients with ASCT was associated with lower levels of residual disease and prolonged PFS [138]. In parallel, another group showed that among 45 patients who underwent ASCT, those who remained MRD-positive by MFC at 3 months post-transplant (42%) had shorter PFS than patients with undetectable disease [139]. More recently, Paiva have shown in a series of 295 prospective patients with MM that the absence of residual clonal plasma cells – immunophenotypic remission – was the most relevant independent prognostic factor among patients receiving HDT/ASCT [140]. Accordingly, even within those patients that were negative by immunofixation 3 months after ASCT, MRD was detected in 36% (53/147) of them, with a similar prognostic differentiation when compared to the overall series of patients. In fact, both MRD-negative/IFE-negative and MRD-negative/IFE-positive patients had a significantly better outcome than MRD-positive/IFE-negative cases, which reflects the higher prognostic value of the immunophenotype investigation of MRD or protein measurements [140]. A limitation of these and other studies is that the results are derived from relatively small series of patients, or exclusively patients who are eligible for transplantation, or moreover, were carried out before the introduction

of novel agents for MM therapy [141]. It is therefore important to evaluate the clinical relevance of achieving deeper levels of response and to compare them with the conventional definition of CR in an era of novel agents.

In 2011, a prospective study examined the prognostic impact of attaining CR (negative immunofixation *plus* <5% plasma cells in BM), versus stringent CR (sCR) (CR plus normal sFLC ratio), and immunophenotypic response (iCR or IR) after induction therapy in 102 elderly patients (>65 years) with MM ineligible for high dose therapy. In this study, 43% of the patients achieved CR, 30% achieved sCR and 30% achieved iCR [142]. In line with previous reports [143,144,145], their results confirm that achievement of conventional CR by the end of induction chemotherapy results in extended survival: i.e., among those 102 patients, those achieving iCR, stringent CR, or CR showed a significantly better outcome than those who achieved partial response in terms of 3-year rate of PFS (90%, 69%, 60%, and 35%, P < .001) and time to progression (TTP) (96%, 71%, 68%, and 37%, P < .001), as well as a trend toward longer OS (94%, 94%, 93%, and 70%, P = .08) (figure 6). Further, they also investigated whether incorporating the IR status into the sCR criteria represent a deeper level of remission and whether this had an improved patient outcome. There was no significant difference in survival between patients with sCR and CR, however, patients in iCR showed significantly increased PFS (median not reached; 95% at 3 years versus 35 months, respectively; P = .02) and TTP (P = .003) compared with those in sCR or CR, suggesting increased sensitivity of MFC to detect MRD (figure 7).

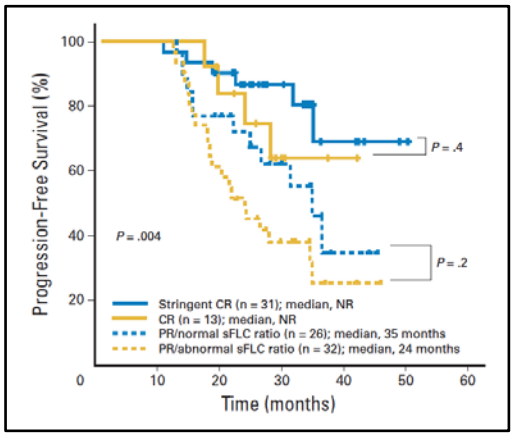

Figure 6. Progression free survival according to the response status after induction therapy in patients with sCR versus CR and PR (with normal sFLC ratio) versus PR (with abnormal sFLC ratio) [142].

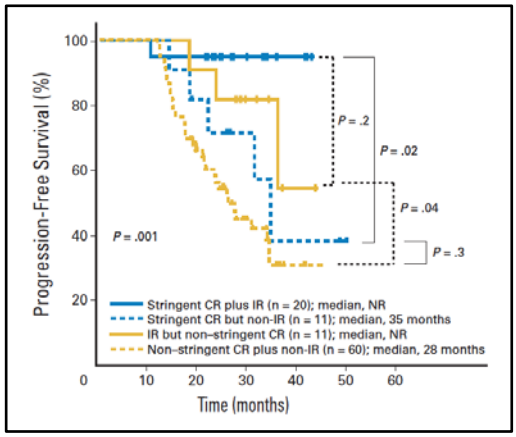

Figure 7. Progression free survival with respect to the depth of response attained after induction therapy in patients with iCR, sCR, non-stringent CR but IR, and non-stringent CR plus non-IR [142].

An important question is whether this ability to detect MRD by MFC has clinical implications for PFS and OS, and whether it may inform therapy and allow us to tailor therapeutic decisions. Along with the presence of baseline high-risk cytogenetics by fluorescent in situ hybridization, persistent MRD by MFC at day 100 after HDT/ASCT was the only other independent factor that predicted for unsustained CR and a poor outcome, proven by a median 39-month OS [146].

Another study by Rawstron assessed MRD using MFC in a large cohort of uniformly treated patients following induction and at 100 days post ASCT, as well as post-induction therapy in a non-transplantation group [129]. They evaluated two induction regimens and found 25% of the patients becoming MRD negative receiving CTD (cyclophosphamide, thalidomide and dexamethasone), compared to 13% of the patients receiving CVAD (cyclophosphamide, vincristine, doxorubicin and dexamethasone). This superiority was maintained after ASCT, with 71% of the patients becoming MRD negative in the CTD-group, compared to 54% in the CAVD group. These data suggest that ASCT remains a highly effective component of myeloma therapy. They demonstrated a 2.8 fold increase in MRD negativity using MFC after ASCT in CTD-treated patients and a 4.2 fold increase in CVAD-treated patients [129]. Paiva assessed MRD using MFC after a number of different induction regimens. The highest rates of MRD negativity were noted with bortezomib-containing regimens, but in all instances, a further improvement was seen after ASCT [147].
In the study of Rawstron, presence of MRD after ASCT was associated with significantly inferior PFS and OS [129]. These data are similar to those reported previously after conventional chemotherapy induction and ASCT [139,140]. When outcome was assessed in patients according to MRD status after induction as well as after ASCT, it was clear that PFS was best in those patients who achieved MRD negativity after induction. This study, along with the study of Paiva [140], suggests the role of ASCT should only be questioned in patients who achieve MRD negativity after induction. An overview of the different studies that examined MRD detection using MFC is presented in attachment 4.

3.2.2. Polymerase chain reaction for MRD detection

Molecular monitoring of disease by using polymerase chain reaction (PCR) is commonly used in chronic myelogenous leukemia, acute lymphoblastic leukemia and acute promyelocytic leukemia to help determine prognosis and guide therapy [148,149,150]. Several PCR techniques have been described for the use of MRD monitoring in MM. Use of PCR allows the amplification of single cells, providing a sensitive method to detect MRD. However, MRD testing in myeloma by PCR has proved to be challenging for several reasons. The immunoglobulin (Ig) heavy and light chains (IGH, IGK and IGL) loci have significant somatic hypermutation, with an average of 92.2% homology to the germline sequence for IGH sequences, 93.9% for IGK, and 93.4% for IGL [151]. For this reason, standard primers designed against specific regions of the Ig genes occasionally fail to bind to the patient template DNA sufficiently well to result in amplification. As a result of these somatic hypermutations at the binding site of commercial primers, an attempt was made to develop highly sensitive molecular assays for individual Ig rearrangements through the design of allele-specific oligonucleotide (ASO) primers [82]. PCR or even quantitative PCR can subsequently be performed using these primers, allowing highly sensitive monitoring of the patient's clone.
Most early studies that examined the role of PCR monitoring for MRD in MM used ASO-PCR methods [152,153,154,155,156,157,158]. In these studies, that were performed before the era of more effective therapy regimens (see above), molecular MRD measurement by qualitative PCR was found not to be useful, as nearly all patients were positive for residual disease at the molecular level, with exception of a small number of patients who achieved "molecular" remission after ASCT. An early study performed in a small number of patients demonstrated persistent molecular remission using qualitative, non-ASO-PCR, suggesting the possibility of cure, particularly after allogeneic SCT [159]. Corradini described molecular remission with a qualitative PCR approach using ASO-primers in significantly more patients after allo-SCT compared to autologous-SCT. Several studies have also demonstrated the prognostic significance of MRD negativity by PCR after allo-SCT [157,160,161,162]. The studies that examined PCR for MRD monitoring in MM are listed in attachment 5.
More recently, semi-quantitative fluorescent and real-time PCR techniques, primarily using ASO primers, were introduced to correlate the level of the clone with clinical disease manifestations and outcomes [163,164,165,166,167]. These methods have allowed very sensitive MRD monitoring in order of 10^{-6} and permit trends in level to be followed rather than binary positive of negative results. Using ASO RQ-PCR, Korthals showed that low MRD (<0.2% S IgH/β-actin) measured before HDT and auto-PBASCT was significantly associated with improved median PFS and OS compared with high MRD [168]. Ladetto documented persistent molecular remissions using either qualitative nested ASO-PCR or ASO-RQ PCR in patients with MM treated with bortezomib, thalidomide and dexamethasone as consolidation after auto-

PBSCT, with no patient in molecular remission relapsing after a median follow-up of 42 months [169]. An update of this study showed after a median follow-up of 65 months, a 5-year OS of 100% for patients achieving MRD negativity; no patients achieving molecular remission has died [170].

3.2.3. Comparison of PCR and MFC for MRD monitoring

Both techniques have been compared in several small studies. Sarasquete retrospectively examined MRD by immunofixation, PCR and MFC in 32 patients with MM in CR or near CR after HDT and ASCT [82]. The ASO RQ-PCR was only applicable in 75% of patients; in contrary, 90% of the samples could be analyzed by MFC. In 25% of cases, PCR and MFC resulted in discrepant results. In all cases, PCR was positive with negative MFC, no cases were positive by MFC and negative by PCR. The discordance rates for either PCR or MFC with immunofixation were 38%, with the more sensitive method varying from case to case. PFS was significantly longer in patients with PCR $\leq 10^{-4}$, but not significantly longer in patients with negative immunofixation or MFC $<10^{-4}$, although there was a trend toward significance with MFC. Thus, in this small series of patients, MRD negativity by ASO RQ-PCR predicted longer PFS, whereas PFS was not significantly different for patients with negative immunofixation and negative MFC. On the contrary, Lioznov found that MFC and ASO-PCR correlated extremely well in 69 samples, with only one sample unavailable for ASO-PCR and none unavailable by MFC [171]. Zhao compared multiple methods for MRD monitoring in 121 BM samples [172]. Immunohistochemistry was the most effective method for detecting residual disease, with a 96% detection rate, followed by limited (3-color analysis) MFC at 72%. Cytogenetics (15%), FISH (50%) and detection of IgG and kappa light chain gene rearrangements by qualitative PCR with a limited set of non-ASO primers (60%) were less successful. Although this study is an interesting comparison of multiple techniques, the sensitivity of both MFC and PCR are likely limited due to the use of older methodology. Additionally, the majority of patients had residual disease at the time of BM examination, and no comparison with serologic studies such as immunofixation was made. Paiva compared immunofixation, sFLC, and immunophenotyping by MFC in 102 patients >65 years diagnosed with MM [142]. Seven percent of the patients with no MRD by MFC (sensitivity $\leq 10^{-4}$ to 10^{-5}) after induction therapy remained positive by immunofixation initially, although all subsequently became immunofixation negative. Discrepant results were common among all methods tested. Those patients with immunophenotypic response had significantly increased PFS and time to progression compared to those with CR (immunofixation negative and < 5% plasma cells on BM biopsy) or sCR (CR and normalization of the sFLC ratio); however, no OS benefit was noted.

3.2.5. Instability of immunophenotype in plasma cell myeloma

Antigenic shift has been well described in hematopoietic disorders, particularly in B-lymphoblastic and myeloid leukemia's [175,176]. The establishment of a baseline immunophenotype to follow in subsequent analyses is routine in MRD monitoring, although little information has been reported describing antigen stability in MM. Recognition of lack of stability in immunophenotype may be important, especially in antigen-directed treatment decisions (rituximab (anti-CD20 mAb), alemtuzumab (anti-CD52 mAb), and imatinib mesylate (anti-c-kit)) and when specific phenotypes are used to monitor MRD (see further). Cao and Gupta reported immunophenotypic instability of several antigens in MM [177,178]. Cao determined the expression frequency and stability of 2 potential therapeutic targets in MM, i.e. CD20 and CD52, along with the frequently aberrantly expressed CD56 antigen. Of the 56 patients evaluated, 23 (41%) showed immunophenotypic changes including CD56 in 6 cases, CD20 in seven cases and CD52 in 17 cases. Combined CD56 /CD52 change was seen in 3 cases and combined CD20/CD52 in 4 cases. Loss of CD20 and CD52 expression following immunotherapy with rituximab or alemtuzumab is common [179]. In another study, Gupta found changes in the immunophenotype of plasma cells in 18 out of 23 cases (78%) with MM and found up and down regulation of CD52 (6 and 5 patients), CD117 (8 and 2 patients) and CD20 (1 and 0) in a few cases, respectively. Down-regulation of aberrantly expressed antigens may influence MRD detection, particularly when more than 1 antigen is simultaneously down regulated. More recently, a study by Spears examined 45 MM patients by four-color flow cytometry for shifts in CD19, CD20, CD38, CD45 and CD56, and cytoplasmatic light chain expression [180]. Immunophenotypic changes were observed in 31% of the cases (14/45). An overview of the specific marker changes in their study

is presented in table 6. Of those cases showing an immunophenotypic shift, three cases demonstrated a reversion to a normal plasma cell immunophenotype, despite maintaining light chain restriction. This finding is of importance in MRD analysis, since it may be impossible to identify the neoplastic cell population if light chain analysis was not employed, as recommended by the European Myeloma Network [25]. However, the study of Spears focused on a relatively limited set of commonly assessed antigens, and it is likely that baseline assessment of a broader panel of antigens, such as CD27, CD28 and CD117 would provide a more robust immunophenotypic fingerprint and enable identification of a residual neoplastic plasma cell population at follow-up.

	Immunophenotypic shift				
Marker	Pts with changes/ Total pts (%)	(+)* to (−)	(−) to (+)	Change in level of expression	Multiple changes
CD19	5/45 (11.1)	0	4	1	0
CD20	2/45 (4.4)	1	1	0	0
CD45	9/45 (20.0)	1	5	2	1†
CD56	5/45 (11.1)	3	0	2	0

*(+) includes partial (+) and subset (+); †From (−) to partial (+) to (−).

Table 6. Summary of specific marker changes in the study of Spears [180].

A more cost-effective approach to MRD analysis would be to start with a limited panel of markers (including CD19 and CD56), which would allow detection of MRD in the majority of cases and if negative, employ a broader panel to exclude neoplastic plasma cells that had reverted to a normal CD19+/CD56- pattern, as in their three encountered cases.

Since there is a frequent change in the immunophenotype of neoplastic plasma cells (both up and down regulation) in MM, MRD evaluation using a wide panel of antibodies would be desirable, and the use of only the markers that were aberrantly expressed at diagnosis may not be useful. This becomes more important in those patients who are treated with monoclonal antibodies like rituximab, alemtuzumab and imatinib mesylate.

3.2.4. Conclusion

All together, these results confirm the great clinical relevance of MRD investigation by MFC and PCR in patients with MM. In line with this, the International Myeloma working group has updated the response criteria to define sCR, which now requires the absence of clonal/aberrant plasma cells by MFC (with ≥ 4 colors) together with a normal sFLC ratio (plus all other criteria required for the definition of CR) [23]. Although PCR strategies are more sensitive, a major drawback is the inability to generate clonal IgH rearrangements from presenting BM specimens. This failure is primarily because of the loss of primer binding sites as a consequence of somatic hypermutation. Moreover, PCR strategies are expensive and time-consuming and many routine-laboratories will be unable to provide results in real-time for clinical decision-making. On the other hand, flow monitoring is possible without the knowledge of the presenting immunophenotype and also provides a means of assessing the quality of the specimen, as normal plasma cells are not detectable in the peripheral blood [173].

With the introduction of 8-color MFC together with new software and analytical approaches, it is expected that the sensitivity of immunophenotyping may reach a level similar to (if not greater than) that of ASO-PCR, increasing its utility in monitoring the effects of new therapeutic agents and protocols.

4. MONOCLONAL ANTIBODY-BASED THERAPY AS A TREATMENT STRATEGY IN MULTIPLE MYELOMA

Over the past 5-10 years, the knowledge of the biology of MM has greatly increased, defining the disease as being heterogeneous with a large and variable number of cytogenetic and molecular abnormalities. Also the interaction of the malignant plasma cells with their microenvironment is better understood. New drugs that interfere with this interaction such as thalidomide, lenalidomide and bortezomib, along with ASCT, have been introduced into clinical practice and have a dramatic impact on the prognosis of MM patients. Today, the life expectancy of an average MM patient is over 5 years but despite this progress, the disease remains incurable.
A new anticancer strategy is the use of monoclonal antibodies (mAb) that operate through a completely different mechanism of action. Ideally, targets for therapeutic mAb should be specifically expressed on cancerous cells but not on normal cells (figure 8). In fact, mAb are an interesting therapeutic option in MM because they are specific to a tumor associated target and have been successfully employed in the treatment of patients with other hematologic diseases (f.i. rituximab in chronic lymphatic leukemia).
Based on immunophenotypes of the plasma cells determined by using MFC, we should distinguish anti-body based therapies for all patients from therapies for particular entities or subpopulations of MM patients. A short overview of the potential application of mAb as therapeutic agents to treat MM will be discussed (table 7).

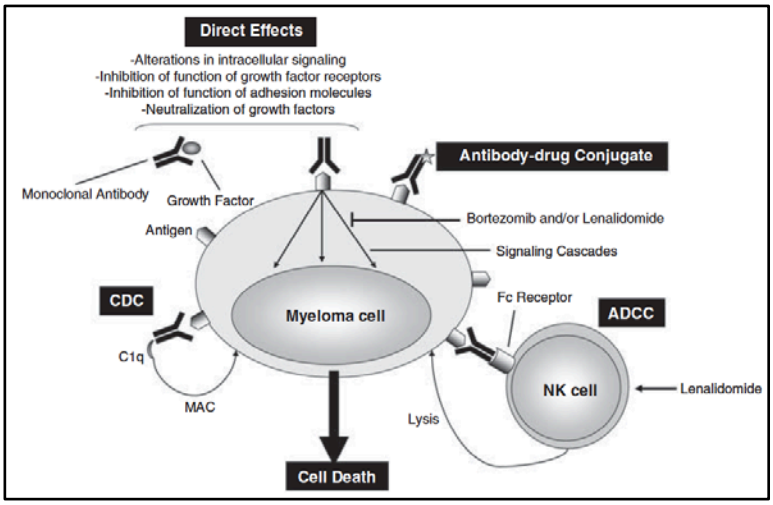

Figure 8. Mechanism of action of mAb. mAb have direct effects on the myeloma cell via modulation of the activity of the targeted antigen. Furthermore, complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) may contribute to mAb induced tumor cell death. The process of ADCC is achieved through activation of Fc receptors on myeloid and NK effector cells by tumor cell-attached immunoglobulins. CDC is dependent on the interaction of the antibody Fc domains with the classic complement-activating protein C3q resulting in the accumulation of C3b, which acts as an opsonin and promotes phagocytosis. C3b also binds to C3 convertase to form a C5 convertase, leading to the induction of the membrane attack complex [185].

4.1. SYNDECAN-1 (anti-CD138 mAb)

As discussed above, CD138 is a cell surface protein, which serves as a receptor for epidermal growth factor ligands. Binding of EGF ligands stimulates cell growth. Immunohistochemical and flow cytometric analysis of patient MM cells has shown that CD138 is expressed in a large majority of cases. Within the hematopoietic compartment, CD138 expression is confined to normal plasma cells, with no expression on hematopoietic stem cells, while expression of CD138 on MM cells is significantly higher than on normal plasma cells. Almost all MM cells, even after exposure to multiple therapies, express the antigen, making it a useful target at any stage of the disease. Different monoclonal antibodies specific to CD138 have been reported. Preliminary data of clinical phase 1/2 studies show evidence and clinical activity of anti CD138-antibodies [185].

4.2. NEURAL CELL ADHESION MOLECULE (anti-CD56 mAb)

Expression of CD56 is found on several cancers including the majority of myeloma cases. Normal plasma cells lack CD56 expression, whereas it is normally found on natural killer cells and a subset of T-lymphocytes. Lorvotuzumab consists of a potent cytotoxic maytansinoid attached to a CD56-binding mAb. Lorvotuzumab has a potent activity as single agent against myeloma cell lines in the presence of stromal cells and in a myeloma mouse model. In combination with lenalidomide, synergistic activity was demonstrated in a myeloma mouse model. In combination with bortezomib, the molecule was found to be additive-to-synergistic against human myeloma xenografts in mice, whereas the combination in vitro showed antagonism. A phase I study with CD56+ relapsed/refractory myeloma patients showed a good toleration and an antimyeloma activity of lorvotuzumab (>/-18% MR) [189,190]. These trials and other preclinical studies supported investigations of lorvotuzumab as part of a combination regimen. A phase I study evaluated the combination with lenalidomide and dexamethasone in CD56+ relapsed/refractory myeloma. In the first dose level, two out of three myeloma patients achieved a response (one PR and one very good PR) without serious adverse effects [185].

4.3. Anti-D38

CD38 is a type II transmembrane glycoprotein with ectoenzymatic activity involved in the production of calcium-mobilizing compounds. CD38 is highly and uniformly expressed on all myeloma cells. Under normal conditions, CD38 is expressed at relatively low levels on lymphoid or myeloid cells and in some tissues of non-hematopoietic origin. A new human mAb directed against CD38, daratumumab, was developed recently, which induced myeloma killing in mouse models. Association with lenalidomide and bortezomib leads to a significant enhancement of cell killing in myeloma cell lines. On the basis of these preclinical results, daratumumab is currently evaluated in phase 2 safety studies [185].

4.4. Anti-CD20

Several papers have shown that MM includes clonotypic B lineage cells at stages earlier than the compartment of malignant plasma cells in the BM, and the circulating component of the MM clone includes at least two distinct CD19+/CD20+ B-cell compartments as well as CD138+ /CD20+ plasma cells. Pilarski evaluated these three compartments before, during and after treatment of patients with rituximab (anti-CD20), followed by quantifying B-cell subsets over a 5-month period during and after treatment. Overall, all three types of circulating B lineage cells persist despite treatment with rituximab. The inability of rituximab to prolong survival in MM may results from this failure to deplete CD20+ B and plasma cells in MM. In another study, rituximab was tested for maintenance therapy in MM following ASCT. The use of rituximab was associated with an unexpectedly high rate of early relapse. The authors hypothesized a possible role for rituximab in provoking an additional reduction in the normal, residual B-cell activity.
Generally, MM is not considered as a disease adequate for anti-CD20 therapy due to weak and various expression of CD20 in the preponderance of subjects. In contrast, other studies demonstrated that the CD20+ phenotype is associated with patients with t(11,14)(q13;q32) and with shorter survival and that sporadic responses have been achieved in patients with CD20+ myelomatous plasma cells [188].

Target	Agent	Clinical study phase	Single agent (S)/combination (C)
Activin A	Sotatercept	I/II	S
BAFF	Tabalumab (mAb)	I/II	S, C (lenalidomide)
CD38	Daratumumab	I	S
	SAR650984	I	S
	MOR202	I	S
CD40	Dacetuzumab (SGN-40)	Ib	S, C (lenalidomide)
	Lucatumumab (HCD122)	I	S
CD56	huN901-DM1 (C-mAb)	I	S
CD74	Milatuzumab	I/II	S
CD138	BT062 (mAb-DM4)	I	S
CS1	Elotuzumab	II/III	S, C (lenalidomide, bortezomib)
CXCR3	Plerixafor	II	C (bortezomib)
DKK-1	BHQ-880 (mAb)	I/II	S
FGF, PDGF	Dovitinib	I	S
HM1.24	anti-HM1.24 (mAb)		
IGF-1/R	CP-751,871 (mAb)	I	S
	EM164 (mAb)	I	S
IL-6/R	Siltuximab (mAb)	II	S, C (bortezomib)
KIR	IPH101 (mAb)	I/II	S
MUC1	AR20.5 (mAb)	I/II	S
RANKL	Denosumab (mAb)	I/II	S
TRAIL	Apo2L/TRAIL (Apo2 ligand)	I	S
	Mapatumumab	I/II	S
VEGF/R	Bevacizumab (mAb)	II	S
	SU5416	II	S
	Vandetanib (ZD6474)	II	S

Table 7. Monoclonal antibodies targeting tumor cells in MM [186]

4.6. CONCLUSION

For the past years, a lot of therapeutic mAb have entered clinical trials or are in clinical development for MM. However, monoclonal antibodies targeting myeloma cells have not yet been included as part of standard myeloma therapy. An important challenge will be the identification of those patients that will benefit from certain antibody-based therapies. Efficacy of monoclonal antibody therapies is dependent on antigen protein expression and neutralizing mAb are best used in tumors that are at least partly dependent on the antigen or pathway neutralized by the therapeutic mAb. Inclusion of MFC and cytogenetic analysis will result in the identification of subgroups of myeloma patients that will likely benefit from a certain antibody based combination therapy. Therefore, future studies should incorporate analysis of biomarker components that can predict the safety and effectiveness of new monoclonal antibodies.

TO DO/ACTIONS

1) Selection of the appropriate markers for characterization of neoplastic/aberrant plasma cells
2) Introduction/implementation of multicolor multiparameter flow cytometry in the clinical laboratory of hematology

ATTACHMENTS

Attachment 1: Diagnostic criteria of plasma cell neoplasms [4].

Plasma cell neoplasms	Disease definition
Monoclonal gammopathy of undetermined significance	All three criteria must be met: Serum monoclonal protein <3g/100ml Clonal bone marrow plasma cells <10% **and** absence of end-organ damage such as hypercalcemia, renal insufficiency, anemia and bone lesions (CRAB) that can be attributed to the plasma cell proliferative disorder
Smoldering multiple myeloma (also referred to as asymptomatic myeloma)	Both criteria must be met: Serum monoclonal protein (IgG or IgA) ≥3g/100 mL and/or clonal bone marrow plasma cells ≥10% **and** absence of end-organ damage such as lytic bone lesions, anemia, hypercalcemia or renal failure that can be attributed to a plasma cell proliferative disorder
Multiple myeloma	All three criteria must be met except as noted: Clonal bone marrow plasma cells ≥10% Presence of serum and/or urinary monoclonal protein (except in patients with true non-secretory MM myeloma) **and** Evidence of end-organ damage that can be attributed to the underlying plasma cell proliferative disorder, specifically: Hypercalcemia: serum calcium ≥11.5 mg/100ml **or** Renal insufficiency: serum creatinine >1.73 mmol/L Anemia: normochromic, normocytic with a hemoglobin value of >2g/100ml below the lower limit of normal or a hemoglobin value <10g/100 ml Bone lesions: lytic lesions, severe osteopenia or pathologic fractures
Waldenström's macroglobulinemia	Both criteria must be met: IgM monoclonal gammopathy (regardless the size of the M-protein) **and** ≥10% bone marrow lymphoplasmacytic infiltration (usually intratrabecular) by small lymphocytes that exhibit plasmacytoid or plasma cell differentiation and a typical immunophenotype (e.g. surface IgM+, CD5+/-, CD10-, CD19+, CD20+, CD23-) that satisfactory excludes other lymphoproliferative disorders, including chronic lymphocytic leukemia and mantle cell lymphoma **Note:** IgM MGUS is defined as: Serum IgM monoclonal protein <3g/100ml, and bone marrow lymphoplasmacytic infiltration <10% and no evidence of anemia, constitutional symptoms, hyperviscosity, lymphadenopathy or hepatosplenomegaly **Note:** Smoldering Waldenström's macroglobulinemia (also referred to as indolent or asymptomatic Waldenström's macroglobulinemia) is defined as: Serum IgM monoclonal protein ≥3g/100ml and/or bone marrow lymphoplasmocytic infiltration ≥10%, **and** no evidence of end-organ damage such as anemia, constitutional symptoms, hyperviscosity, lymphadenopathy or hepatosplenomegaly that can be attributed to a lymphoplasma cell proliferative disorder
Solitary plasmacytoma	All four criteria must be met: Biopsy-proven solitary lesion of bone or soft tissue with evidence of clonal plasma cells Normal bone marrow with no evidence of clonal plasma cells Normal skeletal survey and MRI of spine and pelvis (except for the primary solitary lesion) Absence of end-organ damage such as CRAB lesions that can be attributed to a lymphoplasma cell proliferative disorder
Systemic AL amyloidosis	All four criteria must be met: Presence of an amyloid-related systemic syndrome (such as renal, liver, heart, gastrointestinal tract of peripheral nerve involvement) Positive amyloid staining by Congo-red in any tissue (e.g. fat aspirate, bone marrow or organ biopsy) Evidence that amyloid is light chain-related established by direct examination of the amyloid (immunohistochemical staining, direct sequencing, and so on) **and** Evidence of a monoclonal plasma cell proliferative disorder (serum of urine M-protein, abnormal free light chain ratio or clonal plasma cells in the bone marrow). **Note:** Approximately 2-3% of patients with AL amyloidosis will not meet the requirement for evidence of a monoclonal plasma cell disorder listed above; the diagnosis of AL amyloidosis must be made with caution in these patients
POEMS syndrome	All three criteria must be met: Presence of a monoclonal plasma cell disorder Peripheral neuropathy **and** at least one of the following seven features: osteosclerotic bone lesions, Castleman's disease, organomegaly, endocrinopathy (excluding diabetes mellitus or hypothyroidism), edema, typical skin changes and papilledema **Note:** not every patient meeting the above criteria will have POEMS syndrome; the features should have a temporal relationship with each other and no other attributable cause. The absence of osteosclerotic lesions should make the diagnosis suspect. Elevations in plasma or serum levels of vascular endothelial growth factor and thrombocytosis are common features of the syndrome and are helpful when the diagnosis is difficult.

Attachment 2: International Myeloma Working Group uniform response criteria [4].

CR	Stringent Complete Response (sCR)	VGPR[a]	PR	SD	PD[b]
Negative immunofixation of serum and urine, ***and***	CR as defined, ***plus***	Serum and urine M-component detectable by immunofixation but not on electrophoresis, ***or***	≥50% reduction of serum M-protein and reduction in 24-hour urinary M-protein by ≥90% or to <200mg/24h	Not meeting criteria for CR, VGPR, PR or PD	Increase of 25% from lowest response value in any one or more of the following:
Disappearance of any soft tissue plasmacytomas, ***and***	Normal FLC ratio, ***and***	≥90% reduction in serum M-component plus urine M-component <100mg/24h	If the serum and urine M-protein are not measurable, a decrease ≥50% in the difference between involved and uninvolved FLC levels is required in place of the M-protein criteria		Serum M-component (absolute increase must be ≥0.5 g/dL), ***and/or***
<5% plasma cells in bone marrow	Absence of clonal plasma cells by immunohistochemistry or 2- to 4-color flow cytometry		If serum and urine M-protein are not measurable, and serum free light assay is also not measurable, ≥50% reduction in bone marrow plasma cells is required in place of M-protein, provided baseline percentage was ≥30%		Urine M-component (absolute increase must be ≥200 mg/24h), ***and/or***
			In addition to the above criteria, if present at baseline, ≥50% reduction in the size of soft tissue plasmacytomas is also required		Only in patients without measurable serum and urine M-protein levels: the difference between involved and uninvolved FLC levels (absolute increase must be >10 mg/dL)
					Only in patients without measurable serum and urine M-protein levels and without measurable disease by FLC levels, bone marrow plasma cell percentage (absolute percentage must be
					Definite development of new bone lesions of soft tissue plasmacytomas or definite increase in the size of existing bone lesions or soft tissue plasmacytomas
					Development of hypercalcemia (corrected serum calcium >11.5 mg/dL) that can be attributed to the plasma cell proliferative disorder

Abbreviations: CR: complete response; VGPR: very good partial response; PR: partial response; SD: stable disease; PD: progressive disease

All response categories (CR, SCR, VGPR, PR, and PD) require 2 consecutive assessments made at any time before the institution of any new therapy; CR, sCR, VGPR, PR, and SD categories also require no known evidence of progressive or new bone lesions if radiographic studies were performed. VGPR and CR categories require serum and urine studies regardless of whether disease at baseline was measurable on serum, urine, both, or neither. Radiographic studies are not required to satisfy these response requirements. Bone marrow assessments need not be confirmed. For PD, serum M-component increases of more than or equal to 1 g/dL are sufficient to define relapse if starting M-component is ≥5g/dL.

[a]Clarifications to IMWG criteria for coding CR and VGPR in patients in whom the only measurable disease is by serum FLC levels: CR in such patients indicates a normal FLC ratio of 0.26 to 1.65 in addition to CR criteria listed above. VGPR in such patients requires a > 90% decrease in the difference between involved and uninvolved FLC levels.

[b]Clarifications to IMWG criteria for coding PD: bone marrow criteria for PD are to be used only in patients without measurable disease by M-protein and by FLC levels, "25% increase" refers to M-protein, FLC, and bone marrow results, and does not refer to bone lesions, soft tissue plasmacytomas, or hypercalcemia and the "lowest response value" does not need to be a confirmed value.

Attachment 3: Expression of various antigens in plasma cells of MGUS and MM (adapted from [24]).

Cluster designation	Normal distribution and function	Expression in plasma cells of MGUS and MM	Diagnostic and prognostic significance	References
CD19	All B-cells, including lymphoblasts, mature B-lymphoid cells and most of plasma cells	MGUS: normal plasma cells express CD19 whereas malignant plasma cells do not MM: only negative of diminished CD19 expression on plasma cells	Facilitate as an identification marker of malignant and physiological plasma cells in combination with CD56. Patients with >5% of normal plasma cells had better PFS and OS compared to patients with < 5% of normal plasma cells. Similarly, presence of >5% normal plasma cells or <95% of malignant plasma cells in MGUS and SMM predicted better PFS compared to patients with ≤5% normal plasma cells or ≥95% of malignant plasma cells	56,85,86,87
CD20	During maturation process of pre-B-cells and negative expression in plasma cells	Some subsets of myeloma patients express CD20 in plasma cells	Associated with poor prognosis	86,88,89
CD27	Aid in differentiation of mature B-cells into plasma cells	MGUS: consistent high expression in plasma cells MM: expression is heterogeneous and low intensity comparing to MGUS	Lack of CD27 associated with shorter PFS and OS.	64,66,67
CD28	T-cell activation	MGUS: only very few cases express CD28 MM: CD28 expressing plasma cells represented as aggressive phenotype. Associates always with tumor expansion	Combination of CD28 and CD117 markers identified three groups of patients with different risk. Patients with CD28-CD117+ plasma cells (good risk group) had better PFS and OS compared to patients with CD28+CD117- plasma cells (poor risk) and patients with CD28-CD117- or CD28+CD117+ plasma cells (intermediate risk)	60,90,91
CD33	Myeloid and monocytic cells	Only few myeloma patients express CD33 on plasma cells and correlate with lower survival	CD33 expression associated with poor OS and higher mortality rate	76,79
CD45	B and T cells, diminished expression in precursor cells and some plasma cells	MGUS: equal distribution of $CD45^+$ and $CD45^-$ plasma cells MM: expression is not well characterized by survival rate is higher for $CD45^+$ patient groups comparing to $CD45^-$ groups. Also CD45 expression demonstrates proliferating compartment of normal, reactive and malignant plasma cells	Patients with CD45 positive expression had better OS than patients with CD45 negative expression	38,39,43,92
CD56	NK and NK-T cells	In plasma cell proliferative disorders CD56 consider as a valuable marker in diagnosis	Possess substantial diagnostic value in plasma cell disorders when combined with CD19 marker. Patients with CD56 negative expression on plasma cells found to have reduced OS compared to patients with CD56 positive expression. Also, CD56 negative myeloma cases strongly associated	28,46,93,94

		MM: Most of the myeloma cases express CD56. However, circulating plasma cells and extramedullary myeloma patients lack CD56 expression	with adverse biological parameters	
CD117	Progenitors of myeloid, erytroid and megakaryocytic lineage	MGUS: 50% of cases express CD117 MM: one third of myeloma patients express CD117 and have better prognosis comparing to their counterpart	CD117 expression on plasma cells predicted better outcome in MM patients. Combination of CD117 and CD28 markers delineated MM patients with different risks; CD117 expression is associated with an altered maturation of the myeloid and lymphoid hematopoietic cell compartments and favorable disease features	71,74,91,95
CD138	Plasma cells	Both normal and malignant plasma cells from MGUs and MM cases express CD138 but the expression of CD38 marker is lower in malignant plasma cells	Universal marker of plasma cells and provides a basis to quantify or to assess disease burden in plasma cell proliferative disorders	28
CD200	Member of the immunoglobulin superfamily and expressed on endothelial cells, neurons, B cell and a subset of T cells	MM- more than 70% of cases do express CD200	Absence of CD200 expression on myeloma cells associated with better PFS	96,97
CD81	Expressed on B-cells including plasma cells and regulates CD19 expression	Less than 50% of MM cases express CD81 on plasma cells and expression is heterogeneous in most of the cases (ranging from 5%-92%)	Patients with CD81 expression on myeloma cells had inferior prognostic outcome (PFS and OS) compared to patients with C81 negative expression.	98
CD221	Tyrosine kinase receptor family, expressed widely on all types of cells	MM- more than 70% and 85% of medullary and extramedullary cases express CD221 on the surface of plasma cells, respectively	Patients with CD221 expression had worse prognosis and CD221+ plasma cells were associated with adverse cytogenetic abnormalities	74,99
CD229	Signaling lymphocytic activation molecules family member	MM- consistent expression on plasma cells	Might represent an attractive diagnostic and therapeutic target for MM	100

Attachment 4: Overview of the studies examining MFC for MRD monitoring in MM.

Authors	No of patients	Method	Sensitivity	Treatment regimen	CR	MRD negativity rate
San Miguel et al [138]	87	4-color MFC	10^{-4}	ASCT	45%	36%
Paiva et al [140]	295	4-color MFC	10^{-4}	ASCT	50%	42%
Liu et al [181]	47	4-color MFC	NR	ASCT	66%	8%
					CR/VGPR	
Mateo et al [91]	685	4-color MFC	NR	ASCT	36%	NR
Kumar et al [184]	132	MFC	NR	Chemotherapy	22% to 47%	46%

MFC: multiparameter flow cytometry; CR: complete response; ASCT: autologous stem cell transplantation; NR: not reported; VGPR: very good partial response.

Attachment 5: Overview of the studies examining PCR for MRD monitoring in MM.

Authors	No of patients	Method	Sensitivity	Treatment regimen	CR	MRD negativity rate
Bird et al [159]	5	PCR (not ASO)	NR	Allo-BMT	100%	100%
Corradini et al [152]	18	ASO nested PCR	NR	auto-SCT or allo-SCT	50%	0%
Björkstrand et al [153]	15	ASO-PCR	NR	Auto-SCT x2	53%	80%
Swedin et al [154]	36	ASO semi –nested PCR	10^{-4} – 10^{-5}	Auto-SCT or allo-SCT	42%	21%
Corradini et al [155]	51	ASO-PCR	10^{-5} – 10^{-6}	Auto-SCT or allo-SCT	71%	7%; 50%
Martinelli et al [156]	26	ASO-PCR	10^{-5}	Allo-SCT	38%	50%
Martinelli et al [157]	229	ASO-PCR	10^{-5}	Auto-SCT or allo-SCT	Allo = 38% Auto = 22.5%	27%
Cavo et al [158]	13	ASO-PCR	10^{-5} – 10^{-6}	Allo-SCT	92%	69%
Ladetto et al [163]	29	Real time PC (not ASO) ASO nested PCR	10^{-4} 10^{-3} – 10^{-4}	Auto-SCT	NR	NR
Davies et al [174]	96	PCR (not ASO)	10^{-4}	Auto-SCT	53%	NR
Novella et al [167]	36	PCR (not ASO) ASO nested PCR	10^{-4} – 10^{-6} 10^{-4} – 10^{-5}	Auto-SCT	24%	NR
Corradini et al [161]	70	ASO-PCR	10^{-6}	Allo-SCT	100%	33%
Fenk et al [166]	11	ASO real time PCR	10^{-4} – 10^{-6}	Auto-SCT or allo-SCT	45%	27%
Bakkus et al [165]	87	ASO-PCR	10^{-4}	Auto-SCT	28%	35%
Raab et al [162]	11	ASO real time PCR	10^{-4} – 10^{-5}	Allo-SCT	27%	65%
Ladetto et al [169]	39	ASO nested Real-time PCR (not ASO)	10^{-6} 5×10^{-6}	Auto-SCT	49%	27%; 15% and NR
Korthals et al [168]	70	Real-time ASO-PCR	10^{-4} – 10^{-5}	Auto-SCT	25% nCR before auto-PBSCT 29% nCR after auto-PBSCT	17% and 21%

Attachment 6: Overview of the studies examining multiple methods for MRD monitoring in MM.

Authors	No of patients	Methods	Sensitivity	Treatment regimen	CR	MRD negativity rate
Rawstron et al [139]	45	3-color MFC PCR (not ASO)	10^{-4} 10^{-3} – 10^{-5}	ASCT	73%	NR 56% (25/45)
Saraquete et al [82]	32	Real time ASO-PCR 4-color MFC	10^{-5} 10^{-4}	ASCT	58%	29% (7/24) 54% (13/24)
Martinez-Sanchez et al [182]	53	Fluorescent PCR (not ASO) 4-color MFC	10^{-3} – 10^{-4} 10^{-4}	ASCT	51%	53% (28/53) 33% (17/51)
Puig et al [183]	170	Real time ASO-PCR 4-color MFC	10^{-5} 10^{-4} – 10^{-5}	ASCT	60%	54% (55/103) 46% (47/103)